

Insights Into the Pathogenicity of Rare Missense GCK Variants From the Identification and Functional Characterization of Compound Heterozygous and Double Mutations Inherited in *Cis*

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OBJECTIVE—To demonstrate the importance of using a combined genetic and functional approach to correctly interpret a genetic test for monogenic diabetes.

RESEARCH DESIGN AND METHODS—We identified three probands with a phenotype consistent with maturity-onset diabetes of the young (MODY) subtype GCK-MODY, in whom two potential pathogenic mutations were identified: [R43H/G68D], [E248 K/I225M], or [G261R/D217N]. Allele-specific PCR and cosegregation were used to determine phase. Single and double mutations were kinetically characterized.

RESULTS—The mutations occurred in *cis* (double mutants) in two probands and in *trans* in one proband. Functional studies of all double mutants revealed inactivating kinetics. The previously reported GCK-MODY mutations R43H and G68D were inherited from an affected father and unaffected mother, respectively. Both our functional and genetic studies support R43H as the cause of GCK-MODY and G68D as a neutral rare variant.

CONCLUSIONS—These data highlight the need for family/functional studies, even for previously reported pathogenic mutations.

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A molecular diagnosis of monogenic diabetes is clinically important because there are implications for prognosis, treatment, and family members.

Consequently, the correct interpretation of a molecular genetic test is paramount, but novel variants continue to be poorly characterized (1–3). As the cost of DNA

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sequencing falls, one of the major barriers to genetic testing will be removed and the correct assignment of pathogenicity to novel variants will become more challenging.

Heterozygous inactivating mutations in glucokinase (GCK) cause a subtype of maturity-onset diabetes of the young (GCK-MODY) (4), which is characterized by stable, mild fasting hyperglycemia, whereas homozygous or compound heterozygous inactivating mutations result in the more severe clinical phenotype of permanent neonatal diabetes mellitus (PNDM) (4). The opposite clinical phenotype of hyperinsulinemic hypoglycemia (GCK-HH) ensues from heterozygous activating mutations (4). In the current study, we explored the pathogenicity of six missense GCK mutations that were identified in combination in three probands referred for GCK-MODY molecular genetic testing. Our aim was to use both molecular genetics and functional studies to ensure correct interpretation of the genetic tests.

RESEARCH DESIGN AND METHODS

Subjects studied

All probands were referred to the U.K. diagnostic screening center for GCK-MODY testing, with corresponding clinical details given in Fig. 1. All subjects gave informed consent.

GCK screening and mutation identification

The pancreatic β -cell isoform of GCK (accession number NM_000162.3) was sequenced using standard protocols. Mutation testing was undertaken in family members to establish cosegregation and phase. Where parental DNA was unavailable, molecular haplotyping using allele-specific PCR was used to determine phase (primer sequences available upon request).

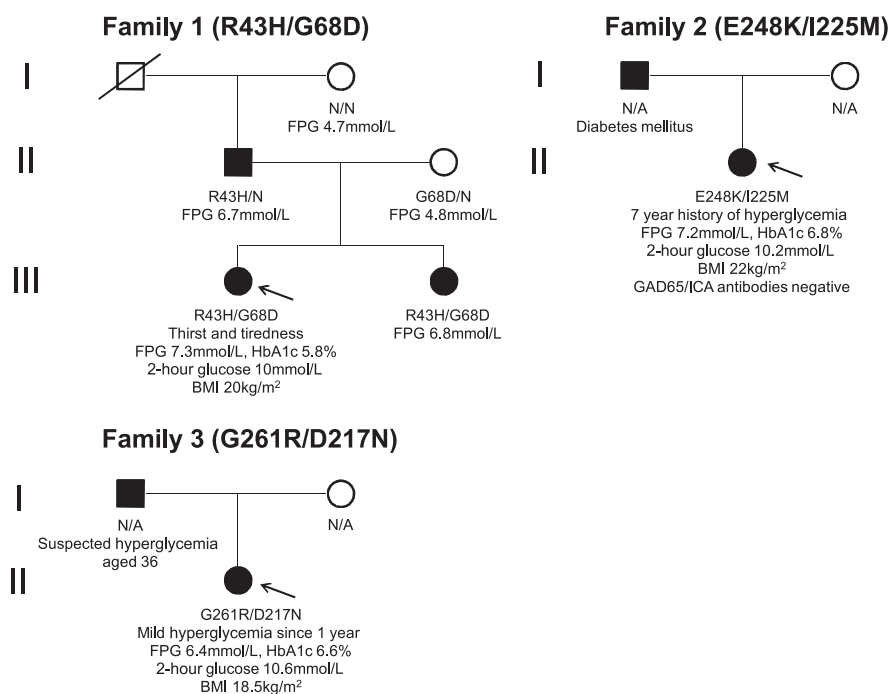


Figure 1—Pedigrees for the three probands. Squares and circles denote males and females, respectively. Filled symbols represent hyperglycemic individuals, and open symbols correspond to those with normoglycemia. Fasting plasma glucose values and mutation status for each family member are given underneath the appropriate symbol. An arrow indicates the proband in each family. N, no mutation; N/A, data not available.

Functional characterization of mutant proteins

Glutathione S-transferase–tagged human wild-type and mutant GCK were generated as described previously (5), as was FLAG-tagged human GCK regulatory protein (6). GCK activity was measured spectrophotometrically using glucose 6-phosphate dehydrogenase–coupled assays (5,7). Pharmacological GCK activator (RO0281675) stimulation, GCK regulatory protein–mediated inhibition (6,8), and thermolability assays (5,9) were also conducted to further probe mutational mechanisms. All statistical analyses were conducted using paired Student *t* tests.

RESULTS—GCK sequencing identified two nonsynonymous mutations in each of the three probands: R43H and G68D (c.128G>A, p.Arg43His and c.203G>A, p.Gly68Asp), E248K and I225M (c.742G>A, p.Glu248Lys and c.675C>G, p.Ile225Met), and G261R and D217N (c.781G>A, p.Gly261Arg and c.649G>A, p.Asp217Asn). Of these six mutations, five had been previously reported in GCK-MODY subjects (R43H, G68D, E248K, I225M, and G261R) but only the latter functionally characterized (4,10). All six

mutations were absent from 400 normal chromosomes and the October 2011 consensus calls of the 1000 Genomes Project (11). Bioinformatic (SIFT and PolyPhen) analysis only provided clear support for pathogenicity for G68D, I225M, and G261R.

Molecular haplotyping demonstrated that for two probands, the mutations were in *cis* ([E248K;I225M] and [G261R;D217N]). In contrast, family studies showed that the R43H and G68D mutations were on separate GCK alleles (Fig. 1). Since both mutations had previously been reported as causal for MODY, the proband would be expected to present with the more severe phenotype of PNDM. However, both the proband and her affected sister (who also inherited both mutations) only exhibited mild fasting hyperglycemia. Moreover, R43H was inherited from the affected father, whereas G68D came from the unaffected mother, thus suggesting that R43H was responsible for the GCK-MODY phenotype. In silico predictions (SIFT and PolyPhen) disagreed with these clinical observations and suggested that G68D was clearly pathogenic, while providing inconclusive results for R43H.

All six mutations were kinetically analyzed as single mutant proteins (Supplementary Table 1): three were inactivating, one (R43H-GCK) exhibited near normal kinetics, and two (G68D-GCK and D217N-GCK) were paradoxically mildly activating.

As basic kinetic analysis of R43H-GCK and G68D-GCK did not demonstrate a molecular mechanism consistent with the clinical data, we explored additional previously established modes of GCK inactivation (9,12). These studies provided evidence that R43H-GCK is unstable at the protein level versus wild-type GCK because R43H-GCK showed significantly lower levels of activity compared with wild type over a range of temperatures in a thermolability assay ($P < 0.05$) (Supplementary Fig. 1), providing a clear mechanism of enzyme inactivation. In contrast, our studies of G68D-GCK showed normal protein stability (Supplementary Fig. 1).

Both double mutants ([E248K;I225M] and [D217N;G261R]) displayed dramatic inactivation and were effectively nonfunctional with drastically reduced turnover numbers and affinity for glucose (Supplementary Table 1).

CONCLUSIONS—Despite heterozygous inactivating GCK mutations being a well-established cause of MODY, the three probands in this study were unusual because mutational screening revealed that they each carried two mutations within this gene, thus raising questions about the pathogenicity of these variants and the correct interpretation of the molecular genetic tests. For two of the three probands, both mutations were present in *cis*. We demonstrated that three (E248K, I225M, and G261R) of these four variants were kinetically inactivating and individually sufficient to cause GCK-MODY, whereas the novel D217N variant showed no evidence of inactivation but instead was paradoxically activating due to increased glucose affinity. However, when studied in combination with G261R, the joint effect of both mutations resulted in a severe impact on enzyme function. Despite the deleterious effect on GCK activity caused by carrying these mutations in *cis*, the mild clinical phenotype observed can be explained by compensation from GCK transcribed from the unaffected allele (13).

In contrast, the two previously reported GCK-MODY mutations R43H and G68D were present in *trans*, meaning the mild hyperglycemia observed could not easily

be explained because two inactivating mutations in *trans* should cause PNDM (4). Our family studies showed that R43H was inherited from the affected father and G68D from the unaffected mother, thus suggesting a pathogenic mutation and rare neutral variant, respectively. Consistent with these observations, our functional studies demonstrated that although R43H-GCK appeared to have similar basic kinetic characteristics to WT-GCK, there was *in vitro* evidence of protein instability, indicating that this variant is pathogenic. For G68D-GCK, there was no evidence of protein instability, and the very mild degree of kinetic activation was not predicted to cause hypoglycemia (Supplementary Table 1), supporting this as a rare neutral variant.

In conclusion, our study indicates that *in silico* evaluation is insufficient when assigning pathogenicity to rare nonsynonymous variants and demonstrates the need for both family and multi-tiered functional studies, even when dealing with previously reported “pathogenic” mutations.

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N.L.B. and K.K.O. collected, analyzed, and interpreted data and wrote the manuscript.

M.v.d.B. collected and analyzed data and critically reviewed the manuscript. N.D.T., E.L.E., K.C., A.B., L.V., J.K.R., and A.R. collected and analyzed data. A.M.S. and K.J.W. collected data. J.G. designed the study and critically reviewed the manuscript. S.E. conceived and designed the study and critically reviewed the manuscript. A.L.G. conceived and designed the study and wrote the manuscript. All authors approved the final version of the manuscript. A.L.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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