Evaluation of mutation screening as a first line test for the diagnosis of the primary hyperoxalurias

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Background. A definitive diagnosis of primary hyperoxaluria type 1 (PH1) and primary hyperoxaluria type 2 (PH2) requires the measurement of alanine:glyoxylate aminotransferase (AGT) and glyoxylate reductase (GR) activities, respectively, in a liver biopsy. We have evaluated a molecular genetic approach for the diagnosis of these autosomal-recessive diseases.

Methods. Polymerase chain reaction (PCR) was used to detect three common mutations in the AGXT gene (c.33_34insC, c.508G>A, and c.731T>C) and one, c.103delG, in the GRHPR gene in DNA samples from 365 unrelated individuals referred for diagnosis of PH1 and/or PH2 by liver enzyme analysis.

Results. One or more of these mutations was found in 183 (68.8%) biopsy proven cases of PH1 and PH2 with a test negative predictive value of 62% and 2%, respectively. 102 (34.1%) of 299 biopsy proven cases of PH1 (OMIM 604285) and 2 (PH2) (OMIM 604296), is now recognized to be the result of mutations in the AGXT and GRHPR genes encoding the enzymes alanine:glyoxylate aminotransferase (AGT) (E.C. 2.6.1.44) and glyoxylate reductase (GR) (E.C. 1.1.1.79), respectively. AGT is liver specific [1] and catalyzes the peroxisomal metabolism of glyoxylate to glycine, while GR plays a role in the reduction of cytosolic glyoxylate and is predominantly, but not exclusively, hepatic in protein distribution [2]. Definitive diagnosis of both diseases currently requires a needle liver biopsy and measurement of AGT and GRHPR activities. This analysis is restricted to a small number of centers worldwide and requires the sample to be shipped frozen. It has been suggested that a molecular approach may be beneficial for the diagnosis of PH1 [3]. This approach may involve sequencing the whole coding region, although this is not necessarily foolproof as mutations may lie in noncoding regions or be synonymous changes which affect splicing. Alternatively, one could use limited mutation analysis as a first line test which may remove the necessity for biopsying all patients.

Conclusion. Limited mutation analysis can provide a useful first line test for PH1 and PH2 in patients in whom primary hyperoxaluria is suspected and in whom secondary causes have been excluded. Those patients in whom a single mutation, or no mutation, is found can then be selectively targeted for liver biopsy.

Key words: primary hyperoxaluria, mutation, screening, molecular diagnosis, glyoxylate reductase, alanine:glyoxylate aminotransferase.

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1The nomenclature used in this paper is based on that recommended by Antonarakis SE, and the Nomenclature Working Group (1998): Recommendations for a nomenclature system for human gene mutations. Hum Mutat 11:1–3, 1998, where “c” denotes cDNA sequence and nucleotide numbering uses the “A” of the ATG translation initiation start site as +1.

The primary hyperoxalurias (PH) are a group of inherited disorders of endogenous oxalate overproduction characterized by markedly elevated urinary oxalate, calcium oxalate stone disease, and early onset of renal failure. The pathogenesis of two of these disorders, types 1 (PH1) (OMIM 604285) and 2 (PH2) (OMIM 604296), critical site as
described in this gene to date (recently reviewed in [9]), including missense, nonsense, splicing, minor insertions and deletions, and major deletions. Some of these changes occur only on the Leu11 polymorphic variant (the so-called minor allele) and it is possible that the changes are nonpathologic if occurring in tandem with the Pro11 polymorphism. For example, the Ile244Thr (c.731T>C) mutation has approximately 50% of normal activity when expressed with Pro11 but less than 5% of activity when expressed in conjunction with Leu11 [5]. Some mutations are relatively common, the Gly170Arg (c.508G>A) mutation accounting for approximately 25% mutant alleles [10] and Ile244Thr (c.731T>C) mutation approximately 9% [11].

Fourteen mutations have been described in the GRHPR gene of which one, c.103delG is relatively common, although this mutation does appear to be restricted to those of Caucasian origin [2, 12].

In this paper we have evaluated a strategy of screening for three common mutations, c.33_34insC, c.508G>A, and c.731T>C in the AGXT gene, and the c.103delG mutation in the GRHPR gene for the molecular diagnosis of PH1 and PH2, respectively. In addition, we have reviewed the residual enzyme activity and clinical presentation of patients homozygous for these mutations to establish whether any clinical or biochemical phenotype is present.

METHODS

Liver and blood samples from 365 patients with suspected primary hyperoxaluria were referred to the University College London Hospitals (UCLH) Primary Hyperoxaluria service, London, UK (N = 329) or to the Biochemical Genetics laboratory, Vancouver, Canada (N = 36). Liver AGT catalytic activity was determined as previously described [13, 14]. Results were expressed as % mean normal activity to allow comparisons between laboratories. Immunoreactive AGT was determined by Western blotting as previously described [13, 15]. Clinical data regarding age of onset of disease, presentation, and date of end-stage renal failure were obtained from details received at time of biopsy. A further four patients with a diagnosis of PH2 made by hyperoxaluria and L-glycericaciduria and found to be homozygous for the c.103delG mutation were included in the phenotype analysis for PH2.

The c.33_34insC, c.508A, and c.731C mutations in AGXT and c.103delG mutation in GRHPR were analyzed by amplification of genomic DNA and restriction enzyme digestion using primers and conditions previously documented [2, 8, 11].

Mutation nomenclature is based on cDNA sequence (GenBank sequences NM_000030 for AGXT and NM_012203 for GRHPR).

RESULTS

Evaluation of screening method for diagnosis of PH1 and PH2

Of 287 patients with biopsy-proven PH1, 179 tested positive for at least one of the three common mutations in AGXT, thus the test sensitivity (test positives/all with PH1) was 62.3%. Of these, 99 (34.5%) were homozygous or compound heterozygous for c.508A, c.33_34insC and c.731C, confirming a diagnosis of PH1. The test had a negative predictive value (true negative/true negatives + false negatives) of 42%. The frequency of the c.33_34insC, c.508A, and c.731C mutations in these 287 PH1 patients was 12%, 27%, and 6%, respectively; the three mutations therefore account for approximately 45% mutant alleles in this gene.

Of the 12 PH2 patients in whom a liver biopsy result was obtained, four tested positive for the c.103delG mutation giving a test sensitivity of 33%; a diagnosis of PH2 could be made by molecular means in three cases homozygous for the change. However, the predictive value of a negative result was only 97.8% largely as a result of the smaller number of biopsies from PH2 patients.

Biochemical phenotype of homozygotes for the common mutations found in the AGXT

The enzyme activity and immunoreactivity found in liver biopsies of individuals homozygous for the three mutations is shown in Figure 1; only c.508A was associated with significant in vitro enzyme activity. As expected for a mutation which leads to a frameshift and premature termination, biopsies from individuals with the 33_34insC insertion mutation all had activity at or below the method sensitivity and the majority were negative for immunoreactive protein. AGT containing the missense mutation c.731C had very little catalytic activity.
and was only weakly positive (less than 25% of control) for immunoreactivity suggesting that an unstable protein is produced. The widest spread of activity was seen with the c.508A mutation. In two cases, one initially presenting with urolithiasis and both subsequently developing end-stage renal failure, activity was at the low end of the normal range and the final diagnosis of PH1 relied on molecular analysis. These two subjects were counted as false negatives for the enzyme test giving enzymology a sensitivity of approximately 99%.

**Biochemical phenotype of homozygotes for the 103delG mutation in GRHPR**

Only three liver biopsies were obtained from patients homozygous for this mutation. GR activities were 0, 3, and 13 nmol/min/mg (reference range 49 to 213). All had AGT enzyme activity within the reference range.

**Age and mode of presentation**

There was no apparent difference in the age of presentation of individuals homozygous for any of the four mutations analyzed, the majority of cases presenting below the age of 10 years (Fig. 2).

For the three AGXT mutations there was no difference in the mode of presentation; renal stones and renal failure being the primary presenting features (Table 1). In the c.103delG homozygotes renal stones were the predominant feature although the number of patients is small.

**DISCUSSION**

Molecular genetics has the potential to provide a non-invasive diagnosis of primary hyperoxaluria. A pragmatic approach which is both affordable and relatively quick would be to offer limited mutation analysis. This approach, when tested on a large group of patients with PH1 and PH2, identified three groups: (1) those in whom two mutations were found and therefore a firm diagnosis was possible (34.1%); (2) a group in whom a single mutation was identified, increasing the clinical support for a diagnosis of PH, but needing confirmation by liver biopsy; (3) the group who were negative for all common mutations and in whom a diagnosis of PH could not be excluded and thus would require a liver biopsy.

With the advent of modern sequencing equipment, it is feasible to sequence the whole gene but even with this approach mutations are not always found [3]. In addition, the significance of previously unrecognized mutations cannot be assumed. AGT itself has a number of polymorphisms, which change the amino acid sequence [8] but have no apparent pathologic effect [7]. Equally, there are others which do and which appear to act in concert with mutations increasing the severity of disease [5]. Finally, the cost of whole gene sequence analysis is not insignificant and would require more staff time and resources to be found. For this reason we decided to evaluate an approach based on what are quantitatively the most frequent mutations causing PH1 and PH2. Two of the mutations in the AGXT gene, c.33_34insC and c.508A, occur in all ethnic groups and are associated with the major and minor alleles respectively. In the present study, these two mutations have allele frequencies of 12% and 27%, respectively, the latter agreeing with earlier figures from this department on a smaller group of patients [10]. The high frequency of c.33_34insC has not been previously described although this mutation has been reported in a variety of ethnic groups [3, 16]. The c.731C mutation, like c.508A, is associated with the minor allele but is commonly found in individuals of Spanish and North African origin [17]. The frequency found in the present study is slightly lower than that previously described by us [11], possibly reflecting the lower number of individuals of North African/Spanish origins in the larger cohort. The c.103delG mutation in PH2 accounts for approximately 37% of cases of the disease [2] but appears to be restricted to those of Caucasian origin [2, 12]. Therefore, depending on the ethnicity of the referring population, it may be necessary to vary the selection of mutations tested for, although unless these occur at a frequency greater than 5% to 10%, little benefit will be gained from expanding the mutation base. For example, in the samples referred to the Canadian laboratory, c.731C represented only 2.8%, whereas c.454A (Phe152Ile) was more common at 6.8%. An alternative strategy currently being explored [Williams, unpublished observations] would be to include screening of individual exons showing a high density of mutations or to extend testing in any symptomatic patient found to be heterozygous for one of the common changes to include a limited number of less common but recurrent mutations.

While a reasonable sensitivity was achieved by mutation analysis for PH1 as a first screen in a patient presenting with hyperoxaluria or symptoms suggestive of endogenous oxalate overproduction, the sensitivity for
PH2 is poor. The sensitivity will fall still further if careful assessment of individual patients is not done to exclude secondary causes of hyperoxaluria. Additional biochemical analyses, such as urinary glycolate, can be used to support a presumptive diagnosis of PH1 but glycolate is normal in approximately one third of cases and has been shown to be raised in some cases which do not have the disease [18]. Conversely, L-glycericaciduria is still of use for the diagnosis of PH2, but it should be noted that the converse is not true (i.e., lack of glycericaciduria cannot exclude PH2 [19]).

The specificity of genetic testing would be expected to equate to the carrier frequency providing the analytical test is reliable. By chance, one might expect a PH1 carrier in 0.02% of the normal population [20]; the carrier frequency of PH2 is as yet unknown but is likely to be similar to, or less than, PH1. However, these figures are only estimates as a patient presenting with stones in later life is less likely to have a metabolic evaluation for their disease. Carriers would not be expected to be symptomatic but there is a very small chance of detecting a mutation in an individual who is a carrier but has hyperoxaluria from some other cause. However, the protocol we propose is an individual who is a carrier but has hyperoxaluria from variation in the number of mitochondria in a particulate cell type; catalytic activity itself gives no indication of how much enzyme is correctly located in the peroxisome, it would function relatively normally and thus one might expect a milder course of disease. However, there was no difference in age of onset of disease in this patient group compared with the other mutations, therefore if one might regard end-stage renal failure and/or death as a mark of severity, of 24 patients who had reached this stage, nine had done so by age of 20 years. This contrasts sharply with the Italian findings [16] where none of their five homozygotes for the c.508A mutation had achieved end-stage renal failure before age 20 years.

The combination of molecular and biochemical analysis is a very powerful tool for the diagnosis of the primary hyperoxalurias. For example, two cases with AGT enzyme activity in the low normal range, were misdiagnosed as “normal” in the absence of molecular results. The apparent normal catalytic activity in biopsies from individuals homozygous for the c.508A mutation may arise from variation in the number of mitochondria in a particular cell type; catalytic activity itself gives no indication of how much enzyme is correctly located in the peroxisomes and therefore potentially functional in vivo, and how much is in the mitochondria and nonfunctional.

**CONCLUSION**

A preliminary screen for limited mutations in the AGXT and GRHPR genes can serve as a useful first line investigation for PH with less risk than a liver biopsy. However, molecular genetics can only offer a diagnosis in approximately one third of cases, liver biopsy is required for the remainder.

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