Functional analyses of HNF1A-MODY variants refine the interpretation of identified sequence variants

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Abstract

Context: While rare variants of the hepatocyte nuclear factor-1 alpha (HNF1A) gene can cause maturity-onset diabetes of the young (HNF1A-MODY), other variants can be risk factors for the development of type 2 diabetes. As has been suggested by the American College of Medical Genetics (ACMG) guidelines for variant interpretation, functional studies provide strong evidence to classify a variant as pathogenic.

Objective: We hypothesized that a functional evaluation can improve the interpretation of the HNF1A variants in our Czech MODY Registry.

Design, Settings and Participants: We studied 17 HNF1A variants that were identified in 48 individuals (33 females/15 males) from 20 Czech families with diabetes by bioinformatics in silico tools and functional protein analyses (transactivation, protein expression, DNA binding, and nuclear localization).

Results: Of the 17 variants, 12 variants (p.Lys120Glu, p.Gln130Glu, p.Arg131Pro, p.Leu139Pro, p.Met154Ile, p.Gln170Ter, p.Glu187SerfsTer40, p.Phe215SerfsTer18, p.Gly253Arg, p.Leu383ArgfsTer3, p.Gly437Val, and p.Thr563HisfsTer85) exhibited significantly reduced transcriptional activity or DNA binding (<40%) and were classified as (likely) pathogenic, 2/17 variants were (likely) benign and 3/17 remained of uncertain significance. Functional analyses allowed for the reclassification of 10/17 variants (59%). Diabetes treatment was improved in 20/29 (69%) carriers of (likely) pathogenic HNF1A variants.

Conclusion: Functional evaluation of the HNF1A variants is necessary to better predict the pathogenic effects and to improve the diagnostic interpretation and treatment, particularly in cases where the co-segregation or family history data are not available or where the phenotype is more diverse and overlaps with other types of diabetes.
Précis:

HNF1A gene variants were functionally studied. Variant’s classification was changed in 59% of variants leading to diabetes treatment optimization in 69% carriers of (likely) pathogenic variants.
Introduction

Maturity-onset diabetes of the young (MODY) is a heterogeneous form of monogenic diabetes and is characterized by autosomal dominant inheritance, onset at a young age and pancreatic beta-cell dysfunction; leading subsequently to inadequate insulin secretion (1). Until now, pathogenic variants in at least 13 different genes are known to cause MODY, and each affected gene defines a specific MODY subtype (2). Pathogenic variants of the *HNF1A* gene, which encodes the hepatocyte nuclear factor 1-alpha (HNF-1A) protein, are associated with common MODY subtype (HNF1A-MODY, OMIM #600496) (3-7). HNF1A-MODY is usually characterized by progressive hyperglycaemia that leads to diabetes with a high risk of developing chronic vascular complications (8,9). However, the use of appropriate therapy at early stages of the disorder may decrease the incidence of complications (10). Subjects with HNF1A-MODY are generally sensitive to oral hypoglycemic agents (OHA) – sulfonylurea derivatives, while some may also require insulin treatment as the disease progresses (10,11).

Typically, HNF1A-MODY diagnosis is established based on fulfilment of clinical criteria for MODY followed by identification of pathogenic variant in the *HNF1A* gene. However, not all clinical criteria must be accomplished (12) and not all observed rare variants in the *HNF1A* gene are causal (13) in HNF1A-MODY patients. Distinguishing benign from pathogenic *HNF1A* variants is obviously crucial for a precise diabetes diagnosis, as is the choice of treatment and genetic counselling/follow-up for preclinical carriers. For this, a five-tier score system is commonly used in clinical diagnostics laboratories to evaluate the pathogenicity of sequence variants. In 2015, the American College of Medical Genetics (ACMG) published guidelines for how to interpret the sequence variants (14). Despite these, there is still a discrepancy in variant interpretation and scoring, especially across laboratories (15). We have recently demonstrated that functional investigations are better at predicting the effects of rare
HNF1A variants compared to bioinformatics in silico tools to distinguish variant risk factors for the development of type 2 diabetes (16). With those findings in mind, we hypothesized that a functional evaluation can improve the bioinformatics interpretation of the pathogenicity of 17 HNF1A variants identified in 46 individuals from 20 families in the Czech MODY Registry, of which the majority had not been previously reported.
Materials and methods

Subjects

We studied 17 *HNF1A* variants that were identified in 48 individuals (33 females/15 males) from 20 Czech families. Inclusion criteria for genetic screening of the *HNF1A* gene were diabetes manifestation before 40 years of age, positive or not available family history of diabetes, detectable endogenous insulin and negative autoantibodies (anti-GAD, anti-IA2, and anti-insulin). Body mass index (BMI) was not used as an inclusion criterion. However, when a BMI over 25 kg/m² was reported, other clinical indicators of MODY were considered in order to be included for the screening. Clinical data (including age at the time of diagnosis, BMI, complications, treatment) and biochemical data was obtained from the diabetologists of the probands (see Supplemental Tables 1-5 for more details (17)). Supplemental Figure 1 shows the pedigrees of the families (17). Study was performed in accordance with the Declaration of Helsinki; all study participants (or their guardians) gave written, informed consent, and the study was approved by the Ethics Committee of the University Hospital Motol and the 2nd Faculty of Medicine, Charles University in Prague.

Genetic analyses

The genomic DNA was extracted from the peripheral blood with a standard salting-out procedure or by using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). All exons and parts of the adjacent introns of the *HNF1A*, *GCK*, and *HNF4A* genes were amplified and sequenced by direct Sanger sequencing as reported previously (18) from all probands. The primer sequences and protocols are available upon request. Of the 20 investigated Czech MODY families we identified 17 different *HNF1A* variants. The schematic positions of these variants in the HNF-1A protein sequence is illustrated in Supplemental Figure 2 (17).
ACMG classification

All HNF1A variants were classified using the ACMG guidelines, which places a variant into one of the five following groups: pathogenic (class 5), likely pathogenic (class 4), uncertain significance (class 3), likely benign (class 2) and benign (class 1) (14). The Alamut v.2.6 (Interactive Biosoftware, Rouen, France) integrated software was used to gather information from PubMed, the Human Gene Mutation Database, ClinVar for the subscore PM5/PP5/BP6, and other population frequency databases, such as the genome aggregation database (GnomAD) for the PM2/BS2 subscore. The in silico prediction tools (SIFT, PolyPhen-2 and Align GVGD) were only used for supportive information (Supplemental Table 6 (17)) for the PP3/BP4 subscore, as suggested by the ACMG guidelines. For a variant to obtain a benign score of BS2 we used a cut-off score >5 for individuals listed in the GnomAD. Missense variants in the dimerization and DNA binding domain are predominant, and therefore these two domains are considered as hot-spots domains in the HNF-1A protein (19). Thus, for a variant to obtain a moderate score of PM1 (variants located in a mutational hot-spot and/or critical and well established functional domain), we referred to the NCB1 data for HNF-1A protein (20), to identify variants located in known residues which interact with DNA. For an unbiased assessment, all 17 variants were selected for functional analyses. Based on earlier reports on effect of pathogenic HNF1A-MODY variants (16,21-26), that pathogenic and MODY causal variants impair HNF-1A activity, DNA binding and localization severely (<40% compared to wild type (WT) HNF-1A) while type 2 diabetes risk variants have a more moderate effect on HNF-1A function (40-60% compared to WT), the PS3 score was applied when a variant performed <40% in a minimum of 2 functional assays. A moderate level of the PS3 score was, however, applied, if a variant performed below this cut off in only one of these assays. According to ACMG guidelines, lack of segregation of a variant indicates a strong benign impact (ACMG score: BS4). However, caution must be taken to rule out any possible phenocopies, unaffected family
members with mild symptoms or age-dependent penetrance. Since HNF1A-MODY may manifest at a later age (>35 years), we used this information only as supportive benign evidence in case of unaffected family members (ACMG score: BS4_supporting).

Functional studies

Human HNF1A cDNA (NCBI Entrez Gene BC104910.1) in pcDNA3.1His/C vector was used as a template for constructing individual HNF1A variants using the QuickChange Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, US), and all constructs were verified by Sanger sequencing. Transiently transfected HeLa cells with WT, empty vector (pcDNA3.1), or variant HNF1A cDNA (23) were used in our functional studies, investigating HNF-1A (i) transcriptional activity using a rat albumin promoter-linked luciferase reporter assay system (16); (ii) protein expression in cell lysates from the transactivation assay by immunoblotting (16); (iii) nuclear localization by fractionation analysis and immunoblotting (27); and (iv) DNA binding ability using electrophoretic mobility shift assay kit (EMSA; LI-COR Bioscience, Lincoln, NE, US) and a cyanine 5 labelled oligonucleotide (Sigma Aldrich, St. Luis, MO, US) containing the HNF-1A binding site in the rat albumin promoter (27). Depending on the functional assay, we used previously published HNF-1A variants p.Leu254Gln and p.Arg263Cys as controls for poor DNA binding or transactivation (23,26). While all variants were assessed for transactivation activity, only variants located in the DNA binding domain, or demonstrated significantly reduced transactivation activity, were assessed for DNA binding effect. See Supplemental Methods for more details (17).

Statistical analysis
The results of functional analyses of individual variants are presented as mean (in percentage) ± standard deviation and relative to WT HNF-1A activity (set as 100%), unless otherwise specified. Experiments were carried out on at least 3 independent occasions unless otherwise specified in the figure legends. Statistical differences between individual variants and WT function were analyzed using GraphPad Prism software (version 8.1.1, GraphPad Software, Inc. San Diego, CA) and raw data (i.e. firefly/renilla ratios) and an unpaired 2-tailed t-test based on n=3. A p-value < 0.05 was considered statistically significant.
Results

**HNF1A gene variants and their ACMG classification prior to functional investigations**

Among the 17 identified variants, 12 variants have not been previously reported in the literature, 3 variants (p.Trp113Leu, p.Lys120Glu, p.Arg131Pro) have been previously reported by us (12,28), and 2 variants (p.Phe177Ser and p.Gly288Trp) have been reported by others (29-31). Only p.Phe177Ser has been functionally investigated before and was found to not affect HNF-1A transcriptional activity (29). The variant p.Gly288Trp was accompanied by the GCK variant p.Glu256Lys in the family CZ743. No additional variants in the GCK nor HNF4A genes were identified in the families tested.

All 17 variants were classified according to ACMG guidelines (Table 1) (14). Most missense variants fell into the group of variants with uncertain significance (class 3), except for p.Gly288Trp, which was classified as benign (class 1) due to its i) presence in 18 individuals in the GnomAD database and ii) lack of segregation in the families that carry this variant. The missense variant p.Arg131Pro was classified as likely pathogenic (class 4) since it was identified de novo. Nonsense and frameshift variants were classified as either likely pathogenic (class 4) or pathogenic (class 5). Due to lack of DNA, co-segregation with diabetes was undetermined for 4 families carrying variants p.Gln130Glu, p.Phe215SerfsTer18, p.Leu383ArgfsTer3 and p.His483Arg (CZ1025).

**Functional studies**

*Altered transcriptional activity and the protein levels of HNF-1A variants*

Compared to the WT HNF-1A activity (set as 100%), the measured levels of transcriptional activity for 11 of the 17 variants were significantly lower (<35%) (Figure 1A). In more detail, all frameshift variants that were investigated as well as the nonsense variant p.Gln170Ter were close to inactive (<5%). The p.Lys120Glu, p.Gln130Glu, p.Arg131Pro, p.Leu139Pro, p.Gln130Glu, p.Arg131Pro, p.Leu139Pro,
p.Met154Ile and p.Gly437Val variants demonstrated <40% activity, and equal to or lower than our MODY control variant p.Leu254Gln, while the p.Gly253Arg, p.Gly288Trp and p.His483Arg variants showed a nonsignificant, mildly reduced transcriptional activity (~43-65%) above the set threshold for pathogenicity (<40%). Finally, p.Trp113Leu, p.Phe177Ser and p.Thr384Lys demonstrated transcriptional activity levels comparable to WT HNF-1A levels.

Next, we evaluated the effect of the individual 17 HNF1A variants on HNF-1A protein expression levels, investigating the cell lysates obtained for the transactivation assay by SDS-PAGE and immunoblotting. Quantification of the HNF-1A-specific bands showed that most of the HNF-1A variants retained relatively normal protein expression levels compared to the levels of WT HNF-1A (Figure 1B). However, reduced protein levels were observed for the p.Gln170Ter, p.Gly253Arg, and p.His483Arg variants (<50% of WT).

Effect of variants on the nuclear localization of HNF-1A

Subsequently, we investigated whether the 17 individual variants could disturb the normal nuclear translocation of HNF-1A (Figure 1C), as compared to WT. The relative nuclear level of the variants, as assessed by cell fractionation and the quantification of HNF-1A-specific bands in the nuclear fractions, was reduced (~45-65%) for the following HNF-1A protein variants: p.Lys120Glu, p.Gln130Glu, p.Arg131Pro, and p.Leu139Pro, while level was strongly reduced for p.Gln170Ter (18%). The remaining variants demonstrated nuclear levels similar to WT (~70-100%).

Variants in the DNA-binding domain severely affect the binding of HNF-1A to a target DNA sequence

Variants located in the DNA-binding domain, or variants demonstrating reduced transcriptional activity <40% (Figure 1A), as our MODY control variant p.Arg263Cys, were selected and
further investigated to assess whether they could impair DNA-binding to an HNF-1A site-containing oligonucleotide (Figure 1D). Variants localized in the DNA-binding domain of HNF-1A (p.Lys120Glu, p.Gln130Glu, p.Arg131Pro, p.Leu139Pro, p.Met154Ile, p.Glu187SerfsTer40, p.Phe215SerfsTer18 and p.Gly253Arg) demonstrated severely reduced DNA-binding ability (<35%). Moreover, compared to WT (set to 100%), the variants p.Gly437Val and p.Thr563HisfsTer85 showed a moderate reduction in DNA-binding ability, estimated to 69% and 76%, respectively. In contrast, the frameshift variant p.Leu383ArgfsTer3 did not reduce the DNA-binding ability of HNF-1A, indicating that impaired DNA-binding is not the loss-of-function mechanism of this variant. The variant p. Gln170Ter was not investigated for DNA-binding because it lacks most of the DNA-binding domain and also showed no activity (1%) on the same rat albumin promoter (transactivation assay). Of the other two variants located in the DNA binding domain (p.Trp113Leu and p.Phe177Ser) the DNA-binding assay was not performed since the transactivation activity, protein expression level and nuclear localization were comparable to WT.

Reclassification of HNF1A variants after functional analyses

Earlier reports (16,21-26) indicate that pathogenic HNF1A variants associated with HNF1A-MODY are usually characterized by severely reduced transcriptional activity, impaired DNA binding, impaired nuclear targeting and/or reduced protein expression levels amounting to ~20-35% compared to WT (100%). Based on this, we used an additive functional score to variants performing <40% in minimum 2 functional assays, in order to gain a PS3 score as described in the ACMG guidelines (Table 1). Applying our functional data to the HNF1A variants allowed for a reclassification of the pathogenicity classes for 10 of the 17 variants (59%). Their new classes of pathogenicity are presented under the column ‘New ACMG classification’ in Table
1. Of those reclassified, the variant p.His483Arg changed from uncertain significance (class 3) to likely benign (class 2). The remaining variants p.Lys120Glu, p.Gln130Glu, p.Leu139Pro, p.Met154Ile, p.Gly253Arg and p.Gly437Val were reclassified from uncertain significance (class 3) to likely pathogenic (class 4), and the variants p.Arg131Pro, p.Phe215SerfsTer18, p.Leu383ArgfsTer3 were reclassified from likely pathogenic (class 4) to pathogenic (class 5).

**Clinical phenotypes in the reclassified HNF1A and non-reclassified variant carriers**

Revisiting the clinical phenotypes of the patients carrying these reclassified variants, we found a good correlation between the phenotypes and the pathogenicity score of the variants. All clinical data are presented in Supplemental Tables 1-5 (17). Only 3 variants remained as variants of uncertain significance (class 3) (p.Trp113Leu, p.Phe177Ser, and p.Thr384Lys) after our functional evaluation. Frequencies of most of these variants were not reported in the population databases and the results of *in silico* bioinformatics prediction programs were inconsistent (Supplemental Table 6 (17)). Segregation of variants p.Phe177Ser and p.Thr384Lys with diabetes in respective families was not sufficiently informative. The carriers of the p.Thr384Lys variant developed diabetes later in life (>35 years) and their BMIs were in the higher range (26-30 kg/m$^2$) at the time of the diabetes diagnosis. In contrast, p.Trp113Leu segregated with diabetes in the family and the lean mother (BMI 21 kg/m$^2$) of the proband treated with insulin prior the genetic testing is now treated with gliclazide with good clinical response.

Functional studies confirmed the classification of the variant p.Gly288Trp as benign. In the CZ904 family, the twins carrying the p.Gly288Trp variant manifested with mild diabetes at 4 years of age and had negative frequently used autoantibodies at diagnosis. Three years later, their diabetes progressed to the need of insulin treatment with C-peptide negativity. A new
autoantibody screening resulted in positive ZnT8 antibodies and their diabetes status was reclassified as type 1. In the family CZ743, the HNF1A gene was investigated first based on our former testing strategy. As the variant p.Gly288Trp did not segregate with diabetes, we continued with genetic testing and we found the p.Glu265Lys variant in the GCK gene. Segregation between this GCK variant and diabetes in this family was evident. In addition, the GCK p.Glu265Lys variant is not present in the GnomAD database, is classified as pathogenic in ClinVar and has been shown to affect protein stability by functional studies (32).

**Treatment implication after reclassification of HNF1A variants**

Diabetes was treated in 25 out of 29 carriers of variants reclassified as likely pathogenic (class 4) and pathogenic (class 5) at the time of genetic testing (Supplemental Table 4 and 5 (17)). In total, our functional assessment and reclassification of HNF1A variants impacted treatment for 20/29 carriers. In more detail, the treatment regime has been changed in 10/14 carriers of likely pathogenic (class 4) variants (Supplemental Table 4 (17)). Of these 10 carriers whose therapy was changed, 7 subjects carrying p.Lys120Glu, p.Leu139Pro, p.Met154Ile or p.Gly437Val were switched from insulin to OHA or combination of insulin and OHA, and 2 subjects (carrying p.Leu139Pro or p.Gly437Val) were switched from diet to OHA. A diet regime was initiated in one subject carrying p.Gly253Arg variant. The carrier of p.Gln130Glu, as well as one of the p.Met154Ile carriers, initially treated with insulin, remained with the same treatment upon their request and consultation with their diabetologists. Moreover, 10/15 carriers of pathogenic (class 5) variants, were also changed treatment strategy. Briefly, 2 carriers of p.Gln170Ter and 2 carriers of p.Leu383ArgfsTer3 and p.Thr563HisfsTer85, respectively, were switched from insulin to OHA. The treatment was optimized in 3/5 subjects carrying p.Glu187SerfsTer40 variant: in 2 subjects from family CZ780 the treatment with OHA metformin has been successfully changed to sulphonylurea derivates (Supplemental Table 5).
The proband from CZ1231 was switched from diet to OHA administration. Three subjects carrying p.Arg131Pro, p.Gln170Ter, or p.Phe215SerfsTer18, respectively, had not adequate reaction to OHA, and remained thus on insulin treatment. A diet regime was initiated in 3 subjects carrying the pathogenic (class 5) variant, p.Gln170Ter, and have not manifested with diabetes yet.
Discussion

We set out to test whether a functional investigation could improve the interpretation of the \textit{HNF1A} variants that were identified in families in the Czech MODY Registry. Normal HNF-1A transcription factor function is dependent on normal transactivation activity, which is dependent on the ability to bind target promoters (DNA) and on sufficient cellular (nuclear) protein level. Of the 17 variants investigated, missense variants located in the DNA binding domain impacted more measurable of normal HNF-1A function (transactivation/DNA binding/nuclear localization) than the missense or nonsense/frameshift variants located in the transactivation domain, as reported by others before (33,34).

Based on our findings, 10 of the 17 variants tested could be reclassified; 6 out of 9 variants initially classified as uncertain significance (class 3) were reclassified as likely pathogenic (class 4), 3 variants were reclassified from likely pathogenic (class 4) to pathogenic (class 5), and 1 variant was reclassified from uncertain significance (class 3) to likely benign (class 2). In most cases, the clinical characteristics of the variant carriers also supported this reclassification. Our functional investigations predominantly helped in cases where segregation data with diabetes was not available (in families carrying p.Gln130Glu, p.Phe215SerfsTer18, p.His483Arg (CZ1025), p.Leu383ArgTer3), in probands carrying p.Thr563HisfsTer85 and p.Arg131Pro variants that originated \textit{de novo} (parents were non-carriers and healthy, paternity has been proven), and when the phenotype alone could not clearly distinguish between MODY and type 2 diabetes.

\textit{Variants with likely pathogenic or pathogenic effects}

The \textit{de novo} variant p.Arg131Pro was identified in a young man who was genetically tested for MODY after incidentally being diagnosed with hyperglycemia. As proof of the pathogenic effect, the p.Arg131Pro variant revealed severely reduced transcriptional activity and DNA
binding (both 10% compared to WT), and showed impaired nuclear localization. Arg131 is highly conserved among vertebrates and its importance has previously been highlighted within the protein sequence for normal HNF-1A function (35). The other missense variants affecting Arg131 (by p.Arg131Gln and p.Arg131Trp) have also previously been associated with HNF1A-MODY and reported to cause reduced transcriptional activity/DNA binding ability (23,36). Interestingly, the clinical presentation of the p.Arg131Pro variant also correlates with a severe diabetes phenotype. The proband carrying p.Arg131Pro manifested with rapidly progressed hyperglycemia. After the genetic diagnosis, the treatment with glibenclamide (OHA) was initiated but the subject did not maintain good glycemic control. Currently, a combination of insulin and glibenclamide with a dose of insulin 1 IU/kg/day (HbA1c 53 mmol/mol (7%)) is administrated instead.

Among the reclassified missense variants in our study, the variants p.Lys120Glu, p.Gln130Glu, p.Leu139Pro and p.Met154Ile showed severely reduced protein function in minimum 2 of the investigated functional assays, (below the applied cut-off values of <40%) (Table 1). Variant p.Gly253Arg showed low transactivation activity with an accompanied low protein expression level. The high standard deviation between replicates in the transactivation assay may possibly be due to technical problems of transfecting or expressing this variant. Other types of experiments showed the comparable nuclear localization of p.Gly253Arg to WT and decreased DNA binding. These variants affect highly conserved amino acids among vertebrate species (Supplemental Figure 6 (17)). All of these variants (except for p.Gln130Glu, where family genotypes were not available) segregated with diabetes in the respective families. As our functional data support a damaging effect of these five individual variants, they were reclassified from uncertain significance (class 3) to likely pathogenic (class 4), implicating them as cause of HNF1A-MODY in carriers.
The missense variant p.Gly437Val displayed low levels of transcriptional activity (~10% of WT HNF-1A activity), while only mildly reduced DNA binding (~70%) and protein levels (~60%). It is unclear how this affected residue (Gly437), which is not highly conserved, can result in such a dramatic loss of transcriptional activity when changed to Val437. Previous detailed analysis of HNF-1A transactivation have indicated the requirement of loss of larger regions of transactivation domain to mediate a substantial loss in activity (22). However, based on our functional findings, cosegregation with diabetes, and the good response to sulfonylurea derivates in the affected family, the variant is hence reinterpreted as likely pathogenic (class 4).

One nonsense and 2 frameshift variants (p.Gln170Ter, p.Glu187SerfsTer40, p.Phe215SerfsTer18) that cause the premature termination of the HNF-1A protein all lead to a complete loss-of-function of HNF-1A in terms of transcriptional activity (lacking parts of the DNA-binding domain and completely lacking the transactivation domain). The p.Gln170Ter nonsense variant also lacks the HNF-1A nuclear localization signals B (amino acid 197-205) and C (amino acid 271-282) (22) and was functionally shown with low nuclear levels (18%) by our assay. This variant also demonstrated reduce cellular protein level (45%). Lastly, the frameshift variants p.Leu383ArgfsTer3 and p.Thr563HisfsTer85 demonstrated severely reduced transcriptional activity, most likely due to their late localization in the protein sequence and affecting the transactivation domain alone. Although segregation data were not available for the variants p.Phe215SerfsTer18 and p.Leu383ArgfsTer3, combining our functional findings with the ACMG criteria was sufficient for the reclassification of these two variants. Therefore, all frameshifts and nonsense variants were classified as pathogenic (class 5).

Variants with uncertain significance
Of the 17 variants, 3 variants (p.Phe177Ser, p.Trp113Leu and p.Thr384Lys) remained classified as uncertain significance (class 3) after functional evaluation. In our hands, the p.Phe177Ser variant did not affect normal HNF-1A function, as was reported by Chi et al. (29), and neither did the p.Trp113Leu variant. Clinical information on carriers of the p.Trp113Leu, p.Phe117Ser and p.Thr384Lys variants included a higher BMI in the range of 28-30 kg/m², which is not a typical characteristic of an HNF1A-MODY phenotype. The p.Thr384Lys variant was found in a family in which 2 of the 3 affected family members have late-onset diabetes (37 and 45 years of age). In addition, one family member had diabetes despite not carrying the p.Thr384Lys variant making the cosegregation analysis inconclusive. Thus, the available clinical data support our functional findings that these 3 variants, although affecting highly conserved HNF-1A residues, might be associated with a mild type 2 diabetes phenotype rather than being the cause of HNF1A-MODY.

Likely benign and benign variants

Our functional analyses supported the ACMG classification of the variant p.Gly288Trp as benign and non-causative for HNF1A-MODY (Table 1). The variant was identified in two unrelated families and did not segregate with diabetes in these families. Moreover, in one of these families (CZ743), we also found the pathogenic GCK variant (p.Glu256Lys) in the proband and affected family members, showing a clear co-segregation with diabetes. A previous report on a Croatian family carrying this variant also reported non-segregation with diabetes (31). Thus, carrying the benign HNF1A variant p.Gly288Trp has no clinical significance, which is important knowledge for precise genetic counseling and treatment decisions. The p.His483Arg variant was reclassified from uncertain significance (class 3) to likely benign (class 2) based on our functional analyses and family history. This variant, which was identified in 2 unrelated Czech families, only moderately reduced the HNF-1A
transcriptional activity (~50%), which was most likely correlated with reduced protein level (~40%). Large variance of the replicates in the transactivation assay may be due to technical issues, as for the p.Gly253Arg variant. In addition, a 2-year follow-up of the proband carrying the p.His483Arg variant showed repeatedly normal HbA1C and normoglycemia on diet, and his father (p.His483Arg carrier) did not have diabetes.

Study limitations

Our study has some limitations. The potential presence of causal variants in other MODY-associated genes, apart from HNF1A, GCK and HNF4A, was not screened for. Furthermore, in some families, relatives were not available for genetic testing.

In summary, this is the first large and detailed genetic and functional investigation of HNF1A variants in the Czech MODY population. Our data are also the first to support the necessity of using additive scores during functional interpretation, for correct HNF1A variant pathogenicity evaluation for precision medicine. A functional evaluation should henceforth be included in the diagnostic work-up of any HNF1A variant in medical genetic departments, and particularly for variants where the family history or clinical data are not available or inconclusive. This would aid clinicians in determining the correct treatment for patients, since patients carrying pathogenic HNF1A variants may benefit from treatment with OHA.
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Disclosure summary: The authors have nothing to disclose.

Data availability

All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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Figure 1: Summary of the data obtained from functional studies.

A: Transcriptional activity of the HNF-1A protein variants in HeLa cells. HeLa cells were transiently transfected with both the wild-type (WT) or mutant HNF1A variants and the firefly (pGL3-RA) and renilla (pRL-SV40) reporter plasmids. The firefly luciferase activity was measured in the cell lysates and the activity was normalized to the renilla luciferase activity. The activity levels are presented relative to the activity levels of WT HNF-1A (100%). Each bar represents the mean of 3 independent experiments (n=3) ± SD. P-values were obtained by unpaired student t-test on firefly/renilla ratios for individual variants compared to the WT ratios. *p<0.05; **p<0.01; ***p<0.001. B: Protein expression levels of the HNF-1A protein variants in HeLa cells. The cell lysates of the transiently transfected HeLa cells collected during the transactivation assays were analyzed by SDS-PAGE and immunoblotting using HNF-1A specific antibodies. The protein levels of HNF-1A were normalized to those of the loading control (actin). The levels are presented as the percentage of the HNF-1A variant protein level relative to that of the WT (100%). Each bar represents the mean of 2 independent experiments (n=2). Representative western blots are shown in Supplemental Figure 3 (17). C: Nuclear localization of the HNF-1A protein variants in HeLa cells. The WT or the HNF1A variants were transiently transfected into HeLa cells followed by cellular fractionation. The nuclear and cytosolic fractions were analyzed by SDS-PAGE and immunoblotting using an HNF-1A-specific antibody. Two housekeeping proteins, topoisomerase IIα and α-tubulin, were used as nuclear and cytosolic protein markers, respectively. The HNF-1A/topoisomerase and HNF-1A/α-tubulin ratios were used to calculate the relative subcellular localization of HNF-1A in nuclear and cytosolic compartments, respectively. The bar of wild type nuclear HNF-1A level shows mean percentage with variability (SDV) based on all individual western blots runs.
during the study. Other bar represents the mean percentage (%) of quantified nuclear HNF-1A protein level, based on 2 independent cellular fractionation experiments (n=2). Representative western blots are shown in Supplemental Figure 4 (17). D: Assessment of the DNA binding ability of the HNF-1A protein variants using electrophoretic mobility shift assay (EMSA). The DNA binding abilities of the HNF-1A protein variants with a Cy5-labeled DNA oligo that corresponded to the HNF-1A binding site in rat albumin was assessed by EMSA. The HNF-1A protein included was from nuclear fractions isolated from transiently transfected HeLa cells. The bound complexes were analyzed on a 6% DNA native retardation gel. The quantification of the intensity of the bound complexes was performed by densiometric analysis using the Image Lab software. Each bar represents the mean percentage of bound complexes (WT set to 100%) of 2 independent EMSA experiments (n=2). Representative gels are shown in Supplemental Figure 5(17). MODY control variants included in individual assays are shown in black-lined pattern.

Table 1: Summary of the ACMG classification and functional studies of the HNF1A variants.

HNF1A variants with description of changes at the DNA and protein levels were classified according to the ACMG guidelines and were thereafter functionally characterized. Applying the functional data to the HNF1A variants caused a reclassification of the pathogenicity classes, which are presented under the column ‘New ACMG classification’. ACMG 2015 classification: class 1 - benign; class 2 - likely benign; class 3 - uncertain significance; class 4 - likely pathogenic; class 5 – pathogenic.

ACMG evidence: PVS1-null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multiexon deletion) in a gene where loss of function is a known
mechanism of disease; PS2 De novo (both maternity and paternity confirmed) in a patient with
the disease and no family history; PS3, well-established in vitro or in vivo functional studies
supportive of a damaging effect on the gene or gene product (a variant performed <40% in a
minimum of two functional assays in our study; moderate PS3 well-established in vitro or in
vivo functional studies supportive of a damaging effect on the gene or gene product (a variant
performed <40% in only one of our assays); PM1, variants located in a mutational hot-spot
and/or critical and well established functional domain; PM2, absent from controls (or at an
extremely low frequency if recessive) as shown in the Exome Sequencing Project; PM5, a novel
missense change at an amino acid residue where a different missense change was determined
to be pathogenic and has been seen before; PP1, cosegregation with disease in multiple affected
family members in a gene definitively known to cause the disease; PP3, multiple lines of
computational evidence that support a deleterious effect on the gene or gene product
(evolutionary impact, splicing, etc.); BS2, observed in a healthy, adult individual for a recessive
(homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full
penetrance expected at an early age; BS3, well-established in vitro or in vivo functional studies
show no damaging effect on protein function or splicing; BS4, the lack of segregation in
affected family members. We collected information related to the variant segregation (SEG) in
families (NA, not available; DN, de novo, * partial segregation, see Supplemental Figure 1(17))
and the occurrence in databases, such as GnomAD = Genome aggregation database; HGMD =
human gene mutation database and ClinVar. Functional studies include the analysis of HNF-
1A transactivation, protein expression, DNA binding and nuclear localization. The results are
presented as a percentage relative to that of WT HNF-1A (set as 100%) from 2 or 3 independent
experiments.
Figure 1: Summary of the data obtained from functional studies.
Table 1: Summary of the ACMG classification and functional studies of the HNF1A variants.

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