# Updated Genetic Testing of Primary Hyperoxaluria Type 1 in a Chinese Population: Results from a Single Center Study and a Systematic Review*

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**Summary**: Primary hyperoxaluria type 1 (PH1) is a rare but devastating autosomal recessive inherited disease caused by mutations in gene *AGXT*. Pathogenic mutations of *AGXT* were mostly reported in Caucasian but infrequently in Asian, especially in Chinese. To update the genotypes of PH1 in the Chinese population, we collected and identified 7 Chinese probands with PH1 from 2013 to 2017 in our center, five of whom had delayed diagnosis and failed in kidney transplantation. Samples of peripheral blood DNA from the 7 patients and their family members were collected and sequencing analysis was performed to test the mutations of gene *AGXT*. Western blotting and enzyme activity analysis were conducted to evaluate the function of the mutations. Furthermore, a systematic review from 1998 to 2017 was performed to observe the genetic characteristics between Chinese and Caucasian. The results showed that a total of 12 mutations were identified in the 7 pedigrees. To the best of our knowledge, 2 novel variants of *AGXT*, p.Gly41Trp and p.Leu33Met, were first reported. Bioinformatics and functional analysis showed that only 7 mutations led to a reduced expression of alanine-glyoxylate amino transferase (AGT) at a protein level. The systematic review revealed significant population heterogeneity in PH1. In conclusion, new genetic subtypes and genetic characteristics of PH1 are updated in the Chinese population. Furthermore, a genotype-phenotype correlation is found in PH1.

**Key words**: primary hyperoxaluria type 1; gene sequencing; *AGXT*; Chinese population

Primary hyperoxaluria (PH) is an autosomal recessive inherited disease characterized by oxalate overproduction, and the patients often present with hyperoxaluria, nephrolithiasis, nephrocalcinosis and chronic renal failure[1–3]. To date, 3 types of PH have been identified, including PH1 (OMIM 259900, pathogenic gene: *AGXT*), PH2 (OMIM 260000, pathogenic gene: *GRHPR*), and PH3 (OMIM 613616, pathogenic gene: *HOGA1*). Among them, PH1 is the most common and severe type. PH1 is caused by deficiency of the liver-specific, peroxisomal enzyme alanine-glyoxylate amino transferase (AGT), which is encoded by *AGXT*[4]. Human *AGXT* is located on chromosome 2 (2p37.3), with 11 exons spreading across 10 kb (GenBank M61755 to M61763 and M61833)[4].

Genetic testing of *AGXT* is a robust but less

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*This work was supported by the grants from the Special Project of Ministry of Health (No. 201302009) and the National Natural Science Foundation of China (No. 81700300).

invasive tool for the diagnosis of PH1[5]. More than 150 variants of *AGXT* have been reported in the human gene mutation database (HGMD) and primary hyperoxaluria mutation database (Accessed 10 Dec 2015). Most variants are missense mutations, which affect the folding and targeting of the AGT protein. In European and North American populations, p.Gly170Arg (G170R) is the most common variant that is responsible for 25%–40% of PH1 cases. In 1998, Toussaint concluded that more than 20% of PH1 patients respond to the treatment with pyridoxine (VB6)[6] and further studies confirmed that these patients carried the G170R variant[7, 8]. Patients carrying two other common missense variants, p.Ile244Thr and p.Phe152Ile, also showed a response to VB6 treatment[9], which was less efficacious in these patients than in those carrying the G170R variant though. VB6 is not effective for most mutations except for only a few subtypes of PH1 mentioned above. Combined kidney-liver transplantation is recommended for PH1[10].

PH1 is considered to be an extremely rare disease with the prevalence of $(1–3)\times10^{-6}$ only[11]. Due to insufficient understanding of PH1, up to 30% cases are diagnosed very late until end-stage renal disease (ERSD) occurs or, even worse, after failed kidney transplantation[12]. Most studies that examined the gene mutations of PH are from Western European, North Africa and Middle East[12]. The epidemiology and characteristics of the *AGXT* genotype and outcomes, however, remain unclear in Asia, especially in China. To update the genotypes of PH1 in the Chinese population, we collected and identified 7 Chinese probands with PH1 from 2013 to 2017 in our organ transplantation center, five of whom had delayed diagnosis and failed in kidney transplantation (KT). Furthermore, a systematic review from 1998 to 2017 was performed to observe the genetic characteristics between Chinese and Caucasian.

## 1 MATERIALS AND METHODS

### 1.1 Study Subjects

This study was approved by the local institutional review board on human subject research at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (IRB ID: TJ-C20160114), and it conformed to the guidelines set forth by the Declaration of Helsinki. Written informed consent was obtained from all the patients and control participants. All donors were from organ donation after citizen death.

### 1.2 Direct DNA Sequencing Analysis for Mutations

Seven PH1 patients who were treated at our center between 2013 and 2017 were collected. Human genomic DNA of the blood samples from the 7 patients and their family members was extracted as described previously[13]. All exons, intron-exon boundaries, and untranslated regions (UTRs) of *AGXT* were screened via polymerase chain reaction (PCR) to find rare variants. The PCR products of DNA extracted from 6 patients were purified by agarose gel electrophoresis and sequenced directly using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA).

### 1.3 Bioinformatic Analyses for Variants

HGMD (http://www.hgmd.cf.ac.uk/ac/index.php) and hyperoxaluria mutation database (www.uclh.nhs.uk/OURSERVICES/SERVICEA-Z/PATHBIOMED/CBIO/Pages/Phmdatabase.aspx) were used to catalogue the missense mutations in *AGXT* which had been reported. Conservatism analysis for variants was performed by Centre for Integrative Bioinformatics VU (http://www.ibi.vu.nl/programs/pralinewww/). The degree of harmfulness of variants was predicted by Variant Effect Predictor online (http://asia.ensembl.org/Homo_sapiens/Tools/VEP?db=core). The Sorting Tolerant From Tntolerant (SIFT) and Polymorphism Phenotyping (PolyPhen) scores were graded to show the degree of harmfulness of variants. The ExAC data set (Exome Aggregation Consortium, http://exac.broadinstitute.org/) was used to observe the heterogeneity of variants in different populations.

### 1.4 Western Blotting

Human Hela cells were cultured in 12-well plates for 24 h and transfected with 1 μg either p3xFLAG-CMV-10-AGXT-Wt or p3xFLAG-CMV-10-AGXT-Mut including 12 mutants, while the empty vector (p3xFLAG-CMV-10) was used as a negative control. Forty-eight h later, the transfected cells were collected and incubated in ice-cold Tris/HCL&NaCl&EDTA&Nonidet (TNEN) lysis buffer (50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 2.0 mmol/L EDTA, 1.0% Nonidet P-40) with 1 mini tab of EDTA-free protease inhibitors (Roche, USA) and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) for 30 min at 4°C. The insoluble fraction was pelleted by centrifugation at 12 000 × g for 15 min at 4°C. The supernatants (100 μL) were mixed with 25 μL 5× Laemmli buffer [0.3 mol/L Tris-HCl, 6% SDS, 60% glycerol, 120 mmol/L dithiothreitol (DDT), and proprietary pink tracking dye], and heated at 100°C for 10 min. Then, 10 μL samples were subjected to SDS-PAGE (10%). After electrophoresis, proteins were transferred onto a 0.45 μm polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The membrane was probed with an anti-DDDDK-tag mouse monoclonal antibody (M185-3L, 1:6000, Medical & Biological Laboratories, Japan), followed by incubation with HRP-conjugated secondary goat anti-mouse antibody (1:5000, Biosharp, China). The protein signal was visualized by a Super Signal

West Pico Chemiluminescent substrate according to the manufacturer's instructions (Pierce Chemical Co., USA). Human α-tubulin (1:5000) was used as a loading control. Each assay was performed in triplicate and repeated at least three times. Since limited samples could be loaded on one gel, we ran two SDS-PAGE gels simultaneously for total 16 samples [including 12 mutations, 2 negative control (NC) and 2 wide type (WT) samples] for each experiment.

**1.5 Enzyme Activity Analysis**

The protocol of enzyme activity analysis was mentioned in previous studies[14, 15]. Briefly, human Hela cells were cultured in 6-well plates for 24 h and transfected with 2 μg either p3xFLAG-CMV-10-AGXT-Wt or p3xFLAG-CMV-10-AGXT-Mut including 12 mutants. After 48 h, transfected cells were collected and incubated in accordance with what was mentioned above. The supernatant (150 μL) was incubated at 37°C in a medium containing 50 mmol/L glyoxylate (Catalog Number: XW02981241, Urchem, China), 50 mmol/L L-alanine (Catalog Number: L800640-25g, Macklin, China), 150 μmol/L pyridoxal 5-phosphate (PRP) (Catalog Number: P816234-1g, Macklin, China) and 100 mmol/L potassium phosphate (Sinopharm Chemical Reagent Co., Ltd, China), pH 8.0, in a final volume of 200 μL. After 120 min, the reaction was terminated by adding 100 μL 3.0 mol/L trichloroacetic acid (Sinopharm Chemical Reagent Co., Ltd, China) to the cooled mixture. Denaturated proteins were removed by centrifugation for 5 min at 13 000 r/min at 4°C.

The enzyme activity of *AGXT* was represented by pyruvic acid which is the product of the enzymatic reaction. Pyruvic acid was detected by Agilent 1100 Series LC/MSD Trap SL (Agilent, USA), and pyruvic acid (5 mg/mL) (Catalog Number: Wts2097-20mg, China) used as standard sample. This test method is qualitative.

**1.6 Search Strategy and Selection Criteria**

For the systematic review, we searched PubMed and EMBase Database (January 1, 1998 to December 31, 2017) with the terms ["AGXT gene" OR "Primary hyperoxaluria type 1" AND "mutation"] or ["AGXT gene" OR "Primary hyperoxaluria type 1" AND "variant"]. We also searched two main Chinese databases: Wanfang and Weipu Database in order to review the reported *AGXT* mutations in the Chinese population. Papers in English or Chinese language were retained. All Chinese papers selected had English abstracts. Papers without *AGXT* genetic testing, without clinical PH1 phenotypes or without population information were excluded.

**1.7 Statistical Analysis**

Experimental data from at least three independent experiments was presented as means±standard error (S.E.). Statistical analysis was performed with Student's *t*-test using SPSS version 17.0 software (SPSS, USA). A *P*-value of 0.05 or less was considered to be significant. For Western blot analysis, the images from three independent experiments were scanned with Quantity One 4.6.8 (Basic) (Bio-Rad, USA) and quantified. The means from three independent experiments were compared between two different groups with Student's *t*-test.

## 2 RESULTS

**2.1 Clinical, Biochemical and Genetic Characteristics of Seven PH1 Probands**

A total of 1247 adult KTs were performed from 2013 to 2017 in our center, and 7 patients were diagnosed as having PH1, which was confirmed after detection of mutations of *AGXT* gene by sequencing (fig. 1A). The prevalence of PH1 in our center was approximately 6/1000, much higher than previous reports ($1/10^6$ to $3/10^6$)[11]. Among the 7 probands, KT failed in 5 and the other two gave up KT due to a definite diagnosis. The clinical characteristics of patients are showed in table 1.

After *AGXT* genetic testing, the 7 PH1 pedigrees were subjected to detection of total 12 variants including p.Ala186Val, p.Arg197Gln, p.Arg36His, p.Arg112Term, p.Arg111Term, p.Gly41Trp, p.Lys12Gln fs, p.Arg360Gln, p.Leu33Met, p.Gly350Asp, p.Ile340Met and Lys209Asn. HGMD and primary hyperoxaluria mutation database revealed that 2 variants, p.Gly41Trp and p.Leu33Met, were newly reported to be associated with PH1 (the sequence results are shown in fig. 1B). The 2 mutations were conserved in different species (fig. 1C, score=7 evaluated by Centre for Integrative Bioinformatics VU). We also analyzed the possible effects of 7 missense variants, using SIFT and PolyPhen

**Table 1 Manifestations and biochemical features of patients with PH1**

| Patient ID* | Gender | Age (years) | Age of onset (years) | SCr (μmol/L) | Nephrolithiasis |
|---|---|---|---|---|---|
| Ⅰ21 | Female | 38 | 18 | 730 | Yes |
| Ⅱ3 | Female | 44 | 22 | 125 | Yes |
| Ⅲ3 | Male | 35 | 22 | 1103 | Yes |
| Ⅳ3 | Male | 48 | 43 | 515 | No |
| Ⅴ3 | Female | 20 | 20 | NA | Yes |
| Ⅵ3 | Male | 44 | 44 | NA | Yes |
| Ⅶ3 | Female | 37 | 31 | NA | Yes |

*The patient ID was named by family number combined with member number in the family.

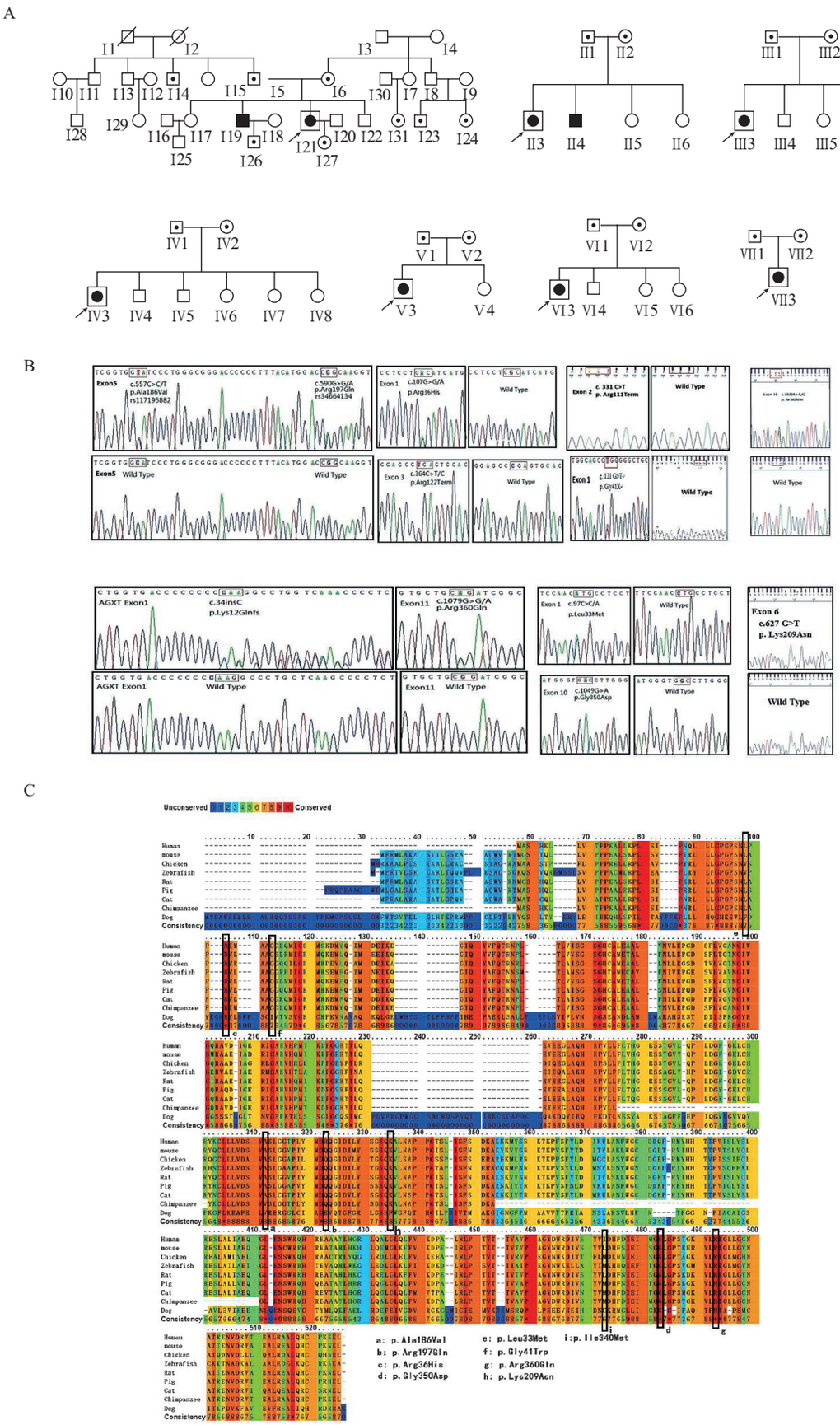


**Fig. 1** Identification of rare mutations within *AGXT* gene by sequencing

A: the pedigree plot for the 7 patients with PH1; B: the sequencing results of mutations within *AGXT* gene of the 7 patients with PH1; C: the conservatism of 12 mutations in the 7 patients. Mutation p.Arg36His, p.Arg360Gln, and p.Gly350Asp of AGXT are highly conserved throughout evolution.

**Table 2 The critically analysis information of variable locus in *AGXT***

| Sample ID | Exon | Location (GRCh37/hg19) | DNA variation | Protein variation | Type | MAF | SNP | SIFT | PolyPhen |
|---|---|---|---|---|---|---|---|---|---|
| Ⅰ21 | Exon 10 | chr2:241817545 | c. 1049 G>A | p. Gly350Asp | Homozygous | 0.00006 | rs180177156 | 0 | 1 |
| Ⅰ21 | Exon 1 | chr2:241808379 | c. 97 C>A | p. Leu33Met | Heterozygous | / | / | 0.24 | 0.13 |
| Ⅱ3 | Exon 1 | chr2:241808389 | c. 107 G>A | p. Arg36His | Heterozygous | 0.00002 | rs180177162 | 0 | 0.985 |
| Ⅱ3 | Exon 3 | chr2:241810066 | c. 364 C>T | p. Arg122* | Heterozygous | 0.0001 | rs180177210 | / | / |
| Ⅲ3 | Exon 1 | chr2:241808403 | c. 121 G>T | p. Gly41Trp | Heterozygous | / | / | 0.19 | 0.999 |
| Ⅲ3 | Exon 2 | chr2:241808752 | c. 331 C>T | p. Arg111* | Heterozygous | 0.000009 | rs180177202 | / | / |
| Ⅳ3, Ⅵ3 | Exon 5 | chr2:241812428 | c. 557 C>T | p. Ala186Val | Heterozygous | 0.0114 | rs117195882 | 0.05 | 0.227 |
| Ⅳ3, Ⅵ3 | Exon 6 | chr2:241812461 | c. 590 G>A | p. Arg197Gln | Heterozygous | 0.0114 | rs34664134 | 1 | 0 |
| Ⅳ3 | Exon 10 | chr2:241817516 | c.1020 A>G | p.Ile340Met | Homozygous | 0.1653 | rs4426527 | 1 | 0 |
| Ⅴ3, Ⅵ, Ⅶ | Exon 1 | chr2:241808315 | c. 34 insC | p. Lys12Glnfs | Heterozygous | / | rs398122322 | / | / |
| Ⅴ3 | Exon 11 | chr2:241818138 | c. 1079G>A | p. Arg360Gln | Heterozygous | / | rs180177161 | 0 | 1 |
| Ⅶ | Exon 6 | chr2:241813426 | c. 672G>T | p.Lys209Asn | Heterozygous | / | / | 0 | 1 |

online software (table 2).

The results showed that these 2 novel mutations were harmless (p.Gly41Trp: SIFT score=0.19, PolyPhen score=0.999 and p.Leu33Met: SIFT score=0.24, PolyPhen score=0.13). In order to further identify the function of mutations, we examined the regulation of mutations at the protein level by Western blotting analysis. The results showed that the mutations p.Lys12Glnfs, p.Arg111*, p.Arg122* and p.Ala186Val caused the 100% loss of the AGT protein expression. Mutations p.Leu33Met, p.Ile340Met and Arg360Gln significantly reduced 30.7%, 15.7% and 16.7% of the *AGXT* protein expression respectively ($P$<0.0001, fig. 2A and 2B).

Furthermore, we examined the regulation of mutations at the enzyme activity level by mass spectrum analysis. The mass spectrum analysis was qualitative and the results were consistent with Western blotting analysis. Mutations p.Lys12Glnfs, p.Arg111*, p.Arg122* and p.Ala186Val caused the 100% loss of the AGT protein activity. Mutations p.Arg197Gln, p.Arg36His, p.Gly41Trp, p.Arg360Gln, p.Leu33Met, p.Gly350Asp and p.Ile340Met partly caused the loss of the AGT protein activity (fig. 3). The enzyme activity results were consistent with the Western blotting results.

### 2.2 Genotype-phenotype Correlation in PH1

In our study, we totally tested 7 PH1 probands

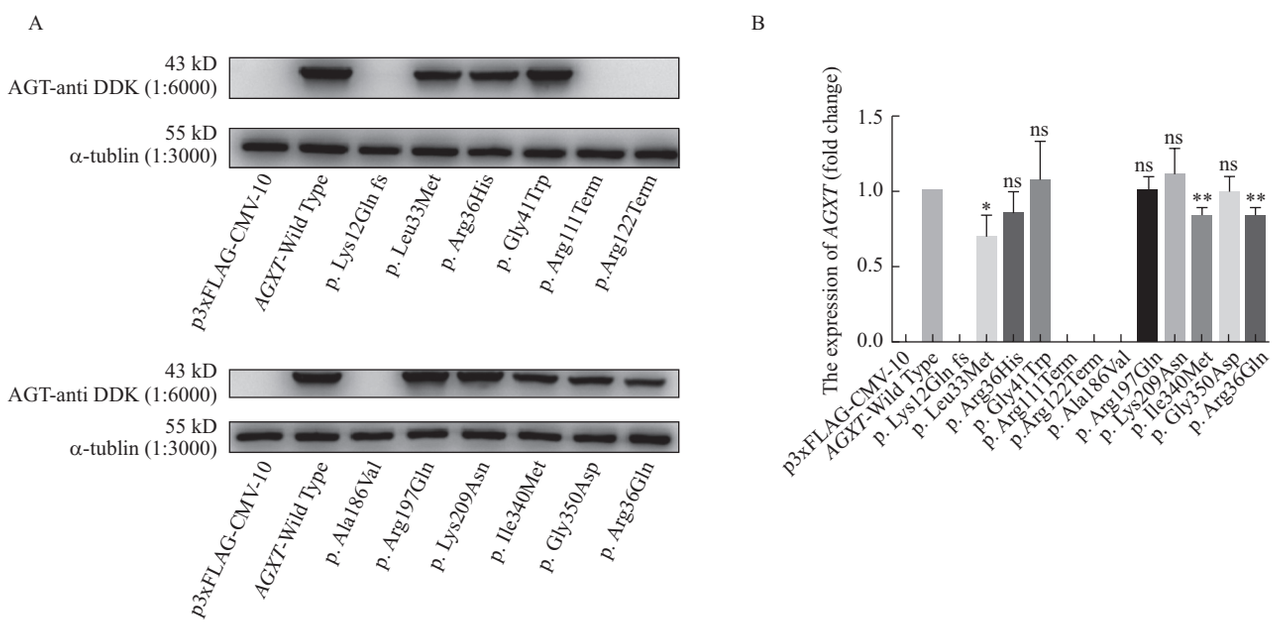


**Fig. 2** Rare mutations within *AGXT* regulated expression of AGT protein

A: Western blot analysis of *AGXT* mutations. Mutations p.Lys12Gln fs, p.Arg111Term, p.Arg122Term and p.Ala186Val caused the 100% loss of the AGT protein expression. Mutations p.Leu33Met, p.Ile340Met and Arg360Gln significantly reduced 30.7%, 15.7% and 16.7% respectively of the *AGXT* protein expression. α-tublin was used as a loading control. B: Western blot analysis was quantified. For three independent experiment, the target protein AGT was exposed 1 or 2 seconds per image, while α-tublin was exposed properly according to the different circumstances every time. Protein AGT and α-tublin were exposed independently from the different part of the same gel for every independent experiment. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* *AGXT*-Wilde Type; ns: no significance

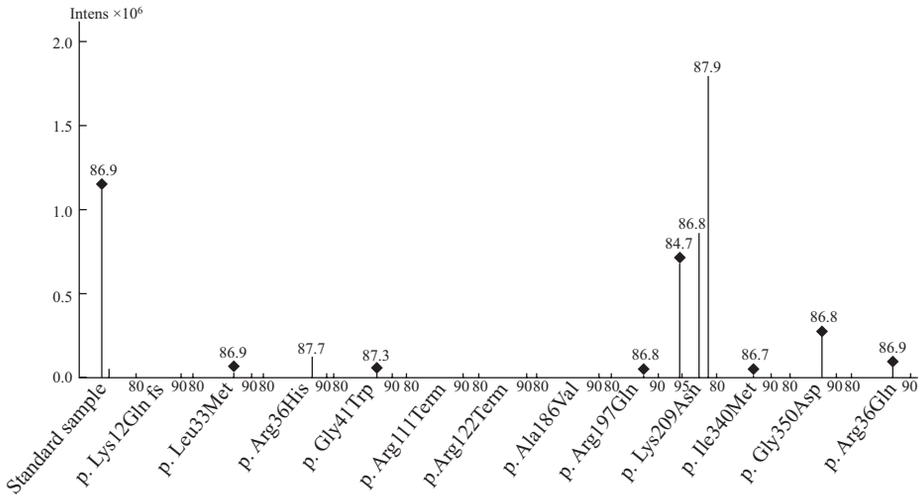


**Fig. 3** Rare mutations within *AGXT* regulated enzyme activity of AGT protein
The enzyme activity of AGXT was represented by pyruvic acid and measured using mass-spectrography analysis. The optimized parameter of the mass spectrum was determined as follows: sampling method: LC-MS; ion source: ESI; detection mode: negative ion; scan range (m/z): 20–200; ion source temperature (℃): 240; atomization gas flow rate (PSI): 40; drying gas flow rate (L/min): 10; capillary voltage (V): 1500; capillary outlet voltage (V): 109; scanning mode: Auto MS/MSn

and their pedigrees. It was found that there was a genotype-phenotype correlation in PH1. First, frameshift or terminate mutations were associated with severe phenotypes. In our study, the patients with onset of symptoms such as nephrolithiasis were significantly younger when they carried frameshift or terminate mutations compared with other mutations (mean age: 24 years *vs.* 35 years). Second, multi-mutations caused severe phenotypes. In our study, patient I21 carried 3 missense mutations including a homozygous mutation p.Gly350Asp and a heterozygous mutation p.Leu33Met and his age at onset of symptoms was 18 years old. However, I19 (the brother of patient I21) who carried a homozygous mutation p.Gly350Asp only had relatively mild phenotypes and have not developed ESRD till now (41 years old).

### 2.3 Genetic Characteristics of PH1 Patients in Chinese Population

To date, PH1 was most reported in Caucasian but rarely in Chinese. In order to update the genetic characteristics of PH1 patients in the Chinese population, we performed a systematic review (1998–2017) to observe the genetic characteristics between Chinese and Caucasian. In total, we screened 336 abstracts and finally 56 studies were included (fig. 4A). These 56 studies provided clear race information and exact genotypes of *AGXT* which were screened by sequencing testing. Totally 66 mutations were identified among Caucasian and 22 among Chinese. The mutations of *AGXT* identified in Chinese population were significantly different from those in Caucasian (fig. 4B).

Firstly, only 7 mutations including p.Lys12Glnfs, p.Arg122*, p.Ser275Argfs, p.Arg333*, p.Ile340Met, p.Gly350Asp and p.Arg360Gln were overlapped between the two populations. Secondly, in the Caucasian population, 6 mutations including p.Gly170Arg, p.Ile244Thr, p.Phe152Ile, p.Lys12Glnfs, p.Gly190Arg and p.Pro11Leu/Glnfs were very common, which were identified in at least 5 studies, but they didn't appear in Chinese population. Interestingly, the 3 most common mutations p.Gly170Arg, p.Ile244Thr and p.Phe152Ile were rare in the Chinese population. Thirdly the mutations of *AGXT* identified in the Chinese population were quite sporadic, which is inconsistent with common mutations identified in multiple studies.

## 3 DISCUSSION

Genetic basis of PH is believed to be clear but still a number of suspected PH cases weren't confirmed by genetic testing[5], especially in China. PH1 has been considered as an extremely rare disease with the prevalence of 1/1000000 in previous studies[11]. However, the prevalence of PH1 was higher in our study, reaching 1/1000, which we speculate is due to lack of genetic testing to confirm PH1 before the KT. As we know it, isolated KT is not recommended for PH1 patients. In China, PH1 patients are more likely to be misdiagnosed with common chronic kidney diseases and even receive isolated KT owing to the lack of sequencing testing. From 2013 to 2014, a total of 455 patients accepted KT in our center and the rate of graft dysfunction (including patient death) in one year was 5.05% (23 patients). Among them, 13.04%

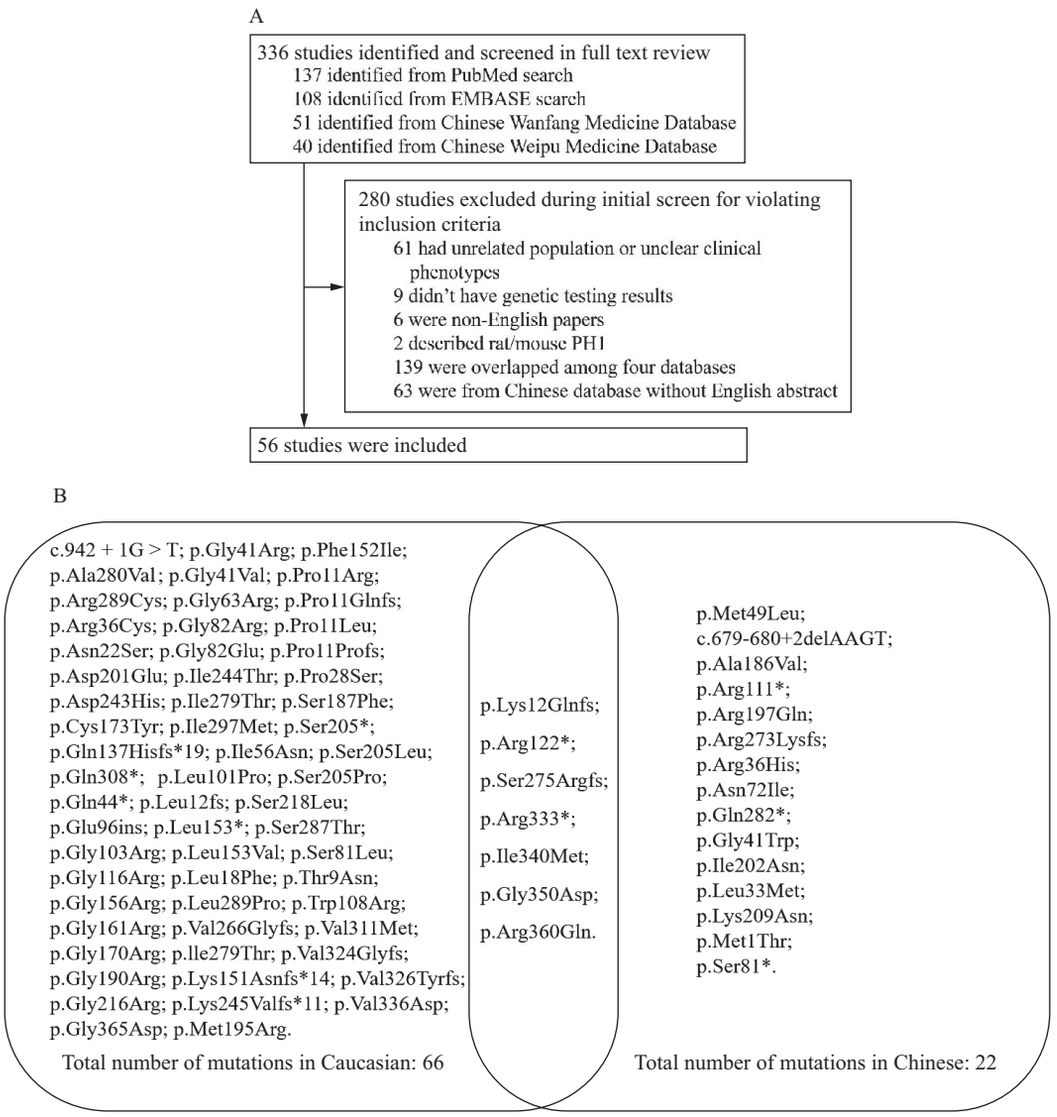


**Fig. 4** Data selection and mutations in Han population and Caucasian
A: search strategy and selection criteria; B: list of mutations in Han population and Caucasian. Seven clinical related mutations were overlapped in both populations.

(three patients) were PH1. Since genetic testing was ignored, KT was performed and failed. From 2015 to 2016, two patients waiting for KT were suspected to have PH1 and they subsequently received sequencing analysis and were diagnosed with PH1. Therefore, we consider that sequencing analysis is very necessary for avoiding KT failure due to PH1.

Remarkably, the findings in our study showed that the three most common missense variants of *AGXT* reported in the Caucasian population—G170R, I244T, and F152I—were extremely rare in the Chinese population. Some reports pointed that it is only necessary to test selected exons of *AGXT* where common mutations are found, instead of the entire gene[12, 16–18]. However, owing to the significant heterogeneity in the Chinese population, we believe that it is more appropriate to test all exons of *AGXT*. Furthermore, the mutations of *AGXT* identified in the Chinese population were quite sporadic and there was a lack of very frequent mutations. Population heterogeneity was significant in PH1. According to previous studies, 20% PH1 patients who carried p.G170R, p.F152I, and p. I244T could respond to VB6 treatment[6, 9]. Unfortunately, it is very hard to evaluate if those sporadic and low frequent mutations are sensitive to VB6 treatment. Future studies with larger sample sizes are needed to further validate genetic characteristics relevant to PH1 in the Chinese population.

Additionally, in the current study, it was proved that there was a genotype-phenotype correlation in PH1. PH1 is caused by deficiency of the liver-

specific, peroxisomal enzyme AGT[19]. Therefore, severe mutations such as frameshift or terminate mutations could cause haploinsufficiency of AGT and clinical PH1 phenotype. Similarly, multi-mutations in a patient were also found to be associated with severe phenotypes. However, except for those severe mutations and the most common mutation p. G170R, no comparable knowledge is available for the remaining missense mutations[20]. Moreover, it is difficult to discriminate true mutations from rare polymorphisms. In addition, unlike autosomal dominant inherited diseases which are caused by one mutation, autosomal recessive diseases such as PH1 are the manifestation of at least two mutations. Most of the variant pathogenicity classifiers such as SIFT, PolyPhen-2, CADD, MetaLR and the latest M-CAP are effective to evaluate the pathogenicity of single-site missense variants[21]. Genotype-phenotype correlation is fairly complicated, which is considered to be a reason for the genetic heterogeneity of PH1. Several recent reports have highlighted the exorbitant genotype-phenotypic heterogeneity of patients with PH1[12]. Currently, the factors contributing to this heterogeneity are unclear. In our study, the Western blot results also showed that a considerable proportion of mutations seemed to be mild mutations. Therefore, a large number of controls need to be tested to discriminate pathogenic mutations from polymorphisms.

It is noteworthy that the present study was limited by its small sample size. In addition, due to the limited reports on PH1 in Chinese population, many variants were not identified in the Chinese population in this study, including some variants which are also common in other populations. Future studies with larger sample sizes are needed to further validate genetic characteristics relevant to PH1 in the Chinese population.

**Conflict of Interest Statement**

All the authors declare no conflicts of interest.

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(Received May 15, 2018; revised Sep. 6, 2018)