Opposite clinical phenotypes of "glucokinase disease": description of a novel activating mutation and contiguous inactivating mutations in human glucokinase (*GCK*) gene.

Abbreviated title: Biostructural analysis of GCK disease

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Abbreviations: *GCK*, glucokinase gene; GK, glucokinase protein; GK-WT, glucokinase wild-type; GSIR, glucose stimulated insulin release; GSIR-T, threshold for glucose stimulated insulin release; FPG, fasting plasma glucose; IFG, impaired fasting glucose; OGTT, oral glucose tolerance test; IVGTT, intravenous glucose tolerance test; FPIR, first phase insulin response.

Disclosure Statement

The authors have nothing to disclose.

Abstract

Glucokinase (GK) is essential for glucose-stimulated insulin release from pancreatic β-cell, serving as glucose sensor in humans. Inactivating or activating mutations of glucokinase lead to different forms of "Glucokinase Disease", i.e. Monogenic Diabetes of Youth (GCK-MDY), Permanent Neonatal Diabetes (inactivating mutations) and Congenital Hyperinsulinism, respectively. Here we present a novel *GCK* activating mutation (p.E442K) found in an infant with neonatal hypoglycaemia (1.5 mmol/l) and in two other family members suffering from recurrent hypoglycemic episodes in their childhood or adult life. In contrast to the severe clinical presentation in the index case, functional studies showed only a slight activation of the protein (relative activity index of 3.3). We also report on functional studies of two inactivating mutations of the *GCK* (p.E440G and p.S441W), contiguous to the activating one, that lead to GCK-MDY. Interestingly, adult family members carrying the GK pE440G mutation show a unusually "progressive" diabetic phenotype.

Word count: 186??

INTRODUCTION

Glucokinase enzyme (GK) has unique functional and structural properties for acting as the pancreatic β-cell "glucose sensor". GK plays thus a role in glucose-stimulated insulin release (GSIR) (1), and regulates the threshold for GSIR (GSIR-T) (1). The high control of GK on β-cells function is best illustrated by the profound impact on GSIR-T of mutations of glucokinase gene (*GCK*). Indeed, GSIR-T in carriers of inactivating *GCK* mutations increases, leading to the mild fasting hyperglycemia in subjects with heterozygous mutations or to severe diabetes like in the case of individuals with homozygous or compound heterozygous mutations. The resulting clinical phenotype of partial glucokinase deficiency is GCK-Monogenic Diabetes of Youth (GCK-MDY), also known as Maturity Onset Diabetes of the Young 2 (MODY2) (2), while complete glucokinase deficiency leads to permanent, neonatal diabetes mellitus (GCK-PNDM) (3,4). On the contrary, the GSIR-T in carriers of activating mutations of *GCK* decreases, causing hypoglycemia (GCK-HI) due to inappropriate insulin secretion when plasma glucose is below the normal GSIR-T, featuring mild or severe forms of the disease (5-8). These different GK linked disorders of glucose metabolism (GCK-MDY, GCK-PNDM and GCK-HI) are the three different forms of "glucokinase disease".

"Glucokinase disease" can be caused by missense *GCK* mutations located anywhere in the primary sequence with no major "hot spots" defined (9,10). However GCK-HI mutations cluster in the small domain of GK protein, where the allosteric activator site is located (11)

In this manuscript we present a novel *GCK* activating mutation that, in spite of presenting the lowest relative activity index (%AI) and the highest predicted GSIR-T of all naturally occurring GK activating mutations described so far, leads to severe neonatal hypoglycaemia (1.5 mmol/l). Furthermore, we also report functional studies of contiguous inactivating mutations in *GCK* that lead to the hyperglycaemic form of the "glucokinase disease" (GK-MDY).

RESULTS

Identification of missense mutations in the GCK gene

Family trees of the three patients carrying the *GCK* mutations studied in this report are depicted in figure 1. Denaturing gradient electrophoresis (DGGE) experiments showed abnormal patterns of PCR products of exon 10 of the *GCK* in all affected families' members available for analysis. We identified three missense mutations in the heterozygous state. A novel mutation in codon 442, with lysine substituting for glutamic acid (p.E442K) in the proband of family GCK-HI-1 (subject III-2 in Fig. 1a), as well as in her affected brother and mother (subjects III-1 and II-1 in Fig. 1a). A single nucleotide change resulting in a tryptophan for serine substitution at codon 441 (p.S441W) in the proband of family FE-11 (subject III-1 in Fig. 1b), in his younger brother, his mother and maternal grandmother. This mutation had been previously described in another Italian family (10). Finally, a novel mutation resulting in a substitution of glutamic acid by glycine at codon 440 (p.E440G) was found in the proband of family FI-10 (subject III-1 in Fig. 1c), in his younger brother, in his father and in a paternal uncle. None of the mutations were found in 100 healthy controls' chromosomes.

Metabolic features of families with GK diseases

Family GCK-HI-RM-1 (E442K). The proband with hypoglycaemia (Subject III-2 in Fig. 1a) was born at the 41st week of gestation and presented at 1st day of life with plasma glucose (PG) of 1.5 mmol/L. Her birth weight was 2,840 g and the clinical examination unremarkable. Family history disclosed that the maternal grand father, the mother as well as the eldest brother presented with recurrent, symptomatic "hypoglycemic-like" episodes characterized by cold sweating, pallor, fatigue, hunger, and tachycardia; in addtion, the mother and the brother of the index case showed fasting plasma glucose of 2.2-3.0 mmol/l in several occasions. At the age of 3 weeks (weight: 4,200 g) the proband was referrred to the Metabolic Unit of Bambino Gesù Pediatric Hospital for further investigation. At that time her plasma glucose ranged between 1.9 and 4.3 mmol/L. She had normal plasma values of ammonia, lactate, triglycerides (122 mg/dl) and total cholesterol (130 mg/dl). The urine excretion of α-ketoglutarate, another marker of HI due to gain-of-function mutations of glutamate dehydrogenase gene (GLUD1) (12), was normal, as well as blood acylcarnitines, aminoacids, and serum transferrin IEF. Abdominal and brain ultrasonography were also normal. Following i.m. glucagon (1 mg.), plasma glucose (PG) rose

from 2.2 to 6.0 mmol/L; simultaneous baseline evaluation of plasma cortisol (23 μg/dl), IGF1 (237 ng/ml) and ACTH (35 pg/ml) were all normal. These clinical investigations combined excluded some causes of hypoglycemia. Low dose diazoxide therapy (2 mg/kg/d) was started in the proband which normalized blood glucose and was continued for 3 months. The patient, now 6 years old and on diet therapy only, did not suffer of any other symptomatic hypoglycemia; in contrast the patient's elder brother experienced in the same time interval two unexplained syncopal episodes.

Family FE-11 (S441W). All the affected members of this family (Fig. 1b) presented impaired fasting glucose (IFG) (6.5-6.9 mmol/l), and all were treated only with diet. The oral glucose tolerance test (OGTT) carried out in the proband (Fig. 1b, subject III-1) showed IGT and low plasma insulin at 30' (18 μU/ml with corresponding plasma glucose of 10 mmol/l) (Table 1), a result which is typically found in GCK-MDY patients.

Family FI-10 (E440G). The proband and his younger brother presented impaired fasting glucose (IFG) (6.1 and 5.8 mmol/l, respectively) (Fig. 1c, subjects III-1 and III-2). Intravenous glucose tolerance test (IVGTT)-derived first-phase insulin response (FPIR) of children carrying the p.E440G mutation showed unexpectedly high figures of 300 and 241 μU/ml. These values respectively exceed the 97th (260.7 μU/ml) and the 90th (223.7 μU/ml) centile of normal Italian children of corresponding pubertal status (13). This high FPIR was observed again in both brothers when the IVGTTs were repeated two years later (308 and 215 μU/ml, respectively) (Table 1). In addiiton, adult family members carrying the mutation showed a diebetic phenotype, not typical of GCK-MDY, with proband's father presenting high fasting plasma glucose (10.4 mmol/l) and a paternal uncle treated with oral hypoglycemic agents; unfortunately two other paternal uncles, one treated with OHA and another with insulin were not available for genetic analysis (Fig. 1).

Kinetic analysis of recombinant wild type and mutant glucokinase.

Kinetic properties of WT and mutant GK are shown in Table 2. Mutation GK-E442K showed a higher affinity for glucose ($S_{0.5}$= 4.43 mmol/l) than GK-WT and a relative activity

index (%AI) which was over 3 times higher (Table 2), indicating that K442 is an activating mutation. On the contrary, mutations GK-E440G and GK-S441W showed a lower affinity for glucose, ($S_{0.5}$ = 10.85 and 15.89 mmol/l, respectively), as compared to GK-WT. The lowest affinity for the second substrate $MgATP^{2-}$, catalytical activity and cooperativity index (Hill number - n_H) were found in mutation GK-S441W (Table 2). The %AI of GK- E440G and GK-S441W were almost 60% and 90% lower than GK-WT. Consequently, the calculated GSIR-T of MDY-causing mutations GK-E440G and GK-S441W was higher than GK-WT (5.82 and 6.63 mmol/l vs. 5 mmol/l respectively), while calculated GSIR-T of the activating mutation GK-E442K was lower (4.14 mmol/l) than GK-WT (Fig. 2).

Prediction of structural effects of naturally occurring glucokinase mutations

We introduced the mutated residues into the closed active and super-open inactive GKB (Glucokinase Beta cell-specific) structure models described by Kamata et al (14) and then compared mutated GK-G440, GK-W441 and GK K442 with GK-WT. In Kamata's model the side chain of Glu 442 (E442) is exposed to the solvent in both closed and super-open conformations. The mutant Lys residue in GK-HI-causing K442 would also be exposed to the solvent in both conformations (Fig. 3a). However, the positive charge of the side chain of the Lys 442 residue may interact with the negative charge of Glu 216 residue (E216), stabilizing the closed conformation. In addition, in the super-open conformation (Fig. 3a), the side chain of Lys 442 would be closer to Ala 454 residue (A454), destabilizing the super-open conformation. As a result, the structure of K442 enzyme would favor the closed, active conformation, and would consequently lead to higher glucose affinity.

The change of Glu 440 residue (E440) by Gly residue (G440) (Fig. 3b) would destabilize the structure of the protein, since Gly residues are prone to modify the conformation of a particular structure. This interpretation would explain the observed reduction in glucose and $MgATP^{2-}$ affinity. In the case of the S441W mutation (Fig. 3c, W441), the new bulkier tryptophane residue would be projected toward the inside of the protein, destabilizing it because of its interaction with the α5 helix. This would cause a dramatic reduction in glucose affinity, as observed in *in vitro* experiments.

DISCUSSION

In this report we present a novel activating *GCK* mutation (p.E442K) in a newborn with severe neonatal hypoglycemia. She is now 6 years old, in good general condition and treated with diet therapy only. Her older brother and mother, also bearing the same *GCK* mutation, did not present hypoglycemia in the neonatal period but repeated episodes of symptomatic hypoglycemia later in life. Functional studies of mutated protein showed an enzyme with almost a two-fold increase in glucose affinity compared to wild type GK. The E442 residue is located in a loop between β13 and α13 domains of GK. According to Kamata's model (14), this loop plays an important role in the conformational change of the GK from the super-open to the closed active conformation of the enzyme. Although E442 does not participate directly in the binding to the allosteric activator (compound A in Kamata's model) (14), the K442 mutation favours the transition to the closed conformation of the enzyme, leading to increased glucose affinity (Fig. 3a). The structural analysis along with the calculated %AI and GSIR-T (3.3 and 4.14 mmol/l, respectively) obtained from the experimental data, indicate that the activation of GK caused by the E442K mutation is the cause of hypoglycaemia in the proband and in other members of the family carrying the mutation (Figure 1a).

This mutation is a good example of the discrepancy one may find between the severity of the clinical phenotype at presentation and the data resulting from the functional analysis "*in vitro*" of the mutated enzyme. Indeed, the results of the *kinetic* analysis showed that the mutation E442K presents the lowest %AI as well as the highest GSIR-T of all naturally occurring *GCK* activating mutations described to date (11,15). This is in line with the relatively mild symptoms of hypoglycemia showed in affected members in childhood and adult life, but in contrast with the severe clinical onset presented by the proband. We can only speculate that the proband could have some transient immaturity of glucoregulatory pathways which contributed to lower her plasma glucose levels. However, we do not believe that this may be ascribed to the relatively low birth weight of the proband, whcih was in line with that reported in other patients with activating GCK mutations born from affected mothers (16).

We also described two inactivating *GCK* mutations (p.E440G, p.S441W) contiguous to the activating p.E442K. Patients carrying the mutation p.S441W present a phenotype highly characteristic of GCK-MDY patients, i.e. IFG combined with impaired glucose tolerance (IGT) with low values at tests evaluating (early) insulin release (17). In contrast, the two young and lean brothers carrying the mutation p.E440G showed high plasma insulin levels at 30' of OGTT and in two independent IVGTT tests (Table 1). Alike the family previously reported with GCK-MDY (mutation p.L184P) (18), we also observed some metabolic heterogeneity within the different E440G carriers of family FI-10 (Fig. 1). As a matter of fact, though functional and structural studies of GK-E440G (Table 2) fully explain the basic metabolic features of the proband and his brother, they are not sufficient to clarify the severe diabetic phenotype observed in adult family members carrying the mutation. Thus, the co-existence of other unknown genes implicated in glucose metabolism or unrecognized environmental factors should be considered in this kindred.

MATERIAL and METHODS

Subjects

The proband with hypoglycemia (Subject III-2 in Fig. 1a) was referred to the Metabolic Unit of Bambino Gesù Children's Hospital for diagnostic workup. Routine laboratory exams were all normal with the exception of insulin, which was inappropriately high (12 μU/ml) for the corresponding plasma glucose. Family history disclosed that her elder brother and mother (subjects III-1 and II-1 in Fig. 1a) suffered repeated episodes of symptomatic hypoglycemia and the maternal grandfather (deceased, not tested) had "hypoglycemia-like" symptoms (i.e. sweatiness, headache, hunger and weakness).

The diabetic probands studied in this report were identified during the course of an Italian nation-wide project aimed at selecting patients with monogenic diabetes (MD) (subjects III-1 in Fig. 1b and 1c) out of subjects referred to pediatric diabetes clinics for incidental hyperglycemia (10,19).

Metabolic studies

Probands of families with (GCK-MDY) (subjects III-1 in Fig. 1b and 1c) underwent two tests as part of the protocol for studying subjects with incidental hyperglycemia: a standard OGTT with serum insulin determinations and an IVGTT. IVGTT was performed injecting 0.5 g of glucose per kg/body weight. Blood samples were taken at -15, 0, and 1, 3, 5, 10 minutes after the glucose injection for plasma insulin determination. FPIR was calculated as the sum of insulin immunoreactivity at minute 1 and 3 and the result compared to those obtained in normal Italian children according to their pubertal stage (13). Insulin assay was not centralized. However other IVGTT tests performed in the same center of family FI-10, on probands with mutations in the GCK always elicited low FPIR (see Table 1). All tests were approved by the local institutional ethics committee and a written informed consent was obtained from the parents of the probands.

Molecular genetic studies

Genomic DNA was extracted from peripheral lymphocytes. The complete coding sequence of the *GCK* was amplified by the polymerase chain reaction (PCR) and analysed by denaturing gradient electrophoresis (DGGE) as previously described (10). In the proband clinically defined as having hyperinsulinemic hypoglycemia and his relatives, screening of *GCK* gene was performed first, based on the autosomal-dominant mode of inheritance of hypoglycaemia (5, 11). We excluded the possibility of mutations of the glutamate dehydrogenase gene (*GDH*) because of the normal plasma ammonia concentrations (20). The PCR products showing abnormal electrophoretic pattern were subjected to direct sequencing by an ABI DNA sequencing apparatus 373A (Perkin Elmer Applied Bio Systems, Foster City, CA). Mutations were confirmed in all affected family members available for analysis.

Site-directed mutagenesis

Mutations p.E440G, p.S441W, p.E442K on *GCK* were introduced into the wild type human pancreatic *GCK* using the Quick Change site-directed mutagenesis kit from Stratagene (La Jolla, CA), (oligonucleotides sequences available upon request). Plasmid pUC-GlkB was used as a template in the PCR reactions. All plasmids were sequenced to confirm that only the desired mutation had been introduced. Mutated plasmids (pUC-GlkB-E440G, S441W and

E442K) were digested with *Eco*RI and *Sal*I and the insert subcloned into plasmid pGEX-6P-1 (Amersham Pharmacia), to allow its expression in *Escherichia coli* as a glutathionyl S-transferase (GST) fusion protein. Purified recombinant GST-GK was routinely screened for purity by SDS-PAGE.

Kinetic and structural analysis of the GK protein

Studies of the kinetic properties of GK wild type (GK-WT) and GK-E440G, GK-S441W and GK-E442K in the presence of 2 mmol of dithiothreitol per liter of reaction mixture were performed spectrophotometrically as described previously (21). At least three different preparations of GK-WT and GK-mutants were made and analysed. We used non-linear kinetics according to the Hill equation to determine the affinity of the enzyme for glucose, and the Hill coefficient that characterizes the sigmoidal glucose dependency of GK. In order to measure the glucose phosphorylation capacity of the enzyme we used the relative activity index, which was calculated according to the formula previously reported (11). The structural analysis of the activating mutation was carried out using the crystal structure of human GK (14).

Mathematical modelling

We used a minimal mathematical model (1,11,21) to assess the impact of GK mutations on GSIR. We determined the impact of blood glucose levels on GK expression for both wild type and mutated alleles by using the expression coefficient for either allele: $e = (S^{nH} \times 2)/(S^{nH} + S_{0.5}{}^{nH})$, where S refers to the glucose level at threshold, nH is the Hill coefficient for cooperativeness with glucose, the numerical value of 2 indicates that half-maximal induction is achieved at glucose $S_{0.5}$, and $S_{0.5}$ refers to the concentration of glucose needed to achieve the half-maximal rate of phosphorylation (1, 11).

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LEGENDS TO FIGURES.

Fig. 1 Family-trees of families GCK-HI-1, FE-11 and FI-10. Patients with no mutations detected in *GCK* are denoted as n/n; those carrying the mutations are denoted as n/m; nt= not tested. Individuals identified by symbols with vertical white lines have IFG. Individuals with symbols filled in black have diabetes. Individuals with horizontal black lines have hyperinsulinism and hypoglycemia. Current treatment is indicated below patients' symbols.

Fig. 2 Threshold for glucose stimulated insulin release (t-GSIR) as a function of the relative activity index of glucokinase protein wild type (GK-WT) and naturally occurring mutations GK-E440G, GK-S441W and GK-E442K.

Fig. 3 Close-up of the structural model of the GK-E440, GK-S441, GK-E442 (wild type), GK-E440G, GK-S441W (GCK-MDY naturally occurring mutation) and GK-E442K (HI mutation). The key α5 and α13 helixes of glucokinase are indicated in the left structures of **Panel a,** (super-open conformation of glucokinase) (14) and **Panels b, c** (closed conformation) (14). An enlargement of the region of interest (dotted square) is shown in each panel. Mutated residues are shown in red. The interacting residues are in green, cyan and magenta. Crystal coordinates from the closed active (1V4S) and super-open inactive (1V4T) conformation of GlkB (14) were visualized using the Pymol Molecular Graphics System version 0.97 (Delano Scientific LLC).

Table 1: Results of the metabolic studies performed in the probands with GCK-MDY.

			IVGTT	OGTT					
Family / mutation	AGE (years)	BMI (Kg/m^2)	FPIR (μU/ml)	FPG (mmol/L)	IRI / bas. (μU/ml)	IRI / 30 min. (μU/ml)	PG / 30 min. (mmol/L)	IRI / 120 min. (μU/ml)	PG / 120 min. (mmol/L)
FI-10 / E440G									
Subject III-1									
1997	10	17.5	308	6.1	13.9	180	10	50	7
1999	12		310						
Subject III-2									
1997	8	17.2	241	5.8	7	70	9.5	11.1	5.1
1999	10		215						
FE-11 / S441W									
Subject III-1	10	n.a	n.a	6.7	5	18	10	20	7.9
MI-34 / S441W									
Proband (9)	9	16.2	96	7.6	13	n.a.	12.8	n.a.	8.2

(9); The results of this probamd presented in this table have been previously reported in Massa et al. Diabetologia 44:898-905. n.a.; not available.

The following FPIR values have been obtained in probands with GCK mutation who underwent IVGTT in the same Center of family FI-10: proband FI-2, mutation S383fsdelC1=37 (<1st centile; ref 18); proband FI-22, mutation Y108H=54 (=1st centile); proband FI-14, mutation L276P=56 (<3rd centile); proband FI-18, mutation F150Y=80 (<25th centile); proband FI-4, mutation N231fsdelA=92 (=25th).

Table 2: Functional characteristics of the recombinants glucokinase proteins wild type (GK-WT), and the naturally occurring mutations GK-E440G, GK-S441W and GK-E442K. Data are means ± SE from three separate enzyme preparations.

	$S_{0.5}$ *(mmol/l)*	n_H *(unitless)*	ATP_{Km} *(mmol/l)*	K_{cat} *(sec^{-1})*	**Relative Activity Index** *(%AI)*
WT	7.97±0.31	1.71±0.07	0.45±0.05	65.57±7.19	1
E440G	10.85±0.68	1.52±0.03	0.74±0.04	51.55±4.64	0.41
S441W	15.89±2.49	1.38±0.07	1.12±0.14	20.72±4.56	0.11
E442K	4.43±0.17	1.6±0.05	0.72±0.1	41.98±6.20	3.33

Figure 1

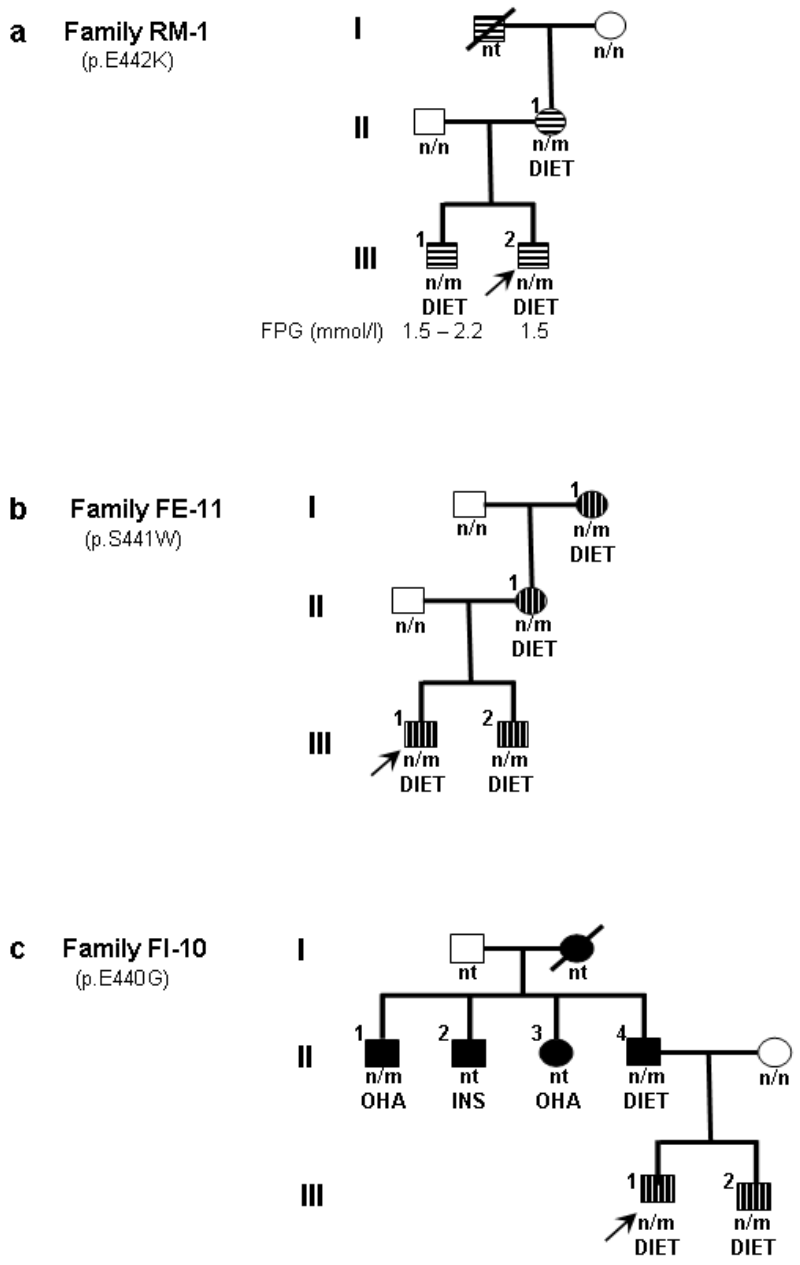

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Figure 2

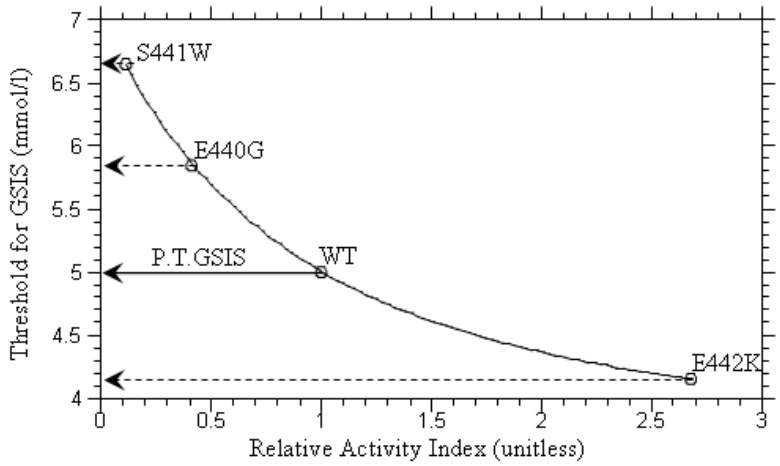

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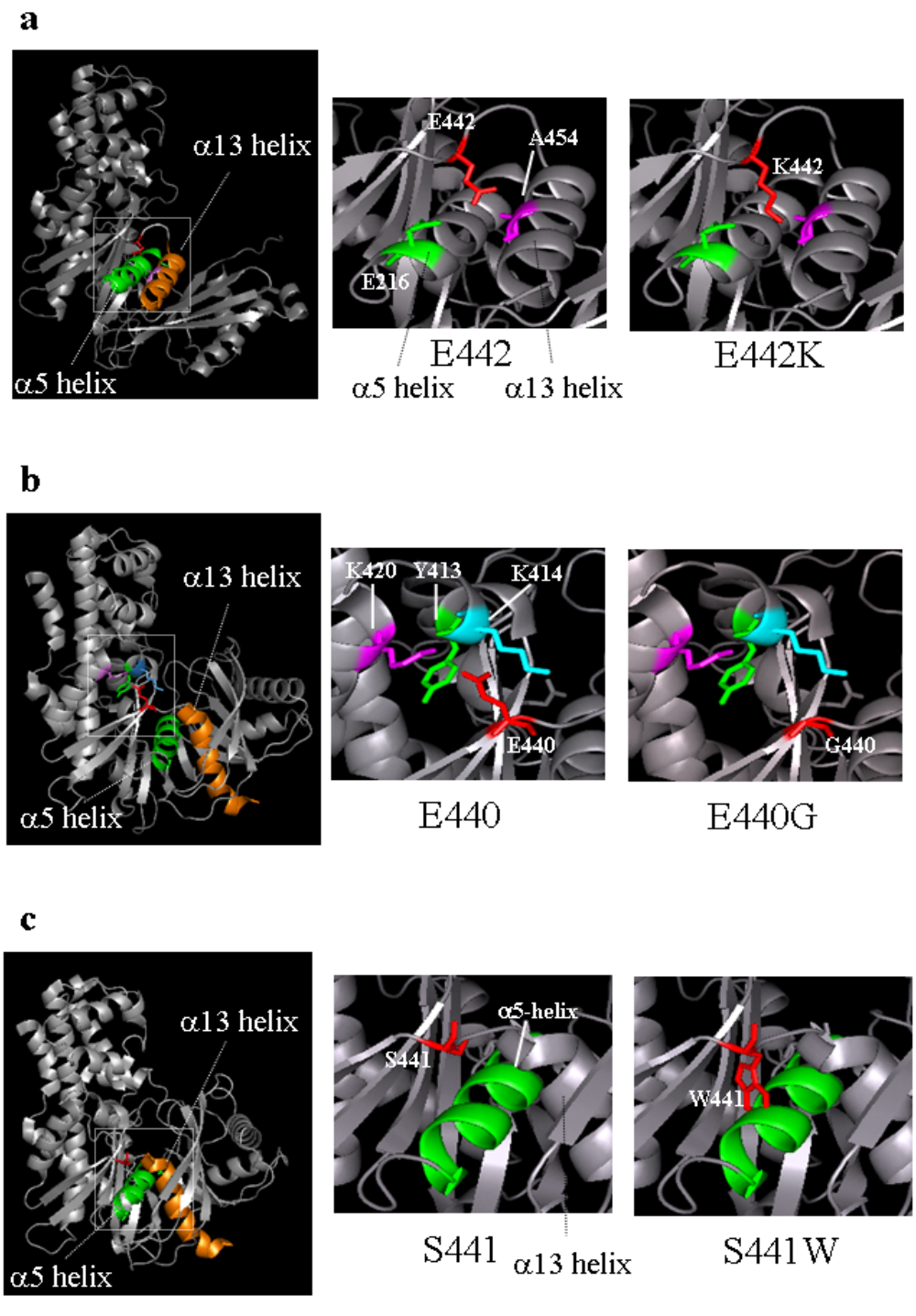
Figure 3



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