

Novel de novo mutation in SUR1 presenting as hyperinsulinism in infancy followed by overt diabetes in early adolescence

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Running Title: Inactivating SUR1 mutation and childhood diabetes

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ABSTRACT

Background: Congenital hyperinsulinism, usually associated with severe neonatal hypoglycemia, may progress to diabetes, typically during the 4th decade of life in non-pancreatectomized patients.

Clinical data and methods: A 10.5y old female presented with new onset, antibody-negative diabetes (HbA_{1c}-10.6%). She was born large for gestational age (5Kg) to a non-diabetic mother, and developed frequent hypoglycemic episodes, which persisted until age 3y, responding initially to intravenous glucose and later to oral sweets. Currently, she is fully pubertal, obese (BMI-30.2kg/m²), with a partially controlled convulsive disorder (since age 1y) and poor school performance. Glucose levels were >11.1mmol/l throughout 72 hours of continuous glucose monitoring, with low insulin secretion during IVGTT. *KCNJ11* and *ABCC8* mutation analysis was performed and the mutation identified was characterized in COSm6 cells.

Results: A novel, *de novo* heterozygous *ABCC8* (SUR1) mutation (R370S) was identified in the patient's DNA but not in that of either parent. Co-transfection of Kir6.2 and mutant SUR1 demonstrated that the mutated protein is expressed efficiently at the cell surface but fails to respond to MgADP, resulting in minimal channel activity. Interestingly, the heterozygous channel (WT:R370S) responded well to glibenclamide, a finding that lead to the successful initiation of sulfonylurea therapy.

Conclusions: This new *ABCC8* mutation is associated with neonatal hyperinsulinism progressing within 10 years to insulinopenic diabetes. Consistent with in-vitro findings, the patient responded to sulfonylurea treatment. The mechanism causing the relatively rapid loss in β -cell function is not clear, but it may be due to mutation-induced increased β -cell apoptosis related to increased metabolic demand.

KEY WORDS: SUR1, heterozygous, de novo, hyperinsulinism, diabetes mellitus,.

Congenital Hyperinsulinism (CHI) is a disorder of glucose metabolism characterized by dysregulated secretion of insulin resulting in hypoglycemia (1). Most cases are attributed to defects in the pancreatic β -cell ATP-sensitive potassium (K_{ATP}) channels, which are critical for coupling glucose metabolism to membrane electrical activity and insulin release (2, 3). K_{ATP} channels are activated (opened) by intracellular adenosine diphosphate (ADP) and inhibited (closed) by ATP, the product of increased glucose metabolism. Closure of the K_{ATP} channel leads to membrane depolarization, which activates voltage-dependent calcium channels, leading to an influx of calcium and subsequently insulin granule exocytosis (3, 4).

Cloning and reconstitution studies demonstrate that the K_{ATP} channel is a hetero-octameric complex of two subunit types: the sulfonylurea receptor SUR1 and the inward rectifying potassium channel Kir6.2 (3, 5, 6). Given the key role of the K_{ATP} channel in insulin secretion, it is not surprising that inhibiting and activating mutations in the genes encoding the subunits of this channel can result in CHI (7, 8) and neonatal diabetes (9, 10) respectively. More interestingly, a dominantly inherited SUR1 mutation has been reported in a Finnish family, leading to CHI early in life and impaired insulin secretion or mild diabetes in adulthood (11, 12).

Here we report a novel, *de novo* heterozygous mutation in the SUR1 gene (*ABCC8*) (R370S) causing CHI in early infancy followed by overt diabetes presenting at 10.5 years of age, suggesting that this mutation facilitates the development of early-onset non-autoimmune diabetes.

CASE REPORT

A 10.5 years old female presented with fasting hyperglycemia (12.2 mmol/l),

obesity (BMI 30.2 kg/m²), and aggressive behavior. She was the last born for non-consanguineous Palestinian parents with 7 older, healthy siblings. Both parents developed type 2 diabetes during their 5th decade of life. Her birth weight was significantly large (4950g) in spite of normal gestational OGTT. From the second day of life until age 3y she had recurrent hypoglycemic convulsions (blood glucose 1.9-2.7 mmol/l) that were initially treated by intravenous glucose infusions and later by administration of sweet drinks at home. Hypoglycemia was not documented after 3y of age, but convulsions were never adequately controlled despite antiepileptic treatment (carbamazepine, hydantoin). EEG at age 1y revealed diffuse epileptic activity, while brain CT scan was normal. In spite of normal developmental milestones, her current school performance is poor, she behaves aggressively and uses insulting words. Her menarche was at 10y of age. Hyperphagia and obesity have been a concern since early childhood.

Except for few pink striae on the abdomen her physical exam was unremarkable for a fully pubertal adolescent. Laboratory investigations showed a blood glucose level of 12.2 mmol/l, moderate glucosuria and undetectable anti-GAD (<1.0 u/ml) and anti-insulin autoantibodies. Thyroid function tests and prolactin were normal and a mildly elevated urinary free cortisol (299 nmol/24h; normal <250 nmol/24h) was easily suppressed by 90% to 37.5 nmol/24h after one day of 0.5 mg dexamethasone every 6 h. Neurological evaluation, EEG and brain MRI were normal. Hydantoin replacement by topiramate achieved better epileptic control.

METHODS

Clinical studies. Prior to any anti-diabetic treatment, the Continuous Glucose Monitoring System (CGMS[®], Minimed, Sylmar, CA) was applied for 48 hours

during which the patient had a low carbohydrate diet. An intravenous glucose tolerance test (IVGTT) using 25% dextrose solution (0.5 gm/kg) was performed following a 10 hour fast. Glucose and insulin levels were determined at -5, 0, 1, 3, 5, 10 minutes.

Genetic studies. A was extracted from leukocytes of the patient, her parents, and one brother. For the patient, all coding exons and exon/intron boundaries of *KCNJ11* and *ABCC8*, coding for Kir6.2 and SUR1 components of K_{ATP} channel respectively, were PCR amplified and sequenced as previously described (13, 14). Genetic markers were used to confirm parenthood and the relevant DNA segment was sequenced in both parents. DNA from one brother and 65 unrelated Jerusalem Palestinians were analyzed by a specifically designed primer mismatch assay, which creates a new SDU1 restriction site on the mutant allele.

Functional Studies (Recombinant K_{ATP} channel experiments). *Construction of SUR1 and Kir6.2 plasmids.* The R370S point mutation of SUR1 was introduced into hamster SUR1 cDNA in the pECE plasmid using the QuikChange site-directed mutagenesis kit (Stratagene). The mutation was confirmed by DNA sequencing (15). Mutant clones from at least two independent PCR reactions were analyzed to avoid false results from undesired mutations introduced by PCR. Rat Kir6.2 cDNA is in the pCDNA1 vector.

Chemiluminescence Assay. Cell surface expression level was determined by a chemiluminescence assay using SUR1 containing a flag epitope (DYKDDDDK) at the N-terminus (fSUR), as previously described (16). Briefly, COSm6 cells grown on 35mm dishes were transfected with 0.6 μ g fSUR1 and 0.4 μ g Kir6.2 cDNA per 3 μ g of Fugene6 at ~70% confluency. At 48-72 hours post-transfection cells were fixed with 2% paraformaldehyde, incubated in M2 anti-FLAG antibody (10 μ g/ml), then horseradish peroxidase-conjugated anti-

mouse antibody (Jackson Immuno Research, Inc., 1:1000 dilution), followed by luminol. Luminescence signal was quantified using a Turner 20/20 luminometer.

$^{86}\text{Rb}^+$ efflux assay. COSm6 transiently expressing K_{ATP} channel subunits were loaded with $^{86}\text{RbCl}$ (1 μ Ci/ml) overnight. Before measurement of $^{86}\text{Rb}^+$ efflux, cells were incubated for 30 min at room temperature in Krebs-Ringer solution with metabolic inhibitors (2.5 μ g/ml oligomycin and 1 mM 2-deoxy-D-glucose). At selected time points, the solution was aspirated from the cells, and replaced with fresh solution. At the end of a 40 min period, cells were lysed. The $^{86}\text{Rb}^+$ in the aspirated solution and the cell lysate was counted. The percentage efflux at each time point was calculated as the cumulative counts in the aspirated solution divided by the total counts from the solutions and the cell lysates.

Electrophysiology. Inside-out patch-clamp recording of COSm6 cells transiently expressing channel subunits was performed as previously described (4, 16, 17) using Axopatch 1D amplifier and Clampex 9.2 software. Recordings were made at -50mV membrane potential in symmetrical K-INT solution containing (in mmol/l) 140 KCl, 10 K-HEPES, and 1 K-EGTA (pH 7.3 with KOH). For measuring MgADP, diazoxide and glibenclamide responses, MgCl_2 was added for a free concentration of ~1mmol/l. Data were analyzed using pCLAMP software. Statistical analysis was performed with Origin 6.1 using independent two-population two-tailed Student's t test.

Clinical therapeutic trials. Following the in vitro study results, oral hypoglycemic agents repaglinide (NovoNorm[®] 1 mg before meals for one day), and glibenclamide (5 - 10 mg bid for consecutive 4 days) were administered, without insulin. Fasting and postprandial glucose levels were recorded and compared to pretreatment values.

RESULTS

Clinical Studies. As CHI was evident in infancy, continuous blood glucose monitoring was performed to exclude occult hypoglycemic episodes. The CGMS[®] revealed 48h of continuous hyperglycemia (glucose 9.2-20 mmol/l) with no hypoglycemic events (Fig. 1A). Following a 12 h fast, while glucose was consistently high (12.2 mmol/l), insulin was 285 pmol/l, cortisol 338 nmol/l, growth hormone 81.4 pmol/l, and ammonia 44 μ mol/l (reference range 10-60). β -OH-butyrate, acetoacetate and free fatty acids were elevated at 232, 101 and 460 μ mol/l respectively. IVGTT revealed some incremental insulin secretion, up to 582 pmol/l that failed to restore euglycemia (Fig. 1B), but suggested a possible role for insulin secretagogues as a treatment modality.

Genetic analysis. The increased birth weight, recurrent hypoglycemias and consequent neurological damage suggested neonatal hyperinsulinism, despite the current reduced insulin secretion. A novel heterozygous missense mutation was detected in exon 7 of the *ABCC8*; AGG to AGC at the 1111 nucleotide position (Fig. 2A) predicting an arginine to serine mutation at the 370th amino acid of the SUR1 protein (*R370S*). Exon 7 was sequenced in both parents' DNA and was found to be entirely normal with no evidence of mosaicism. One sibling and 130 alleles from controls of the same ethnic background were also negative for the mutation (Fig. 2B). Parenthood was confirmed by genetic markers.

Expression studies. In order to confirm the functionality of our patient's mutation several expression studies were performed. Co-transfection of Kir6.2 and mutant SUR1 (SUR1-R370S) in COSm6 cells demonstrated normal channel expression on the cell membrane (Fig. 3A). The effect of metabolic inhibition on different channel states was studied by ⁸⁶Rb⁺ efflux assays. Upon metabolic inhibition, WT channels were rapidly activated giving rise to over

90% cumulative efflux at the end of the 40-min incubation period (Fig. 3B). On the other hand, the mutant channel only gave rise to <10% efflux. Inside-out patch clamp recordings revealed that the homozygote mutant channels completely lacked response to MgADP, in contrast to the stimulatory effect of MgADP on WT channels (Fig. 3C). When cells were made "heterozygous" by expressing both wild-type and mutant (WT:R370S) proteins to imitate our clinical setting, the metabolic inhibition-induced channel activity observed was greatly reduced and MgADP-induced current was reduced by more than 50% of the WT (Fig. 3D). These results corresponded to *in vivo* membrane depolarization in the fasting state causing hyperinsulinemic hypoglycemia. Similar responses were seen when applying the K_{ATP} channel opener diazoxide, which could reverse the inhibitory effect of 0.1 mM ATP on wild-type channels, but failed to stimulate the homozygous mutated channels (Fig. 3E). In the "heterozygous" cells, (WT:R370S) the channel was partially stimulated by diazoxide (Fig. 3F). The sulfonylurea, glibenclamide, given at 10nmol/l and 100nmol/l blocked the WT channels, but also had a similar blocking effect on the mutant channels, in homozygous and heterozygous states (Fig. 3G, H).

Therapeutic trials by insulin secretagogues. Based on some retained insulin response to IVGTT and the response of the mutated channel to glibenclamide, we studied our patient's response to insulin secretagogues. Repaglinide (1 mg), which closes K_{ATP} channels, binding to a site distinct from sulfonylureas (18), was given before meals and in 2 consecutive trials it resulted in a decrease of the sixty-minute postprandial glucose levels from 9.7-10.5 mmol/l to 5-8.3 mmol/l (Fig. 1C). Similarly, glibenclamide 5 mg twice daily also lowered postprandial glucose levels from 8.3-11 mmol/l to 7.2-7.8 mmol/l (Fig. 1D) and a further increment in the glibenclamide dose seemed even more effective.

DISCUSSION

Here we report a patient with a *de novo* heterozygous mutation in the SUR1 gene (*R370S*) resulting in congenital hyperinsulinism followed by overt diabetes becoming evident before 10.5y of age. *ABCC8* gene mutations have been known to cause autosomal recessive and dominant hyperinsulinism (7, 11, 19). Although the amino-acid location of the *R370S* mutation could not predict its dominant effect, we demonstrated normal membrane expression, a finding common to previously-reported dominant SUR1 gene mutations (16, 19). As the K_{ATP} channel is a hetero-octamer, containing 4 SUR1 subunits, a normally processed and translocated heterozygous mutant SUR1 subunit, such as *R370S*, would be expected to be present in 15/16 of the channels on the membrane and thus may alter their function. Conversely, mutated SUR1 proteins that do not reach the membrane will not result in abnormal channel activity in the heterozygous state, since all channels reaching the membrane would contain 4 normal SUR1 subunits.

Activating SUR1 mutations were recently found to cause neonatal diabetes but without antecedent hyperinsulinemic hypoglycemia (10). Huopio et al reported a dominant inactivating *ABCC8* mutation, *E1506K*, which caused hyperinsulinemic hypoglycemia followed by hypoinsulinemic diabetes, but when compared to our patient, overt diabetes presented later, during middle age, in non-pancreatectomized *E1506K* patients, and neonatal hypoglycemia was mild (11, 12). Studies of predominantly Ashkenazi Jews with recessive *ABCC8* mutations suggest that the natural history of the disease is one of clinical remission despite pancreas preservation. In some cases the remission progressed to impairment of glucose tolerance (20).

While the molecular mechanisms causing *ABCC8* mutation-mediated CHI and neonatal diabetes have been clarified, the molecular mechanisms resulting in the progression from hyper- to hypoinsulinism

are not known. A slow, progressive loss of β -cell mass due to increased β -cell apoptosis may be responsible for both the remission in CHI and for the progression to diabetes (20-22). Interestingly, the heterozygous *HNF4A* mutation causing the maturity-onset diabetes of the young (MODY1) was recently found to cause a phenotype similar to that described here, with transient neonatal hyperinsulinemic hypoglycemia followed by diabetes later in life (23), implying the possibility of similar underlying pathophysiology.

Our data in COS cells suggest equal biogenesis efficiency (data not shown) and surface expression of the wild-type and mutant *R370S* proteins at the plasma membrane. Thus, it is unlikely that the decrease in functional β -cell mass is related to apoptosis induced by misfolding of the mutant SUR1 protein, as has been suggested for neonatal diabetes-associated insulin gene mutations (24). Kassem et al have previously shown increased β -cell apoptosis in patients with recessive *ABCC8* mutations (21). This apoptosis could be precipitated by increased metabolic stress, due to continuous stimulation of insulin secretion resulting in oxidative stress mediated apoptosis. Alternatively, apoptosis could be initiated by chronically elevated cytoplasmic calcium levels, as have been seen in β -cells of patients with CHI (25). Studies of Kir6.2 and SUR1 knockout mice models yielded variable phenotypes of glucose tolerance ranging from normoglycemia to diabetes, but this experimental model also appears to be associated with increased beta-cell apoptosis and progression from hypoglycemia to diabetes, albeit at a different rate (26-29).

Regardless of the precise mechanism, enhanced apoptosis appears to be a generic response to inactive K_{ATP} channels and not related to the specific mutation. Why then did our patient develop diabetes at such a young age (10.5 years)? It is likely that the obesity and the early puberty present in our case played an important role. Both factors

contribute to insulin resistance that may have unmasked an insulin secretion defect caused by the *ABCC8* mutation. Her constant intake of sweet drinks during childhood may have caused β -cell exhaustion. Furthermore, both parents developed type 2 diabetes during their 5th decade, suggesting the presence of a relatively heavy genetic burden for this disease. However, none of her 7 older siblings developed adolescent or early adult diabetes and adolescent-onset type 2 diabetes is rare in Palestinians despite a high prevalence of severe obesity, making it likely that the *ABCC8* mutation contributed significantly to both the obesity and the diabetic phenotype.

Our *in vitro* findings had significant implications for clinical management. Initially, our patient was treated with insulin. However, the surprising response of the mutated R370S K_{ATP} channel to sulfonylureas *in vitro* suggested the use of these medications for glycemic control. The good response to glibenclamide enabled our patient to be treated with oral medications rather than insulin injections, a significant advantage given her poor compliance. In addition, the partial response of WT:R370S heterozygous channel to diazoxide suggests that this treatment modality might have been effective in our patient, perhaps preventing hypoglycemia and improving the

neurological outcome. In retrospect, adequate treatment with diazoxide might have also prevented the need for constant glucose loading and decreased the risk of obesity, perhaps preventing or delaying the onset of diabetes.

In conclusion, we describe a new *de-novo* mutation in the *ABCC8* gene, in a patient with CHI in infancy followed by overt diabetes in childhood. This clinical phenotype gives another perspective to the crucial role of the SUR1 protein in β -cell function. The expression studies performed on the mutated SUR1 confirmed channel dysfunction and indicated a possible mechanism for dominant CHI. The clinical consideration of atypical diabetes at this young age and the *in-vitro* studies had an important impact on patient management, suggesting the use of oral sulfonylurea treatment which resulted in improved glycemic control. The similarity between the phenotype described here and the phenotype related to *HNF4A* mutations (MODY1) emphasizes the complex interrelationship between hyperinsulinemic hypoglycemia and early-onset hypoinsulinemic diabetes. Our findings suggest a critical role for the *ABCC8* R370S mutation in the early presentation of diabetes in this patient.

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FIGURE LEGENDS

Figure 1.

Clinical studies and trials: A. Continuous Subcutaneous Glucose Monitoring using MiniMed CGMS Sensor[®]. Measurements were made every 10 seconds and averaged over 5 minutes. During 48 hours, hyperglycemia was constant, with no hypoglycemic events. B. Intravenous glucose tolerance test revealing a retained, although insufficient, insulin secretion capacity and failure to restore the euglycemia. C. A therapeutic trial with repaglinide 1 mg before meals. An example of 1 out of 2 consecutive trials: The 2 hour postprandial glucose levels following repaglinide administration decreased from 9.7-10.5 mmol/l to 5-8.3 mmol/l. D. A therapeutic trial with glibenclamide 5 mg twice daily lowered postprandial glucose levels from 8.3-11 mmol/l to 7.2-7.8 mmol/l.

Figure 2.

The R370S mutation: A. Sequence analysis reveals a heterozygous G→C mutation in the SUR1 gene resulting in the substitution of arginine for serine at the 370 amino acid of SUR1 protein. B. Detection of the mutation in the proband, but not in either parents or the healthy sibling, by primer mismatch SUD1 restriction analysis of PCR-amplified genomic DNA. The mutant is digested whereas the wild type is not. Direct sequencing of DNA from both parents failed to reveal any evidence of mosaicism.

Figure 3.

Expression studies: COS cells were untransfected (unt) or transiently transfected with Kir6.2 and SUR1 of WT, R370S, or WT and R370S at 1:1 equal molar cDNA ratio (WT: R370S) and subjected to various expression and functional analyses.

(A) Surface expression level of K_{ATP} channels assessed by chemiluminescence assays. The relative chemiluminescence units of untransfected, WT and R370S samples are 234±8 (n=11), 4788±509 (n=14) and 4736±485 (n=15), respectively. The WT and R370S expression levels are not significantly different ($p>0.5$).

(B) Channel response to metabolic inhibition assessed by ⁸⁶Rb⁺ efflux assays. Untransfected cells exhibited background efflux (<10% in 40 min) whereas cells expressing WT channels exhibited >90% efflux, which was inhibited by 1μM glibenclamide. Cells expressing the R370S mutant had near background flux levels and cells expressing a mixture of WT and R370S showed intermediate flux level between WT and the mutant.

(C) Representative current traces of WT and R370S channels in response to MgADP stimulation obtained by inside-out patch clamp recording. In contrast to WT channels which were activated by ADP, the mutant failed to be stimulated.

(D) Averaged response of channels to MgADP stimulation. The current in 0.1mM ATP or 0.1mM ATP/0.5mM ADP were expressed as percent of that observed in K-INT solution. In 0.1mM ATP/0.5mM ADP, the % current of WT, WT:R370S, and R370S was 64.3±7.9 (n=10), 23.4±4.7(n=6), and 5.4±2.3 (n=7), respectively; the values of both WT:R370S and R370S are significantly lower than that of WT ($p<0.05$ and 0.005 respectively).

(E) and (F) Same as (C) and (D) except that channel response to diazoxide was examined. The percent current in 0.1mM ATP/0.3mM diazoxide for WT, WT:R370S and R370S was 43.0±5.2, 22.8±3.0, and 3.9±1.0, respectively. Again, both the simulated heterozygous and homozygous expression conditions gave significantly less response than WT ($p<0.001$).

(G) Representative current traces showing channel response to 10nM glibenclamide in K-INT. Note the inhibition was irreversible.

(H) Averaged channel response to 10 or 100 nM glibenclamide. The percent current in 10nM glibenclamide was 53.3 ± 6.0 (n=8), 70.6 ± 8.1 (n=4), and 55.4 ± 4.8 (n=5) for WT, WT:R370S and R370S, respectively; these values are not significantly different ($p > 0.1$). In 100nM glibenclamide, the percent current was 34.0 ± 4.3 (n=10), 28.4 ± 5.3 (n=5), and 30.9 ± 4.3 (n=5), respectively; the values are not significantly different ($p > 0.5$).

Figure 1. Clinical Studies and Trials

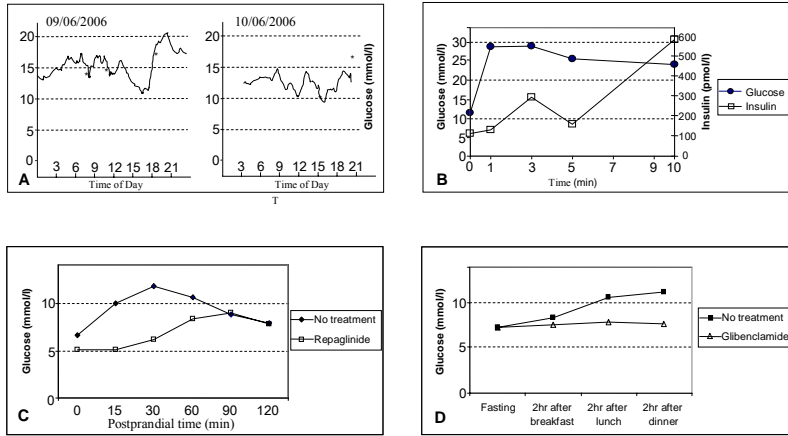


Figure 2. The R370S mutation

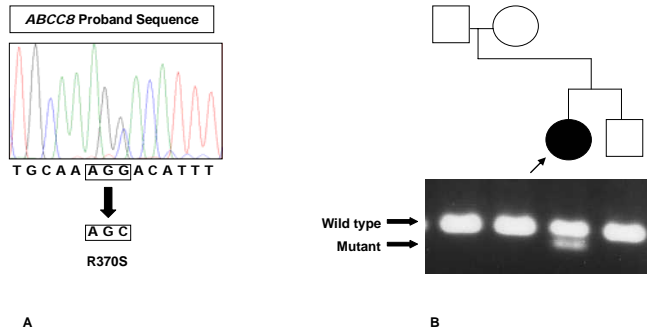


Figure 3. Expression studies on the R370S mutation

