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Inherent lipid metabolic dysfunction in glycogen storage disease IIIa

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ABSTRACT

We studied two patients from a nonconsanguineous family with life-long abnormal liver function, hepatomegaly and abnormal fatty acid profiles. Abnormal liver function, hypoglycemia and muscle weakness are observed in various genetic diseases, including medium-chain acyl-CoA dehydrogenase (MCAD) deficiency and glycogen storage diseases. The proband showed increased free fatty acids, mainly C8 and C10, resembling fatty acid oxidation disorder. However, no mutation was found in *ACADM* and *ACADL* gene. Sequencing of theamylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase (*AGL*) gene showed that both patients were compound heterozygotes for c.118C > T (p.Gln40X) and c.753_756 del CAGA (p.Asp251Glufsx29), whereas their parents were each heterozygous for one of these mutations. The AGL protein was undetectable in EBV-B cells from the two patients. Transcriptome analysis demonstrated a significant different pattern of gene expression in both of patients' cells, including genes involving in the PPAR signaling pathway, fatty acid biosynthesis, lipid synthesis and visceral fat deposition and metabolic syndrome. This unique gene expression pattern is probably due to the absence of AGL, which potentially accounts for the observed clinical phenotypes of hyperlipidemia and hepatocyte steatosis in glycogen storage disease type IIIa.

1. Introduction

Recurrent abnormal liver function is one of the most frequently observed presentations in clinical practice [1,2]. This problem may persist from early childhood into adulthood and is both frustrating and costly to patients and their families. Identifying the cause of liver injury is one of the most challenging tasks facing clinicians in developing countries once common etiologies, such as viral hepatitis, drug-related liver injury, autoimmune hepatitis and nonalcoholic fatty liver disease, have been excluded [3]. Rare genetic metabolic disorders exist but are often missed or misdiagnosed in patients with atypical clinical presentations, who may not report the specific symptoms or events suggestive of the disease. It is difficult to identify which of the hundreds of inborn genetic metabolic disorders implicated in liver conditions is actually responsible for the symptoms. Immediate correct diagnosis is also hampered by the lack of availability of laboratory tests for specific serum biological markers and the overlapping clinical presentations of different diseases [4,5]. Unlike Western countries, developing countries do not routinely carry out many screening tests on neonates [6]. This, together with the scarcity of amended nutrition/feeding and the lack of a centralized referral system, makes diagnosis and treatment extremely difficult for affected patients. Molecular diagnosis is increasingly becoming the principal approach for confirming clinical hypotheses and providing the best possible evidence for diagnosis [7,8].

Patients with persistent abnormal liver function, lipid profiles and muscle weakness may have one of many genetic disorders, including fatty acid oxidative disorders (FAOD) and glycogen storage disorders [9,10]. Patients with FAOD may present a broad spectrum of clinical symptoms, ranging from a complete absence of symptoms to extreme sleepiness, poor appetite, nausea, diarrhea

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and hypoglycemia [10]. Severe complications, including hepatic encephalopathy, myopathy, cardiomyopathy and neuropathy, may also be observed [11]. There is considerable genetic and biochemical heterogeneity among patients with FAOD, with at least 12 genetic etiologies identified, resulting indifferent enzyme or transporter deficiencies [12]. Glycogen storage diseases are characterized by inherited abnormalities of glycogen metabolism in the liver, muscle and other tissues; 11 distinct glycogen storage diseases have been described (types 0 to X) [7,13,14]. The overlapping of clinical presentations made them difficult to differentiate. The differentiation of GSD subtypes was essentially based on the enzyme biochemistry and molecular diagnosis. We describe here two GSD IIIa patients with obscure clinical phenotypes mimicking FAOD and our investigation of the biological interaction between glycogen degradation and fatty acid oxidation in these patients.

2. Methods

2.1. Patients and ethic declaration

We studied two Han Chinese patients (P1 and P2) born to non-consanguineous parents (Fig. 1A). Both patients had displayed lethargy, diaphoresis, abnormal liver function, muscle weakness and hepatomegaly since early childhood. Both tested negative for HBsAg, anti-HCV antibody and antinuclear (ANA) and antimitochondrial (AMA) antibodies, and both had normal ceruloplasmin, ferritin and immunoglobulin levels. A detailed clinical description will be presented in the Table 1. These patients were referred to us as chronic hepatitis of unknown etiology. With the written consent of the parents, we carried out genetic and biochemical characterization for these patients and their relatives. This study was approved by the IRB of Ruijin hospital, Shanghai Jiaotong University School of Medicine.

2.2. Genomic DNA extraction and molecular biology

Genomic DNA was extracted from peripheral blood leukocytes with a Genomic DNA Purification Kit (Qiagen, Germany). We amplified all the coding exons and their flanking intron–exon junctions for the candidate disease-causing genes, including the acyl-CoA dehydrogenase gene, the *C*-4 to *C*-12 straight chain gene (*ACADM*) encoding MCAD, the acyl-CoA dehydrogenase gene, the long chain gene (*ACADL*) encoding long-chain acyl-CoA dehydrogenase (LCAD), and the glucose-6-phosphatase (*G6PC*), amylo-alpha-1, 6-glucosidase and 4-alpha-glucanotransferase (*AGL*) genes. PCR products were sequenced with a Big-Dye Terminator sequencing kit and an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences of the PCR primers used are available on request.

2.3. Cell culture and western blots

PBMCs were isolated from peripheral blood samples and transformed with Epstein-Barr virus (EBV) to establish stable cell lines, as previously described [15]. The EBV-transformed B lymphocytes (EBV-B cells) were maintained in RPMI 1640 culture medium (Gibco 61,870) with 10% fetal calf serum (Gibco 10,099–141). They were then sonicated in cell lyses buffer and the debris was removed by centrifugation. The supernatant (80 μg of protein) was subjected to gel electrophoresis and the resulting bands were transferred to a nitrocellulose membrane, as described elsewhere [16]. The membrane was then probed with a rabbit polyclonal antibody directed against AGL (Abcam, ab71423) and then with a peroxidase-labeled secondary antibody against rabbit IgG (KPL, 04-15-06), for detection of the AGL protein. Actin served as a loading control.

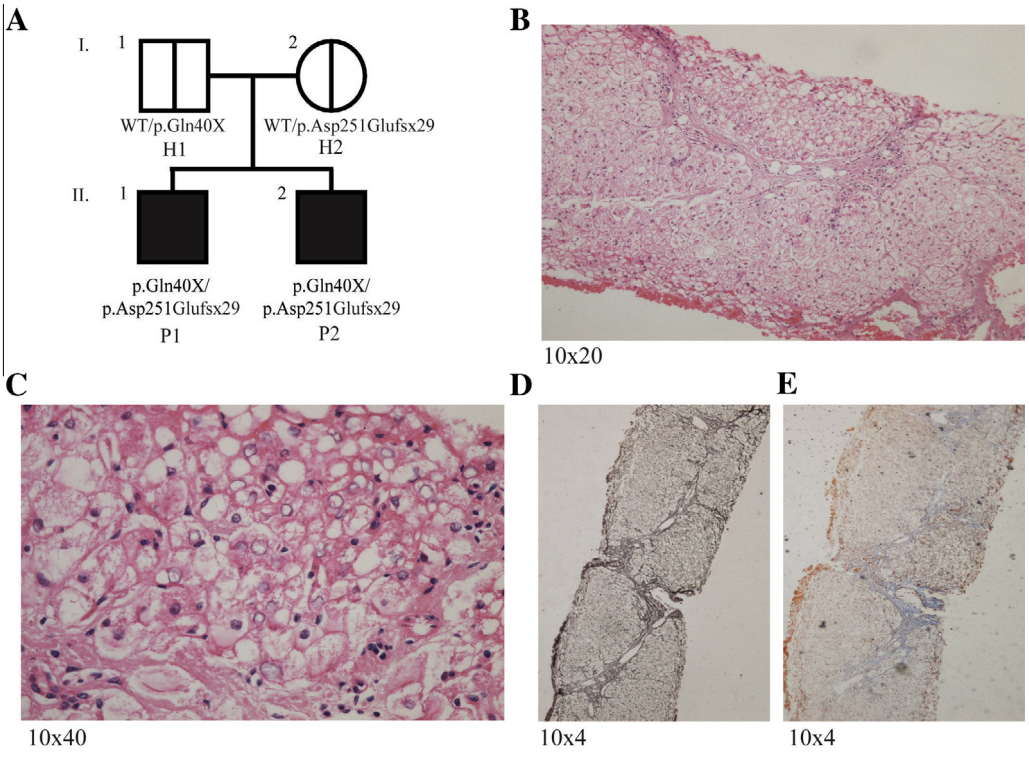


Fig. 1. The pedigree and liver histological features of the patients with AGL deficiency. (A) Family pedigree and genotype of the *AGL* gene. P1 and P2 indicate the two patients with compound heterozygous mutations, H1 and H2 indicate the heterozygous parents. (B–E) Pathological examination of a liver biopsy sample from P1. Images of the HE-stained tissue at a magnification of 10 × 20 (b) or 10 × 40 (C). Reticulin staining (D) and Masson staining (e) at a magnification of 10 × 4.

2.4. DNA microarray analysis

Total RNA was extracted from EBV-B cells with Trizol reagent (Invitrogen), purified on an RN easy column (Qiagen) and quantified by UV absorption (Nanodrop). RNA quality was checked with a 2100 Bioanalyzer and the RNA 6000 Lab Chip R (Agilent Technologies). The RNA was then labeled with Cy3 and hybridized with the Whole Human Genome Microarray 4×44 K (G4112F, Agilent). Arrays were scanned with an Axon 4000B Microarray Scanner System (Axon Instruments, SBC Shanghai, China). The data were analyzed with Gene Spring GX7.3.1 software (Agilent, SBC Shanghai, China).

2.5. Microarray data analysis

Pathway analysis: We used the KEGG [17,18], Biocarta and Reatome programs [19] to search for biological pathways with potentially different levels of gene expression between the patients and healthy controls. We then used Fisher's exact test and χ^2 tests to identify the pathways significantly affected, using a significance threshold defined by the *P*-value and false discovery rate (FDR). The enrichment coefficient Re was calculated as previously described [18–20]. We used the KEGG database to construct the network of genes as a function of the relationships between the genes, proteins and compounds in the database [17,21–24].

3. Results

3.1. Abnormal MS/MS metabolic profiles mimicking MCAD deficiency

P1 was a 16-year-old boy born to nonconsanguineous parents. He had displayed lethargy and mild muscle weakness with abnormal results in liver function tests (LFTs) since early childhood. His brother (P2) had similar problems (Fig. 1A). P1 was referred to us for an exacerbation of lethargy and persistent dull pain in the upper right abdomen after the exclusion of common liver diseases by local physicians. Physical examination revealed an enlarged liver, but no apparent neurologic abnormality in terms of muscle tension, reflex and balance. Laboratory tests showed high ALT and AST levels, mild fasting hypoglycemia, high triglyceride and cholesterol concentrations and high creatine kinase levels (detailed in Table 1). P1 also presented hyperammonemia, but had a normal activated partial thromboplastin time (APTT) and prothrombin time (PT). The hypoglycemia was not due to abnormal insulin secretion, because P1 had normal fasting insulin and *C* peptide concentrations. Ultrasound showed an enlarged liver with a fatty infiltrate. Histological examination of the liver revealed steatosis, similar to that observed in nonalcoholic fatty liver disease, and liver cirrhosis (Fig. 1B–E). Acylcarnitine analysis of blood spots by tandem mass spectrometry (MS/MS) showed that P1 and P2 had significantly higher than normal fasting octanoylcarnitine and decanoylcarnitine levels. Hypoglycemia, high CK levels, muscle weakness and high levels of *C*8 and *C*10 compounds on MS/MS (Table 2) are high-specificity biomarkers of MCAD deficiency. However, an analysis of the organic acids present in urine showed that the concentrations of 5-hydroxyl hexanoic acid, hexanoylglycine and other medium-chain dicarboxylic acids (data not shown) were not high, which was inconsistent with MCAD deficiency. We therefore determined to validate the diagnosis of MCAD deficiency by sequencing the coding regions of the *ACADM* gene and their

Table 2
MS/MS analysis of fatty acid metabolites from the dry blood spots of two patients with AGL deficiency

Metabolites (normal range)	P1	P2
C6 (0.01 ~ 0.15 μmol/l)	0.32↑	0.12
C8 (0.01 ~ 0.25 μmol/l)	1.36↑↑↑	0.62↑↑
C10 (0.01 ~ 0.4 μmol/l)	2.08↑↑↑	0.56↑
C12 (0.01 ~ 0.2 μmol/l)	0.56↑	0.26↑
C10:1 (0.02 ~ 0.35 μmol/l)	0.99↑↑	0.39↑
C12:1 (0.00 ~ 0.15 μmol/l)	0.52↑↑	0.12
C14:1 (0.01 ~ 0.2 μmol/l)	0.57↑↑	0.32↑
C14:2 (0.00 ~ 0.1 μmol/l)	0.23↑	0.13↑
C16:1 (0.01 ~ 0.2 μmol/l)	0.21	0.19
C8/C2 (ratio < 0.02)	0.05↑	0.04↑

Table 1
The clinical and genetic differences between MCAD, GSD-1a and GSD-IIIa patients, and the clinical features of P1.

Characteristics	Reference value	P1	MCAD	GSD-Ia	GSD-IIIa
Development		Delayed	Typically normal development	Delayed development	Delayed development
Fasting blood glucose (mmol/l)	4–6	3.5↓	↓	↓↓↓	↓
Urine ketones	–	++++	– –	Absence of severe ketosis	↑↑↑
Total triglycerides (mmol/l)	0.56–1.76	2.34↑	+	+++	+
Uric acid (μmol/l)	160–430	437	Acute process: Hepatomegaly and acute liver disease (no inflammatory encephalopathy with hyperammonemia, liver dysfunction, and fatty infiltration)	Chronic, hepatomegaly	Chronic, hepatomegaly
Lactate (mmol/L)	0.5~1.7	1.5			
ALT (IU/l)	10–64	144↑			
AST (IU/l)	10–42	148↑			
AKP (IU/l)	38–121	240↑			
Albumin (g/l)	32–55	41			
Ammonia (μmol/l)	9–33	45.2↑↑			
LDH (IU/ml)	91–192	309↑	18% chronic muscle weakness	Lack of muscle symptoms	Common muscle symptoms
Myoglobin (ng/ml)	<70	207,900↑↑			
CK-MB (ng/ml)	0.3–4	84,000↑↑			
CK (IU/l)	22–169	3917↑↑↑			
pH	7.35–7.45	7.33↓	Not common	Common	Not common
HCO_3(mmol/l)	22–27	21↓			
Echocardiogram		Normal	No cardiac involvement	No cardiac involvement	Potential
Neurological abnormalities		Low transmission rate	Only in patients with metabolic decompensation	Common	Common
Inheritance mode and gene			AR, *ACADM*	AR, *G6PC*	AR, *AGL*
Clinical penetrance			Incomplete	Complete	Complete

ALT: alanine transaminase; AST: aspartate aminotransferase; AKP: alkaline phosphatase; LDH: lactate dehyrogenase; CK: creatine kinase; CK-MB: Creatine phosphokinase-MB); AR: autosomal recessive.

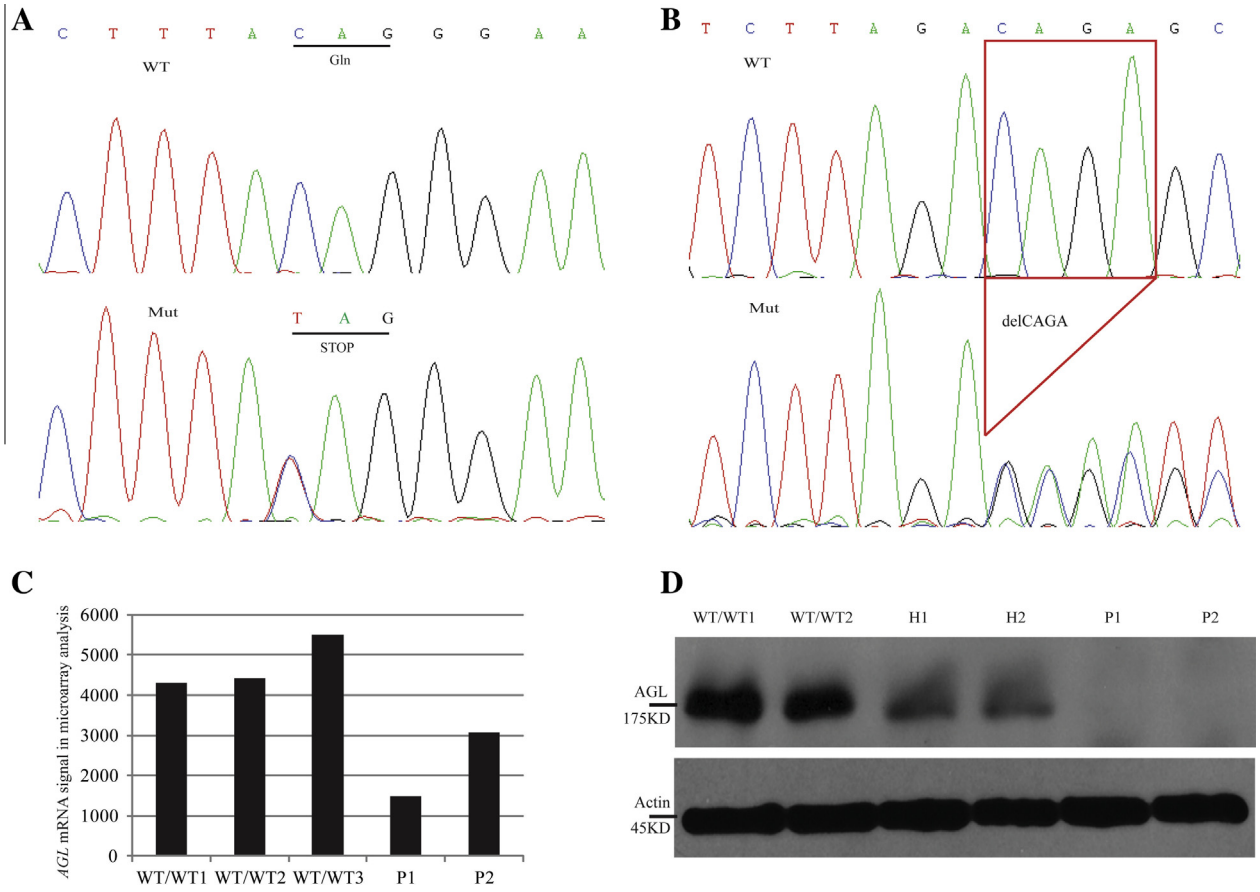


Fig. 2. Molecular characterization of P1 and P2. (A and B) Electropherogram showing the heterozygous mutations of *AGL* found in P1 and P2. The upper panels correspond to the wild-type sequence, and the lower panels correspond to the mutations. The p.Gln40X mutation is due to a change of nucleotide from *C* to *T*, introducing a TAG (STOP) codon. The red box shows the deletion of four nucleotides (CAGA). (C). Levels of *AGL* mRNA in the transcriptome analysis of EBV-B cells from three healthy controls, P1 and P2. (D) Western blot showing AGL protein levels in the EBV-B cells from two healthy controls, the parents (H1 and H2) and the two patients (P1 and P2).

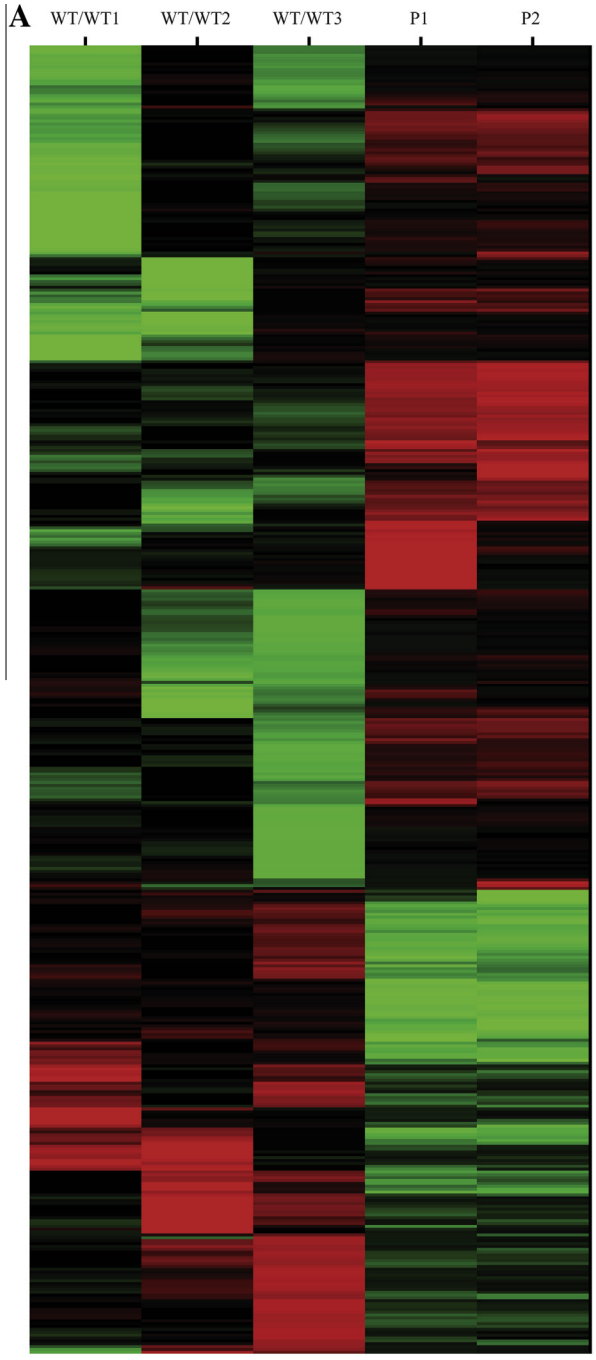
flanking introns. No mutation of this gene was found in either P1 or P2. This excluded the possibility of MCAD deficiency in these two patients. The *ACADL* gene sequence was also found to be wild-type in both patients. In summary, the abnormal MS/MS profiles and liver disease of P1 and P2 were not due to genetic disorders of the fatty acid oxidation pathway.

3.2. Identification of compound heterozygous AGL mutations in P1 and P2

We continued to investigate these two patients, to obtain genetic evidence of inborn errors responsible for their abnormal liver function and hypoglycemia. We neglected one important clue: the high concentration of ketones in the urine of P1, which is important to differentiate between FAOD and GSD. We carried out a muscle biopsy for P1 and the specimen was positively stained with PAS reagent (data not shown). We investigated the liver biopsy specimen again and found that it contains PAS positive staining material and sensitive to diastase digestion (data not shown). No abnormalities were noted on cardiac ultrasound. A clinical diagnosis of GSD was made. We then sequenced the genes responsible for the two most likely types of GSD, *G6PC* and *AGL*. No mutation was found in *G6PC* in P1, but we found two heterozygous mutations in the *AGL* gene: a nonsense mutation in exon4, c.118C > T (p.Gln40X) (Fig. 2A), and a frameshift mutation in exon7, c.753_756 Del CAGA (p.Asp251Glufsx29) (Fig. 2B). We then sequenced these genes in other members of the family. P2 carried the same mutations as P1. Their father carried a heterozygous p.Gln40X mutation, and their mother carried a heterozygous p.Asp251Glufsx29 mutation. The pattern of segregation in the family confirmed that P1 and P2 had an autosomal recessive genetic disorder, GSD IIIa. We also sequenced 100 unrelated healthy controls from the Han Chinese population. All were wild-type for both mutant alleles, suggesting that these variants are not frequent polymorphisms. These variants were also absent from public databases (NCBI, 1000 genomes). The p.Gln40X mutation has been reported [25], whereas p.Asp251Glufsx29 has never before been reported. In summary, we found that P1 and P2 carried compound heterozygous *AGL* mutations that could account for their clinically abnormal liver function.

3.3. Molecular characterization of AGL in two patients

We investigated the biological effects of the mutations identified further, by carrying out a whole-genome microarray analysis with EBV-B cells from two healthy controls, the parents and both patients. The two patients had a weaker *AGL* mRNA signal than their parents and the healthy controls (Fig. 2C), consistent with the nonsense-mediated decay (NMD) of nonsense or frameshift mutations. Both mutations were predicted to lead to a premature stop codon, potentially resulting in a truncated protein without a C-terminus, but nonsense-mediated mRNA decay prevented the production of these aberrant truncated AGL proteins. We further investigated AGL protein levels by western blotting with a rabbit polyclonal antibody recognizing the C-terminus of AGL. A 175kD band was detected in the healthy controls,



B Selected up-regulated genes in AGL deficiency patients

Gene	Fold	description
HSD11B1	10.8959	hydroxysteroid (11-beta) dehydrogenase 1 (HSD11B1), transcript variant 2
ACSL6	10.482	long chain fatty acyl CoA synthetase 2 (LACS2) mRNA, complete cds.
ACACB	4.84085	acetyl-Coenzyme A carboxylase beta (ACACB)
MPO	3.81681	myeloperoxidase (MPO), nuclear gene encoding mitochondrial protein
GSTT2	3.75494	glutathione S-transferase theta 2 (GSTT2)
IRS1	3.63978	insulin receptor substrate 1 (IRS1)
HSD17B12	3.59763	hydroxysteroid (17-beta) dehydrogenase 12 (cDNA clone IMAGE:4281565), complete cds.
ACPP	3.19418	acid phosphatase, prostate (ACPP)
FA2H	3.14329	fatty acid 2-hydroxylase (FA2H)
MCAT	3.13147	malonyl CoA:ACP acyltransferase (mitochondrial) (MCAT), nuclear gene encoding mitochondrial protein, transcript variant 2
CYP2C9	3.08826	cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9)
ABCA1	3.04545	ATP-binding cassette, sub-family A (ABC1), member 1 (ABCA1)
GCKR	2.66697	glucokinase (hexokinase 4) regulator (GCKR)
CPT1C	2.57744	carnitine palmitoyltransferase 1C (CPT1C)
PPARG	2.47392	peroxisome proliferator-activated receptor gamma (PPARG), transcript variant 3
ALDH8A1	2.38092	aldehyde dehydrogenase 8 family, member A1 (ALDH8A1), transcript variant 1
ME1	2.36058	NADP-dependent malic enzyme mRNA, complete cds.
CAV1	2.33529	caveolin 1, caveolae protein, 22kDa (CAV1)
SCD5	2.32278	stearoyl-CoA desaturase 5 (SCD5), transcript variant 1
PHYHIP	2.29027	phytanoyl-CoA 2-hydroxylase interacting protein (PHYHIP)
CYP27B1	2.22546	cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1), nuclear gene encoding mitochondrial protein
CYP1A1	2.05764	cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1)

C Selected down-regulated genes in AGL deficiency patients

Gene	Fold	description
GLT1D1	12.4945	glycosyltransferase 1 domain containing 1 (GLT1D1)
ALDH3B2	8.98416	aldehyde dehydrogenase 3 family, member B2 (ALDH3B2), transcript variant 1
LRP12	7.50049	low density lipoprotein-related protein 12 (LRP12)
UGT2B15	4.03682	UDP glucuronosyltransferase 2 family, polypeptide B15 (UGT2B15)
MAPK10	3.95359	mitogen-activated protein kinase 10 (MAPK10), transcript variant 3
PRKCH	3.78517	protein kinase C, eta (PRKCH)
UGT2B11	3.75119	UDP glucuronosyltransferase 2 family, polypeptide B11 (UGT2B11)
AGL	3.40184	amylo-1, 6-glucosidase, 4-alpha-glucanotransferase (AGL)
ADH1C	3.06301	alcohol dehydrogenase 1C (class I), gamma polypeptide (ADH1C)
PLIN	2.85652	perilipin (PLIN)
SLC2A2	2.67145	solute carrier family 2 (facilitated glucose transporter), member 2 (SLC2A2)
ALDH1A3	2.54009	aldehyde dehydrogenase 1 family, member A3 (ALDH1A3)
UPP1	2.48692	uridine phosphorylase 1 (UPP1), transcript variant 2
ECHDC3	2.39081	enoyl Coenzyme A hydratase domain containing 3 (ECHDC3)
LIPG	2.32245	lipase, endothelial (LIPG)
PPAP2B	2.31437	phosphatidic acid phosphatase type 2B (PPAP2B), transcript variant 1
UGT2B7	2.23188	UDP glucuronosyltransferase 2 family, polypeptide B7 (UGT2B7)
ACSL6	2.01621	acyl-CoA synthetase long-chain family member 6 (ACSL6), transcript variant 2

Fig. 3. Transcriptome analysis of the EBV-B cells from P1 and P2. (A) Cluster analysis of the mRNA transcripts from the EBV-B cells of three healthy controls, P1 and P2. Two groups of genes display differential expression between the patients and the healthy controls. The upregulated genes are indicated in red, and the downregulated genes are indicated in green. Selected metabolic genes, either upregulated (B) or downregulated (C), observed in patients with AGL deficiency.

but no AGL signal was detected in either of the patients. The parents, who were heterozygous, had intermediate levels of AGL protein (Fig. 2D). Thus, both P1 and P2 display complete AGL deficiency.

3.4. Differential gene expression between GSD-III patients and healthy controls

Abnormal lipid metabolism is not rare in patients with GSD-IIIa. We investigated the mechanisms if the abolition of AGL function would potentially interfere with the regulation of lipid metabolism. We performed a microarray analysis with the Whole Human Genome Microarray 4 × 44 K (G4112F, Agilent) and EBV-B cells from the two patients and two controls. Using the threshold of a two-fold difference in expression between GSD III and the healthy control, we found that 1526 transcripts were differentially expressed in the cells of patients with GSD III; 984 of these genes were upregulated and 542 were downregulated (data not shown). Principal component analysis yielded a clear separation of the two groups (data not shown). The cluster map was organized into two sections, corresponding to upregulation and downregulation, according to the level of gene expression in GSD III

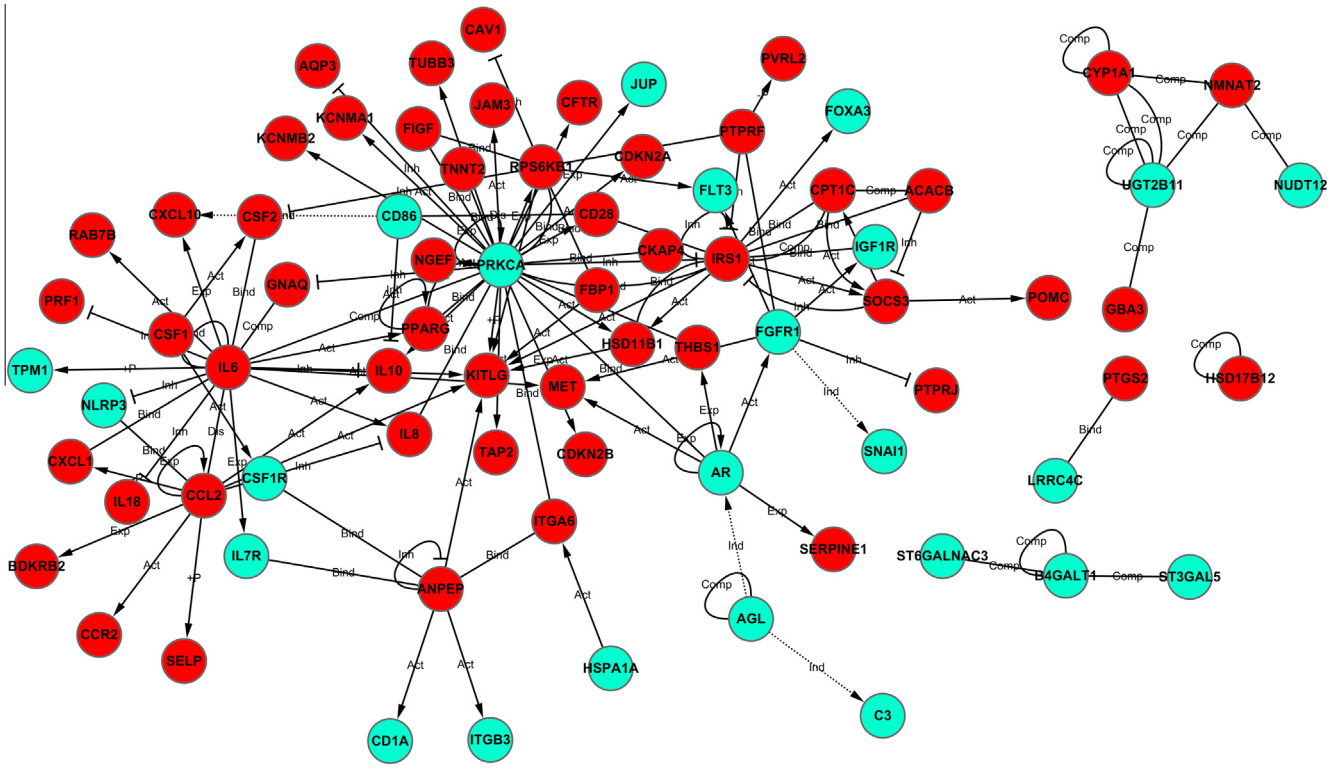


Fig. 4. Pathway analysis of genes up- and downregulated in the patients with AGL deficiency. Genes in red circles are upregulated, whereas those in blue circles are downregulated. The major affected pathways include xenobiotics metabolism by cytochrome P450, the low-density lipoprotein (LDL) pathway during atherogenesis, nuclear receptors in lipid metabolism and toxicity, the PPAR signaling pathway, drug metabolism by cytochrome P450, visceral fat deposition and metabolic syndrome.

patients with respect to three healthy controls (Fig. 3A). Many genes involved in fatty acid and lipid metabolism displayed significant differential regulation between the patients and the healthy controls.

3.5. Unsaturated fatty acids and fatty acid biosynthesis are the most strongly affected in patients

We then investigated the metabolic pathways affected by AGL deficiency. We found that metabolism of xenobiotics by cytochrome P450, the low-density lipoprotein (LDL) pathway during atherogenesis, nuclear receptors in lipid metabolism and toxicity, the PPAR signaling pathway, the drug-metabolizing cytochrome P450, visceral fat deposits and the metabolic syndrome differed significantly between patients and controls (Fig. 3). The biosynthesis of fatty acids in general, and of unsaturated fatty acids in particular, was the most strongly affected pathway (Fig. 3B and C). Mutiple genes encoding PPAR signaling pathway molecules were over expressed in the GSD III patients: *ME1*, *PPARG*, *ACSL6*, *CPT1C* and *MCAT*. Several genes encoding proteins involved in lipid/fatty acid biosynthesis signaling were also upregulated in the patients: *ACACB*, *SCD5*, *HSD11B1* and *HSD17B12*. The expression of several genes encoding proteins involved in starch and sucrose metabolism (such as *AGL*, *UGT2B4*, *UGT2B7* and *UGT2B15*) and the glycolysis/gluconeogenesis pathway (such as *ADH1C*, *ALDH1A3* and *ALDH3B2*) was downregulated in GSD III patients. Using the KEGG database to build the affected gene network [17,21–24], we found that the aberrant expression of *PRKCA*, *CCL2* and *PPARG* played a key role in determining the overall level of abnormal metabolic gene expression in GSD III patients (Fig. 4).

4. Discussion

Glycogen storage diseases (GSDs) are characterized by inherited abnormalities of glycogen metabolism in the liver, muscle, and brain and are classified into types 0 to X [8]. The synchronous activities of glycogen phosphorylase and debranching enzyme are required for glycogen degradation [26]. An impairment of debranching enzyme activity leads to the accumulation of an abnormal form of glycogen, limiting dextrin levels in the liver, heart and skeletal muscle [14]. GSD III is biochemically and clinically heterogeneous [7], but patients with GSD-III usually present hepatomegaly, hypoglycemia, hyperlipidemia and high serum transaminase levels, as in our index cases. The presence of two loss-of-function mutations was confirmed, as no AGL protein was observed in cells from the patients. In total, 26 mutations of the *AGL* gene have been reported in 19 Chinese patients, but none of these mutations is present at high frequency [27–31]. The frequency of *AGL* mutation carriage is unknown in China, but we suspect that most patients with AGL deficiency remain unidentified.

Hyperlipidemia, of unknown cause, was observed in the patients with GSD. This condition is often reported in patients with glycogen storage disease (GSD) types Ia and Ib. A few investigators have evaluated lipid metabolism in children with GSD III, who also frequently display hyperlipidemia and there is increasing evidence that hyperlipidemia and hepatocyte steatosis are two of the most common clinical manifestations of GSD III [7,9,13,32,33]. However, the cause of these problems remains unclear. On the basis of our gene expression profiling in GSD III patients, we suggest that hyperlipidemia in these patients may result from the upregulation of PPARγ or the PPAR signaling pathway. Many studies have shown that PPARγ plays a key role in the regulatory network underlying

adipocyte differentiation and function [34–36]. Mutations of the gene encoding the PPARγ and its downstream target genes result in lipodystrophy. Increases in PPARγ gene expression promote lipogenesis and lipid accumulation in hepatocytes [37–39]. So, PPARγ or PPAR signaling pathway upregulation may contribute to hyperlipidemia and hepatocyte steatosis in GSD III patients. Alternatively, an increase in lipid/fatty acid biosynthesis and the flux of free fatty acids from a larger mass of visceral adipose tissue may also lead to hyperlipidemia and hepatic steatosis [40,41]. We found that several genes involved in lipid/fatty acid biosynthesis were upregulated in GSD III patients: e.g., *ACACB*, *HSD11B1*, *ABCA1*, *HSD17B12* [42–47].

In summary, we have identified a new *AGL* mutation in a family severely affected by GSD III. We also found that glycogen storage diseases may give rise to a pathological acylcarnitine profile, for which further exploration is required, in a larger group of GSD patients. Gene expression profiling showed that *AGL* mutation led to an upregulation of genes encoding chemokines and molecules involved in the PPAR signaling pathway and lipid/fatty acid biosynthesis, potentially accounting for the hyperlipidemia and hepatocyte steatosis observed in the patients.

Notes for future study

1. We studied the EBV-B cells, but not liver biopsy hepatocytes might provide the bondfide phenotype of metabolic abnormalities of AGL mutation.
2. Although we found some key genes up-regulated/downregulated. We didn't investigate the mechanism. We didn't complement this phenotype. We even didn't measure ccl-2 chemokines level in the GSD-IIIa patients.

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