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# Selected Exonic Sequencing of the *AGXT* Gene Provides a Genetic Diagnosis in 50% of Patients with Primary Hyperoxaluria Type 1

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***Background:*** **Definitive diagnosis of primary hyperoxaluria type 1 (PH1) requires analysis of alanine:glyoxylate aminotransferase (AGT) activity in the liver. We have previously shown that targeted screening for the 3 most common mutations in the *AGXT* gene (c.33_34insC, c.508G>A, and c.731T>C) can provide a molecular diagnosis in 34.5% of PH1 patients, eliminating the need for a liver biopsy. Having reviewed the distribution of all *AGXT* mutations, we have evaluated a diagnostic strategy that uses selected exon sequencing for the molecular diagnosis of PH1.**

***Methods:*** **We sequenced exons 1, 4, and 7 for 300 biopsy-confirmed PH1 patients and expressed the identified missense mutations in vitro.**

***Results:*** **Our identification of at least 1 mutation in 224 patients (75%) and 2 mutations in 149 patients increased the diagnostic sensitivity to 50%. We detected 29 kinds of sequence changes, 15 of which were novel. Four of these mutations were in exon 1 (c.2_3delinsAT, c.30_32delCC, c.122G>A, c.126delG), 7 were in exon 4 (c.447_454delGCTGCTGT, c.449T>C, c.473C>T, c.481G>A, c.481G>T, c.497T>C, c.424-2A>G), and 4 were in exon 7 (c.725insT, c.737G>A, c.757T>C, c.776 + 1G>A). The missense changes were associated with severely decreased AGT catalytic activity and negative immunoreactivity when expressed in vitro. Missense mutation c.26C>A, previously described as a pathological mutation, had activity similar to that of the wild-type enzyme.**

***Conclusions:*** **Selective exon sequencing can allow a definitive diagnosis in 50% of PH1 patients. The test offers a rapid turnaround time (15 days) with minimal risk to the patient. Demonstration of the expression of missense changes is essential to demonstrate pathogenicity.**



Primary hyperoxaluria type 1 (PH1)[1] (OMIM 259900) is an autosomal recessive disorder of glyoxylate metabolism caused by deficiency of alanine:glyoxylate aminotransferase (AGT; EC 2.6.1.44). AGT is a pyridoxine 5′-phosphate–dependent enzyme that is liver specific *(1)* and usually located within the peroxisome *(2)*. This intracellular location allows efficient removal of potentially toxic glyoxylate via conversion to glycine. In the absence of AGT, glyoxylate accumulates and is converted to oxalate, which is excreted by the kidney and leads to hyperoxaluria. Insoluble calcium oxalate salts crystallize in the kidney, leading to urolithiasis and nephrocalcinosis, thereby decreasing renal function, and ultimately leading to end-stage renal failure and systemic oxalosis if treatment is not initiated *(3)*.

*AGXT*,[2] the gene encoding the AGT protein, comprises 11 exons over 10 kb and maps to chromosome 2q37.3 *(4)*. The cDNA contains an open reading frame of 1179 nucleotides encoding a 392-residue polypeptide that homodimerizes to yield a protein of 86 kDa *(5)*. Wild-type AGT exists as 2 main genetic variants, with either a proline (major allele) or leucine (minor allele) residue at position 11 in the protein sequence *(6)*. The minor *AGXT* allele has a frequency of 15%–20% in European and North American populations *(6)* but a much higher frequency, ~50%, among PH1 patients *(7)*. The Leu11 variant has several measurable effects on the properties of recombinant AGT protein expressed in vitro. The Leu11 variant encodes a protein with ~50% of the activity of the more common Pro11 allele, and the Leu11 protein has a de-

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[1] Nonstandard abbreviations: PH1, primary hyperoxaluria type 1; AGT, alaninine:glyoxylate aminotransferase.

[2] Human gene: *AGXT*, alanine:glyoxylate aminotransferase (oxalosis I; hyperoxaluria I; glycolicaciduria; serine-pyruvate aminotransferase).

creased dimerization rate at increased temperatures *(8)*. There is also evidence that the presence of Leu11 potentiates the effect of some mutations *(8, 9)*.

The test with the highest sensitivity for the diagnosis of PH1 is analysis of AGT activity in a liver biopsy sample. This procedure is not without risk to the patient, however, and molecular testing may thus offer a less hazardous alternative. The more than 50 mutations that have been described in the gene to date include missense point mutations, nonsense mutations, splice site mutations, minor insertions and deletions, and major deletions [reviewed in Ref. *(10)*]. Direct testing for the 3 most common mutations, c.33_34insC, c.508G>A, and c.731T>C, has allowed a molecular diagnosis to be made in 34.5% of PH1 cases *(11)*. That these 3 mutations along with one third of the other documented mutations are found in exons 1, 4, and 7 suggests that these exons may be a hot spot for mutation and thus be worth targeting. This report describes the outcome of DNA sequencing of these 3 exons in 300 liver biopsy–proven PH1 patients. The diagnostic value of this wider approach as a 1st-line test for PH1 was assessed by comparing the mutation-detection rates to a screen for the 3 common mutations only *(11)*. In addition, all of the missense changes detected in these exons (both novel and previously published but uncharacterized mutations) were evaluated by their expression in vitro on the backgrounds of the major and minor alleles.

## Materials and Methods

### SAMPLES

Liver and blood samples from patients with suspected PH were obtained after their referral to the UCL Hospitals Primary Hyperoxaluria Diagnostic Service for PH1 diagnosis. A PH1 diagnosis was made in all patients by measuring AGT activity in a liver biopsy sample *(12)*. The project was approved by the R&D Committee of UCL Hospitals. The residual liver sample remaining after PH1 diagnostic testing was anonymized, and implied consent was assumed for blood samples received for molecular testing.

### DNA ANALYSIS

Genomic DNA was isolated from peripheral blood leukocytes with the QIAamp DNA Blood Mini Kit (Qiagen) in accordance with the manufacturer's instructions. DNA was isolated from the nuclear pellet of liver sonicates (~5 mg of starting tissue) by adding 600 μL of a solution containing 25 mmol/L EDTA and 20 g/L sodium dodecyl sulfate; 10 μL proteinase K (stock solution 10 g/L) was added before incubating for 2 h at 60 °C. The DNA solution was cooled to room temperature, 200 μL of 10 mol/L ammonium acetate was added, and the mixture was vortex-mixed. After centrifugation at 11 000*g* for 3 min, the supernatant was decanted into another tube, and the DNA was precipitated with 600 μL isopropanol. After another centrifugation step, the pellet was washed with 70% ethanol, air dried, and suspended in Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8). Samples were stored at −20 °C before analysis.

Intronic primers, based on the genomic sequence of *AGXT* in a chromosome 2 contig (GenBank accession no. NT_005416), were used to amplify exons 1–4 and exon 7 (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue7). PCRs typically contained 100 ng of DNA and were performed with a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems). PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) before cycle sequencing with a BigDye Terminator v3.1 Ready Reaction Kit (Applied Biosystems) with suitably positioned internal primers. Sequence analysis was carried out with an ABI 310 Genetic Analyzer (Applied Biosystems). Numbering of mutations is based on cDNA sequence NM_000030 (AGXT), with nucleotide 1 denoting the 1st coding base. Nomenclature follows HUGO/HGVS recommendations (http://www.genomic.unimelb.edu.au/mdi/mutnomen/).

### ANALYSIS OF SPLICE SITE MUTATIONS

RNA was extracted from liver by homogenization in Tri Reagent (Sigma-Genosys) according to the manufacturer's instructions. Extracted RNA (0.5–1 μg) was reverse transcribed with Sensiscript® reverse transcriptase (Qiagen), and cDNA was amplified in 2 overlapping fragments (see Table 1 in the online Data Supplement). Products were sequenced with M13 universal primers after TA cloning into the pCR® 2.1 vector (Invitrogen).

### CONSTRUCTION AND ANALYSIS OF MUTANTS

Oligonucleotide primers containing the desired mutation were designed to anneal to the same DNA segment on opposite strands of the plasmid template and were synthesized by Sigma-Genosys. Plasmid constructs of the pTrcHisA expression vector containing *AGXT* cDNA corresponding to the major and minor alleles were kindly provided by Professor C. Danpure (Department of Biology, University College London). Mutant plasmid constructs were generated by site-directed mutagenesis with a QuikChange® Site-Directed Mutagenesis Kit (Stratagene). All mutant constructs were sequenced along the entire length of the coding region to confirm the presence of the desired mutation and the absence of any spurious changes. *Escherichia coli* BL21 (DE3)–competent cells (Stratagene) were transformed with mutant plasmid, and production of histidine-tagged recombinant AGT protein was induced as described previously *(8)*. Crude bacterial extracts were prepared with Bugbuster™ (Novagen) in accordance with the manufacturer's instructions. After overnight dialysis in a T3 dialysis membrane (Pierce and Warriner) against 20 mmol/L potassium phosphate buffer, pH 7.0, extracts were analyzed for AGT catalytic activity and immunoreactivity as described previously *(12)*. Total protein was quantified by measuring the absorbance at 280 nm. For comparison, wild-type controls

for the major and minor alleles and a negative control consisting of the vector with no *AGXT* cDNA insert were also expressed.

## Results

### DNA SEQUENCING OF EXONS 1, 4, AND 7

We detected 29 sequence variants by DNA sequencing of exons 1, 4, and 7, including the most common mutations, c.33_34insC, c.508G>A, and c.731T>C. Of the 15 novel mutations, 4 were in exon 1 (c.2_3delinsAT, c.30_32delCC, c.122G>A, and c.126delG), 7 were in exon 4 (c.447_454delGCTGCTGT, c.449T>C, c.473C>T, c.481G>A, c.481G>T, c.497T>C, and c.424–2A>G), and 4 were in exon 7 (c.725insT, c.737G>A, c.757T>C, and c.776 + 1G>A; Table 1). We detected an additional 6 missense changes that have previously been reported, although not expressed in vitro. These mutations are c.26C>A *(13, 14)* and c.106C>T *(15)* in exon 1, c.466G>A *(16)* and c.518G>A *(17)* in exon 4, and c.697C>T and c.698G>A *(17)* in exon 7.

We detected 2 nonsense mutations in exon 7, both of which affected the tryptophan residue at position 246. These mutations were a novel c.737G>A mutation that was found in association with the minor allele and the previously documented c.738G>A mutation *(17)*. The c.447_454delGCTGCTGT mutation in exon 4 and the c.725insT mutation in exon 7 would be expected to lead to frame shifts and premature termination at codons 164 and 254, respectively. Two mutations (c.424–2A>G and c.776 + 1G>A) were found in the splice acceptor site of intron 3 and the splice donor site of intron 7, respectively, and would be expected to disrupt splicing. A sequence analysis of the aberrant cDNA transcripts found that the c.424–2A>G mutation produced a loss of 12 nucleotides from the beginning of exon 4, whereas the c.776 + 1G>A

**Table 1. Mutations detected in exons 1, 4, and 7 of the *AGXT* gene by DNA sequencing and the associated allele of the *AGXT* polymorphism.[a]**

| Mutation | Effect | No. of homozygotes | No. of heterozygotes | Liver AGT activity, % | Allele |
|---|---|---|---|---|---|
| Exon 1 | | | | | |
| c.2–3delinsAT | Met1Asn | 0 | 1 | 0 | Major |
| c.33delC | Frameshift | 1 | 2 | 6 | Major |
| c.30_32delCC | Frameshift | 1 | 1 | 6 | Major |
| c.33_34insC | Frameshift | 28 | 27 | 8 (0–14) | Major |
| c.106C>T | Arg36Cys | 0 | 2 | 9 | Minor |
| c.117_118insCA | Frameshift | 0 | 1 | NA | Major |
| c.121G>A | Gly41Arg | 0 | 6 | NA | Both |
| c.122G>A | Gly41Glu | 0 | 1 | NA | Major |
| c.126delG | Frameshift | 1 | 0 | 3 | Major |
| Exon 4 | | | | | |
| c.447_454del | Frameshift | 6 | 0 | 6 | Major |
| c.424–2A>G | Missplicing | 1 | 0 | 8 | Major |
| c.449T>C | Leu150Pro | 0 | 1 | NA | Minor |
| c.454T>A | Phe152Ile | 0 | 14 | 12 | Minor |
| c.466G>A | Gly156Arg | 0 | 5 | NA | Major |
| c.473C>T | Ser158Leu | 0 | 1 | 6 | Major |
| c.481G>A | Gly161Ser | 0 | 2 | 15 | Minor |
| c.481G>T | Gly161Cys | 0 | 3 | NA | Minor |
| c.497T>C | Leu166Pro | 0 | 1 | NA | Minor |
| c.508G>A | Gly170Arg | 41 | 74 | 23 (0–72) | Minor |
| c.518G>A | Cys173Tyr | 0 | 1 | NA | Major |
| Exon 7 | | | | | |
| c.697C>T | Arg233Cys | 1 | 1 | 6 | Minor |
| c.698G>A | Arg233His | 1 | 1 | 9 | Minor |
| c.725insT | Frameshift | 0 | 1 | NA | Major |
| c.731T>C | Ile244Thr | 13 | 4, 1 | 11 (0–36), NA | Minor, Major |
| c.737G>A | Trp246stop | 0 | 1 | NA | Minor |
| c.738G>A | Trp246stop | 0 | 1 | NA | Major |
| c.757T>C | Cys253Arg | 0 | 1 | 9 | Minor |
| c.776 + 1G>A | Missplicing | 0 | 1 | NA | Major |

[a] Liver AGT activity is expressed as percentage of mean control activity and is shown for individuals homozygous for a mutation or with a null allele (e.g., termination codon or missplicing mutation) on the second allele. The activity ranges for the 3 common mutations are shown in parentheses. Only mean activity is shown for other mutations. The functional sensitivity of the assay is ~10% of the mean control activity. NA, Not applicable.

mutation led to the addition of 24 nucleotides at the end of exon 7.

Interestingly, 1 patient who was a compound heterozygote for the c.508G>A and c.731T>C mutations was heterozygous for the major allele of the *AGXT* polymorphism. Both of these mutations have been associated to date only with the minor allele. The PCR product was cloned into the pCR2.1 vector, and sequencing of the individual alleles confirmed the association of the c.731T>C mutation with the major allele in this case. The liver AGT activity for this individual was 14% of that of the mean control value and was clearly consistent with a diagnosis of PH1.

### EXPRESSION STUDIES OF THE MISSENSE MUTATIONS

The novel missense mutations, as well as the previously reported but uncharacterized mutations, were expressed on the backgrounds of the major and minor alleles. With the exception of the c.26C>A change, all missense changes were associated with severely decreased catalytic activity and negative immunoreactivity when expressed on the presenting polymorphic allele (Table 2). For the major-allele mutations, the effects were equally deleterious whether they were expressed on the background of the minor or major allele. In contrast, for all but 2 of the minor-allele mutations (Arg36Cys and Leu150Pro), catalytic activity was significantly greater when the mutations were expressed on the major allele than on the minor allele ($P < 0.01$). In addition, immunoreactivity was positive, suggesting that the presence of the major allele confers a degree of stability.

**Table 2. Specific catalytic activities and immunoreactivities of mutant recombinant AGT proteins of mutations expressed on the backgrounds of the major and minor alleles of the *AGXT* polymorphism.[a]**

| | AGT specific activity, $\mu mol \cdot h^{-1} \cdot (mg\ protein)^{-1}$ | |
|---|---|---|
| **Mutation** | **Minor** | **Major** |
| Met1Asn | 1.09 (0.21) (−) | 1.19 (0.07) (±) |
| Arg36Cys | 3.3 (0.45) (−) | 7.9 (3.2) (±) |
| Gly41Glu | 0.91 (0.14) (−) | 1.03 (0.14) (±) |
| Leu150Pro | 1.13 (0.18) (−) | 1.18 (0.08) (−) |
| Gly156Arg | 1.28 (0.22) (−) | 1.24 (0.13) (−) |
| Ser158Leu | 0.89 (0.02) (−) | 1.3 (0.47) (−) |
| Gly161Ser | 1.37 (0.30) (−) | 73.6 (13.3)[b] (+) |
| Gly161Cys | 1.52 (0.79) (−) | 46.0 (5.6)[b] (+) |
| Leu166Pro | 1.66 (0.31) (−) | 78.2 (14.6)[b] (+) |
| Cys173Tyr | 1.59 (0.63) (−) | 1.84 (0.23) (−) |
| Arg233Cys | 1.19 (0.18) (−) | 72.0 (4.3)[b] (+) |
| Arg233His | 1.69 (0.26) (−) | 73.9 (3.4)[b] (+) |
| Cys253Arg | 1.13 (0.10) (−) | 23.7 (4.4)[b] (+) |
| Wild-type AGT | 318 (12.5) (+) | 499 (6.1) (+) |
| Negative control | 0.98 (0.27) (−) | |

[a] Results are presented as the mean (SD) of 3 separate experiments. Detection (+) and lack of detection (−) of immunoreactive AGT protein are indicated.

[b] Activity is significantly different ($P < 0.01$) from the activity of the negative control and from the activity of the same mutation expressed on the minor allele.

The c.26C>A change (Thr9Asn) demonstrated 138% of the mean wild-type AGT activity when expressed on the major allele. This change has previously been described as a pathological mutation *(13, 14)*, but in our study this change was actually detected in an unaffected family member and did not track with the disease. Analysis of 76 controls showed that this variant was present in 1 of 158 alleles, an observation consistent with the change being a polymorphism with no pathological significance.

### EVALUATION OF THE UTILITY OF EXON SEQUENCING FOR PH1 DIAGNOSIS

This study enables an assessment of the value of DNA sequencing of exons 1, 4, and 7 for first-line testing. We previously reported that limited restriction-digest screening for the 3 common mutations (c.33_34insC, c.508G>A, and c.731T>C) detected 1 mutation and 2 mutations in 62% and 34.5% of PH1 patients, respectively *(11)*. In the current study, DNA sequencing of exons 1, 4, and 7 detected at least 1 mutation in 224 of the 300 patients, yielding a test sensitivity of 75%. Two disease-causing mutations were identified in 149 of 300 patients. Thus, the test has a diagnostic sensitivity of 50%, which offers a 15% increase in sensitivity over restriction-digest screening for the 3 common mutations alone (95% CI, 10.7%–18.7%; $P < 0.001$).

## Discussion

In this study, DNA sequencing of exons 1, 4, and 7 of the *AGXT* gene detected 26 mutations in addition to the 3 most common mutations (c.33_34insC, c.508G>A, and c.731T>C). Fifteen novel mutations were detected, with exon 4 showing the highest density of mutations. This result is in contrast to that of a previous study with a smaller group of patients, in which exon 7 showed the highest mutation density *(17)*. Analysis of the published crystal structure of AGT *(18)* indicates that exons 4 and 7 code for amino acid residues within the large N-terminal domain of the protein that are not directly involved in the active site. The Ser158 residue encoded by exon 4 is hydrogen bonded via a side-chain hydroxyl group to the exocyclic hydroxyl O3 of the pyridoxal ring of the pyridoxine 5′-phosphate cofactor; hence, the Ser158Leu mutation is likely to disrupt cofactor binding. The negative immunoreactivity of the mutant recombinant protein would suggest, however, that this mutation, like the other missense mutations, primarily affects folding of the protein in a way that leads to decreased stability.

Sequence analysis of cDNA revealed that the c.424–2A>G and c.776 + 1G>A mutations, which occur in splice site consensus sequences, cause missplicing. Missplicing did not alter the reading frame but would disrupt the amino acid sequence via the loss of amino acid residues 142–145 in the case of c.424–2A>G and the addition of 8 amino acid residues after residue 282 in the

case of the c.776 + 1G>A mutation. Both of these alterations in the primary structure of the protein would be likely to interfere with folding and lead to protein instability. For the frameshift mutations, as with the nonsense mutations, the resulting truncated mRNA transcripts would most likely be degraded by nonsense-mediated mRNA decay *(19)*.

Of all the novel mutations identified, c.447_454delGCTGCTGT was the most common and was detected exclusively in association with the major allele in 6 unrelated patients, all of whom were homozygous and of Asian origin. The previously documented c.454T>A mutation *(20)* was found in a heterozygous state in 14 unrelated individuals, a frequency lower than the frequencies previously found in cohorts of Canadian and Italian patients *(11, 20)*.

The effects of the c.508G>A, c.731T>C, and c.454T>A minor-allele mutations have previously been reported to be potentiated when expressed on the minor allele, compared with their expression on the major allele *(8)*. These findings appeared to be due to a synergistic effect of the Leu11 polymorphism and led the authors to speculate that these mutations would have no pathological phenotype if they occurred on the background of the major allele. Interestingly, the majority of the minor-allele mutations identified in this study were associated with higher residual AGT catalytic activity and detectable immunoreactivity when they were expressed on the major allele, suggesting that the Pro11 variant can stabilize the protein. AGT activity was still significantly lower than that of the wild type, however, and it would seem likely that the mutations would confer disease in vivo, regardless of the associated polymorphic allele. This theory is supported by the detection of the c.731T>C mutation on the background of the major allele in a proven PH1 patient in this study (Table 1).

When mutation detection is used to diagnose inherited disease, it is essential to demonstrate the functionality of any detected novel sequence changes. Of the 50 or so mutations identified in the *AGXT* gene, however, few have been characterized to date. Pathogenicity has usually been implied if the change is absent in 100 wild-type alleles or if heterozygosity for the change is demonstrated in the parents of the patient. As is seen with the c.26C>A change, however, this strategy is not wholly successful. Given that this change affected a nonconserved amino acid residue, perhaps such a change could also be an indicator of nonpathogenicity, perhaps requiring a higher level of proof (i.e., expression studies).

We have previously shown that selected mutation screening for the 3 common mutations (c.33_34insC, c.508G>A, and c.731T>C) as a 1st-line test avoids the need for liver biopsy in 34.5% of patients *(11)*. In the current study, we evaluated the diagnostic value of targeted DNA sequencing of the exons where these mutations reside. This wider approach affords a significant increase in diagnostic sensitivity to 50% of PH1 patients. Although this sensitivity is lower than that of liver AGT activity [estimated to be >99% *(11)*], it is achieved by means of a relatively noninvasive outpatient procedure. The sensitivity of genetic analysis could be increased by sequencing the entire *AGXT* gene, and 2 studies have suggested this approach as a possible suitable replacement for liver enzyme studies *(21, 22)*. In both studies, however, sequencing of all of the exons of the *AGXT* gene was applied to only small patient cohorts, and both disease-causing mutations were identified in only 24 of 29 probands. Thus, the sensitivity of whole-gene sequencing is less than that of liver biopsy analysis, and ~20% of patients would be left without a definitive diagnosis. For these patients, a liver biopsy will still be necessary, and whole-gene sequencing will have substantially delayed the diagnosis and increased the cost. Therefore, targeted sequence analysis of exons 1, 4, and 7 offers the most convenient 1st-line test available, and liver biopsy analysis can be offered to those patients who test negative in the former test and for whom a high clinical suspicion for PH1 exists. The turnaround time for such a test would be ~15 days. In cases in which a novel mutation is found, the ideal next step would be to conduct expression and/or family studies to demonstrate functionality.

An advantage of mutation detection over liver biopsy analysis is the knowledge obtained of the causative mutations, which can then be used for diagnosis of siblings and prenatal diagnosis, because such information will not be reliant on linkage analysis *(23)* and will identify de novo mutations *(24)*. In addition, knowledge of the genotype may be useful in guiding patient treatment. For example, the c.508G>A and c.454T>A mutations both produce AGT protein and are associated with responsiveness to pyridoxine *(13, 22)*. By contrast, patients with null mutations, such as c.33_34insC and c.447_454delGCTGCTGT, which produce no AGT protein, may be unresponsive to pyridoxine therapy and hence be more likely to require liver transplantation for effective treatment.

In conclusion, targeted DNA sequencing of exons 1, 4, and 7 of the *AGXT* gene as a first-line test for the diagnosis of PH1 in symptomatic individuals offers a substantial increase in diagnostic sensitivity over screening for only the 3 common mutations. Use of this approach avoids the need for liver biopsy in 50% of PH1 patients and can provide a quicker diagnosis at lower cost than whole-gene sequencing.

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