Five cases of isolated glycerol kinase deficiency, including two families: failure to find genotype:phenotype correlation

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Abstract

Little is understood of the genotype/phenotype correlations in X linked glycerol kinase deficiency (GKD) where most cases are caused by extensive deletions of Xp21, which often include genes flanking the GK locus. Few cases of isolated GKD have been investigated where the phenotype is not influenced by neighbouring genes. In this paper, we present the mutation data from four confirmed and one suspected case of non-deletion, isolated, X linked GKD and therefore extend the base of patients that can allow an assessment of genotype/phenotype correlations for this disease. The mutations found were two terminations leading to premature truncation of the GK polypeptide chain, one insertion, and an amino acid substitution. Phenotypic variation was observed in two families, where there was more than one affected subject carrying the same mutation, confirming previous studies that suggest there is no correlation between disease severity and genotype. Furthermore, the nature of the mutation in different families does not appear to influence the spectrum of phenotypic variation. In addition, one coding polymorphism in exon 3 has been found. The characterisation of the gene structure has been completed and shows that instead of 19 exons, there are 21 exons. (J Med Genet 2000;37:434–441)

Keywords: glycerol kinase deficiency; genotype/phenotype correlation

Glycerol kinase deficiency (GKD) is an X linked disorder resulting from isolation or mutation of the gene for the enzyme glycerol kinase (E.C.2.7.1.30). Normally, the enzyme catalyses the phosphorylation of dietary glycerol to glycerol-3-phosphate, which is used in the synthesis of lipids. At the mitochondrial outer membrane, competition between glycerol kinase and hexokinase is believed to be important for the regulation of the balance between carbohydrate and lipid metabolism. Loss of enzyme activity is characterised by a raised level of free glycerol in both the urine (hyperglycerolaemia) and the serum (hypoglycaemic coma) of an affected person. The severity of the disease is highly variable; the severe infantile form results from a contiguous gene deletion syndrome involving loss of the gene for glycerol kinase (GK) with all or part of the genes for Duchenne muscular dystrophy (DMD) or congenital adrenal hypoplasia (AHC) or both. In contrast, isolated GKD is not associated with AHC or DMD, and can manifest as an adult, benign form or a juvenile form. The juvenile form shows phenotypic variation both between subjects from different families and within families. In some cases it can be similar in the degree of severity to the infantile form. This has led to the proposal that genetic background and environmental factors have important modifying influences.

The gene for GKD is one of a family that includes intronless testis specific autosomal copies. The X linked gene is the only member of the family to have introns. In total, there are 21 exons, two of which are subject to alternative splicing, and a common transcript size of 2 kb in somatic tissues. In this study we have looked at the cause of isolated GKD in four confirmed non-deletion families and a single suspected case. All patients and families were referred for biochemical analysis following episodes of hypoglycaemic coma, with seizures also occurring in two subjects. On confirmation of the diagnosis, molecular techniques were used to define the types of mutation present. RT-PCR was used to sequence the whole gene product of two patients. The remaining cases were investigated using a combination of exon specific amplification, SSCP analysis, and sequencing. The data have been combined with a survey of other published cases to look at the distribution of mutations across the gene structure. This confirms that each represents a novel, family specific mutation. One polymorphism in exon 3 has also been defined.

Materials and methods

PATIENTS

Patients 1 and 2 are non-identical twin boys. One brother showed no apparent symptoms, whereas the second was diagnosed after several seizures. Patients 3 and 4 were both referred for biochemical analysis after suffering episodes of hypoglycaemic coma following periods of illness/fasting. These four cases were biochemically confirmed with glycerol kinase deficiency in the absence of raised creatine kinase activity or adrenal malfunction. These results suggested that the patients had isolated glycerol kinase deficiency with no large associated deletions of the X chromosome. Patient 4 had reduced levels of GK enzyme activity in skin cell cultures. Southern blot hybridisation experiments, previously described for cases 1, 2, and 3, established that the GK gene was not deleted.

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Glycerol kinase mutations determine the gene structure are delineated beneath the schematic.

Given below in kb, based on sizes of PCR products between exons. Genomic clones (cosmids, PACs, and a YAC) used to three signals have been sequenced and are included in the annotation to GenBank entry X78211. The intron sizes are initiating atg. The exact size of exon 19 is undetermined, as there are three potential polyadenylation signals. Two of the positions by the Vs. The numbers of bases coding are shown above the exons. Exon 1 has 114 bases of 5'UTR before the

Figure 1 Schematic of the Xp glycerol kinase gene structure. Exons are represented by the numbered boxes and intron tre. An additional phenol:chloroform extrac-

protocol supplied by the HGMP resource cen-

pared from the PAC clones using the standard

cosmid clones. Two PAC clones were isolated

in the middle of the genomic structure and was previously determined to lie approximately

PCR AMPLIFICATION OF EXONS

Primer pairs for exon amplification were as previously described7 except for exon 1 (primers GK33, ctc gtc acc tgc ccc ccc c, with GK26B, aca gcc acc ccc ccc gtc ccc cc g), exon 8A (primers GKE8AA, aat agt tca aca gga tgg tta g, with GKE8AB, aaa gcc caa agc aca tca cag t), exon 9 (primers GKE9A, tgc ttt ctt tgt ctt gtc tgc, with GKE9B, gca ctc tga act aag agg), exon 9A (primers GKE9AA, aat atc cta tgg ctc tt ctc taa, with GKE9C, taa gca cca aat aga aaa a), exon 10 (primers GKE10A, ttg ctc tct tac ttc tat ttc tcc ct, with GKE10B, tta aac cct gat aag gat tga g), exon 11 (primers GKE11A, atc tta ttt ccc act act g, with GKE11B), exon 17 (primers GKE17A, with GKE17C, gat gaa ttc tgg cct tgc t), exon 18 (primers GKE18AA, aat agt ttc aca gga tgg cgt cat g, with GKE18AB, aat gcc caa agc aca tca cag t), exon 19 (primers GKE19A2, tgc ttt ccc acct ttg atg gta, with GKE19R, gaa gat atg gag tgc gca aat gaga ggg). PCR reactions were carried out in the presence of 250 mmol/l primers, 200 mmol/l dNTPs, and 1 × buffer (10 mmol/l Tris-HCl, pH 9.0, 1.5 mmol/l MgCl2, 50 mmol/l KCl, 1% Triton X-100, and 0.1% gelatin) with 0.5 units of “Super Taq” Taq polymerase (HT Biotechnol-

ogy Ltd, Cambridge, UK). Each 50 µl reaction contained 100 ng of total human DNA. Cycling conditions for all PCRs were 95°C for 2.5 minutes followed by 35 cycles of 95°C for 20 seconds, Tanneal for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The Tanneal used for exon 19 primers was 57°C, and for all otherprimer pairs it was 55°C.

The sequence from intron 3 was amplified using primers ISF (atg tgt tgg ctt tct gga cc) and ISR (aag caa aag ctt ggt cct tg). PCR reactions were carried out as above, using an annealing temperature of 58°C.

PCR products were purified before SSCP or sequence analysis using the high pure PCR product purification kit from Boehringer Man-

nheim GmbH.

RT-PCR

RNA was prepared from cultured fibroblast lines (10⁶ to 10⁷ cells) using Tri-reagent (Sigma).

or altered in the DNA from any of these patients. Cell lines were established for all four cases.

Patient 5 presented at 2 years of age, when he was found in a hypoglycaemic coma after a 17 hour fast. There was no significant past history apart from a tendency to be sweaty and irritable before breakfast. He responded rapidly to intravenous glucose. During the coma his blood sugar was 1.1 mmol/l, serum bicarbonate was low (13 mmol/l), urinary organic acid screening showed very high glycerol levels, and serum triglyceride levels were very high (8.8 mmol/l). Glycerol kinase deficiency was later confirmed by finding very high levels of plasma glycerol (4.6 mmol/l) and a glycerol kinase enzyme activity of only 12% in skin fibroblasts. Complex glycerol kinase deficiency was ruled out by finding normal creatine kinase levels, normal adrenal function, and normal karyo-

type. A cell line was established for this patient. Nine other family members were subsequently assessed. None of them had any symptoms suggestive of GK deficiency but the proband’s maternal grandfather (II.1) and great uncle (II.3) had very high plasma triglycerides and plasma and urinary glycerol levels. They also had raised cholesterol levels, and II.1 has since died shortly after birth. Raised urinary glycerol levels just above the upper limit of normal. DNA from all nine family members was made available for investigation.

Patient 6 was the offspring of a consanguin-

eous marriage, suffered neonatal seizures, and died shortly after birth. Raised urinary glycerol was suspected and DNA was sent for further investigation of the glycerol kinase gene. No cell line was established for this patient.

SCREENING THE PAC LIBRARY FOR GENOMIC CLONES

The RPCI1 library was screened by PCR using primers for glycerol kinase exon 7. This exon was previously determined to lie approximately in the middle of the genomic structure and was not represented in the previously isolated cosmid clones. Two PAC clones were isolated by this method, 7e22 and 2l17. DNA was prepared from cultured fibroblast lines (10⁶ to 10⁷ cells) using Tri-reagent (Sigma). RNA was prepared from cultured fibroblast lines (10⁶ to 10⁷ cells) using Tri-reagent (Sigma).
The RNA was treated with Rnase free DNase before first strand cDNA synthesis using the Promega Reverse Transcription System. The cDNA was used in subsequent PCR reactions with intronic primers (GK exon 7) and GAPDH primers to test for DNA contamination and for cDNA quality respectively. The 5' portion of the GK transcript was amplified using primers GK33 with GK30, and the 3' portion was amplified using primers GK29 with GK15.7 PCR reactions were carried out using 1 µl of RT product in 20 µl for 35 to 40 cycles at an annealing temperature of 55°C.

SSCP ANALYSIS OF EXONS
Aliquots (3 µl) of PCR product for each exon were denatured in formamide loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), snap cooled on ice, then run on 8% acrylamide, 10% glycerol, 0.5 × TBE buffer.

Figure 2. Analysis of patient 5 and his extended family. (A) Sequence difference between patient 5 (P) and a normal control (N), on the complementary strand of exon 9A. (B) TspRII digest of PCR product for exon 9A, confirming the inheritance of the normal (lower) and abnormal (upper) bands in two male sibs from a heterozygous mother. An unrelated subject is shown (N) and the marker is a 1 kb ladder. (C) Extended pedigree. Living subjects shown have been tested at the DNA level and biochemically, as indicated in the key.
on 8 × 10 cm minigels. Gels were run at 4°C in 0.5 × TBE at 1 W for three to four hours. Bands were visualised by silver staining.10

DNA SEQUENCING

Exons were sequenced with radioactive 33P using the Amersham Thermo Sequenase radio-labelled terminator cycle sequencing kit and run on standard denaturing gels in glycerol tolerant buffer supplied by the manufacturer, or by fluorescent labelling using the Amersham Pharmacia Thermo Sequenase dye terminator cycle sequencing kit. Fluorescent sequences were separated on an ABI 373A DNA sequencer.

Results

REFINEMENT OF THE GENE STRUCTURE

Previous analysis of the glycerol kinase deficiency gene on the X chromosome has shown that it consists of at least 19 exons.7 Isolation of genomic clones (PAC 7-e-22 and PAC 2-l-17) containing the entire gene from the RPCI1 PAC library11 has permitted the completion of the structure. This has shown that exon 9 is in fact two separate exons of 36 bp and 68 bp with an intron between nucleotides 879 and 880 (EMBL entry X78211) (as also found by Sjarif et al5) and that the originally published boundary between exons 10 and 11 was incorrectly assigned owing to the presence of a coligation within the 5' portion of exon 11 in cosmid clone 2110. The correct boundary has been established between nucleotides 990 and 991. Exon 10 is thus 43 bp and exon 11 is 81 bp. An additional 18 bp exon described in a fetal brain transcript,6 which lies between exons 8 and 9, and could not be mapped in an Xp derived YAC clone,7 has subsequently been observed as a genuine exon in RT-PCR products from patient cell lines (study).4 Furthermore, an equivalent 18 bp exon has been observed in transcripts from fetal mouse brain.12 The position and sequence of this exon was confirmed from the PAC genomic clones. Thus the total number of exons is 21. The gene structure is shown schematically in fig 1.

ANALYSIS OF PATIENTS WITH ISOLATED GKD

Cell lines were available for five subjects (patients 1, 2, 3, 4, and 5) from four independent families, all with confirmed GKD. DNA only was obtained for a sixth patient with suspected GKD. Patients 1, 2, and 3 have been described previously, and are known to show no major deletions or rearrangements of the GKD locus.6 The remaining patients were referred as isolated cases of GKD following biochemical assessment of enzyme activity.

Initially RNA was prepared from all the cell lines, to amplify the GK transcript by RT-PCR. Samples from patients 4 and 5 only gave prod-

Figure 3  Analysis of patient 4 by RT-PCR and sequencing. (A) Results of two RT-PCR experiments (lanes RT1 and RT2), with the three bands from the patient (a, b, c) compared with the single band in the normal control (lane C). (B) Sequence of the genomic fragment from intron 3 shows the creation of the splice junction on the left, and an internal polymorphism specific to the patient RT-PCR product is on the right, confirming the origin of the genomic PCR fragment. (C) Protein translations for the RT-PCR products, with early termination for the abnormal transcripts. The insertion is shown in italics.
ucts. There appears to be a genuine deficit of the GK transcript in the other patient samples, as RT-PCR controls with GK primers using normal subjects, or controls on the patient cDNA with primers for other expressed genes give the expected PCR products.

Sequencing of the cDNA PCR product from patient 5 showed a T to C base substitution at the first nucleotide of exon 9A (nucleotide position 880 on EMBL entry X78211) that results in C256R (GTG to GCG) (fig 2A). This was confirmed by genomic PCR and sequence analysis. The base change also destroys a TspRI site in the genomic DNA (fig 2B), offering an independent mode of assessment for this mutation. The RFLP was not seen in a screen of 40 other X chromosomes from normal subjects. Three generations of the proband’s family were tested by both methods. The base change was found in the two other males who had the benign form of the condition; the grandfather (II.1) and the great uncle (II.3), but not in the males at a 50% prior risk, who all had normal glycerol levels. The three female obligate carriers were heterozygous for the mutation, as expected (fig 2C). As in a previous study of patients with isolated GKD, the cDNA prepared from

Figure 4 (A) Mutation analysis in patients 1 and 2. The sequence of the positive strand of exon 14 shows the codon change from CAA in the normal control (N) to TAA in the patient (P) (only one brother is shown). (B) Patient 3 (P) is compared with a control (N) on the complementary strand for exon 14. The T to A highlighted represents a change in the codon from AGA to TGA. (C) Results for the family of patient 6 sequenced on the complementary strand of exon 3. His mother is heterozygous for the base at position 279 (T+C), his father has the same sequence as that previously published (C), and patient 6 has the T. (D) The presence of the alternative codon is confirmed by digestion with AvaII. The marker (M) is a 1 kb ladder. Additional lanes show two unrelated female controls (F1 and F2).
patient 5 contained a mixture of alternative transcripts with and without the 18 bp insertion (exon 8A) originally described in a fetal brain clone. The two forms occurred in approximately equal amounts. Most of the transcripts also contained the other alternatively spliced exon, exon 18, as the RT-PCR product was of the appropriate size for the larger cDNA, and this was the major sequence ladder observed by direct sequencing.

RT-PCR product representing the 5' portion of the GK transcript from patient 4 appeared to be abnormal as it gave three bands (fig 3A). Each PCR product was purified and reamplified with nested primers (GK31 with GK29) before sequencing. The smallest band represented a normal transcript with exons 1 to 7. The middle band was identical to exon 1 and exon 2, a unique sequence of 212 bp replaced exon 3, and exons 4 to 7 were normal. The largest band matched exons 1 to 7, with the same unique sequence this time inserted between exons 3 and 4. Comparison with sequences deposited from a Xp22 contig (AC005913) shows that the additional 212 bp insertion were used to amplify the intronic sequence and confirm a mutation which creates a new splice site, as well as a polymorphism within the inserted sequence (fig 3B).

Genomic PCR products for individual exons were prepared from patients 1, 2, 3, and 6. A preliminary screen by SSCP was carried out for the first four sets of samples with exons 2, 3, 4, 5, 6, 7, 8, 11, 12, 13, 14, 15, 16, 17, and 18. This analysis gave reproducible band shifts for patient 1 and patient 2 in exon 14, for patient 3 in exon 11, and for patient 6 in exon 8. Sequencing of exon 14 from patients 1 and 2 showed the presence of a premature termination codon Q403X (C1321T) (fig 4A). Similarly, patient 3 was found to have a transversion in exon 11, which also introduced a stop codon (A1042T, R310X) (fig 4B). No other changes were detected in these patients after sequencing ~50% of the GK gene at the genomic level. The band shift seen in exon 8 for patient 6 arises from a base change in the 3' flanking intron (IVS8+17 C to A) and probably represents a polymorphism within a region of 59 bp consisting predominantly of poly A (GenBank entry AJ252557). Therefore, the remainder of the gene was systematically sequenced. The only alteration in the coding sequence is silent. This is a transition in the third base of codon 55 (CAG to CAA) in exon 3 (fig 4C), which is heterozygous in the mother of the patient. This latter change removes a restriction site for AvaII (fig 4D). Potentially, such silent base changes can alter exonic splicing elements, but in a screen of 40 X chromosomes, five were found with the CAA codon (not shown). As it is unlikely that such a high frequency of mutation would exist within a normal population, this suggests that the nucleotide change represents a polymorphism.

**Discussion**

The C256R change seen in the DNA from patient 5 is likely to represent the true mutation in this family, firstly because it segregates with
the biochemically affected males, and secondly it alters a highly conserved amino acid. The residue at this position is always cysteine in the human testis specific genes, the X linked gene of other mammals (rat and mouse), and the putative glycerol kinase genes of *S cerevisiae* and *C elegans*. In *Mycoplasma genitalium* it is valine and in the bacterial glycerol kinase genes it is also non-polar (either I or A). Similarly, the equivalent position in the xylulose kinases for *E coli* and *H influenzae* is occupied by a glycine. This amino acid lies close to D259, the residue equivalent to D245 in *E coli* glycerol kinase, which forms part of the glycerol binding pocket at the active site of the bacterial enzyme. The introduction of a charge at this position may be disrupting the three dimensional organisation of the enzyme active site, resulting in a decreased level of enzyme activity.

With patient 4, no exonic mutations were evident, but the pattern of cDNA species observed following RT-PCR is consistent with a mutation in intron 3 creating an additional splice junction, and this has subsequently been confirmed. The introduction of the novel exon causes premature termination of the gene product by introducing a frameshift into the ORFs of both abnormally sized transcripts (fig 3C). In this case it is unclear whether the disease arises as the result of simple haploinsufficiency or owing to interference by the alternative products. In bacteria, it is known that GK can be present as part of a protein complex and may also form homodimers or homotetramers. The rat glycerol kinase protein was initially isolated as a dimer and reported as an ATP dependent transporter of activated glucocorticoid receptor complexes. Currently, the detail of potential sites of interaction within mammalian GK is unknown. In the familial cases 1 and 2, and patient 3, the GK gene mutations were shown to be terminations occurring within the first two thirds of the exonic structure. This may explain the failure of RT-PCR, as other gene transcripts have been found to be subject to a high rate of degradation when similarly mutated.

No evidence for gene mutation was found in the genomic DNA of patient 6, who was referred as a suspected, rather than a confirmed, case. However, a polymorphism in exon 3 was detected. Additional differences were confined to intronic sequences and are not predicted to be deleterious.

With this study, the total number of independent cases of GKD that have been analysed at the nucleotide level is brought to 11. The distribution and the nature of the mutations are illustrated in fig 5. There is no obvious clustering within the gene and no single type of mutation appears to be more common (two splice site, two partial deletions, three terminations, and four missense mutations). All the missense mutations are at highly conserved residues. Patient 5 has been discussed above. The other three residues involved in substitutions, T278, D440, and W503, all occur in blocks of amino acids which show absolute identity between known glycerol kinases, including many from bacteria. Furthermore, the equivalent regions of bacterial gluconokinases and xylulokinases are conserved at these residues. In the cases of splice site mutations, deletions, and terminations, all result in premature termination of the protein, with or without novel C-terminal sequences.

As has been stated previously, each family has a unique mutation, with no correlation between the genotype and the observed phenotype. Indeed, the severity of the disease is highly variable within a single family, between subjects of the same generation and across generations. This has several implications for diagnostic purposes. Firstly, it is not possible to predict the severity of the disease in potential affected family members. This means that prenatal diagnosis is not straightforward in isolated GKD, but assessment of the neonate to determine whether dietary modification is required is important for disease management. Secondly, where possible, the grandparental origin of the mutation is a useful indicator to allow identification of extended family members who are at risk of being obligate carriers. Lastly, isolated GKD within a family usually comes to light when a child has a sufficiently severe phenotype for referral to biochemical and genetics specialists. Among the cases here, and previous reports, many subjects reach adulthood without any acute episodes that would merit such investigations. This may indicate that the actual number of cases of isolated GKD is higher than the published reports suggest. At least some of these patients are being treated for the wrong condition, as the test for raised blood triglyceride also detects raised blood glycerol, but does not distinguish between the two. Conversely, as exemplified by the three brothers in the second generation of the pedigree for case 5, a mixture of hypercholesterolaemia and triglyceridaemia can occur in GK families and, therefore, raised blood triglyceride is attributable to the raised blood glycerol. As a result, it is important not to confuse symptoms of a more serious condition with benign adult GKD.

The lack of phenotype/genotype correlation means that there are probably modifiers elsewhere in the genome that contribute to the severity of isolated GKD. These may include polymorphic variants of the constituents of the mitochondrial pathways that regulate the production of ATP, for example, genes for the enzymes in the dihydroxyacetone phosphate shuttle, or the voltage dependent anion channels (VDAC), of which several have been mapped in both mouse and man. These are proposed to be the site of mitochondrially bound GK and are known to bind the enzyme hexokinase, which competes with GK at the outer mitochondrial membrane. So far, only one mouse model has been constructed by a knockout of the murine gene. In this mouse, raised plasma free fatty acids have been implicated in the severe phenotype which shows high mortality within the first three to four days post partum. However, it is unclear whether free fatty acids have a role in modifying the severity of human GKD. Furthermore, it may be necessary to develop a more extensive
model by selectively introducing mutant chromosomes onto other genetic backgrounds. This would allow the mapping of potential modifying genes, and could include animals carrying the amino acid substitutions observed in reported human cases of GKD.

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