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ORIGINAL ARTICLE

**Avihu Boneh · Stanley H. Korman · Kenichi Sato
Junko Kanno · Yoichi Matsubara · Israela Lerer
Ziva Ben-Neriah · Shigeo Kure**

A single nucleotide substitution that abolishes the initiator methionine codon of the *GLDC* gene is prevalent among patients with glycine encephalopathy in Jerusalem

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Abstract Glycine encephalopathy (GE) (non-ketotic hyperglycinemia) is an autosomal recessive neurometabolic disease caused by defective activity of the glycine cleavage system. Clinically, patients present usually in the neonatal period with hypotonia, encephalopathy, hiccups and breath arrests with or without overt seizures. GE is considered rare, but its incidence is relatively high in several geographical areas around the world. We report a novel mutation causing GE in six extended Arab families, all from a small suburban village (population 5,000). A methionine to threonine change in the initiation codon of the glycine decarboxylase gene led to markedly reduced glycine decarboxylase mRNA levels and abolished glycine cleavage system activity.

Keywords Glycine encephalopathy · Non-ketotic hyperglycinemia · Glycine decarboxylase · Mutation · Initiation codon

A. Boneh · I. Lerer · Z. Ben-Neriah
Department of Human Genetics,
Hadassah-Hebrew University Medical Centre,
Jerusalem, Israel

S. H. Korman
Department of Clinical Biochemistry,
Hadassah-Hebrew University Medical Centre,
Jerusalem, Israel

K. Sato · J. Kanno · Y. Matsubara · S. Kure
Department of Medical Genetics,
Tohoku University School of Medicine,
Sendai, Japan

A. Boneh (✉)
Metabolic Service, Genetic Health Services Victoria,
The Murdoch Children's Research Institute,
Royal Children's Hospital, Flemington Road,
Melbourne, Victoria, 3052 Australia
E-mail: avihu.boneh@ghsv.org.au
Tel.: +61-3-83416252
Fax: +61-3-83416390

Introduction

Glycine encephalopathy (GE; or non-ketotic hyperglycinemia, OMIM 238300) is an autosomal recessive neurometabolic disease caused by defective activity of the glycine cleavage system (Hamosh and Johnston 2001). This complex is made of four proteins that work in concert in the metabolic process of glycine: a P protein, a pyridoxal-phosphate-containing glycine decarboxylase (GLDC), a T protein, a tetrahydrofolate requiring aminomethyltransferase, an L protein, a lipoamide dehydrogenase and a lipoic-acid-containing H protein. The P protein, T protein and H protein are encoded by the *GLDC*, *AMT* and *GCSH* genes, respectively. Defects in the P protein, the GLDC, are the most common causes of GE and lead to more than 80% of GE cases.

Classical GE typically presents in the first days of life. Assisted ventilation is needed to overcome severe and prolonged apnoeic episodes with or without overt seizures. Babies present also with hypotonia, lethargy and hiccups. Most children who survive the neonatal period are severely disabled. Despite the almost universally similar clinical outcome of the disorder, the number of disease-causing mutations in the genes encoding the P and the T proteins is steadily growing, and a clear genotype–phenotype correlation is not possible at this stage.

The GE is considered rare, but its prevalence is relatively high in several geographical areas around the world (von Wendt et al. 1979; Kure et al. 1998; Applegarth et al. 2000). In the last 15 years, we have been treating several children with GE from six Arab families from a suburban village near Jerusalem. Some of these children have been previously reported (Boneh et al. 1996). We report the finding of a single mutation in the initiation codon of the P protein causing GE in all these families.

Patients and methods

Patients

We analysed DNA samples from 31 individuals from six families, including eight patients (Table 1). The parents of patients in five families were first cousins (Fig. 1). The parents in family 4 were unrelated but originated from families 1 and 2. Five of these patients (families 1 and 3) have been previously reported (Boneh et al. 1996). All patients except one presented with hypotonia and respiratory abnormalities on days 3–4 of life, followed in all patients by apnoea, poor reflexes and, in three patients, hiccups. Seizures were not a prominent feature of their initial clinical presentation, but some patients had seizures later. Patient III:1 and patient III:4, both from family 2, deceased at 14 and 9 years of age, respectively. The patient in family 5 (patient 1), a female, fed poorly and did not wake up for feeds on day 2 of life, and when hospitalised on day 3, she was markedly hypotonic and did not awaken even in response to painful stimuli. Primitive reflexes could not be elicited. Convulsions were observed on day 4. Neither apnoea nor hiccups were noted at any stage. CSF and plasma glycine levels were

Table 1 The *GLDC* M1T mutation in six glycine encephalopathy (GE) families in Jerusalem

Family	Member	Genotype
Family 1	III:2	Heterozygote
	III:4	Heterozygote
	IV:1	Heterozygote
	IV:2	Heterozygote
	IV:3	Heterozygote
	IV:4	Heterozygote
	IV:5	Heterozygote
	V:1	No mutant allele
	V:2	No mutant allele
	V:3 (proband)	Homozygote
	V:5	Homozygote
	V:4	No mutant allele
Family 2	II:1	Heterozygote
	II:2	Heterozygote
	III:1 (proband)	Homozygote
	III:2	Heterozygote
	III:3	Heterozygote
	III:4	Homozygote
	III:6	Heterozygote
	III:7	Heterozygote
	IV:1	Homozygote
Family 3	Father	Heterozygote
	Mother	Heterozygote
	Brother	Heterozygote
	Patient	Not determined
Family 4	Father	Heterozygote
	Mother	Heterozygote
	Patient	Homozygote
Family 5	Father	Heterozygote
	Mother	Heterozygote
	Patient	Homozygote
Family 6	Father	Not determined
	Mother	Not determined
	Patient	Homozygote

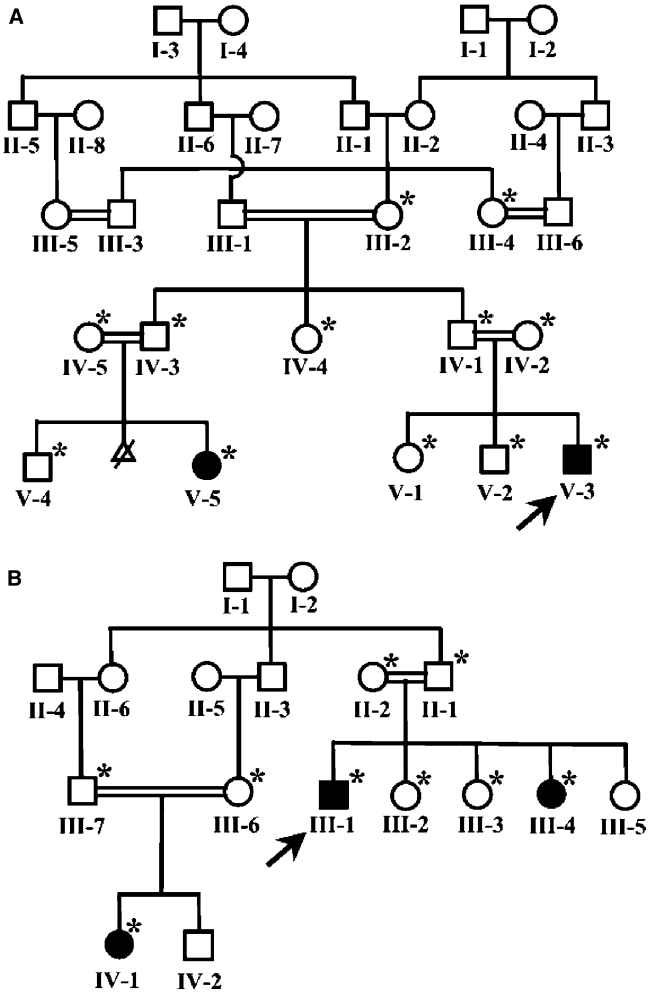

Fig. 1 Family trees of families 1 and 2 are shown. Note consanguinity. *Asterisks* denote individuals from whom DNA was analysed. The probands in both families are marked with an *arrow*

119 and 808 μmol/l, respectively, with a ratio of 0.15. The patient in family 6 (patient 2), a male, was noted to be sleepy, not crying, flaccid and sucking poorly on day 2 of life. On day 3, CSF and plasma glycine levels were 210 and 2,187, respectively, with a ratio of 0.10. He required mechanical ventilation but later regained spontaneous respiration and resumed oral feeding. He has not had seizures. Although he seemed to develop normally in the first months of life, at 18 months of age, he had axial hypotonia, could not walk and said single syllables but no words. He had not had seizures. All other patients were severely disabled.

Sequencing analysis

We have amplified 25 exons of the *GLDC* gene by PCR, as described (Takayanagi et al. 2000), as well as nine exons of the *AMT* gene and five exons of the *GCSH* gene. Exon Amplicons were subjected to 2.5% agarose gel electrophoresis, and bands with the expected size

were cut out for DNA purification. Nucleotide sequences were determined by the dye-terminator cycle sequencing kit (PE biosystems, Foster City, CA, USA) using the PE1F primer as a sequencing primer.

Multiplex RT-PCR analysis

Lymphoblast cell lines were established from two patients: patient II:1 from family 5 (patient 1) and patient I:1 from family 6 (patient 2). Total RNA was prepared from each cell line, and 10 μg of each was used for reverse transcription in 20 μl of mixture, as described (Kure et al. 1992b). GLDC cDNA fragment with 600 bp was amplified with a set of primers GLDC-D1, 5′-TGGCCAAGCGATTAGAAACACACT-3′; GLDC-D2, 5′-TATCAAATGCTCTGGGGAGAGGCAT-3′ while LPT cDNA fragment (772 bp) was amplified by a pair of PCR primers using LPT21F, 5′-AGGCGCCCGCACCACGCCCA-3′; LPT17R, 5′-TCCTGATCCCTTGGTAAGGGCTC-3′. Both PCRs was performed in a single tube (30 μl of PCR mix) using Multiplex PCR Kit (Qiagen, Germany) according to the manufacturer's protocol. A total of 2 μl of reverse transcription mixtures was subjected to multiplex PCR analysis, and 10 μl of PCR products were size-separated on 2.5% agarose gel electrophoresis. The intensity of each band and the ratio of the GLDC/LPT bands were calculated by the NIH Image software.

Assay of the glycine cleavage system activity in lymphoblasts

Enzymatic activity of the glycine cleavage system in lymphobalst cell lines (patients 1 and 2) was determined by the decarboxylation reaction using [1−^{14}C]glycine, as described (Kure et al. 1992a). The assay was performed in triplicate, and means and standard deviations were determined.

Results

Identification of the M1T mutation in GLDC exon 1

We screened the *GLDC*, *AMT* and *GCSH* genes for mutations using DNA obtained from the proband in family 1 (patient V:3). A homozygous single-base substitution from T to C (c.2T > C) was found in exon 1 of the *GLDC* gene. This substitution changes the obligate/consensus initiation ATG codon (encoding methionine) to ACG (normally encoding threonine) and is hence termed the M1T mutation, as shown in Fig. 2. The parents of the proband were heterozygous for this mutation. The M1T mutation was then screened for in all six families with GE from this village and was detected in all of them. The results of the genotyping are shown in Table 1. All patients in these families were homozygous for the M1T mutation while all obligate carriers were heterozygous for the mutation. A total of 122 control alleles from the same population were analysed, and no M1T mutant allele was identified in these DNA samples.

Expression of the mutant transcripts

We considered the possibility that the mutant GLDC mRNA would be unstable by a mechanism of nonsense-mediated mRNA decay because the M1T mutation abolished the initiator codon. To examine the level of the GLDC mRNA expressed in lymphoblasts, we performed a multiplex RT-PCR analysis using *LPT* mRNA as a control. The *LPT* gene encodes a housekeeping enzyme, lipoyl transferase, that generates holo-H-protein by conjugating lipoic acid to apo-H-protein (Fujiwara et al. 1999). Both cDNA fragments, derived from *LPT* and *GLDC* genes, were successfully amplified in a single reaction mixture (Fig. 3). Ratios of cDNA band intensity (GLDC/LPT) were calculated to be 0.95, 1.12, 0.14 and 0.08 in control 1, control 2, patient 1 and patient 2 lymphoblast cell lines, respectively, indicating that GLDC mRNA levels were markedly reduced in the tested patients' cell lines.

Glycine cleavage system activity in lymphoblasts

Glycine decarboxylation activity of lymphoblasts was 0.1 ± 0.20 and 0 ± 0.15 (normal range 0.61–16) nmole of

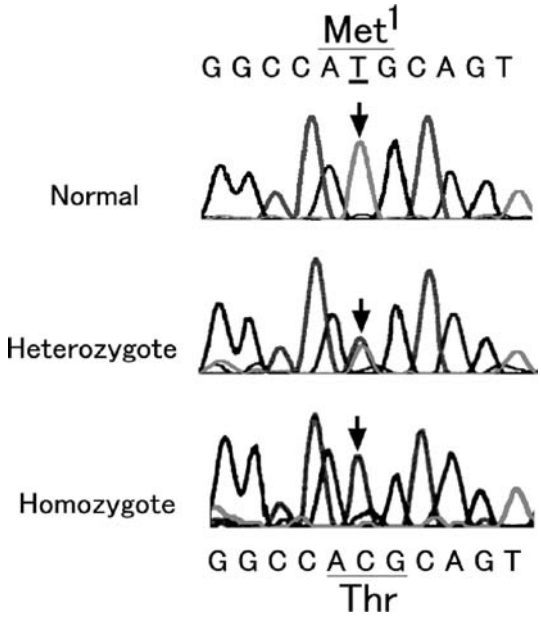

Fig. 2 Sequencing chromatogram of the M1T mutation. PCR fragments containing *GLDC* exon 1 were amplified from genomic DNA from a control subject, an obligate heterozygote and a patient, and was sequenced by the dye-terminator method. The M1T mutation sites are indicated by *arrows*

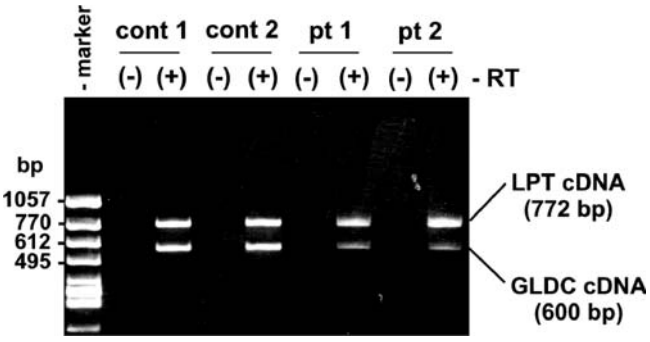

Fig. 3 Multiplex RT-PCR analysis of GLDC mRNA expressed in patients' lymphoblasts. Total RNA samples were prepared from two control subjects (*cont 1 and 2*) and two patients (*pt 1 and 2*) and subjected to reverse transcription and multiplex PCR (*RT + lanes*). As a negative control, the same amount of total RNA was used for multiplex PCR amplification without reverse transcription (*RT – lanes*)

$^{14}CO_2$-formed/mg protein/h in patients 1 and 2 lymphoblast cells, respectively, indicating that the enzyme activity was completely abolished in these patients.

Discussion

GE is a rare disorder, but its exact world-wide incidence is not known. A high incidence of GE has been reported in certain ethnic groups and geographical locations. In Finland, the incidence of GE is 1 in 55,000 new-born babies, but it is even higher in northern Finland, with an incidence of 1 in 12,000 (von Wendt et al. 1979). The incidence of GE in British Columbia, Canada, is 1 in 63,000, also probably higher than the expected world-wide incidence (Applegarth et al. 2000). A high incidence of GE has been previously reported in an Arab village in Israel (Kure et al. 1998). Moreover, single mutations have been reported as a cause of GE in some of these populations. Thus, a single mutation, Ser564Ile, has been found to be the cause of the high incidence of GE in Finland (Kure et al. 1992b). Likewise, a single mutation in the T protein, His42Arg, was found to be the cause of the high incidence in the Arab village in northern Israel (Kure et al. 1998).

The current report adds to the list of single mutations leading to a high incidence of GE in isolated populations. A single novel mutation caused GE in eight patients (and one deceased proband from whom no DNA was available for analysis) from six families, all from a small geographical area near Jerusalem. The inhabitants of this suburban village (approximately 5,000 people) live as large kindreds, as is common to many communities in this geographical area (Jaber et al. 2000; Zlotogora et al. 1996) (for example, see Fig. 1). Consanguinity in this population is common and has been estimated at ~33% of all marriages (Jaber et al. 2000). Interestingly, DNA analysis of another family from a different area near Jerusalem revealed another causative mutation (Korman et al. 2004). Thus, based on the theoretical calculations presented previously (Zlotogora et al. 1996), it may be surmised that the mutation reported here occurred around five generations ago.

Mutations affecting the ATG start codon have not been widely reported. In theory, there are several potential consequences of such mutations, ranging from a near-normal protein product to a complete absence of the protein or a truncated protein, the extent of which being dependent on the location of the next ATG sequence.

It has been suggested that initiation codons are recognised most efficiently when they are located in a favourable context, as defined by the Kozak consensus sequence ACCatgG (Kozak 1986). Different permutations of non-ATG codons have varying capacities to substitute as initiation codons, but their efficiency is questionable. Indeed, our results indicate that the mRNA level was reduced in the patients' cell lines.

The mechanism by which a moderate reduction of the level of the mutant mRNA leads to severe dysfunction of the gene product remains unknown. Given that our patients had null GCS activity in the enzymatic analysis, one would have expected the level of the GLDC mRNA to be very low or negligible if one would attribute the null activity to the level of the GLDC mRNA only. However, although the level of GLDC mRNA in the RT-PCR analysis was markedly reduced, it was not negligible, suggesting that the reduction of the GLDC mRNA was not the primary or only cause of null enzymatic activity. One possible explanation for this finding is that lack of recognition of the first initiator methionine by the translation complex may lead to instability of the mutant GLDC mRNA. Further analysis using in vitro expression of mutant GLDC RNA is required to elucidate this possibility.

The other possible mechanism relates to the targeting of the gene product to the mitochondria. The next ATG codon in the GLDC gene at which translation could initiate is located 231 nucleotides downstream. It is situated in-frame in a partially favourable context (GAGAGatgCT). Use of this codon for initiation of translation would result in deletion of 77 amino acid residues with loss of the mitochondrial leader peptide. The GLDC is a component of the multi-enzyme system that operates in mitochondria in concert with three other GCS components. Thus, it may be predicted that as a result of the M1T mutation, which abolished the initiator methionine and could be expected to lead to the truncation of the mitochondrial leader peptide of the GLDC precursor, the mutant GLDC would not enter into the mitochondria nor interact with other GCS components. This would render the protein product non-functional and may provide another explanation to the null GCS activity in the lymphoblast cell line and severe symptoms of the homozygous patients.

An initiation codon mutation was also reported in the ornithine delta aminotransferase, causing gyrate atrophy

of the choroid and retina (Mitchell et al. 1988). As in our patients, the protein product of the mutated gene was expected to truncate 138 amino acids of the ornithine amino transferase enzyme, thus eliminating the entire mitochondrial leader sequence and abolishing enzyme activity (Mitchell et al. 1988). Likewise, Frank et al. (1999) reported two mutations in the initiation codon of the protoporphyrinogen oxidase gene, leading to variegate porphyria due to a non-functional protein. By contrast, a mutation in the translation initiation codon of the mitochondrial acetoacetyl-CoA thiolase gene did not affect the gene-product mRNA levels and did not completely abolish enzyme activity, yielding a residual activity of 15% (Fukao et al. 2003). Cremonesi et al. (2004) have reported a mutation in the ATG initiation codon of the L-ferritin gene that disabled protein expression but did not cause iron deposition in the brain and did not lead to any haematological or neurological symptoms. Moreover, mutations in the ATG initiation codon have also been shown to be markers of common haplotypes. For example, the mitochondrial T3308C mutation in the initiation codon of the NADH-dehydrogenase subunit 1 seems to be an ancient marker of a common West African haplogroup (Rocha et al. 1999). Likewise, in a large phylogeographic analysis of mitochondrial DNA haplogroups in China, the T12338C mutation in the initiation codon of the NADH-dehydrogenase subunit 5 was shown to be a common polymorphic change in various populations across China (Kong et al. 2004). It seems, therefore, that mutations in the translation initiation codon do not necessarily cause a severe deficiency of the gene product, and the effects of these mutations need to be evaluated specifically for each gene, as previously suggested (Fukao et al. 2003).

In summary, we have found a novel mutation, M1T, in the initiation codon of the glycine carboxylase gene, leading to reduced RNA levels, abolished enzyme activity and clinical evidence of GE in several families from a small geographical area. The identification of a common mutation in this population is of significance in that it will enable fast, easy and reliable genetic screening and prenatal diagnosis for interested couples in this community in the future.

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