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Mutations in glucokinase and other genes detected in neonatal and type 1B diabetes patient using whole exome sequencing may lead to disease-causing changes in protein activity

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ABSTRACT

Monogenic diabetes is caused by mutations that reduce β-cell function. While Sanger sequencing is the standard method used to detect mutated genes. Next-generation sequencing techniques, such as whole exome sequencing (WES), can be used to find multiple gene mutations in one assay. We used WES to detect genetic mutations in both permanent neonatal (PND) and type 1B diabetes (T1BD).

A total of five PND and nine T1BD patients were enrolled in this study. WES variants were assessed using VarioWatch, excluding those identified previously. Sanger sequencing was used to confirm the mutations, and their pathogenicity was established via the literature or bioinformatic/functional analysis. The PND and T1BD patients were diagnosed at 0.1–0.5 and 0.8–2.7 years of age, respectively. Diabetic ketoacidosis was present at diagnosis in 60% of PND patients and 44.4% of T1BD patients. We found five novel mutations in five different genes. Notably, patient 602 had a novel homozygous missense mutation c.1295C > A (T432 K) in the glucokinase (*GCK*) gene. Compared to the wild-type recombinant protein, the mutant protein had significantly lower enzymatic activity (2.5%, $p = 0.0002$) and V_{max} (1.23 ± 0.019 vs. 0.33 ± 0.016, respectively; $p = 0.005$). WES is a robust technique that can be used to unravel the etiologies of genetically heterogeneous forms of diabetes. Homozygous inactivating mutations of the *GCK* gene may have a significant role in PND pathogenesis.

1. Introduction

Diabetes mellitus is a metabolic disorder involving hyperglycemia that results from insulin deficiency, insulin resistance, or both and can cause many complications such as retinopathy, neuropathy, nephropathy, and cardiovascular disease. Approximately 2% of all diabetes patients have monogenic diabetes, which is caused by single-gene mutations that reduce pancreatic β cell function or increase insulin resistance. Unfortunately, this form of diabetes is often misdiagnosed as either type 1 or type 2 diabetes mellitus [1]. Furthermore, monogenic diabetes is commonly classified into neonatal diabetes mellitus (ND), maturity-onset diabetes of the young (MODY), and other rare forms of

Abbreviations: ND, neonatal diabetes; PND, permanent neonatal diabetes; T1BD, type 1B diabetes; WES, whole exome sequencing

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genetic mutation-related diabetes, making differential diagnosis difficult depending on the age of onset [2].

The onset of ND, for example, is typically within the first 6 months of life, and the disease can manifest transient or permanent symptoms. In fact, transient ND often spontaneously remits before 18 months of age, while permanent ND (PND) persists and requires life-long therapy. Notably, approximately 70% of all transient ND cases are caused by the overexpression of an imprinted region on chromosome 6q24 [3], whereas approximately 25% are due to activating mutations of the potassium voltage-gated channel, subfamily J, member 11 (*KCNJ11*) or ATP binding cassette subfamily C member 8 (*ABCC8*) genes [4]. The remaining 5% of cases are known to be caused by mutations of the insulin (*INS*) gene, the ZFP57 zinc finger protein (*ZFP57*) gene, or other unknown causes [5]. PND is also known to be caused by activating mutations of *KCNJ11* [6] or *ABCC8* [7], mutations of *INS* [5,8], or homozygous or compound heterozygous mutations of glucokinase (*GCK*) [9]. Interestingly, patients with K_{ATP} channel (*KCNJ11* or *ABCC8*) mutations are typically more responsive to a high-dose oral sulfonylurea treatment and are able to discontinue insulin therapy [10,11].

Type 1B diabetes (T1BD) is a non-autoimmune disease and accounts for about 10% of all type 1 diabetes diagnoses. Mutations of *KCNJ11* and *INS* have also been associated with T1BD in a Japanese population study [12]. However, the genetic similarities and differences between these T1BD patients and those with ND have not been fully established.

Sanger sequencing is currently the standard method used to detect gene mutations in monogenic diabetes, whereby candidate genes are sequentially tested according to incidence and phenotype of the disease. Because monogenic diabetes is an umbrella term for multiple genetic etiologies, its genetic diagnosis and comparison to other forms of diabetes is challenging. Next-generation sequencing uses massive parallel sequencing and simultaneously analyzes millions of DNA fragments from a single sample. It is high-throughput and able to sequence an entire genome in a short time period [13]. Recently, whole exome sequencing (WES) was used to diagnose various Mendelian disorders, including monogenic diabetes [14,15]. Thus, with the advent of this new technology, the genetic comparison of various forms of diabetes has become possible.

In the present study, we utilized WES to detect genetic mutations in both PND and T1BD patients. To our knowledge, this is the first time next-generation sequencing has been used to analyze PND and T1BD patients in parallel, allowing for their direct comparison.

2. Material and methods

2.1. Subjects

A total of 5 PND patients and 9 T1BD patients were enrolled in this study. The age at diagnosis ranged from one month to 2.7 years (Table 1). The diagnosis was made according to clinical presentation and laboratory confirmation. PND was defined as diabetes mellitus diagnosed before 6 months of age, requiring insulin therapy, persisting beyond 18 months of life, and without detectable anti-GAD65 and anti-IA2 antibodies. T1BD was defined as diabetes mellitus diagnosed at or after 6 months of age, requiring insulin therapy, with a C-peptide level < 0.7 mmol/l (2.1 ng/ml) at random or < 1.1 mmol/l (3.3 ng/ml) at the peak by a glucagon test, and without anti-GAD65 and anti-IA2 antibodies. This study was approved by the Institutional Review Board at the Mackay Memorial Hospital. Study participants and/or their guardians gave informed consent.

2.2. WES data analysis

Genomic DNA was extracted from fresh or frozen peripheral blood leukocytes using the Gentra Puregene method (Qiagen, Gaithersburg, MD, USA). DNA was fragmented using the Covaris S2 system (Covaris, Woburn, MA, USA). Targeted capture, massive parallel sequencing, and analysis were performed at the National Center of Genome Medicine (NCGM; Academia Sinica, Taiwan). We used a SureSelect Human All Exon kit V5 (Agilent Technologies, Santa Clara, CA, USA) for exome capture and enrichment. Enriched PCR products were clustered on a cBot platform and then sequenced on an Illumina HiSeq2000 (Illumina Inc., San Diego, CA, USA) with 100 bp paired end reads. We mapped paired-end-reads to a reference human genome (UCSC NCBI37/hg19) with the Burrows-Wheeler Alignment (BWA) tool. Bioinformatic analysis was performed using in-house freeware and the Integrative Genome Viewer of Broad Institute [16]. Variants were identified and assessed in VarioWatch of GenePipe [17]. We excluded the variants already identified in the dbSNP and 1000 Genomes project. We evaluated the possible functional significance of the remaining variants using SIFT [18]. All suspiciously pathogenic mutations were confirmed by Sanger sequencing.

Table 1
The demographics of the permanent neonatal diabetes and type 1B diabetes patients in this study.

Patient	Sex	Diabetes	Age at diagnosis (yr)	DKA at diagnosis
602	F	PND	0.1	No
91	M	PND	0.3	Yes
377	M	PND	0.3	No
548	F	PND	0.5	Yes
5429	M	PND	0.5	Yes
3153	F	T1BD	0.8	No
401	M	T1BD	1.0	Yes
178	M	T1BD	1.2	Yes
172	F	T1BD	2.0	Yes
981	M	T1BD	2.2	No
176	F	T1BD	2.3	No
211	M	T1BD	2.4	No
227	M	T1BD	2.7	Yes
294	M	T1BD	2.7	No

Abbreviations: M, male; F, female; T1BD, type 1B diabetes; PND, permanent neonatal diabetes; DKA, diabetic ketoacidosis.

2.3. Cloning full-length GCK and constructing mutant GCK-T432K

Using the human liver cDNA library (Clontech Co.) as a template, approximately 1.4 kb of the full-length human liver *GCK* cDNA was PCR-amplified using the forward primer 5′-GAATTCATGGCGATGGATGTCACAAGG-3′ and the reverse primer 5′-CTCGAGTCACTGGCCCAGCATACAGG-3′ (Fig. 1A). The amplified *GCK* cDNA fragment was then cloned into a yT&A vector (Yeastern Co.). The sequence of this cloned cDNA vector was confirmed by DNA sequencing. To construct the *His-GCK* fusion gene, the cloned *GCK* DNA was subcloned into the pET30a expression vector (Novagen Co.) (designated pET30-*GCK*). To construct the glutathionyl S-transferase fusion gene, the cloned *GCK* DNA was further subcloned into the pGEX-KG expression vector (Amersham-Pharmacia Co.) (designated *GST-GCK*). All of the plasmid constructs were confirmed by DNA sequencing.

To construct the *GCK-T432K* mutant gene, the pET30-*GCK* plasmid DNA was used as a template for PCR-mediated site-directed mutagenesis using a set of primers (5′-CATGCCAGCGTGCGCAGGCTTAAGCCCAGCTGCGAGATCACCTTC-3′ and 5′-GAAGGTGATCTCGCAGCTGGGCTTAAGCCTGCGCACGCTG GCATG-3′) and a QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). This allowed us to mutate the threonine (T) amino acid residue at position 432 to a lysine (K) residue (*T432K*). The resulting mutant plasmid construct (designated pET30-*GCK-T432K*) was confirmed by DNA sequencing. The *GCK-T432K* DNA fragment was then subcloned into a pGEX-KG expression vector (designated *GST-GCK-T432K*), and the resulting plasmid construct was also confirmed by DNA sequencing.

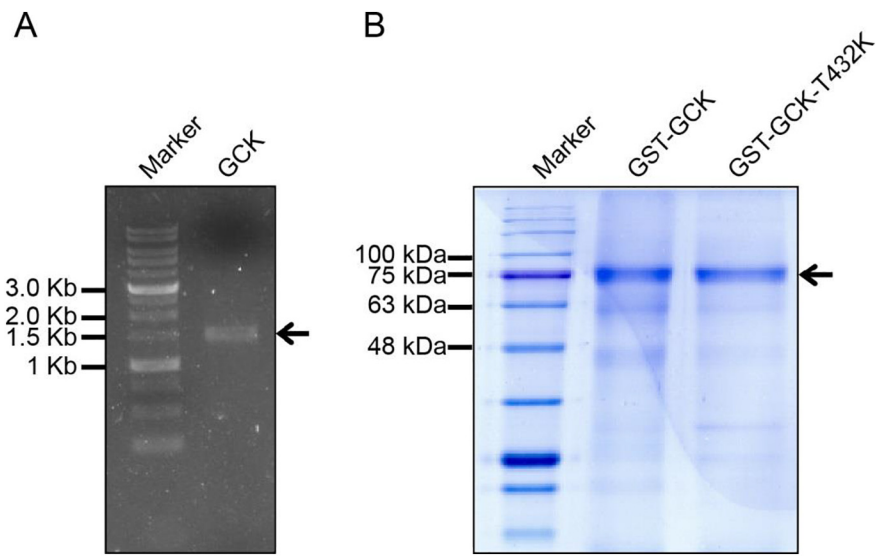

Fig. 1. Size analysis of the glucokinase (GCK) gene and recombinant protein products. (A) The DNA fragment of the GCK gene is approximately 1.4 kb in size as shown on a 1% agarose gel (arrow). (B) Purified GST-GCK (wild-type) and GST-GCK-T432K (mutant) proteins were analyzed by 10% SDS-PAGE, followed by Coomassie blue staining (arrow).

2.4. Expression and purification of GST-GCK and GST-GCK-T432K recombinant proteins

Both the *GST-GCK* and *GST-GCK-T432K* plasmid constructs were respectively transfected into *Escherichia coli* BL21 (DE3). After the bacteria were incubated in 20 ml of 2YT medium containing 0.4% glucose overnight, the cultures were diluted 1/10 in 200 ml of 2YT medium and incubated at 37 °C until an OD_{600nm} of 1.0 was obtained. A final concentration of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the bacterial culture to induce the expression of GST-GCK or GST-GCK-T432 K recombinant proteins, and the cultures were incubated at 22 °C for 6–16 h. The GST-GCK and GST-GCK-T432K recombinant proteins were purified using glutathione-conjugated sepharose beads [19]. A total of 200 ml of each bacterial culture was pelleted, re-suspended in 8 ml of lysis buffer (0.1 M PBS, 5 mM DTT, 1% triton X-100), and broken using a French Pressure cell press. After centrifugation, the bacterial lysates were mixed with the glutathione-conjugated sepharose beads and incubated at 4 °C for 1–1.5 h. After the beads were spun down and washed with lysis buffer 3–4 times, the GST-GCK and GST-GCK-T432 K recombinant proteins were eluted with elution buffer (50 mM Tris/HCl (pH 8.0), 200 mM KCl, 10 mM glutathione, 5 mM DTT) and kept in storage buffer (30% glycerol, 50 mM glucose, 10 mM glutathione, 5 mM DTT, 200 mM KCl, 50 mM Tris/HCl (PH 8.0)) at −80 °C [19,20]. The purity of the GST-GCK and GST-GCK-T432K recombinant proteins was confirmed by 10% SDS PAGE (Fig. 1B).

2.5. GCK activity

Using a hexokinase colorimetric assay kit (Biovision Inc., San Francisco, CA), GCK activity was determined spectrophotometrically at OD_{450nm} on a spectrophotometer at room temperature. The enzyme activity was calculated from the NADH standard curve using the equation as below:

$$\text{Hexokinase activity (nmol/min/ml, mU/ml)} = \frac{B}{\Delta T \times V \times \text{dilution factor}}$$

where B is the amount of NADH in the standard curve (nmol), ΔT is the reaction time (min), and V is the sample volume added to the reaction well (ml).

To evaluate enzyme kinetics, the Michaelis–Menten equation was used to calculate the V_{max} and K_M of both GST-GCK and GST-GCK-T432 K recombinant proteins using Sigma Plot 12.0 software. Glucose (0.1 to 25 mmol/l) was then incubated with the optimal concentration of GST-GCK or GST-GCK-T432 K recombinant proteins. At least three repeats were performed in each test.

2.6. Statistical analysis

The results are presented as the mean ± standard deviation (SD). The statistical significance between two different groups was evaluated by the Mann-Whitney *U* test.

3. Results

3.1. WES analysis of PND and T1BD patients

The age at diagnosis ranged from 0.1 to 0.5 years in PND patients and from 0.8 to 2.7 years in T1BD patients (Table 1). Diabetic ketoacidosis was present at the initial diagnosis in 60% of the PND patients and 44.4% of the T1BD patients. Using WES, we found 5 unique pathogenic mutations in 5 genes in our 14 patients. All mutations were confirmed by Sanger sequencing and were confirmed to be pathogenic

Table 2
The five novel mutations detected in patients with permanent neonatal diabetes or type 1B diabetes.

Patient	Diagnosis	Gene	cDNA	Protein	Consequence	Disease caused	References
602	PND	*GCK*	1295C > A	T432K	Pathogenicity confirmed in the study	PND	This study
91	PND	*KCNJ11*	602G > A	R201H	Pathogenic	PND	[21]
548	PND	*INS*	265C > T	R89C	Pathogenic	PND	[8]
981	T1BD	*GLIS3*	298G > C	V100L	Pathogenic[a]	Not reported in literature	
176	T1BD	*INSR*	1324A > G	S442G	Pathogenic[a]	Not reported in literature	

Abbreviations: T1BD, type 1B diabetes; PND, permanent neonatal diabetes; GCK, glucokinase; KCNJ11, potassium voltage-gated channel subfamily J member 11; INS, insulin; GLIS3, GLIS family zinc finger 3; INSR, insulin receptor.
[a] By SIFT [18].

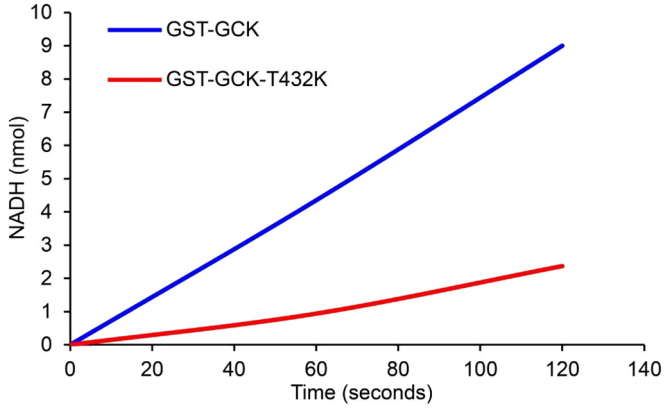

Fig. 2. The enzymatic activity of GST-GCK and GST-GCK-T432K. The rate of NADH production generated by GST-GCK and GST-GCK-T432K is shown. The reactions were performed at room temperature with a total of 5 μg of protein in each reaction.

according to the current literature, our study, and/or SIFT analysis (Table 2).

3.2. GCK activity analysis

Notably, patient 602 had a novel homozygous missense mutation c.1295C > A (p.Thr432Lys) in exon 10 of the *GCK* gene. We speculated that this mutation could be the primary cause of PND in this patient. To test this, we investigated the in vitro effects of this T432 K mutation on GCK activity.

The enzymatic activity of GST-GCK-T432 K was significantly lower than that of GST-GCK (66.1 ± 16.5 vs. 2604.9 ± 831.1 nmol/min/ml; p = 0.0002) (Fig. 2, Table 3). The V_{max} (nmol/min), determined with the Michaelis–Menten equation, of the mutant GCK was also significantly lower than that of the wild-type GST-GCK (0.33 ± 0.016 vs. 1.23 ± 0.019; p = 0.005) (Fig. 3, Table 3).

4. Discussion

With the recent advent and application of next-generation sequencing, the genetic comparison of various disease is possible in a quick and efficient manner. In this study, we observed unique mutations in five different genes in five individual patients with PND or T1BD using WES. Our results suggest that WES is a powerful tool that can be used to identify mutations in monogenic diabetes even with its heterogeneous genetic etiologies. To our knowledge, this is the first time WES has been used to genetically analyze and compare PND and T1BD patients.

The diagnostic rates of clinical genetic disorders with heterogeneous etiologies are 5–15% by karyotype analysis [22], 15–20% by chromosomal microarray analysis [23], 3–47% by Sanger sequencing for single-gene [15], 31.4% by next-generation sequencing, and 24.8% by WES [15]. The diagnostic rate of the present study was 36%. Excluding the T1BD patients, we found three mutations in five cases of PND, a diagnostic rate of 60%. These superior diagnostic rates for all the patients and the PND subset are likely due to stringent phenotyping.

One of the patients in this study was observed to have a mutation in the *GCK* gene. GCK phosphorylates glucose, producing glucose-6-phosphate and facilitating glucose storage (as glycogen) as well as its disposal via glycolysis in hepatocytes. Glycolysis mediates an increase in ATP and a decrease in Mg-ADP concentrations, leading to the closure of K_{ATP} channels and membrane depolarization. These changes subsequently trigger the opening of calcium channels and promote calcium entry into β cells and insulin exocytosis [24]. Therefore, GCK and K_{ATP} channels play a key role in glucose sensing and insulin secretion in β cells [25]. Mutations in the *GCK* gene could cause significant disruption of these processes [9]. In order to further investigate this particular *GCK* mutation, we used cloned liver *GCK* cDNA and constructed mutant *GCK-T432K* DNA to generate wild-type GST-GCK and mutant GST-GCK-T432 K recombinant proteins. The activity of these proteins was then analyzed, and the mutant appears to have only 2.5% of the enzymatic activity of the wild-type GST-GCK. This explains why the PND developed early in this patient, who was diagnosed at 0.1 year after birth and was homozygous for this *GCK-T423K* mutation.

Notably, an *E. coli* expression system was used to express the GST-GCK and GST-GCK-T432 K recombinant proteins in this study. Unfortunately, proteins expressed with this particular system may have misfolding, impaired disulfide bonds, and/or incorrect post-translation modifications [26]. However, the GST-GCK activity measured here appears to be similar to that of purified rat liver GCK [19,27], rat islet GCK [19,28], and tag-free GCK [19,29]. This suggests that our GST-GCK recombinant protein may be similar to native GCK with regards to conformation and function. GST-GCK and GST-GCK-T432K were also expressed in the same system for comparison. Therefore, while this expression system has some limitations, our results are reliable and comparable to previous studies for the wild-type.

Other limitations also exist. First, we used a human liver cell cDNA library to clone the *GCK* gene. Human GCK has three isoforms (one in the pancreas and two in the liver). It is, therefore, unclear whether the T432K mutation similarly reduces GCK kinase activity in β cells. Second, we only focused on the *GCK* mutation and did not confirm the novel mutations in the *GLIS3* and *INSR* genes in other patients. While additional work is necessary to evaluate the causative nature and function of these other mutations, the present study did identify multiple novel gene mutations involved in PND or T1BD and provides a foundation for further exploration.

Furthermore, our focus on the novel *GCK* mutation may also directly influence current treatment options for PND. Glibenclamide, a well-known sulfonylurea antidiabetic drug, was previously reported to be only partially effective in a child bearing a homozygous *GCK T168A* mutation [30]. This mutation had reduced glucokinase activity down to 2%, thus causing disease-related issues that could not be remedied by glibenclamide treatment. It is likely that the efficacy of glibenclamide in patients with *GCK* mutations depends on the residual activity of mutant GCK. Therefore, baseline GCK activity should be measured before sulfonylurea treatment.

In conclusion, we utilized WES to detect genetic mutations in both PND and T1BD patients. Our analysis indicates that WES is a robust technique that can be used to unravel the etiologies of genetically heterogeneous diseases and may lead to more effective personalized therapy for the patients. Using our WES data, we also identified a novel

Table 3
Enzyme activity and kinetics of GST-GCK and GST-GCK-T432*K* at room temperature.

	nmol/min/ml	U/mg	V_{max} (nmol/min)	K_M
GST-GCK	2604.9 ± 831.1	4.34 ± 1.17	1.23 ± 0.019	0.046 ± 0.007
GST-GCK-T432K	66.1 ± 16.5	0.33 ± 0.082	0.33 ± 0.016	0.16 ± 0.07
p[a]	0.0002	0.0001	0.005	0.007

Data are the mean ± SD.
[a] GST-GCK vs. GST-GCK-T432K.

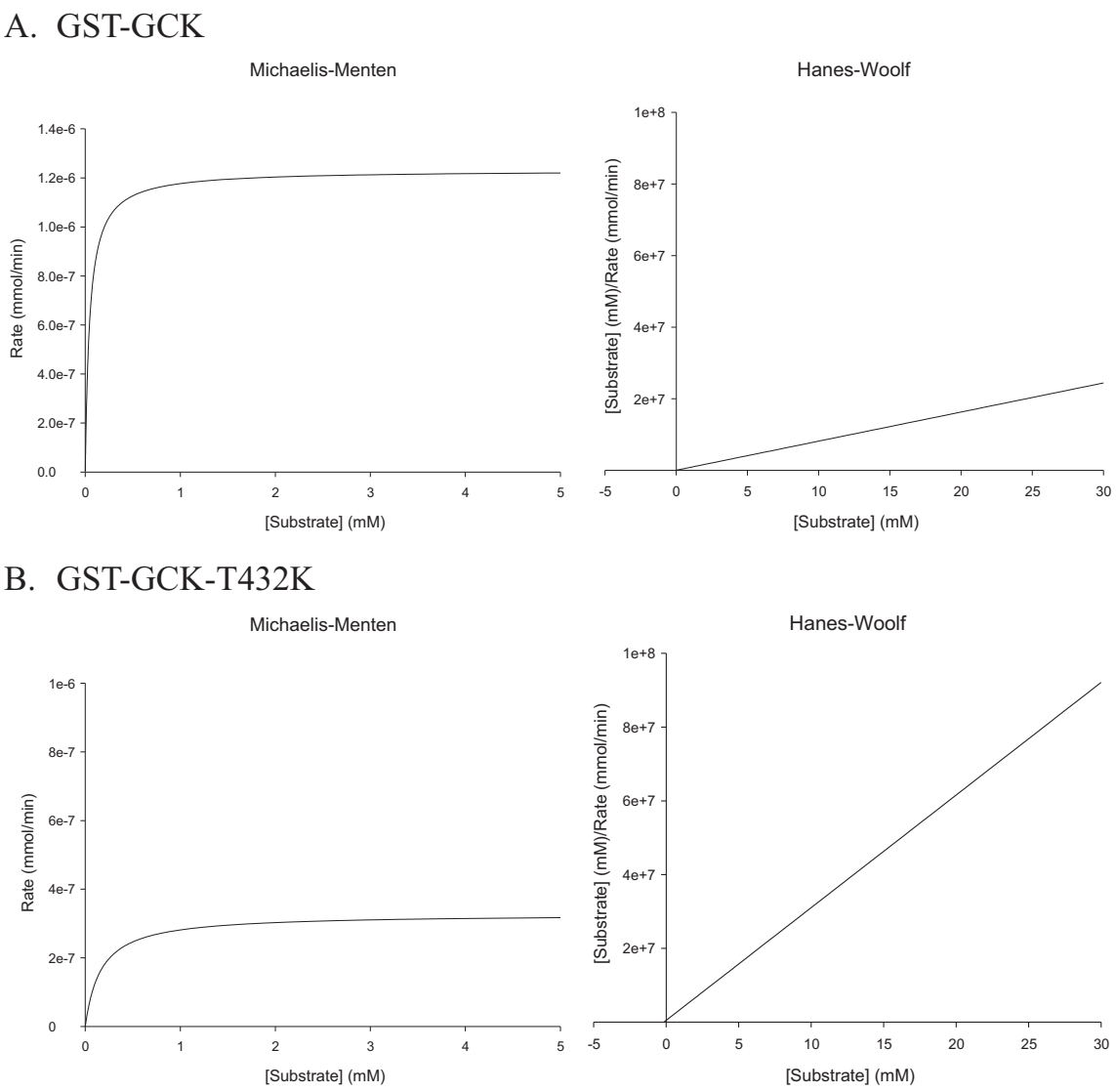

Fig. 3. The Michaelis–Menten saturation curve and Hanes–Woolf plot for GST-GCK and GST-GCK-T432K.

homozygous inactivating mutation in the *GCK* gene and evaluated the mutation-mediated changes in protein function that may be related to PND pathogenesis in the affected patient. Taken together, while the molecular diagnosis of monogenic diabetes still presents numerous challenges, this study provides insight into the diagnostic power of WES and how the identified mutations can be studied and individually treated.

Disclosure statement

The authors have nothing to disclose.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Duality of interest

We declare no competing interests.

Contribution statement

YJL and YLJ designed the study. DCL, CYH, YWT, and WSY performed the experiments. WHT, FSL, CLL, HWY, TYC, and CHL contributed materials and analyzed the data. DCL and CYH drafted the report. YJL and YLJ edited the report. DCL and CYH contributed equally. YLJ and YJL contributed equally.

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