INTRODUCTION

Fabry disease (FD, OMIM #301500) is a rare X-linked recessive genetic lysosomal storage disease caused by deleterious mutations in the GLA gene encoding the lysosomal enzyme α-galactosidase A (GLA) (Desnick et al., 2003). Currently more than 900 related mutations were reported, and different mutations can result in different degrees of loss of α-Gal A activity. Due to mutation of the gene encoding GLA, part or all of the enzyme activity is lost, resulting in lysosomal accumulations of globotriaosylceramide, and other neutral glycosphingolipids in various cells and tissues. FD is often a multiorgan disease due to a large amount of accumulation in human body such as heart, kidney, pancreas, skin, lungs,
and nervous system eventually causes a series of organ lesions (Echevarria et al., 2016; Germain, 2010; Scriver, 1995). The patients with Fabry’s disease are mainly male, and the incidence rate is 1:40,000–60,000. According to the different levels of GLA activity in patients, the clinical manifestations and severity of FD are diversified. In general, male patients are more serious than female patients (heterozygous), and most patients die from cardiovascular and cerebrovascular complications or uremia at 40–50 years old (Echevarria et al., 2016; Germain, 2010; Scriver, 1995).

Inflammatory processes observed in lysosomal storage disorders have already been reported. The inflammatory response in the central nervous system (CNS) primarily involves microglial cells (resident dendritic cells) and astrocytes in many lysosomal storage disorders. The main symptom of CNS involved in FD is stroke which is caused by the inflammatory processes acting on vascular and cardiac pathways. It is easy to understand that the CNS is the primary target for many lysosomal storage disorders due to its intrinsic peculiarities (highly differentiated cells with limited chances of replication) (Tuttolomondo et al., 2011; Tuttolomondo, Pecoraro, Simonetta, Miceli, Arnao, et al., 2013; Tuttolomondo, Pecoraro, Simonetta, Miceli, Pinto, et al., 2013).

So far, more than 750 missense mutations have been reported in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=GLA). Missense mutations affecting any one of the cysteines that form the disulfide bonds of the wild-type GLA are associated with the classic FD phenotype, and show negligible or very low residual enzyme activity (Ashley, Shabbeer, Yasuda, Eng, & Desnick, 2001; Ashton-Prolla et al., 2000; Blanch, Meaney, & Morris, 1996; Elstein, Altarescu, & Beck, 2010; Eng, Resnick-Silverman, Niehaus, Astrin, & Desnick, 1993; Galanos et al., 2002; Okumiya et al., 1995; Rodriguez-Mari, Coll, & Chabas, 2003; Schafer et al., 2005; Shabbeer, Yasuda, Luca, & Desnick, 2002; Topaloglu et al., 1999; Yasuda et al., 2003).

In the present study, we identified a novel mutation c.280T>C (Cys94Arg) in GLA in a Chinese family with nephropathy. The aim of this study was to characterize the clinicopathologic features and confirm the pathogenicity of this mutation that causes FD.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance and patients’ information

This study was approved by the local Ethics Committees and written informed consent was obtained from all patients participating in the study. This Chinese family was recruited from FD patient organization in China, and this family was geographically localized in Shanxi Province. Patients’ medical records were reviewed and evaluated, clinical and physical examinations were performed. Percutaneous renal biopsies were done by nephrologists in the hospital. Based on all medical records, clinical presentation, data given by examinations and pathologic findings, the patients were diagnosed as FD.

2.2 | Sequencing analysis

Genomic DNA was extracted from peripheral blood samples using the DNeasy Blood & Tissue Kit (Qiagen, Cat No. 69506) according to the manufacturer’s instructions. All coding regions and exon–intron splice junctions of the GLA gene (GenBank reference sequence NG_007119.1) were analyzed using polymerase chain reaction (PCR) amplification in combination with Sanger sequencing using the primers described previously (Shabbeer, Robinson, & Desnick, 2005). PCR products were purified using the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Cat. No. B518131).

2.3 | RT-PCR and qRT-PCR analysis

To evaluate the transcript variants of GLA by RT-PCR and qRT-PCR in the blood cells from patient and three healthy volunteers, total RNA was extracted with Trizol following the instructions of the supplier (Invitrogen, Cat. No. 15596-018). First-strand cDNA synthesis was performed using the M-MLV reverse transcriptase RNase H Minus-kit from Promega. The primer pair for qRT-PCR of GLA were used: Fw: 5’-GAAGGATGCAGGTTATGAGTACCT-3’ and Rv: 5’- ATCTGCATAAATCCCTAGCTTCAG-3’. For normalization, the expression of GAPDH (Forward: CGGAGTCAACGGATTTGGTCGTAT; Reverse: AGCCCTTCTCCATGGGTGTTGACAG) was used.

2.4 | Analysis of evolutionary conservation of amino acid residues and structure prediction of the mutant protein

Evolutionary conservation of amino acid residue alteration was analyzed by comparing across different species. The crystal structure of the GLA mature human form is available in complex with its ligand galactose (Garman & Garboczi, 2004; Guce, Clark, Rogich, & Garman, 2011). The homology modeling programs Swiss-Model (http://swissmodel.expasy.org) was used to develop an appropriate model to mimic the effects of the mutated region. The structures were displayed by PDB-Viewer software.

2.5 | Site-directed mutagenesis of GLA

Single base mutation was individually introduced by PCR. The expression plasmid harboring the wild-type cDNA of GLA has been done before (Li et al., 2019). Design primers
according to the instructions for site-directed mutagenesis reagents (QuikChange® Site-Directed Mutagenesis Kit).

### 2.6 Cell culture and transient transfection

HEK293T and HeLa cells were maintained in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C and 5% CO₂. Cells were transfected by the Polyetherimide (Polyscience, Cat. No. 23966-2) according to the manufacturer’s instructions. Transfected cells were incubated for 24–48 hr posttransfection.

### 2.7 Western blotting

Cells were grown to about 90% confluency in six well plates, washed twice with ice cold PBS and resuspended in modified radio-immunoprecipitation lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Na-desoxycholate, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM PMSF). Cell suspensions were passed through a 0.45 mm needle 10 times and incubated for 15 min on ice, followed by sonication. The lysates were cleared by centrifugation at 12,000 g for 10 min at 4°C. The samples were resuspended in 5x SDS sample buffer and heated at 98°C for 5–10 min. For western blot analysis, proteins were resolved in 12% or 15% SDS polyacrylamide gels and transferred to NC membrane (GE) using the wet blot transfer over 3 hr (I = 300 mA). The blots were probed with primary antibodies, then washed with TBST (10 mM Tris/ HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) three times and incubated with horseradish peroxidase-conjugated secondary antibody (Thermo). The blots were washed again with TBST and signals were visualized using chemiluminescence (ECL) system (GE, Cat. No. RPN2232). The following antibodies were used: GFP mouse monoclonal antibody (proteintech, Cat. No. 66002-1-Ig), GAPDH mouse monoclonal antibody (proteintech, Cat. No. 60004-1-Ig).

### 2.8 GLA enzyme activity assay

HEK293T cells were cultured and transfected with plasmids containing either wild-type or mutant GLA, respectively. At 48 hr posttransfection, cells were collected after centrifugation at 168 g using bench top centrifuge. Every 5 million cells were resuspended with 1 ml extraction buffer followed by sonication. The lysates were cleared by centrifugation at 15,000 g for 10 min at 4°C. The GLA enzyme activity of the supernatants was measured according to the manufacturer’s instructions (Solarbio, Cat. No. BC2575) (Li et al., 2019).

### 2.9 Statistical analysis

All data are presented as the mean ± SD from at least three separate experiments. The p values were determined by two-tailed Student’s t test. p < .05 was considered as being significant.

### Table 1

Clinicopathologic features of the patient described in this study

| Subject   | Age/sex | Clinical diagnosis     | α-galactosidase A | c.280T>C mutation | Kidney biopsy findings |
|-----------|---------|------------------------|-------------------|-------------------|------------------------|
| Patient II-1 | 38/F    | Chronic nephritic syndrome | Decreased (11.68 nmol hr⁻¹ mg⁻¹) | Present | Consistent with FD |

### Figure 1

Pedigree and sequencing of patients from the Chinese families. The patient involved in this study is pointed by an arrow. Sanger sequencing analysis performed on the genomic DNA from indicated patients. The gene variation is shown by black arrow.
RESULTS

3.1 Identification of GLA gene variant in probands with FD manifestations

The patient included in this study with clinical manifestations of FD was recruited from one Chinese family. The clinicopathologic features of the patient including enzymatic and genetic analysis results are summarized in Table 1. The pedigree of the family is shown in Figure 1. The control blood samples were collected from three healthy volunteers. The blood sample of two affected females (II-1 and II-2) were obtained and Sanger sequencing were performed for GLA gene. Sanger sequencing revealed that these two females contains the heterozygous novel GLA mutation (c.280T>C, p.Cys94Arg) (Figure 1). The renal biopsy results from patient II-1 was evaluated. Her biopsy specimen contained 11 glomeruli which showed two global and two segmental glomeruloscleroses. The mesangial cells and stroma showed mild to moderate hyperplasia in focal segments, but no obvious proliferation of endothelial cells. We observed vacuolar degeneration of basement membrane without obvious thickening and vacuolar degeneration of epithelial cells with one balloon adhesion; tubulointerstitial lesions were mild to moderate, with obvious vacuolar degeneration and granular degeneration of tubular epithelial cells accompanied by multiple focal atrophy and infiltration of multiple focal mononuclear cells in the interstitium with fibrosis (Figure 2a). The arterioles became thicker. Electron microscopy showed an increase number of lysosome and a large number of medullary bodies and zebra bodies, considered as Fabry’s disease nephropathy (Figure 2b).

To identify the mRNA expression level of GLA in patient samples, qRT-PCR was performed. Total RNA was isolated from the blood samples of patient II-1 and three healthy volunteers, and qRT-PCR was normalized to that of GAPDH. The results clearly showed that the level of GLA mRNA was reduced to two third in the samples from patient compared to control (Figure 3). This result suggests that the mRNAs of mutant GLA were not stable in the cells and imply an insufficient normal GLA in the patient.

FIGURE 2 Light and electron microscopic findings of renal biopsies from patients II-1. (a) Glomeruli with global or segmental glomeruloscleroses (H&E stain, 400×). (b) An increase number of lysosome and a large number of medullary bodies and zebra bodies in visceral epithelial cells (electron microscopy)

FIGURE 3 qRT-PCR analysis performed on total RNA obtained from blood samples of patient II-1 and three healthy volunteers individuals. Levels were normalized to the amount of GAPDH. Data represent the mean ± SE of three independent measurements performed in triplicate (*p < .05)
3.2 Sequential and structural analysis

A previous X-ray crystallographic study revealed that the human mature GLA structure corresponds to a homodimeric glycoprotein. Each monomer is composed of two domains: a domain 1 (D1), that contains the active site at the center of the β strands in the (β/α)8 barrel and a C-terminal domain 2 (D2), that contains antiparallel β strands (Garman & Garboczi, 2004). Evolutionary conservation analysis of amino acid residues showed that the impaired amino acid residues C94 was most highly evolutionary conserved among GLA proteins from different species (Figure 4a).

Molecular modeling showed that the impaired amino acid residues C94 was involved in the formation of disulfide bound with C52 and close to the enzyme activity site (D92, D93). The missense mutation (c.280T>C, p.C94R) disturbs the formation of disulfide bridge between C52 and C94 and might affect the enzyme activity, indicating this mutation was likely causative mutation predisposing to FD (Figure 4b).

3.3 Abnormalities of the enzyme activity and nuclear shape in mutant GLA transfected cells

To examine whether the mutated protein exhibits any residual enzyme activity, an expression construct pEGFP-GLA full length wild-type and pEGFP-GLA mutant were prepared and expressed in HEK293T cells. The similar transfection

![Graphical representation](image-url)
efficiency of those transfectants was observed by western blot analysis and GAPDH was used as loading control (Figure 5a). The GLA enzyme activity of GFP alone, GFP-GLA-WT and GFP-GLA-MT transfected cells were detected using the kit from Solarbio (Cat No. BC2575). Before comparing the enzyme activities for GFP-GLA-WT and GFP-GLA-MT transfected cells, the activity of GFP alone transfected cells was subtracted as endogenous enzyme activity according to manufacturer’s guidelines. The enzyme activity of HEK293T cells transfected with mutant GLA construct was significantly lower with a relative GLA enzyme activity down to 40% compared to that of GLA wild-type transfected cells (Figure 5b). This result indicated that the missense mutation reduced the enzyme activity of GLA.

To identify whether the mutant GLA has altered localization inside the transfected cells, we introduced the mutation into wild-type GFP-tagged full length GLA and expressed the corresponding proteins in HEK293T cells. In GFP alone transfected cells, GFP signal was detected in both nucleus and cytoplasm. In both GFP-GLA-WT and GFP-GLA-MT transfected cells, the GLA fusions were uniformly expressed in the cytoplasm without any alteration of the localization. Surprisingly, nuclear shrinkage was observed in the GFP-GLA-MT transfected cells indicating that the overexpression of the impaired proteins might affect the nuclear shape (Figure 5c). The altered nuclear shape might result from the abnormal protein which need to be confirmed by further study.

**FIGURE 5** Enzyme activity of the mutant GLA. (a) Western blot analysis were used to show the similar transfection efficiency for HEK293T lysates transfected with GFP, GFP-GLA-WT or GFP-GLA-MT plasmids. GAPDH was used as loading control. (b) The result of enzyme activity assay from HEK293T cells transfected with wild-type or mutant GLA plasmids. Data were presented as the mean ± SD from three independent experiments. Asterisks represent significance (*p < .05). (c) HEK293T were transfected with GFP alone, GFP-GLA-WT and GFP-GLA-MT plasmids and the localization of wild-type and mutant GLA were studied by immunofluorescence. Bar: 5 μm

4 | DISCUSSION

In this study, we identified a novel GLA mutation (c.280T>C, p.Cys94Arg) in a Chinese family diagnosed as Fabry’s disease nephropathy. GLA mutation in coding sequence can result in amino acid changes, which is likely to alter protein conformation and subsequent functional defect of the GLA enzyme (Duro et al., 2014).
Evolutionary conservation analysis of amino acid residues showed that this changed amino acid residue is most highly evolutionary conserved among GLA proteins from different species, indicating the mutation was likely pathological (Figure 4a). The structure of GLA is a homodimer with each monomer containing a (β/α)₈ domain with the active site and an antiparallel β domain (Garman & Garboczi, 2004). The mature GLA monomers have 12 cysteine (Cys) residues, at positions 52, 56, 63, 90, 94, 142, 172, 174, 202, 223, 378, and 382 in the polypeptide chain (Garman & Garboczi, 2004). An additional cysteine (Cys12) is removed from the monomer during the posttranslational enzyme maturation, as part of the signal peptide. The Cys90 and Cys174 residues are unpaired. The other 10 cysteine residues form five disulfide bridges at positions 52–94, 56–63, 142–172, 202–223, and 378–382 are important for the stabilization of the three-dimensional structure of the enzyme; furthermore, the 142–172 disulfide contributes to the GLA catalytic site (Ferreira et al., 2015). It was reported that any cysteine mutation involved in disulfide bridge formation of wild-type GLA structure are associated with classic FD phenotype with negligible or very low residual enzyme activity (Eng et al., 1994, 1993; Filoni et al., 2010; Ploos van Amstel, Jansen, Jong, Hamel, & Wevers, 1994). So far, missense mutations involving the Cys12 or Cys90 residues have never been reported in FD patients (Ferreira et al., 2015). For GLA Cys94, two missense variants have been described changing the wild-type cysteine to serine (Ser) or tyrosine (Tyr), respectively (Blaydon, Hill, & Winchester, 2001; Eng et al., 1997). However, no functional studies were reported to confirmed the pathogenicity of these two variants. In our study, we found a novel GLA mutation c.280T>C (C94R) which affect the formation of disulphide bridge. Furthermore, Cys94 is located in a mutational hot spot. To date, five missense variants have been reported for Asp92 and Asp93, respectively, and four variants found for Trp95. Here, we discovered the third variant for Cys94.

The active site of GLA contains side chains with residues W47, D92, D93, Y134, C142, K168, D170, E203, L206, Y207, R227, D231, D266, and M267, which is extremely sensitive to mutation (Garman & Garboczi, 2002, 2004). Even conservative substitutions around the active site can result in the complete loss of enzymatic activity. Moreover, mutations can lead to folding defects in the GLA polypeptide, wherein the hydrophobic core of the protein is disrupted and the enzyme cannot fold or remain folded in the acidic lysozyme environment (Garman & Garboczi, 2002, 2004). C94 involved in our study is next to the enzyme active sites D92 and D93. Furthermore, the cells transfected with mutant GLA construct showed significantly lower GLA enzyme activity compared with wild-type GLA transfected cells (Figure 5c). The mutant C94R results in the loss of enzymatic activity might due to the impaired binding with its substrate or the loss of its enzyme activity. To address this question, further studies are required. In summary, we report a novel GLA mutation results in the renal phenotype of FD. Our study confirms that c.280T>C is a Fabry-causative mutation and data in this study enrich Fabry mutation database and underlines the significance of family member genotyping and genetic counseling in diagnosis and treatment of the disease.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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