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ORIGINAL ARTICLE

Functional characterization of p.Pro409His variant in *HNF1A*, a hypomorphic mutation involved in pancreatic β-cell dysfunction

Donatella Nocera[1] · Miranda Menniti[2] · Stefania Belviso[2] · Heather Mandy Bond[3] · Delia Lanzillotta[3] · Cristina Barbara Spoleti[2] · Maria Rosaria Guagliardi[1] · Paola Malatesta[1] · Francesco Trapasso[1,3] · Concetta Irace[2] · Nicola Perrotti[1,2] · Rodolfo Iuliano[1,2]

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Abstract

Aims *HNF1A* is a gene coding for the transcription factor HNF1-α, mutated in some forms of MODY and type 2 diabetes mellitus characterized by a strong genetic component. The penetrance of *HNF1A* variants differs considerably; thus, to assess the genetic risk of diabetes in carrier subjects of a *HNF1A* mutant allele, a functional characterization of mutant forms is of paramount importance.

Methods The *HNF1A* gene was sequenced in two patients with partly discordant diabetic phenotype, carrying the p.Pro409His variant. To evaluate the pathogenicity of the variant, we measured the transactivation power of the corresponding P408H HNF1-α mutant mouse form on HNF1-α target promoters.

Results We found a lower but detectable activity of transactivation of the mutant form compared with the wild-type form and we excluded mechanisms of protein degradation or nuclear mislocalization.

Conclusions The *HNF1A* mutation p.Pro409His can be considered a mild variant that confers a moderate risk of type 2 diabetes mellitus in heterozygous carriers.

Keywords HNF1A · Mutation · MODY · Type 2 diabetes mellitus

Introduction

Type 2 Diabetes Mellitus (T2DM) is a heterogeneous group of metabolic disorders, characterized by chronic hyperglycemia, resulting from a deficiency of insulin secretion, insulin resistance of target tissues or both [1]. T2DM is generally recognized as a polygenic and multifactorial disease in which variations in several genetic loci together with environmental triggers are required for its development. However, about 1–2% of T2DM patients suffer from a monogenic disease caused by a rare single-gene defect that strongly contributes to the phenotype with a modest, if any, environmental contribution. The most frequent form of Monogenic Diabetes is the Maturity-Onset Diabetes of the Young (MODY), which is characterized by an autosomal dominant inheritance and a primary defect of pancreatic β-cell function [2].

Mutations in *HNF1A* gene (OMIM 142410) are the most common cause of MODY among the adults [3]. The main clinical feature of *HNF1A*-MODY is a progressive β-cell failure, with decreased glucose-induced insulin secretion, detectable also in *HNF1A* mutated pre-diabetic subjects. Patients with mutations in *HNF1A* show also glycosuria, due to a lower renal threshold for glucose reabsorption, and a large glucose increase (> 5 mmol/l) in response to an Oral Glucose Tolerance Test (OGTT) [4–6]. They are usually very sensitive to the hypoglycemic effects of sulfonylurea

Managed by Massimo Porta.

✉ Nicola Perrotti
perrotti@unicz.it

✉ Rodolfo Iuliano
iuliano@unicz.it

[1] Unit of Medical Genetics, University Hospital Mater Domini, Viale T. Campanella, 115, Catanzaro, Italy

[2] Department of Human Health, University Magna Graecia of Catanzaro, Campus S. Venuta, Viale Europa, località Germaneto, 88100 Catanzaro, Italy

[3] Department of Experimental and Clinical Medicine, University Magna Graecia of Catanzaro, Campus S. Venuta, Viale Europa, località Germaneto, 88100 Catanzaro, Italy

medication, which is now considered first-line treatment of this diabetes form [7].

HNF1-α, the protein encoded by the *HNF1A* gene, is a transcription factor expressed in liver, kidney, intestine, stomach, and pancreas [8]. It regulates a large number of liver-specific genes, as well as pancreatic genes involved in glucose metabolism and beta-cell growth [9]. HNF1-α has three functional domains: an N-terminal dimerization domain; a DNA-binding region that contains a nuclear localization signal; and a C-terminal transactivation domain [10]. HNF1-α binds to DNA as a homodimer or heterodimer with the structurally related HNF1B transcription factor [11].

Mutations of the *HNF1A* gene may cause diabetes through haploinsufficiency (simply loss of function) or by a dominant negative mechanism. Since one *HNF1A* allele is normal in the MODY patients, the level of *HNF1A* expression must play a critical role in determining β-cell function [12].

Mild mutations (hypomorphic variants) of the *HNF1A* gene have also been identified in subjects with common T2DM (polygenic and multifactorial) [13, 14] and gestational diabetes mellitus [15], suggesting that *HNF1A* may be involved in beta-cell dysfunction in different forms of diabetes. Analyses *in vitro* of different *HNF1A* mutations have demonstrated that the variants that co-segregate with MODY cause diabetes due to haploinsufficiency, severely impaired binding and transactivation of HNF1-α target genes (about less than 30% compared to wild type) and/or reduced protein stability. Similar investigations of hypomorphic rare *HNF1A* variants, associated with T2D, have shown a milder effect on HNF1-α function, compared with MODY variants, by reducing HNF1-α transactivation potential to less than 40–60%, while DNA-binding properties have remained intact [16].

In this study, we have investigated by in vitro functional analyses the potential pathogenic effect of the p.Pro409His rare variant of *HNF1A*, identified in two women with different degrees of β-cell failure. The identification of rare *HNF1A* variants with significant functional effect may be important, since it could help the clinical follow-up of prediabetic subjects as well as the choice of anti-diabetic cure.

Materials and methods

Patients and mutation screening

The first patient with the p.Pro409His rare variant of *HNF1A* was diagnosed with diabetes at age 12 years. She was admitted to our hospital because of polydipsia, polyuria, and polyphagia, without weight loss for 6 months. Her BMI was 23.4 kg/m^2, fasting blood glucose was 254 mg/dl (14.1 mmol/l), glycated hemoglobin (HbA1C) was 9.6% (81 mmol/mol), and fasting C-peptide was 3.2 ng/ml (1.0595 nmol/l). The Islet autoantibodies glutamate decarboxylase (GAD) and Islet antigen 2 (IA-2) were negative. The high-sensitivity C-reactive protein (hs-CRP) was 0.73 mg/l. Insulin therapy was initiated before genetic characterization. Her mother had typical T2DM with metabolic syndrome diagnosed in her forties and was successfully treated with metformin to control blood glucose, but she did not carry the mutation. The DNA sample of the father was not available for the genetic analysis.

The second patient harboring the same variant was a 37-year-old woman with a mild phenotype characterized by Impaired Glucose Tolerance detected by large glucose increments in OGTT (fasting plasma glucose 80 mg/dl; 2-h plasma glucose 189 mg/dl) and a history of Gestational Diabetes Mellitus. The patient had a normal weight without signs and symptoms of insulin resistance. In this case, the mutation was inherited from the overweight father who developed a mild fasting age-related hyperglycemia.

The patients were referred for mutational screening of the *HNF1A* gene at the Genetics Unit of *Mater Domini* Hospital in Catanzaro by the Unit of Metabolic Diseases of the same Hospital for a molecular diagnosis of MODY. The genetic test was performed after informed consent in accordance with the Helsinki Declaration and the approval of local Ethics Committee (Regione Calabria-Sezione Area Centro).

All the exons and their flanking sequences of *HNF1A* gene were amplified by polymerase chain reaction (PCR) using primers and experimental conditions described by others [17].

Sequences obtained were compared with the *HNF1A* reference sequence (NM_000545). The analysis of the allele frequency was performed using the ExAc database (http://exac.broadinstitute.org/) [18]. In silico analysis of the pathogenic potential of the uncharacterized mutation was carried out with MirDNMR (https://www.wzgenomics.cn/mirdnmr/) [19].

Site-directed mutagenesis

The construct pcDNA3-mHNF1A encoding the mouse wild-type HNF1-α was used as a template to generate the mHNF1A (P408H) variant using QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The primers used in the reaction were: 5′-tggcctcgctacatggggtcatga-3′ and 5′-tcatgaccccatgtagcgaggcca-3′; mutant bases are underlined. The incorporation of the mutation into the construct was checked by sequencing.

Luciferase assay

HeLa (Human Cervical Carcinoma) and Huh7 (Hepatocellular Carcinoma) cell lines were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, USA) supplemented

with 10% FBS and 1% penicillin/streptomycin (Sigma-Aldrich, Saint Louis, USA). Cells were grown at 37 °C in a 5% CO_2 atmosphere incubator.

Transient transfections were performed with Lipofectamine 3000 kit (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions, on 80% confluent cells plated in 12-well culture dishes, using the mHNF1A wild type or mHNF1A (P408H) variant expression vectors, the pTK-Renilla luciferase vector (Promega, Madison, WI, USA) to control the efficiency of transfection and the following luciferase reporter constructs: pGL3-GLUT2 (that contains base pairs − 1308 to 1346 of the mouse GLUT2 promoter) or β28-LUC (that contains three copies of the β-fibrinogen HNF1-α binding site). The luciferase reporter constructs and the pcDNA3-mHNF1A vector were kindly provided by Dr. Marìa-Angeles Navas (Complutense University of Madrid, Spain). Luciferase activities were measured 24 h after transfection using Dual Luciferase Reporter Assay (Promega, Madison, WI, USA) on a GloMax explorer instrument (Promega, Madison, WI, USA). Values of activity were then calculated as the ratio of Firefly/Renilla readings [20].

Western blot analysis

The protein content of cell extracts was quantified by the Bradford assay (Bio-Rad, Hercules, CA, USA) and 50 µg of each protein extract were subjected to SDS PAGE and Western blot analysis using a standard protocol [21]. Rabbit HNF1α-D7Z2Q (Cell Signaling Technology, Beverly, MA, USA), and α-Tubulin (Santa Cruz Biotechnology, Dallas, TX, USA) primary antibodies, Rabbit IgG HRP-conjugated Antibody (GE Healthcare, Little Chalfont, UK) and ECL kit (GE Healthcare, Little Chalfont, UK) were used to verify the mHNF1A expression and as loading control.

Immunofluorescence assay

3×10^5 HeLa cells were seeded in 6-well dishes and transfected with 2.5 µg of pcDNA3-mHNF1A or pcDNA3-mHNF1A (P408H) constructs u sing Lipofectamine 3000 Kit. After 24 h of transient transfection, the cells were fixed, permeabilized and then incubated with HNF1α-D7Z2Q Rabbit mAb and Alexa Fluor 488 donkey anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR, USA) as described elsewhere [22]. Coverslips were mounted using Mowiol 4-88 Reagent (Sigma-Aldrich, Saint Louis, USA) and images were captured using a Leica DFC 350 FX camera (20 × magnification) and acquired by Leica Application Suite Software (Version 2.8.1).

Statistical analysis

Comparison between the activity of wild-type mHNF1A and P408H mutant form was performed analyzing the fold activity change with respect to empty vector. All data are presented as means ± S.D. of four experimental values. To determine the statistical significance, the Student *t* test was used. Statistical significance was set at $P < 0.05$.

Results

In our genetic unit, we identified by DNA sequencing a rare *HNF1A* variant in two apparently unrelated patients from southern Italy. The *HNF1A* variant detected, c.1226C>A (p.Pro409His), located in the exon 6, was described in a large study on *HNF1A*-MODY patients [23]. This mutation is already annotated in the ExAc database [18] with a frequency of 0.0008% (1/120558), indicating that the variation is not a polymorphism. The resulting P409H amino acid change on the basis of MirDNMR prediction resulted pathogenic for the 14/14 algorithms interrogated, with a threshold value of 9/14 (Table 1).

The P409 residue of human HNF1-α corresponds to that located in the mouse protein at position 408 (Fig. 1a). Thus, the possible pathogenic effect of the mutation was investigated in vitro using a vector expressing the mouse P408H mutant form and comparing its effect with the activity of the wild-type form [24].

The role of P408H mutation was studied measuring the ability of mHNF1A P408H mutant and wild-type form to activate the transcription of two HNF1-α targets: the β28-LUC and GLUT2 promoters in Huh7 and HeLa cells. With both promoters, a significant increase of luciferase activity induced by the transfection with pcDNA3-mHNF1A and

Table 1 Results of in silico analysis of P409H HNF1-α variant obtained with 14 algorithms through MirDNMR software

SIFT	0 (damaging)	Polyphen2_HDV	1.0 (probably damaging)	Polyphen2_HVAR	0.996 (probably damaging)
LRT	0.000 (deleterious)	Mutation Taster	1.000 (disease causing)	Mutation Assessor	2.005 (medium)
FATHMM	− 4.56 (damaging)	Radial SVM	1.106 (damaging)	LR	0.947 (damaging)
VEST3	0.849 (damaging)	CADD	22.7 (damaging)	GERP++	4.81 (conserved)
phyloP100way	7.629 (conserved)	SiPhy_29way	16.956 (Conserved)		

All algorithms predicted a pathogenic effect of this variant

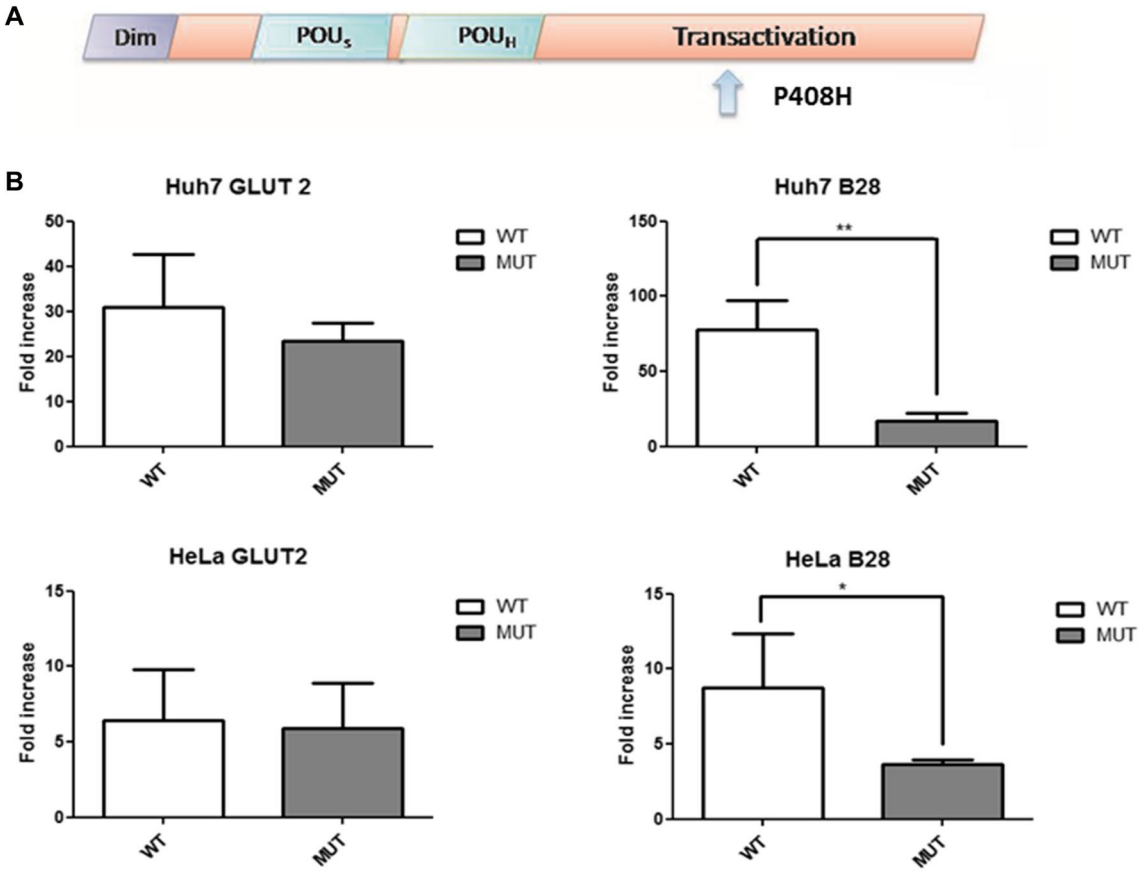

Fig. 1 Transcriptional activity of mHNF1A and P408H mutant. **a** Schematic representation of the functional domains of the HNF1-α transcription factor. The arrow indicates the position of the Pro-408His variant. **b** Transcriptional activity of mHNF1A wild type (WT) and P408H variant (MUT). HeLa and Huh7 cells lines were co-transfected with 200 ng of the reporter constructs pGL3-GLUT2 or pGL3-β28-Luc, 200 ng of pRL-Tk and 50 ng of wild type or mutant pcDNA3 mHNF1A expression vectors. Cells were harvested 24 h after transfection and assayed for luciferase activity (Firefly/Renilla). The fold increase of luciferase activity in cells transfected with wild type or mutant mHNF1A was calculated using the luciferase activity of cells transfected with the same amount of pcDNA3 empty vector as reference value. Student *t* test was used to determine statistical significance (*$P \leq 0.05$, **$P \leq 0.01$)

pcDNA3-mHNF1A (P408H) was observed, although transfection with the mutant form resulted in a significant lower activation of β28-LUC promoter compared to that obtained with the wild-type form (Fig. 1b). No significant difference was detected in the activation of pGL3-GLUT2 promoter by pcDNA3-mHNF1A wild type and mutant form.

Western blotting of protein lysate extracts obtained from HeLa and Huh7 transfected with vectors coding for either the wild type or the mutant form of mHNF1A showed similar levels of expression, suggesting that the mutation had no relevant effects on the stability of the protein (Fig. 2a).

The pathogenicity of some variants of the *HNF1A* gene may depend on the alteration of post-translational mechanisms affecting the proper nuclear localization of the corresponding protein mutants [25, 26]. To assess whether the variant identified could have an effect on the protein ability to move into the nucleus, subcellular localization of the overexpressed wild-type mHNF1A and mutant P408H was analyzed by immunofluorescence in HeLa cells. No difference in cellular localization was detected between mutant and wild-type form, since both proteins localized almost exclusively in the nucleus of HeLa transfected cells (Fig. 2b).

Discussion

Sequence variants of *HNF1A* can be causative of different form of diabetes with a penetrance that varies considerably depending on the mutation and the genetic background [27].

In this study, we report the same rare *HNF1A* variant (p.Pro409His) in two patients with different diabetic clinical conditions. Since this variant has never been functionally analyzed, we decided to perform in vitro functional studies to better define the role of the *HNF1A* mutation in the development of diabetic phenotype.

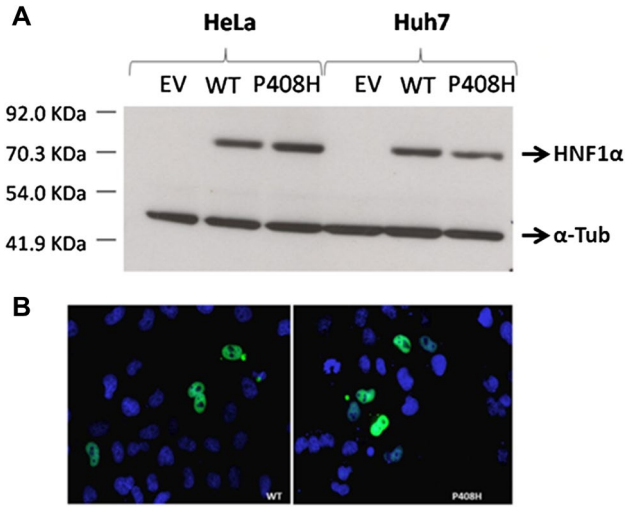

Fig. 2 In vitro characterization studies. **a** mHNF1A protein expression. Western blot of total proteins extracted from HeLa and Huh7 cells transfected with empty vector pcDNA3 (EV), pcDNA3-mHNF1A wild type (WT), and pcDNA3-P408H variant (MUT). α-Tubulin was used as loading control. **b** Localization of wild type and mutant mHNF1A. Monolayers of HeLa cells grown on glass coverslips transfected with expression vectors encoding the mHNF1A wild type and P408H mutant. After 24 h from transfection, cells were fixed and incubated with rabbit anti-HNF1 antibodies. The staining was detected with Alexa Fluor 488 donkey anti-rabbit secondary antibodies (green fluorescence). DAPI staining was performed for nuclear detection (blue fluorescence)

HNF1A MODY-associated variants exert their action by either one of the two different mechanisms, i.e., (a) haploinsufficiency, due to the lack of adequate protein levels resulting from a significant loss of function of the mutated allele and (b) dominant negative effects caused by the interference of the mutant product with the wild-type form leading to the formation of inactive heterodimers [26, 28].

Bioinformatic tools predict the pathogenicity of HNF1-α P409H variant and suggest the necessity of a deeper characterization of the mutation. The level of transactivation reached by the *HNF1A* variants on HNF1-α target genes is the method preferred to test in vitro the pathogenicity of new mutations. Our in vitro results showed that the mouse HNFA1 (P408H), that corresponds to HNF1-α (P409H) human variant, can be considered a variant with a mild effect, since it significantly down-regulated the activity of only one of the two HNF1-α target promoters tested. This is not surprising and could be explained by the interaction of HNF1-α with different co-activators necessary for full transactivation of various target promoters [24, 29]. We present evidence demonstrating that an increased protein degradation or mislocalization of HNF1-α cannot account for the mechanism underlying the downregulation of the reporter construct β28-LUC.

Going back to the interpretation of clinical data, the presence of the *HNF1A* p.Pro409His variant has been found associated with diabetes with very different characteristics, ranging from a mild diabetic phenotype observed in our second patient with gestational diabetes to the more severe diabetic phenotype observed in the younger patient with a typical *HNF1A*-MODY phenotype. The additional maternal predisposition for type 2 diabetes mellitus could have aggravated the genetic susceptibility burden for the diabetic phenotype in the younger patient. Therefore, in this patient, the diabetic phenotype is not exclusively dependent on the p.Pro409His variant. The same variant has also shown a low effect in the father of the second patient, suggesting that it is different from the classical Monogenic Diabetes mutations characterized by dominant disease transmission and deleterious impact.

Based on the combination of clinical and experimental findings, we propose that p.Pro409His is a hypomorphic variant with transactivation activity partially reduced but not absent in vitro that could be responsible for reduced pancreatic beta-cell function and dysglycemia. Since it occurs in complex multifactorial inheritance, the severity of the phenotype can be affected by differences in either the genetic background, environmental factors or both.

It would be interesting to establish the prevalence of *HNF1A* p.Pro409His variant in Calabria region in a sample of diabetic patients and, given the rarity of this variant, to investigate if it came from a common founder.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all patients included in the study.

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