

Relative Expression of a Dominant Mutated *ABCC8* Allele Determines the Clinical Manifestation of Congenital Hyperinsulinism

Ruth Shemer,¹ Carmit Avnon Ziv,² Efrat Laiba,² Qing Zhou,³ Joel Gay,³ Sharona Tunovsky-Babaey,⁴ Show-Ling Shyng,³ Benjamin Glaser,⁴ and David H. Zangen²

Congenital hyperinsulinism (CHI) is most commonly caused by mutations in the β -cell ATP-sensitive K^+ (K_{ATP}) channel genes. Severe CHI was diagnosed in a 1-day-old girl; the mother's cousin and sister had a similar phenotype. *ABCC8* gene sequencing (leukocyte DNA) revealed a heterozygous, exon 37, six-base pair in-frame insertion mutation in the affected patient and aunt but also in her unaffected mother and grandfather. In expression studies using transfected COSm6 cells, mutant sulfonylurea receptor 1 (SUR1) protein was expressed on the cell surface but failed to respond to MgADP even in the heterozygous state. mRNA expression in lymphocytes determined by sequencing cDNA clones and quantifying 6FAM-labeled PCR products found that although the healthy mother predominantly expressed the normal transcript, her affected daughter, carrying the same mutant allele, primarily transcribed the mutant. The methylation pattern of the imprinting control region of chromosome 11p15.5 and *ABCC8* promoter was similar for all family members. In conclusion, differences in transcript expression may determine the clinical phenotype of CHI in this maternally inherited dominant mutation. The use of peripheral lymphocytes as a peripheral window to the β -cell transcription profile can serve in resolving β -cell phenotypes. The severe, dominant-negative nature of the 1508insAS mutation suggests that it affects the functional stoichiometry of SUR1-regulated gating of K_{ATP} channels. *Diabetes* 61:258–263, 2012

Congenital hyperinsulinism (CHI), characterized by dysregulated insulin secretion and hypoglycemia, is most commonly (40–50%) caused by dysfunction of one of the two subunits of the pancreatic ATP-sensitive K^+ (K_{ATP}) channel: the sulfonylurea receptor 1 (SUR1) and the inward-rectifying potassium channel (Kir6.2) (1). Normally, K_{ATP} channels, which are open in unstimulated β -cells, close as the ATP-to-ADP ratio increases, ultimately resulting in insulin exocytosis (2).

Histologically, CHI is either a diffuse disease in which all the pancreatic β -cells are affected or a focal disease in which only some of the β -cells are overactive. Biallelic, recessive

germ line mutations in *ABCC8*, encoding SUR1, or in *KCNJ11*, encoding Kir6.2, lead to diffuse CHI, as do dominant mutations in these genes (3). Focal CHI (4,5) is caused by a paternally inherited K_{ATP} channel gene mutation and a subsequent somatic deletion in the maternal chromosome 11p15-ter in a β -cell precursor. Loss of heterozygosity (LOH) in this region, which includes the K_{ATP} channel genes and imprinted loci involved in controlling cell growth (Fig. 1D) (6), enables the precursor cell, expressing only mutant K_{ATP} channels and paternally imprinted cell growth stimulating genes, to undergo clonal expansion and form the focal lesion.

Here we describe a unique, maternally inherited dominant-negative mutation in *ABCC8* that causes CHI in some but not all family members carrying the mutation. By quantifying allele-specific *ABCC8* mRNA expression in lymphocytes (7), we demonstrate that relative mutated allele expression may predict the clinical phenotype and serve as a peripheral window to the β -cell expression profile.

CASE REPORT

A 1-day-old female (III-2 [individuals are identified by Roman numerals indicating the generation and Arabic numerals indicating the specific individual]) (Fig. 1A), born large for gestational age (4.8 kg) to nonconsanguineous Palestinian parents, developed severe hyperinsulinemic (10.1 mU/L) hypoglycemia (20 mg/dL) without ketonuria (ammonia and lactate were normal). After intravenous glucose infusion (20 mg/kg/min), gastric tube feeding and somatostatin analog (Octreotide; Novartis, Basel, Switzerland) (dose 18 μ g/kg/day) were necessary to achieve stable normoglycemia during 96 h of subcutaneous glucose monitoring (Minimed, Sylmar, CA) and thereafter. At age 2 years, she was gradually weaned off octreotide (<5 μ g/kg/day), but minimal continuous cornstarch intake was still required. The mother's sister (II-6) and paternal cousin (II-2) had similar, albeit milder, clinical presentations. Because a heterozygous six-base pair (bp) in-frame insertion mutation in *ABCC8* exon 37 was found in the cousin (II-2) and her unaffected haploidentical brother (II-3) but not in their mother (I-1) (Fig. 1B and C), it was thought that patient II-2 had focal CHI as a result of paternal uniparental disomy or paternal mutation and LOH (8). Pancreatectomy was not clinically required, and the hypoglycemia resolved spontaneously.

RESEARCH DESIGN AND METHODS

Genetic studies. Leukocyte DNA from family members was sequenced for the relevant segment. For the proband and her father, all *KCNJ11* and *ABCC8* coding exons and the *ABCC8* promoter were sequenced as previously described (9,10).

From the ¹Department of Developmental Biology and Cancer Research, Faculty of Medicine, Hebrew University, Jerusalem, Israel; the ²Division of Pediatric Endocrinology, Department of Pediatrics, Hadassah Hebrew University Medical Center, Jerusalem, Israel; the ³Department of Biochemistry & Molecular Biology, Oregon Health & Science University, Portland, Oregon; and the ⁴Endocrinology and Metabolism Service, Internal Medicine Department, Hadassah Hebrew University Medical Center, Jerusalem, Israel.

Corresponding author: David H. Zangen, zangend@hadassah.org.il.

Received 16 July 2011 and accepted 7 October 2011.

DOI: 10.2337/db11-0984

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db11-0984/-/DC1>.

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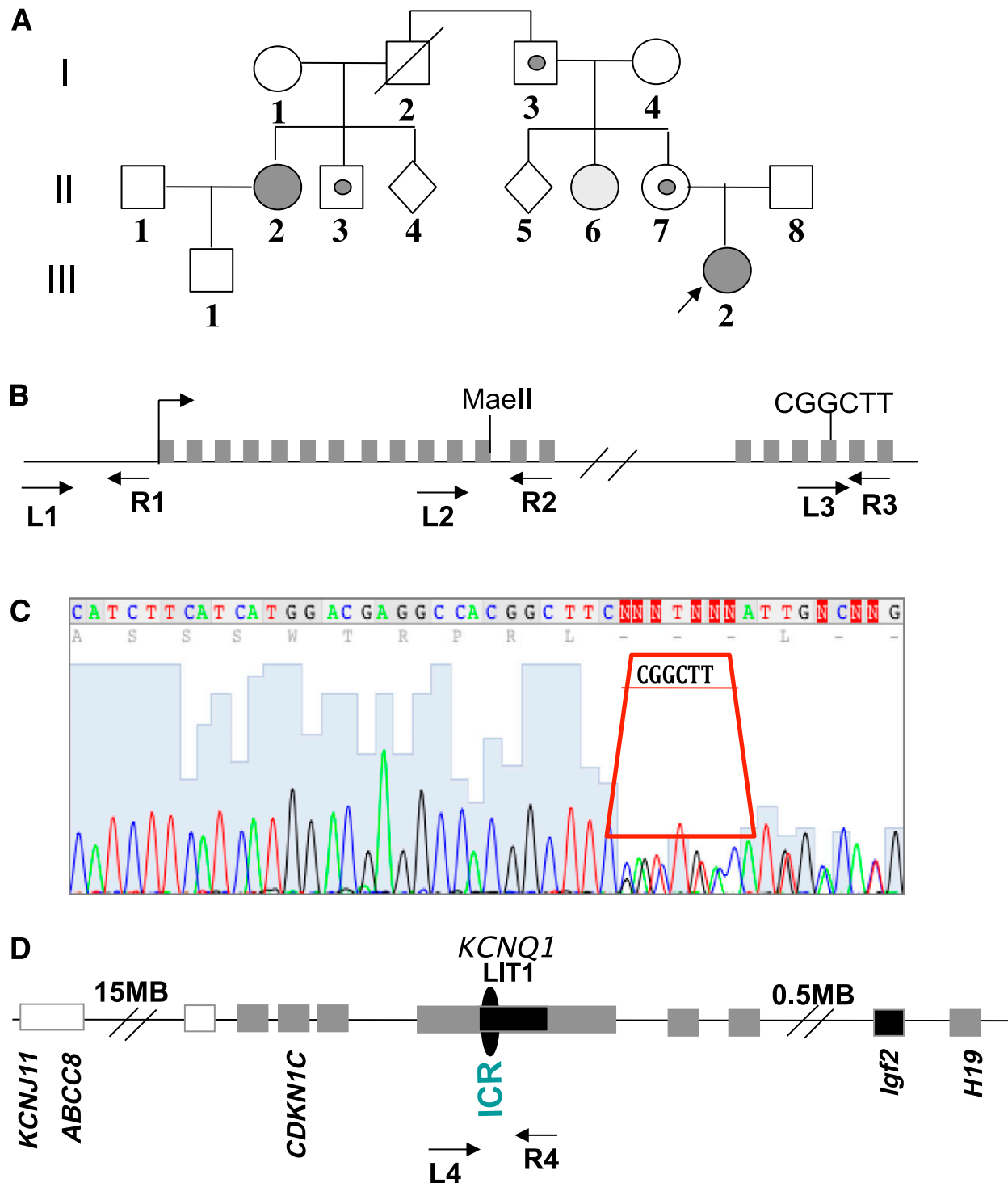


FIG. 1. A: Family pedigree. Circles represent females and squares represent males. Dark gray-filled symbols indicate CHI-affected heterozygotes, the light gray-filled symbol represents heterozygotes with mild phenotype, and symbols with dots represent unaffected heterozygotes. Diamonds represent several healthy male and female siblings. Square with the diagonal slash represents a deceased male. Roman numerals indicate the generation, Arabic numerals indicate the specific individual, and the proband is indicated with an arrow. **B:** A schematic diagram of the *ABCC8* gene. Exons are depicted as boxes and are not drawn to scale. Arrow represents the transcription start site. Exon 12 contains the MaeII restriction polymorphic site. Exon 37 contains the in-frame insertion mutation (1508AS) of six bp (CGGCTT). L1 and R1 indicate the locations of the primers used for bisulfite methylation analysis of the gene promoter region. L2 and R2 indicate the location of the primers used to identify the MaeII polymorphic site and L3 and R3 that of the primers used to identify the mutation. **C:** Sequencing results showing part of exon 37 of the *ABCC8* gene demonstrating the in-frame insertion mutation of six nucleotides (CGGCTT; 1508AS) in the proband's DNA. **D:** A schematic diagram of the imprinted genes located in the chromosome 11p15.5 region. Maternally expressed genes *CDKN1C*, *H19*, and *KCNQ1* are depicted as gray boxes. The paternally expressed gene (*Igf2*) is depicted as a black box. Biallelically expressed genes *KCNJ11* and *ABCC8* are depicted as white boxes. The ICR is located at the *KCNQ1* (*LIT1*) gene sequence. L4 and R4 are primers for the ICR. (A high-quality color representation of this figure is available in the online issue.)

Functional studies. The *ABCC8* insertion mutation (CGGCTT) was introduced into hamster *ABCC8* cDNA in the pECE plasmid using the Quik-Change mutagenesis kit (Stratagene, Santa Clara, CA) and confirmed by DNA sequencing (11). Rat *KCNJ11* cDNA is in the pCDNA1 vector. COSm6 cells were transfected with recombinant SUR1 and Kir6.2 cDNAs using FuGene6 (Promega Corporation, Madison, WI).

Cell surface expression of channels was determined by a chemiluminescence assay, channel activity in intact cells in response to metabolic inhibition (30-min incubation in 2.5 μ M oligomycin and 1 mmol/L 2-deoxy-D-glucose) was assessed using an $^{86}\text{Rb}^+$ efflux assay, and channel response to ATP, MgADP, and diazoxide were monitored using inside-out patch-clamp electrophysiological recordings as previously described (12). Statistical analysis was performed using an independent two-population two-tailed Student *t* test.

mRNA quantitative studies. RNA samples (1 μ g) extracted from two to three different blood samples of each family member using the Tempus Spin RNA Isolation Kit (Applied Biosystems, Foster City, CA) were reverse transcribed.

cDNA samples (and cDNA from normal human β -cells as control) were amplified from the *ABCC8* gene region flanking the mutation (exon 37–39). Purified PCR products were cloned into pGEM vectors, and 20–30 different clones were sequenced for each original RNA sample (pGEM-T Vector System I kit; Promega). Each cDNA sample was also amplified with 6FAM-labeled primer, separated by high-resolution sequencing gel electrophoresis (the mutant was six bp longer), and analyzed using Biosystems Inc. sequencing analyzer.

Bisulfite methylation analysis. After bisulfite treatment (13) of DNA samples, the *KCNQ1* and *ABCC8* promoter regions were PCR amplified. Purified products were cloned into pGEM vectors and sequenced.

Allelic expression of the paternal DNA. cDNA samples of proband and parents were amplified using primers flanking a synonymous single nucleotide polymorphism rs1799857(C/T) in *ABCC8* exon 12. PCR products were digested (MaeII) and separated based on the MaeII restriction site present only on the C allele. The two visible gel bands represent the T allele (186 bp) and the C allele (120 bp). Difference in band intensity was a measure of allelic expression.

Primer sequence and PCR conditions are provided in the Supplementary Data.

RESULTS

Genetic analysis. Increased birth weight, persistent hypoglycemia, and inappropriately high insulin levels suggested CHI. Screening of family members for the exon 37 insertion mutation c4525insCGGCTT, previously identified in an affected maternal cousin, found that the proband (III-2) (Fig. 1A), her affected aunt (II-6), her unaffected mother (II-7), and her reportedly unaffected maternal grandfather (I-3) are also heterozygous for this mutation. This mutation predicts an insertion of AlaSer residues after codon 1508 (Fig. 1B and C).

No other mutations were identified in *ABCC8* or *KCNJ11* exons (or exon/intron boundaries) or in the *ABCC8* promoter in the proband or her father.

Expression studies. To test the mutation functionality, wild-type (WT) *KCNJ11* and mutant *ABCC8* were cotransfected in COSm6 cells. The resulting channels demonstrated nearly normal abundance of the mature and immature forms of SUR1 and ~66 and 87% of normal cell membrane channel expression in the homozygous and heterozygous states, respectively (Fig. 2A and B). $^{86}\text{Rb}^+$ efflux assays showed rapid activation of WT channels, giving rise to >90% cumulative efflux after 40 min of incubation. By contrast, the 1508insAS mutant homozygously expressed in COSm6 cells showed no channel activity (Fig. 2C).

Inside-out patch-clamp recordings revealed that in contrast to the stimulatory effect seen in WT channels, the homozygote mutant channels and the simulated heterozygous channels completely lacked response to either MgADP or diazoxide, indicating a strong dominant-negative effect of the 1508insAS mutation (Fig. 2D and E).

Quantitative analysis of mRNA transcripts. The strong dominant-negative effect of the mutant SUR1 predicted that all carriers should be severely symptomatic. However, four heterozygous individuals were clinically asymptomatic.

To address this unexpected finding, we tested the possibility that the mutant transcript may not be expressed at the same level as the WT transcript in all patients. Lymphocyte-derived cDNAs from five symptomatic and asymptomatic family members were amplified for the fragment of the *ABCC8* gene flanking the mutation site (exon 37–39). PCR products were cloned. Sequencing of >20 clones from each individual showed that although the majority of the clones from the healthy mother (77%) and the healthy maternal grandfather (73%) carried the normal allele, the affected proband showed the opposite, with 66% of the clones being mutated (Fig. 3A). It is interesting that the affected aunt, who had a milder clinical presentation (survived without significant medical therapy) and has been in remission for 10 years with no hypoglycemia, currently expressed 66% normal cDNA alleles. To confirm this finding, the lymphocyte-derived cDNAs were amplified with a 6FAM-labeled primer. Because the mutation is an insertion of six bp, it could be separated by high-resolution sequencing gel electrophoresis. In three independent samples, withdrawn at 12, 18, and 24 months of age (while clinical improvement was taking place), the affected subject predominantly expressed the mutant mRNA (Fig. 3B). In contrast, her unaffected mother showed high relative expression of normal mRNA compared with the mutant mRNA. The aunt with the milder disease, and currently in clinical remission, showed an intermediate expression pattern, similar to the previous cloning studies. The father, who does not carry the mutation, expressed only normal mRNA as expected.

To exclude the possibility of an intronic or promoter mutation affecting expression of the paternally inherited allele, we amplified exon 12 of the parental and patient cDNA, which includes a heterozygous MaeII restriction enzyme polymorphic site. MaeII digestion (Fig. 3C) showed that the father transcribes both alleles at comparable levels. The patient and mother preferentially express one opposing allele (38 and 64%, respectively), confirming the findings of differential expression of the mutation itself. **Bisulfite methylation analysis.** In an effort to explain the differential allelic expression found in our patient and her unaffected family, we searched for epigenetic differences. DNA bisulfite methylation analysis of the *KCNQ1* imprinting control region (ICR) and of the *ABCC8* promoter was performed in lymphocytes of all five family members. The methylation patterns of the *ABCC8* promoter region and the *KCNQ1* ICR (50%) were normal, regardless of the patient's mutation or disease status (Fig. 3D and E) (some data not shown).

DISCUSSION

The unusual finding of a severe, autosomal-dominant *ABCC8* mutation causing CHI in only 50% of the carriers suggested that variable mutant allele expression may determine the disease phenotype. Mutations in the *ABCC8* gene are responsible for 40–50% of CHI cases, focal or diffuse (4,14,15). Previous studies of dominant SUR1 mutations show that mutant channels were expressed on the cell surface, but their response to MgADP was defective; in the heterozygous state, channels had intermediate responses to MgADP (16–18). The mutated protein found in our family, with its predicted two amino acid insertion, is expressed on the cell surface, but the channel does not open in response to MgADP or diazoxide even in the heterozygous state. Thus, to our knowledge, the 1508insAS

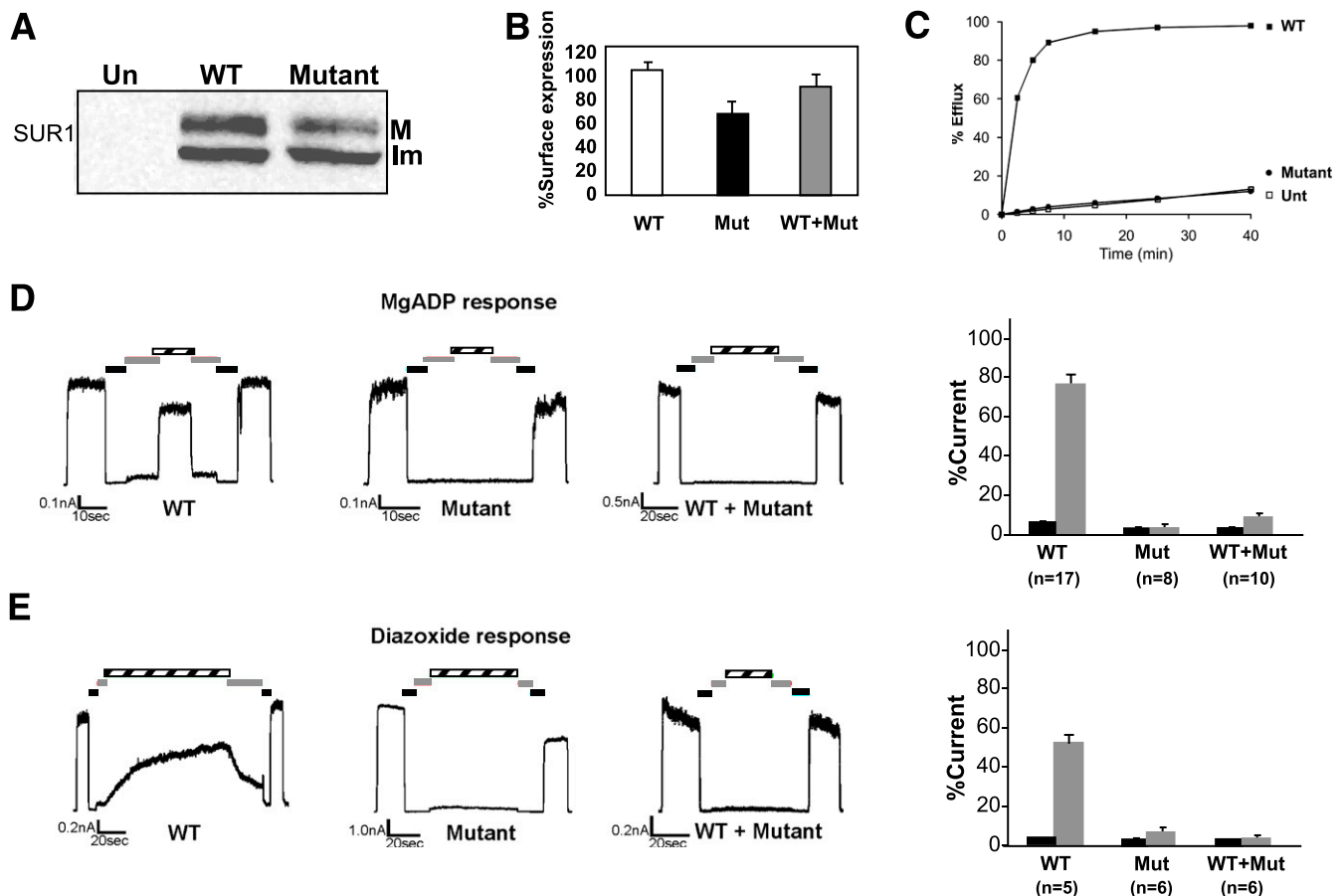


FIG. 2. *A:* Representative Western blot of WT and 1508AS insertion mutant SUR1 coexpressed with Kir6.2 in COSm6 cells. M, mature complex-glycosylated band; Im, immature core-glycosylated band; Un, untransfected COSm6 cells; WT, COSm6 cells transfected with wild type; Mutant, COSm6 cells transfected with the mutant. *B:* Surface expression of K_{ATP} channels assessed by chemiluminescence assays. The relative chemiluminescence signal in percentage of WT, 1508AS insertion mutant (Mut), and heterozygote samples (WT+Mut) are 100 ± 3.5 ($n = 6$), 65.9 ± 3.5 ($n = 6$), and 87 ± 4.8 ($n = 6$), respectively. The expression levels of the mutant in both homozygous and simulated heterozygous states are lower than WT ($P < 0.1$). *C:* Channel response to metabolic inhibition assessed by $^{86}\text{Rb}^+$ efflux assays. Whereas cells expressing WT channels exhibited $>90\%$ efflux, cells expressing the 1508AS insertion mutant had near background flux levels observed in untransfected (Unt) cells ($<10\%$ in 40 min). *D:* Representative current traces of WT and 1508AS insertion mutant channels in response to MgADP stimulation obtained by inside-out patch-clamp recording (left). In contrast to WT channels, which were activated by MgADP, the mutant and the heterozygote expressing both normal and mutant channel failed to be stimulated. Averaged response of channels to MgADP stimulation (right). The current in 0.1 mmol/L ATP (black) or 0.1 mmol/L ATP/0.5 mmol/L ADP (gray) were expressed as percentage of that observed in K-INT solution. In 0.1 mmol/L ATP/0.5 mmol/L ADP, the percent current of WT, 1508AS (μ), and WT: 1508AS (WT+ μ) was 69.60 ± 4.32 ($n = 17$), 3.01 ± 1.96 ($n = 8$), and 7.37 ± 2.23 ($n = 10$), respectively; the values of both 1508AS and WT: 1508AS are significantly lower than that of WT ($P < 0.001$). *E:* Same as (*D*) except that channel response to diazoxide was examined. The percent current in 0.1 mmol/L ATP/0.3 mmol/L diazoxide for WT, 1508AS, and WT: 1508AS was 50.12 ± 4.71 ($n = 5$), 7.31 ± 5.28 ($n = 6$), and 3.03 ± 3.40 ($n = 6$), respectively. Again, both the simulated heterozygous and homozygous expression conditions gave significantly less response than WT ($P < 0.001$). (A high-quality color representation of this figure is available in the online issue.)

mutation is the first CHI mutation exhibiting a nearly complete dominant-negative gating effect. Because the 1510–11 AlaSer residues lie in the interface of the two nucleotide-binding domains (NBDs), duplication of these two residues may disrupt interactions between the NBDs and affect the octameric channel complex during MgADP stimulation.

Initially, the mother's cousin was thought to have focal CHI because only one mutation was found and her haploidentical brother was asymptomatic. In a similar manner, the clinical findings in the aunt, with a paternally inherited mutation, were also consistent with focal CHI. In contrast, our proband, with severe CHI, inherited her mutated allele from her mother, which is not consistent with the two-hit mechanism proposed to cause focal CHI (19,20). Furthermore, three case subjects with focal CHI, usually described as a rare, sporadic event in one family, seemed extremely unlikely (21).

We hypothesized that the marked phenotypic variability in mutation-carrying family members was related to the

relative expression of the mutant allele. Lymphoblastic mRNA was previously used to study the ratio between *HNF1A* mRNA levels and the risk of type 2 diabetes (22); however, lymphoblast cell lines tend to clonalize over time, limiting their use in predicting human pathophysiology (23). Because no pancreatic tissue was available, and given the limitations of lymphoblast-derived studies, we amplified *ABCC8* cDNA fragments derived from lymphocytic *ABCC8* mRNA.

Although our patient consistently expressed mostly the mutant allele in independent mRNA samples, her asymptomatic mother and grandfather primarily expressed the normal transcript. Of interest, the more mildly affected aunt (II-6) (Fig. 1), currently in long remission, had an intermediate expression pattern, suggesting that differential expression of mutated and WT alleles may correlate with disease severity. Given the severe dominant-negative nature of this mutation, the hetero-octameric channel structure and the assumption that mutant and normal SUR1 protein

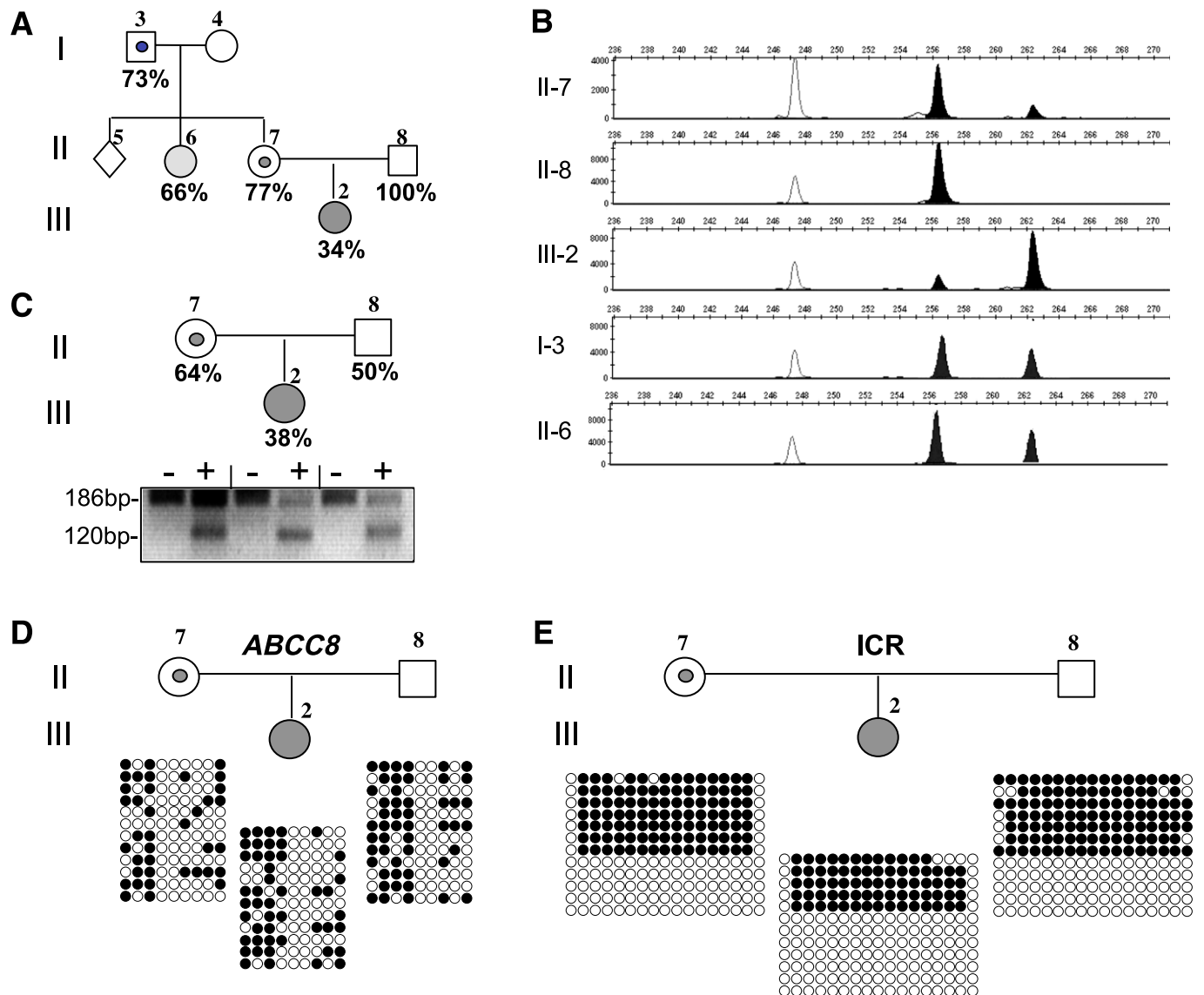


FIG. 3. A: The percentage of clones derived from peripheral blood mRNA samples that express WT alleles out of the total number of clones is shown beneath symbols indicating each individual subject (see RESEARCH DESIGN AND METHODS). For example, of the clones derived from the healthy mother's RNA, 77% contained the normal allele, whereas only 23% contained the mutant allele. In contrast, of the clones derived from her affected daughter, 66% carried the mutant allele and only 34% contained the normal allele. Circles represent females and squares represent males. Dark gray-filled symbols indicate CHI-affected heterozygotes, the light gray-filled symbol represents heterozygotes with mild phenotype, and symbols with dots represent unaffected heterozygotes. **B:** RT-PCR products were separated by high-resolution sequencing gel electrophoresis using 6FAM-labeled primer L3 and primer R3 flanking the mutation (see Fig. 1B). The affected patient (III-2) expressed a relatively high level of mutant mRNA length of 262 compared with normal mRNA length of 256. In contrast, her unaffected mother (II-7) expressed a relatively high level of normal mRNA compared with the mutant mRNA. The father (II-8), who does not carry the mutation, expressed only normal mRNA. The affected aunt (II-6) and unaffected grandfather (I-3) expressed 40 and 26% of the mutant transcript, respectively. **C:** RT-PCR products using primers from a polymorphic site (C/T) in exon 12 of the *ABCC8* gene were (+) digested with MaeII restriction enzyme or (-) not digested. The 186 bp and the 120 bp bands represent the T and C alleles, respectively. The parents and the proband (II-7, -8, and III-2) are heterozygous for the polymorphic site. The relative expression of the undigested (upper) T allele (in percentages, derived from densitometry readings of the bands) is given below the relevant subject in the family tree. DNA methylation pattern of the *ABCC8* promoter region in whole blood cells of the proband and parents (II-7, -8, and III-2) (**D**) and of the *KCNQ1* ICR (LIT1) (**E**). As expected, ~50% of the clones are methylated in the affected proband and unaffected parents (**E**). One row of CpG sites represents an individual clone. ●, methylated CpG site; ○, unmethylated CpG site. (A high-quality color representation of this figure is available in the online issue.)

are randomly recruited into the channels, dysfunction is already expected when one of the four SUR1 molecules is mutated (18). This means that if both the mutant and the normal transcript are equally expressed, the chance of having all four subunits made from the normal transcript is only one-sixteenth (6%). Therefore, if 70% (the normal mother) of the expressed transcript is normal, ~25% of the channels are expected to function normally, enabling normal β -cell function. In marked contrast, if only 30% (our patient) of the transcripts are normal, then only ~1% of the

channels are expected to be normal, abolishing β -cell function. The intermediate expression of the mutant allele in patient II-6 also raises the intriguing possibility that the relative expression of the mutant allele may change over time, thus explaining the clinical remission seen in this patient and in patient II-2. Prospective studies are needed to fully address this question.

In an attempt to understand the mechanism causing interindividual variability in allele expression, we studied two possible epigenetic etiologies. Although *ABCC8* gene's

promoter is known to be a CpG island and, thus, not expected to be regulated by methylation, given its de novo methylation in colon cancer (H. Cedar, unpublished observations), we tested its methylation pattern and that of the adjacent 11p15 ICR (Fig. 1D). Although LOH is thought to be a somatic event restricted to the pancreatic lesion, systemic aberrant methylation of the short arm of chromosome 11 is seen in Wilms tumor and Beckwith-Wiedemann syndrome (24). However, no difference in methylation pattern, either in chromosome 11p15 (ICR) or in the *ABCC8* promoter region, was apparent in any of the family members tested.

Another possibility was that the mutation, located 15 bp from the 37 exon splice donor site, could be variably affected by splice-regulating factors and result in variable splicing and phenotype, as described for cystic fibrosis (25). Splice prediction programs (<http://sroogle/tau/ac/il> and RE-Search) failed to predict any effect on splicing efficiency by the 1508insAS mutation.

In conclusion, we demonstrate that variability in transcript expression may determine the clinical phenotype of CHI. A mechanism that regulates the transcript expression of a mutant allele may protect from or enable a severe phenotype in subjects with the same genotype. It is important that the use of peripheral lymphocytes as a window to the β -cell transcription profile may help explain other unexpected β -cell phenotypes. It may be especially significant given the epigenetic regulation at the telomeric end of chromosome 11p, where several genes of glucose metabolism are encoded. Finally, the unique severe dominant-negative nature of the 1508insAS mutation may provide new insight into the structure-functional relationship of the SUR1 NBD regions on channel gating.

ACKNOWLEDGMENTS

S.-L.S. is supported by National Institutes of Health Grants R01-DK-57699 and R01-DK-66485.

No potential conflicts of interest relevant to this article were reported.

R.S., S.-L.S., B.G., and D.H.Z. researched data and wrote, reviewed, and edited the manuscript. C.A.Z. researched data and wrote the manuscript. E.L., Q.Z., J.G., and S.T.-B. researched data. D.H.Z. is the guarantor of this article.

Parts of this study were presented in abstract form at the 70th Scientific Sessions of the American Diabetes Association, Orlando, Florida, 25–29 June 2010.

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