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Clinical implications of mutation analysis in primary hyperoxaluria type 1

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Background. Primary hyperoxaluria type 1 (PH1) is an inborn error of glyoxylate metabolism with an extensive clinical and genetic heterogeneity. Although over 50 disease-causing mutations have been identified, the relationship between genotype and clinical outcome remains unclear. The aim of this study was to determine this association in order to find clues for improvement of patient care.

Methods. AGXT mutation analysis and assessment of biochemical characteristics and clinical outcome were performed on patients from a Dutch PH1 cohort.

Results. Thirty-three of a cohort of 57 PH1 patients, identified in The Netherlands over a period of 30 years, were analyzed. Ten different mutations were found. The most common mutations were the Gly170Arg, Phe152Ile, and the 33insC mutations, with an allele frequency of 43%, 19%, and 15%, respectively. Homozygous Gly170Arg and Phe152Ile mutations were associated with pyridoxine responsiveness and a preserved renal function over time when treatment was timely initiated. All patients homozygous for the 33insC mutation had end-stage renal disease (ESRD) before the first year of age. In two unrelated patients, a new Val336Asp mutation was found coupled with the Gly170Arg mutation on the minor allele. We also found 3 patients homozygous for a novel Gly82Arg mutation with adverse outcome in 2 of them.

Conclusion. Early detection of Gly170Arg and Phe152Ile mutations in PH1 has important clinical implications because of their association with pyridoxine responsiveness and clinical outcome. The association of a homozygous 33insC mutation with severe infantile ESRD, resulting in early deaths in 2 out of 3 cases, warrants a choice for prenatal diagnostics in affected families.

Primary hyperoxaluria type 1 (PH 1) is a rare autosomal-recessive inborn error of glyoxylate metabolism caused by a functional deficiency of the peroxisomal liver-specific enzyme alanine:glyoxylate aminotransferase (AGT). This enzyme deficiency leads to an overproduction of oxalate and, in most cases, of glycolate. The accumulation of oxalate causes insoluble calcium oxalate depositions in renal tissue and, in case of renal insufficiency, systemic tissue damage caused by oxalate storage. Clinically, symptoms can vary from infrequently occurring renal stones to early onset nephrocalcinosis and end-stage renal disease (ESRD) with severe systemic disease.

PH1 is caused by mutations in the *AGXT* gene encoding AGT. The *AGXT* gene is located at 2q37.3 and consists of 11 exons, ranging from 65 bp to 407 bp [1]. To date, 7 polymorphisms and more than 50 different mutations have been found in the *AGXT* gene. Two different haplotypes have been identified based on the presence or absence of three specific polymorphic variants: the major (frequency 80%) and the minor haplotype (frequency 20%). The latter is defined by a 74 bp duplication within the first intron, and by 32C>T and 1020A>G point mutations, which lead to Pro11Leu and Ile340Met amino acid substitutions. In healthy individuals who are homozygous for the minor haplotype (frequency of 4% in the Caucasian population), 5% of AGT is mistargeted to mitochondria instead of to peroxisomes. In combination with a Gly170Arg substitution, however, this minor haplotype leads to mistargetting to the mitochondria of 90% of AGT. This not only causes a significant reduction of its activity in vivo, but also has consequences for its functioning in glyoxylate metabolism. Several other mutations causing amino acid substitutions have shown to be associated with specific AGT phenotypes: the Gly82Glu substitution abolishes AGT catalytic activity, and the Gly41Arg substitution and Ile244Thr substitution both lead to protein destabilization and aggregation into inclusion bodies [2].

Although several studies have established a relation between genotype and in vitro AGT functioning, the association between genotype and biochemical

Key words: mutation analysis, primary hyperoxaluria type 1, clinical outcome.

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Table 1. AGXT-specific primers used for mutation analysis of PH1 patients

Exon	5′ Primer	3′ Primer
1 and 2	[-21M13]- AAG CAC AGA TAA GCC TCA GG	[M13-Rev]-GAT GGA TCC AGG GCC ATC CC
3 and 4	[-21M13]-GAC ACT CAC GGC CCA CTC TG	[M13-Rev]-GAG CTG TGC TCC AGT CCA CC
5 and 6	[-21M13]-TCA CCT GCT GCC CTC CAT TC	[M13-Rev]-TGC ACA GAG GTG AGG GTC TG
7 and 8	[-21M13]-ACT GAG AGG CTG GTG CTC AG	[M13-Rev]-AGT GCG CCT GTC TCC TCC TG
9 and 10	[-21M13]-CAC CCA TGT CAC TGC CCA CC	[M13-Rev]-CAC CTG GTG CAC AGT CCT GC
11	[-21M13]-AGG CGG GAG GCT GAC GTC AG	[M13-Rev]-CTC CTC TCA CTC TTT CAC AG

[-21M13]: 5′-TGTAAAACGACGGCCAGT-3′.
[M13-Rev]: 5′-CAGGAAACAGCTATGACC-3′.

characteristics in vivo is less evident. One of the most important unanswered questions is the relationship between clinical outcome and genotype. In order to investigate this relationship, we reviewed the biochemical characteristics, as well as the clinical outcome, in 33 patients of a Dutch cohort of all known PH1 patients over a period of 30 years in whom we were able to obtain DNA.

METHODS

Patients

Recently, we described a cohort of 57 patients with PH1 diagnosed between 1970 and 2000 in The Netherlands [3]. From this cohort, we approached all living patients and/or their parents to participate in this study. Furthermore, we isolated DNA from liver biopsy tissue of 2 deceased PH1 patients. Patients who were diagnosed after the year 2000 were also approached, and, after informed consent was received, information on their clinical status was obtained.

Diagnosis of PH1 was made if either deficient AGT activity in the liver samples or the combination of hyperoxaluria and hyperglycolic aciduria (24-hour urine excretion exceeding 0.5 mmol/L oxalate or 0.054 mmol/L oxalate/mmol/L creatinine and 0.14 mmol/L glycolate/mmol/L creatinine, respectively) was demonstrated. Renal function was estimated by the Cockroft formula in adults and the Schwartz formula in children using plasma creatinine values [4, 5]. For patients in whom urinary oxalate could not be measured because of anuria, highly elevated plasma oxalate levels of more than 20 times the upper limit of the reference range (<5 μmol/L) were considered to be caused by PH1 and not by renal insufficiency itself [6]. Patients in whom only hyperoxaluria was established were considered to have PH1 if measurements in liver biopsies demonstrated deficient AGT activities in other family members. When these firm grounds were not present, we decided to include only patients with the following highly suspicious circumstantial evidence for PH1: (*1*) significant response to pyridoxine therapy; and (*2*) severe systemic oxalosis. Renal insufficiency was defined as a creatinine clearance of <60 mL/min/1.73 m^2. ESRD was defined as a creatinine-clearance of <10 mL/min/1.73 m^2 body surface area (BSA). Mutations in the *AGXT* gene were investigated in relation to pyridoxine responsiveness and outcome. Pyridoxine responsiveness was defined by a decline of urinary or plasma oxalate levels of >30% in at least 2 urine samples upon pyridoxine therapy [7]. We calculated the prescribed dosages of pyridoxine per kg body weight in patients in which response of pyridoxine on oxalate excretion was measured. AGT enzyme activities were determined in liver samples as described, and are expressed as percentages of the mean AGT activity determined in control liver samples [8].

Mutation analysis

Genomic DNA was extracted from lymphocytes using the Wizard Genomic DNA purification kit according to the instructions of the supplier (Promega, Madison, WI, USA). Six sets of *AGXT*-specific primers with −21M13 or M13rev extensions were used for the amplification of the entire *AGXT* coding region encoded by exon 1 to 11 (Table 1). Each polymerase chain reaction (PCR) contained 0.4 μmol/L of each primer (synthesized by Pharmacia, Uppsala, Sweden), 10 mmol/L Tris/HCl pH 8.4, 50 mmol/L KCl, 1.5 or 2 mmol/L $MgCL_2$, 0.01% w/v bovine serum albumin (BSA), 0.2 mmol/L dNTP (Pharmacia), and 0.5 μL Taq DNA polymerase (Promega). Two different PCR programs were used to amplify the exons. For exons 1, 2, 3, 4, and 11, the amplification started with 2 minutes of denaturation at 96°C, followed by 5 cycles of 30 seconds at 96°C, 30 seconds at 55°C, and 1 minute at 72°C, and 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C, with a final extension step of 7 minutes at 72°C. For exons 5 to 10, the amplification started with 2 minutes of denaturation at 96°C, followed by 5 cycles of 30 seconds at 96°C, 30 seconds at 55°C, and 2 minutes at 72°C, and 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 2 minutes at 72°C, with a final extension step of 7 minutes at 72°C. The PCR fragments were analyzed by direct sequencing using the Big Dye Terminator Cycle Sequencing Kit or the Big Dye Primer Cycle sequencing kit (Applied Biosystems, Foster City,

Table 2. Patient characteristics

Total number of patients	33
Median age at time of investigation *years*	27 (3–63)
Male/female	15/18
Median age at time of diagnosis *years*	6.6 (0–50)
Urolithiasis	19
Nephrocalcinosis	17
Pyridoxine sensitivity	15
Renal insufficiency at the time of diagnosis	15
ESRD at the time of diagnosis	14
ESRD/renal insufficiency at follow-up	21
Infantile onset	8
Kidney transplantation	12 in 10 patients
Combined liver/kidney transplantation	6
Deceased	2

ESRD, end-stage renal disease.

CA, USA) on an ABI 3100 automated DNA sequencer according to the manufacturer's protocol (Applied Biosystems). Mutation analysis was performed with DNA of the probands and, when available, with DNA of the parents in order to establish the zygosity of the probands.

The study design was approved by the Medical Ethics Committee of the Academic Medical Center, and informed consent was obtained from the participants or their parents.

RESULTS

Clinical data

We identified 57 PH1 patients in The Netherlands over a period of 30 years [3]. For the present study we obtained material for DNA analysis of 33 of these 57 patients, 2 of whom had died at the time of investigation. These 33 patients were the offspring of 26 families. Patient characteristics are given in Table 2; mutational data, biochemical phenotype, and clinical outcome of all patients are presented in Table 3. Consanguinity was reported in one family (family 5, Table 3). The ethnic origin varied: Dutch (23), Turkish (2), Surinam (3), Albanian (4), and mixed Dutch-Turkish (1). The median age at diagnosis was 6.6 (range 0 to 57) years. Twenty (59%) patients were diagnosed under the age of 18, 6 (18%) in the first year of life. At the time of diagnosis, 15 patients (44%) had renal insufficiency, 14 of whom had already developed ESRD. No differences in time of follow-up were found between the patients with a stable renal function and those who developed renal insufficiency [median (range) follow-up: 13.1 (0 to 47) and 7.7 (0 to 46) years, respectively]. AGT activity was measured in the liver samples of 21 patients. The median AGT activity was 9.5% of control values (range 1% to 47%). Pyridoxine therapy was initially started in 16 patients. In 13 of these 16 patients, pyridoxine therapy induced a significant decrease of the oxalate excretion. In two additional patients, pyridoxine was successfully prescribed after isolated kidney transplantation (patients 8 and 18, Table 3).

Kidney transplantation was primary performed in 9 and combined liver-kidney transplantation in 6 patients. Two patients received a second isolated renal graft, 8 and 6 years, respectively, after their first transplantation, and in one patient combined liver kidney transplantation was performed 7 years after the first isolated kidney transplantation. In one patient, isolated kidney transplantation was performed after combined liver kidney transplantation. Renal graft functions were preserved in 5 patients with isolated kidney transplantation for 2, 3, 3, 9, and 14.5 years, respectively (Table 3). Of all 6 patients with combined liver kidney transplantation, one patient died as a consequence of liver failure, and one patient developed severe cirrhosis. One patient showed a deterioration of the renal function; the other 3 patients had a preserved renal and liver function after a follow-up of 1, 4, and 7 years, respectively.

Gene mutations

Eight different mutations were found in 26 unrelated probands (Table 4). We found the Gly170Arg mutation in 20 patients (60.6%), 15 of whom were unrelated. The allele frequency was 44%. Seven patients were compound heterozygous, and 8 were homozygous for this mutation. The minor allele, consisting of the Pro11Leu polymorphism and the 74-bp insertion in the first intron, always co-segregated with this mutation.

Eight patients, 7 of whom were unrelated, carried the Phe152Ile mutation (allele frequency 19%).

The 33insC mutation was found in 5 patients (allele frequency 15%). In 2 patients (patients 15 and 22, Table 3), a novel mutation was identified on the Gly170Arg allele: an amino acid change Val336Asp (1007T>A mutation). In 4 cases (patients 12, 13, 14, 23, Table 3), only 1 heterozygous mutation was found. Of 6 probands, another 7 siblings were diagnosed by screening (Table 3). Sequencing revealed the same mutations in each affected family. In 3 patients of 2 unrelated families, we found homozygosity for a previously unrecognized 121G>A (244G>A) mutation, resulting in a Gly82Arg substitution.

Associations between genotype and phenotype

The Gly170Arg and Val336Asp mutations. Of the 12 patients who were homozygous for the G170Arg mutation (patients 1 to 11 and 15, Table 3), 5 had developed ESRD at time of diagnosis. Pyridoxine responsiveness was observed in 6 of all 7 patients in whom pyridoxine responsiveness was measured. In 1 additional patient with ESRD at time of diagnosis, pyridoxine responsiveness was established after kidney transplantation. The only patient without response upon pyridoxine administration developed renal insufficiency. Remarkably, this patient was also homozygous for a second, previously unrecognized mutation: the Val336Asp mutation. This novel mutation, in association with the Gly170Arg

Table 3. Mutations and clinical characteristics of all patients

Patient (family)	Mutation (allele)	Age at onset/ diagnosis *years*	Age at follow-up *years*	Renal function at diagnosis	Renal function/ outcome at at follow-up *age*	Tx *age*	PyrR	Pyr D *mg/kg*	AGT%
1 (1)	Gly170Arg (m)/Gly170Arg (m)	5/5	13	Normal	Preserved		+	2.5	47.4
2 (2)	Gly170Arg (m)/Gly170Arg (m)	42/42	53	ESRD	Tx preserved[a]	K (44)	ND	ND	ND
3 (3)	Gly170Arg (m)/Gly170Arg (m)	35/35	49	Normal	Preserved		+	14.2	46.9
4 (3)	Gly170Arg (m)/Gly170Arg (m)	6/6	55	Normal	ESRD (51)/Tx preserved[a]	K (52)	+	11.4	14.9
5 (3)	Gly170Arg (m)/Gly170Arg (m)	48/48	53	ESRD	ESRD		ND	ND	ND
6 (4)	Gly170Arg (m)/Gly170Arg (m)	10/16	36	ESRD	Tx preserved[a]	K (22)	ND	ND	ND
7 (5)	Gly170Arg (m)/Gly170Arg (m)	7/7	34	Normal	Preserved		+	7.1	34.0
8 (5)	Gly170Arg (m)/Gly170Arg (m)	25/25	38	Normal	ESRD (34)/Tx preserved[a]	K (36)	+[b]	14.2	ND
9 (6)	Gly170Arg (m)/Gly170Arg (m)	0.5/3.5	17.8	ESRD	Tx decreased function[a]	K(9)K(17)	ND	ND	30.0
10 (7)	Gly170Arg (m)/Gly170Arg (m)	50/50	58	ESRD	Tx decreased function[a]	K(50)K(56)	ND	ND	37.1
11 (7)	Gly170Arg(m)/Gly170Arg (m)	57/57	61	Normal	Preserved		+	6.6	ND
12 (8)	Gly170Arg (m)/Xo (M)	0.4/0.4	11	ESRD	ESRD (4)/Tx preserved[a]	LK(4)K(6)	ND	ND	ND
13 (8)	Gly170Arg (m)/Xo (M)	0.3/0.3	15	Normal	Preserved		+	13.6	5.2
14 (9)	Gly170Arg (m)/Xo (m)	0.5/0.5	3	Normal	Preserved		+	30	6.2
15 (10)	Gly170Arg-Val336Asp (m)/ Gly170Arg-Val336Asp (m)	5/5	8	Decreased	Decreasing		–	4.0	4.5
16 (11)	Gly170Arg (m)/Leu153Val (M)	27/27	39	ESRD	ESRD/Tx-preserved[a]	K(31)LK(38)	ND	ND	16.4
17 (12)	Phe152Ile (m)/Phe152Ile (m)	2/2	31	Normal	Preserved		+	12.5	12.9
18 (13)	Phe152Ile (m)/Phe152Ile (m)	25/30	59	ESRD	Tx decreasing slowly[a]	K(31)	+[b]	14.3	ND
19 (14)	Phe152Ile (m)/Phe152Ile (m)	31/31	37	ESRD	ESRD		ND	ND	ND
20 (14)	Phe152Ile (m)/Phe152Ile (m)	22/22	26	Normal	Preserved		+	2.9	10.0
21 (15)	Phe152Ile (m)/Gly170Arg (m)	1/1	10	Normal	Preserved		+	7.5	10.0
22 (16)	Phe152Ile (m)/Gly170Arg-Val336Asp (m)	4/4	20	Normal	Decreased		+	6.0	10.2
23 (17)	Phe152Ile/Xo (mm)	40/40	44	ESRD	ESRD		ND	ND	16.6
24 (18)	Phe152Ile (m)/33insC (M)	2/2	27	Normal	Preserved		+	6.0	5.9
25 (19)	33insC (M)/33insC (M)	0.1/0.1	8	ESRD	Tx preserved[a]	LK (1.7)	ND	ND	5.2
26 (20)	33insC (M)/33insC (M)	0.5/0.5	0.6	ESRD	Died		ND	ND	1.1
27 (21)	33insC (M)/33insC (M)	0.6/0.6	1.3	ESRD	Died (liver failure)	LK (1.3)	ND	ND	5.0
28 (22)	33insC (M)/Gly170Arg (m)	4/14	23	ESRD	Tx preserved[a]/liver failure	LK (16.5)	ND	ND	ND
29 (23)	33insC (M)/Gly170Arg (m)	6/6	29	Normal	Preserved		+	8.0	16.3
30 (24)	Gly82Arg (m)/Gly82Arg (m)	0.6/0.6	16	Normal	Preserved		ND	ND	ND
31 (25)	Gly82Arg (m)/Gly82Arg (m)	6/6	19	Normal	ESRD (18)/Tx decreasing[a]	LK(19)	–	5.7	3.6
32 (25)	Gly82Arg (m)/Gly82Arg (m)	7/7	21	Normal	Decreased		–	20.0	ND
33 (26)	C173X (M)/IVS1-1G>A (M)	0.2/0.2	14	ESRD	Tx preserved[a]	K(11)	ND	ND	4.1

Abbreviations are: m, minor allele; M, major allele; PyrR, pyridoxine responsiveness ≥30% reduction of U-oxalate under pyridoxine; Pyr D, pyridoxine dosage in mg/kg: minimal dosage with >30% response of U-oxalate or maximum prescribed dosage in case of no response on oxalate excretion; AGT%, percentage AGT-activity in vitro related to AGT-activity in vitro of control tissue; K, kidney transplantation; LK, combined liver-kidney transplantation; ESRD, end-stage renal disease; ND, not determined; Tx, transplantation.

[a]Renal graft function; [b]determined after transplantation.

allele, was also identified heterozygous in another patient who was initially pyridoxine responsive, but showed nevertheless a decline of renal function at follow-up. All but 2 homozygous Gly170Arg patients with a normal renal function at time of diagnosis (and without the additional Val336Asp mutation) preserved their renal function over time. The 2 patients that developed ESRD over time were unfortunately both lost to follow-up in the period between diagnosis and onset of dialysis; both patients had not been treated with pyridoxine, potassium citrate, and/or high fluid intake. After kidney transplantation, both patients were responsive to pyridoxine therapy and preserved their renal function to date with a graft survival of 3 and 14.5 years, respectively (patients 4 and 8, Table 3).

Four out of 6 homozygous Gly170Arg patients who received a renal graft were able to preserve their renal functions after a mean follow-up of 8 (range 3 to 14.5) years.

The Phe152Ile mutation. In 2 out of all 4 homozygous Phe152Ile patients (patients 17 and 20, Table 3), pyridoxine therapy was used successfully in reducing urinary oxalate excretion. Both patients preserved their renal function over time, with a follow-up of 4 and 29 years, respectively. The 2 other patients had ESRD at time of diagnosis. In one of them (patient 18, Table 3), pyridoxine

Table 4. Mutations in the *AGXT* gene in Dutch PH1 patients

Mutation[a]	Exon[b]	Allele[c]	Coding effect
IVS1-1G>A	Intron 1	Major	Splicing defect
33insC (156insC)	1	Major	Frame shift
244G>C (336G>C)	2	Minor	Gly82Arg
454T>A (576T>A)	4	Minor	Phe152Ile
457T>G (579T>G)	4	Major	Leu153Val
508G>A (630G>A)	4	Minor	Gly170Arg
519C>A (641C>A)	5	Major	Cys173STOP
1007T>A	6	Minor	Val336Asp[d]

[a]Nucleotide numbering starts from the first AUG (position 123) according to cDNA sequence of GenBank Accession number NM_000030. Between brackets the notation according to the previously introduced numbering system which starts at the first nucleotide of this cDNA sequence.

[b]Exon-intron numbering according to Purdue et al [18].

[c]Minor allele is defined by the presence of a 74-bp duplication in intron 1 and the 32C>T (Pro11Leu) and 1020A>G (Ile340Met) polymorphic variants, which are absent on the major allele.

[d]The Val336Asp mutation cosegregates with the Gly170Arg mutation on the minor allele.

therapy was initiated at the onset of hemodialysis treatment, resulting in a significant fall in serum oxalate, which continued to decrease after isolated kidney transplantation. However, renal function slowly decreased throughout the follow-up period due to a chronic vascular rejection [9].

Of the 4 heterozygous Phe152Ile patients, one had ESRD at the time of diagnosis (patient 23, Table 3). All other 3 patients were pyridoxine responsive; 2 of them preserved their renal function over time with a follow-up period of 9 and 25 years, respectively (patients 21 and 24, Table 3). The only pyridoxine-responsive patient with a decrease in renal function over time under treatment was compound heterozygous for the Phe152Ile allele and the Gly170Arg+Val336Asp allele (patient 22, Table 3).

The 33insC mutation. All 3 patients who were homozygous for the 33insC mutation developed ESRD within their first year of life. Diagnosis was made at the same time. Two of these patients died within one year after the diagnosis was established (patients 25 to 27, Table 3).

The Gly82Arg mutation. Pyridoxine responsiveness was measured in 2 of 3 homozygous Gly82Arg patients (patients 31 and 32, Table 3) and found negative in both. One of these 2 patients had ESRD at the time of diagnosis and received a combined liver and renal graft (patient 31, Table 3). The other patient showed a decline in renal function over time. In the only patient that preserved renal function over time, pyridoxine responsiveness was not measured (patient 30, Table 3).

Single and double (compound) heterozygous mutations. We found compound heterozygous mutations in 7 patients, 3 of whom had ESRD at the time of diagnosis (patients 16, 28, and 33, Table 3). The course of renal function over time showed a great variation. Two out of 4 patients who were only heterozygous for one mutation also had ESRD at the time of diagnosis (patients 12 and 23, Table 3).

DISCUSSION

We collected data on both the clinical characteristics and genotypes in a large cohort of PH1 patients in The Netherlands. The majority of the detected mutations have previously been found in association with PH1. This makes the *AGXT* mutation screening a useful tool in the diagnostic work-up of PH1 patients in The Netherlands.

The Gly170Arg mutation

In our cohort of Dutch PH1 patients, the Gly170Arg mutation was the most common mutation. Our finding that this mutation was associated with pyridoxine responsiveness is consistent with other reports [10, 11]. Previously, Motley et al [12] have described the molecular basis for the combination of severe hyperoxaluria and hyperglycoluria with the presence of a considerable AGT activity in vitro and pyridoxine responsiveness in these patients. They showed that the Gly170Arg mutation, which is always located on the minor allele, results in increased mistargeting of AGT to the mitochondria instead of peroxisomes, which leads to inactivation of the enzyme in vivo. This mitochondrial mistargeting of AGT already occurs to a small extent (±5%) with AGT encoded by the minor allele because of the presence of a Pro11Leu mutation [13]. This mutation creates a weak mitochondrial targeting sequence in the amino terminus of the encoded AGT. The Gly170Arg mutation increases the efficiency of the mitochondrial targeting sequence by delaying the dimerization of AGT and consequently by preventing the folding of the protein [2, 14]. As a result of this, the amino terminus remains accessible for mitochondrial import. Pyridoxine increases the residual activity of the 10% peroxisomal-located AGT. Previously, there has been discussion whether this relatively favorable biological background (i.e., the pyridoxine responsiveness) is in line with the clinical outcome of patients with this mutation. All homozygous Gly170Arg cohort patients who had a preserved renal function at the time of diagnosis and who were treated accordingly with pyridoxine, a high fluid intake, and potassium citrate were able to preserve renal function throughout the follow-up period. However, 5 homozygous patients had already developed ESRD at the time of diagnosis. Therefore, renal function can be preserved in patients with a Gly170Arg mutation (but without a Val336Asp mutation, as discussed below) after a timely diagnosis and treatment with pyridoxine. This is supported by the course of isolated renal transplantations in Gly170Arg-positive patients in our cohort. In contrast to what might be expected in PH1 patients with isolated kidney transplantation, renal function was preserved in 4 out of 6 of these patients after a median follow-up of more than 7 years, most likely as a consequence of pyridoxine therapy.

The Val336Asp mutation

A novel mutation, 1007T>A (1129T>A), was found in 2 patients and their relatives, which results in the amino acid replacement Val336Asp. The mutation was present on the minor allele and in combination with the frequent Gly170Arg mutation. While Gly170Arg is correlated with pyridoxine responsiveness, the Val336Asp mutation appears to interfere in a negative way with the effect of pyridoxine, and in fact, predisposes to the development of renal insufficiency, at least in homozygous patients.

The Phe152Ile mutation

We found a relatively high number of patients carrying the Phe152Ile mutation. This mutation has previously been described in 6 patients [1, 10, 15]. However, this is the first time that homozygous patients for this mutation are reported. The high prevalence of Phe152Ile in our study, when compared to others, suggests a founder effect. The beneficial effect of pyridoxine in these patients is in line with observations previously made [1].

The 33insC mutation

The 33insC mutation is the third most common mutation found in our study. Patients affected by this mutation in a homozygous condition all suffered from the most severe form of infantile PH1, with ESRD before the first birthday. In the 2 patients who were compound heterozygous for the 33insC mutation, the onset of symptoms and development of ESRD was much later, and the level of AGT activity was higher than in the homozygous patients. In previous studies, the 33insC mutation has also been found [10, 16]. The results of the study by Milosevic et al are in line with our findings [16]. Remarkably, a study by Amoroso et al revealed the opposite effect of the homozygous and heterozygous 33insC mutation: the worst outcome was shown by the heterozygous patient (no pyridoxine responsiveness, no residual AGT activity), in contrast to a milder phenotype in 2 homozygous patients who both appeared to be pyridoxine responsive, and only 1 of whom developed ESRD [10]. This seems hard to explain because the insertion of a nucleotide at residue 33 (156) will result in a reading frame shift and introduces a termination codon at residue 44 (167). The resulting truncated protein would not be predicted to have any enzyme activity, if synthesized at all. The fact that a heterozygous patient for this mutation was responsive to pyridoxine therapy can be explained by the presence of the Gly170Arg mutation.

The Gly82Arg mutation

Although all 3 Gly82Arg homozygous patients had a normal renal function at the time of diagnosis, 2 of them suffered from progressive renal insufficiency over time. Both patients were not responsive to pyridoxine. As far as we know, these 2 families are the first ever described with this mutation. This mutation parallels the previously reported Gly82Glu mutation with respect to the pyridoxine unresponsiveness [2]. Zhang et al showed that binding of pyridoxine to AGT has become impossible under influence of the Gly82Glu mutation as a result of a change in the crystal structure of AGT [17]. It seems likely that the same occurs in the Gly82Arg mutation.

Heterozygous and compound heterozygous mutations

In 4 patients, only 1 heterozygous mutation could be found. Purdue et al and Milosevic et al already showed that in patients who were heterozygous for the Gly170Arg allele and homozygous for the minor allele, only the mutant allele was expressed, indicating that the other allele contained a mutation affecting its transcription or protein stability [12, 16]. However, of the 3 patients heterozygous for the Gly170Arg allele, only 1 was homozygous for the minor allele, suggesting that there may be more alleles that are not expressed. No liver samples were available to test at the transcription level.

Like the true heterozygous patients, all compound heterozygous patients showed a great variation in clinical outcome.

Clinical implications. This study shows a clear association between homozygosity for Gly170Arg and Phe152Ile mutation on one side, and pyridoxine responsiveness on the other side. The clinical course of these patients suggests that early treatment preserves renal function. Therefore, we believe that the combination of these particular mutations, hyperoxaluria, hyperglycoluria, and pyridoxine responsiveness abolishes the need for AGT assessment because this will not reveal anything that might influence the treatment policy. The same counts for homozygous 33insC patients with neonatal onset of disease who do not respond to pyridoxine. In all other cases, AGT assessment remains a necessary tool in the diagnostic work up of PH1.

Mutation analysis might also play a role in the prevention of disease in the relatives of PH1 patients. Family members who are also affected but not yet diagnosed may benefit from an early conservative treatment with pyridoxine. Our study showed that affected siblings who were found by screening had a better outcome compared with the proband, despite the fact that they inherited the same mutation.

Finally, mutation analysis in ESRD PH1 patients might be useful in the decision whether to perform combined liver-kidney or isolated kidney transplantation. Therefore, we believe that even in those PH1 patients who already have developed an ESRD, AGXT mutation analysis can be very useful. It seems defensible only to perform isolated kidney transplantation in homozygous Gly170Arg or Phe152Ile patients. On the other hand, the adverse outcome of the pyridoxine negative 33insC and

Gly82Arg mutations give more argument for a combined liver-kidney, or a timely planned liver transplantation in these patients.

We therefore believe that mutation analysis should be an integral part of the diagnostic work-up of patients.

Limitations of the study

We tried to make a complete overview of PH1 mutations in a recently set up cohort of PH1 patients, comprising the entire spectrum of the disease. Some patients, however, have been lost to follow-up (death, refusal to participate), resulting in a smaller cohort than clinically described before. Other factors like diet and levels of citrate, pH in the urine, may also interplay in the process of renal tissue damage. As clinical data were obtained retrospectively, these factors have not been assessed in our study.

CONCLUSION

Mutation analysis in patients with PH1 has shown to be an important diagnostic tool in PH1 and should be applied in all patients with severe hyperoxaluria. Assessment of a well-known mutation in a patient with severe hyperoxaluria might abolish the need for the invasive liver biopsy in establishing the diagnosis PH1. Moreover, our data have shown that in certain PH1 patients, mutation analysis can also be used as a guide in the therapeutic approach of these patients.

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