



A Novel Missense Mutation in the *GLRB* Gene Causes Hyperekplexia

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ABSTRACT

The purpose of this study is to describe the clinical and genetic characteristics of a Chinese patient with hyperekplexia. We described the clinical feature of a 31-year-old male patient with hyperekplexia. Gene mutation was screened by whole exome sequencing. Furthermore, the plasmid containing wild type or mutant was transiently transfected into HEK293T cells. Realtime PCR and Western blotting were performed to evaluate the expression of *GLRB*. The distribution of *GLRB* was observed by immunofluorescence. One novel p.M99K mutation in the extracellular ligand binding domain of glycine receptor beta subunit was identified in our patient. Through the comparison of different species, it was found that the mutation site and its nearby sequences were highly conserved. Pathogenicity prediction (Polyphen 2 and Mutation Taster) indicated this mutation may be pathogenic. The expression and distribution of *GLRB* were not significant between the mutant and wild types. Our results suggest that the novel *GLRB* mutation may cause hyperekplexia. In addition, we also tried to explore the possible pathogenic mechanism of this mutation. It is essential to conduct more genetic and functional research to improve the understanding of this disease.

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Authors' Contribution

JH and PW conceived and designed experiments. JH and PW performed experiments, analysed data and wrote the manuscript. YS, LW and MD provided technical support, data collection and analysis. All authors provided final approval for submitted and published version.

Key words

Hyperekplexia, Mutation, Glycine receptor β , Startle reaction, Clonazepam

INTRODUCTION

Hyperekplexia is a rare disease. Hyperekplexia is characterized by increased muscle tone (hypertonia) and an exaggerated reaction to abrupt unexpected stimuli (Praveen *et al.*, 2001). It affects newborn children and gradually fades during the first year of life. Nonetheless, older patients with hyperekplexia may still startle easily and exhibit stiffness, which may lead to falls (Russo *et al.*, 2017; Tijssen *et al.*, 1997).

Hyperekplexia results from the dysfunction of the glycinergic neurotransmission system, which is involved in inhibitory neurotransmission. Five genes have been identified as causing the disease (Harvey *et al.*, 2008). Mutations of *GLRA1* gene, which encodes the glycine receptor alpha-1 subunit, are the most common (Harvey *et al.*, 2004). However, the glycine transporter-2 (SLC6A5), gephyrin (GPHN), and collybistin, a RhoGEF for the receptor clustering protein, have yet to be identified as contributing to disease (Dafsari *et al.*, 2019; Davies *et al.*, 2010; Harvey *et al.*, 2004).

The detection of these genes is crucial in making a diagnosis for this disease. We here report a case of hyperekplexia resulting from a novel missense *GLRB* mutation that results in the substitution of methionine at position 99 with lysine (M99K).

MATERIALS AND METHODS

The patient was a 31 years old male from Shanghai. He suffered from recurrent falls for 20 years ago, which could

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be induced by sudden sound and other stimuli. He could not maintain his balance and was easy to fall, resulting in multiple abrasions and sprains. He walked with a wide-based gait for fear of falling. He was conscious at the time of the attacks, and his symptoms disappeared during sleep. He is the only child in his family. His parents were not close relatives, and none of them had similar symptoms. He was born of spontaneous vaginal delivery with a normal birth process and weight (3.2 kg). His intellectual development was normal, but his physical performances were relatively poor during school days.

The physical examination showed the patient had mild hypertension. He presented with a wide-based gait. Tapping his nose tip could induce facial muscle contraction with head back and body stiffness. The tendon reflex of limbs was active. No obvious abnormalities were found in other neurological examinations. The serum creatine kinase level was 412 IU/L (normal range 55-170 IU/L). MRI of the brain with contrast-enhancement revealed an arachnoid cyst in the left temporal area. Ambulatory electroencephalography (AEEG), electromyography (EMG) and other laboratory tests were all normal. Considering the possibility of hyperekplexia, the stiffness and startle symptoms of patients were significantly relieved after treatment with clonazepam.

Mutation analysis

We collected peripheral blood samples from the proband and his parents. We used the Qiagen kit (QIAGEN, Hilden, Germany) to extract DNA from 3mL blood samples and conduct sample quality inspections. We then used next generation sequencing (NGS) to capture and sequence the exons of 3204 genes related to genetic diseases. The variant was analyzed and classified according to ACMG (the American College of Medical Genetics and Genomics) guidelines (Richards *et al.*, 2015). Moreover, we investigated allele frequency (1000 g, dbSNP, and ExAC) and pathogenicity prediction [Polyphen2 and Mutation Taster (<http://www.mutationtaster.org>)]. Sanger sequencing was used to confirm the results of second-generation sequencing.

Culture and transfection of cells

In a humidified incubator with 95% air and 5% CO₂, HEK293T cells (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS and 100 U/mL penicillin/streptomycin. Lipofectamine 3000 (Invitrogen) was used for transfection.

Plasmid construction

The GLRB wild type and mutant plasmids were obtained from Genechem Company (Shanghai, China). To create site-specific mutants within the CV061 vector, PCR-

based site-directed mutagenesis was used. Synthesized and annealed sequences are as follows:

Sense: 5'-ACGGGCCCTCTAGACTCGAGCGC-CACCATGAAGTTTTTATTG-3',

Antisense: 5'-AGTCACTTAAGCTTGGTACC-GATAAATATATAGACCAATATATAAC-3'

Real-time PCR

RNA was extracted from cells with TRIZOL reagent (Invitrogen). PrimeScript 1st Strand cDNA Synthesis Kit (Takara) was used to synthesize the cDNA. SYBR Green Real-time PCR Master Mix (TOYOBO) was used to measure the GLRB level. GLRB mRNA amplification primers were 5'-AGTGGCCCTGGGTATCTCT-3' and 5'-CTTTTGGGGTTGTTTCAGCAT-3'. GLRB mRNA level was compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blotting

GLRB protein expression was determined by Western blotting by using GLRB antibody (1: 1000 dilution, Santa Cruz, sc-365819). GLRB expression was normalized to that of GAPDH (1: 1000 dilution, Cell Signaling Technology, #5174).

Immunofluorescence

After seeding on poly-L-lysine-coated coverslips, HEK293T cells were processed for immunofluorescence. Cells were fixed, eluted with GLRB antibody overnight, and incubated with goat anti mouse fluorescent antibody (P-phycoerythrin, PE) for 30 min. Images were photographed under a fluorescence microscope (Leica).

Swiss-model and UniProt

SWISS-MODEL (<https://swissmodel.expasy.org>) was an automated server for the modeling of homo- and hetero- protein complexes. UniProt (<https://www.uniprot.org>) is a free resource for elucidating the amino acid sequence and function of various protein.

RESULTS

One homozygous variant of the *GLRB* gene, c.T296A (p.M99K), was found in this patient. His parents were both heterozygous carriers of the same variant (Fig. 1). Neither dbSNP (<http://www.ncbi.nlm.nih.gov/snp>) nor 100 genome project (<http://browser.100genomes.org>) or Exome Aggregation Consortium (ExAC) contained this variant. By MutationTaster, c.T296A was predicted to cause disease (0.99 probability, range: 0-1.0). With a score of 0.9667, Polyphen-2 also predicted that the mutation is probably damaging (sensitivity: 0.77; specificity: 0.95).

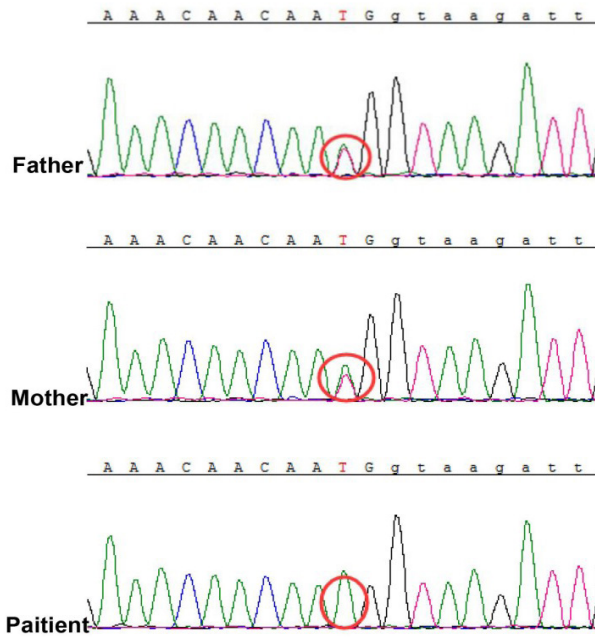


Fig. 1. Next-generation sequencing results from the patient and his parents. The parents are heterozygotes for this variant, and the patient is homozygote. The mutant site (c.T296A) was circled in red.

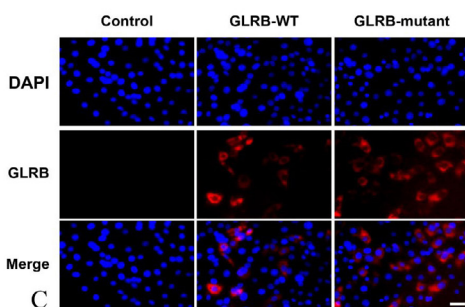
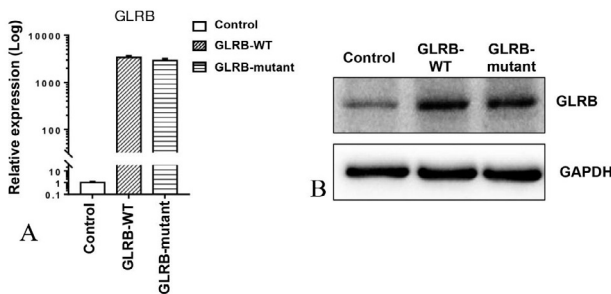


Fig. 2. The mutant GLRB does not affect the expression and distribution of GLRB. The expression of wild type and mutant GLRB were examined by (a) Real-time PCR (b) Western blotting (c) Immunofluorescence analysis. Scale bar = 50 μ m.

Mutant GLRB's mRNA level was not affected by real-time PCR (Fig. 2A). There was no difference in WT and mutant GLRB protein expression (Fig. 2B). Furthermore, immunofluorescence indicated that the distribution of mutant protein did not differ significantly from the control (Fig. 2C). Mutation taster showed strong conservation of amino acid sequence near the mutation site between different species (Fig. 3A). We further used SWISS-MODEL software and UniProt to show the mutation site (Fig. 3B).

Drerio.pro	INSFGSIQETTMDYRVNIFLRQR
Ggallus.pro	INSFGSIQETTMDYRVNIFLRQK
Ptrogodytes.pro	INSFGSIQETTMDYRVNIFLRQK
Mmulatta.pro	INSFGSIQETTMDYRVNIFLRQK
Mmusculus.pro	INSFGSIQETTMDYRVNIFLRQK
Xtropicalis.pro	INSFGSIQETTMDYRVNIFLRQR
Human.pro	INSFGSIQETTMDYRVNIFLRQK
mutated.pro	INSFGSIQETTMDYRVNIFLRQR

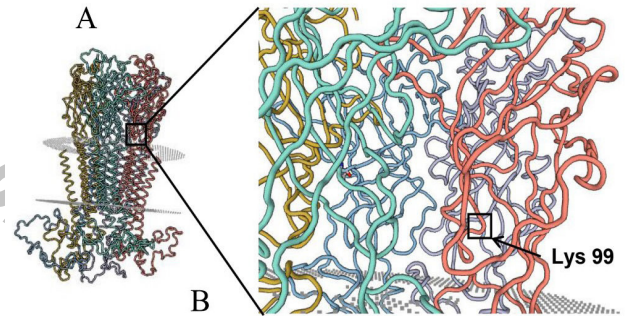


Fig. 3. Modeling of the novel GLRB mutant. A. Sequence alignment of the conserved *GLRB* sequence among different species. The mutation site is labeled in red. B. The p.M99K mutation and surrounding region are shown on the hypothetical 3-D structure of the GLRB (Swiss-model).

DISCUSSION

Hyperekplexia is a relatively rare hereditary nervous system disease, which is related to the dysfunction of the glycinergic neurotransmission system (Zhan *et al.*, 2021). As a result of the lack of understanding of this disease, it is often misdiagnosed as epilepsy or other disorders. So far, a total of 12 patients have been reported in China (Chan *et al.*, 2014; Huang *et al.*, 2018; Li *et al.*, 2017; Poon *et al.*, 2006; Tsai *et al.*, 2004; Yang *et al.*, 2017; Zhan *et al.*, 2020; Zhang *et al.*, 2020). A typical clinical manifestation of hyperekplexia is an excessive startle response after birth. Its inducing factors are often sudden unexpected sound, visual or tactile stimuli, and sometimes even when touching daily operations such as feeding or changing diapers (Koning-Tijssen and Brouwer, 2000). Patients can also have the following abnormal

manifestations, such as limb movement during sleep and myoclonus before falling asleep, umbilical hernia or esophageal hiatal hernia, epilepsy, intellectual or motor developmental delay, congenital hip dislocation, and spastic paraplegia (Bakker *et al.*, 2006). Some patients can be complicated with anxiety and depression (Mine *et al.*, 2015). The diagnosis of hyperekplexia is mainly based on clinical manifestations. Detailed history and physical examination are helpful, especially the patient's neonatal and childhood history. The nose-tapping test can be used as a characteristic diagnostic examination (Shahar *et al.*, 1991). In patients with hyperekplexia, laboratory tests, MRI and EEG are often normal. The diagnosis should exclude the possibility of other diseases. Previous studies suggested that gene mutation detection rates for hyperekplexia were approximately 60% (Davies *et al.*, 2010). Thus, it is necessary to perform genetic analysis for the precise diagnosis. Clonazepam is usually used in treatment, which can compensate for the defects of the GABAergic pathway in the brain stem and enhance the protective response of patients during startle reflex (Zhou *et al.*, 2002). The prognosis of this disease is generally favorable.

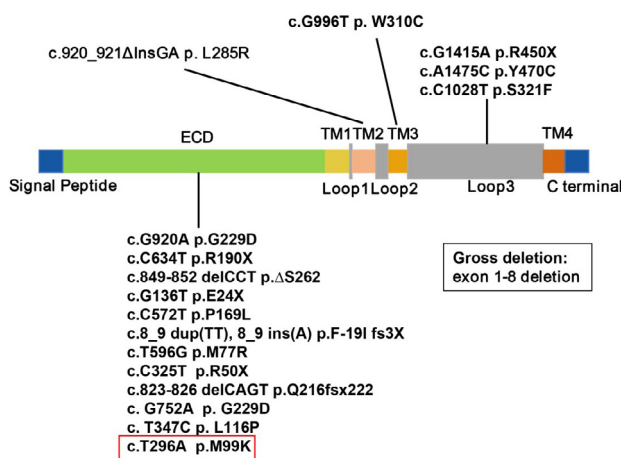


Fig. 4. Summary of GLRB mutations. Diagram of all GLRB mutations (NM_000824). Mutations in black: recessive or compound heterozygous mutations. The mutation detected in this paper is marked in red. ECD, extracellular domain, TM, transmembrane domain.

At present, at least five genes have been identified to be related to hyperekplexia. *GLRA1* is the most frequent mutation gene, encoding glycine transporter subunit $\alpha 1$. *GLRB* is also a major gene of effect (Chung *et al.*, 2013), which encodes glycine transporter subunit β . *GLRA1* binds to *GLRB* to form postsynaptic hetero pentameric glycine (GlyR) receptor with $2\alpha 1:3\beta$ subunit (Yang *et al.*,

2012). The presynaptic glycine transporter-2 (*SLC6A5*) (Alfadhel *et al.*, 2016) as well as two of the GlyR synaptic clustering proteins (*GPHN* and *ARHGEF9*) (Rees *et al.*, 2006) have also been identified as having mutations. To date, more than 20 *GLRB* mutations have been identified as hyperekplexia (Fig. 4) (Chung *et al.*, 2013; Li *et al.*, 2017). Genes encoding *GLRB* are mutated in many ways, including in-frame deletions, two-hit duplications, large repeats-mediated deletions, and splice site mutations that result in exon skipping and missense mutations (Chung *et al.*, 2013). Inheritance of hyperekplexia can be autosomal dominant or autosomal recessive (Harvey *et al.*, 2008). *GLRB* mediated hyperekplexia is mainly in the mode of homozygous recessive or a compound heterozygote inheritance (Chung *et al.*, 2013).

GLRB encodes the glycine receptor β subunit which contains an extracellular domain (ECD), a transmembrane domain (TMD) with four α -helices (TM1-TM4), and a cytoplasmic domain as well as glycine receptor $\alpha 1$ subunit. Interestingly, most *GLRB* mutations are located on the ECD of the protein, not like *GLRA1* mutations which were mostly located on the TMDs (Zhan *et al.*, 2020). UniProt showed position 99 of *GLRB* was also at the extracellular region (<https://www.uniprot.org>). Recent functional studies indicated some *GLRB* mutations either reduced the expression of the glycine receptor on the cell surface or reduced the sensitivity to glycine (James *et al.*, 2013). However, our results showed *GLRB* expression and distribution were not affected obviously. A previous study showed a common mutation M177R that was also located on the ECD did not affect *GLRB* expression, which was consistent with our results (James *et al.*, 2013). We further used the prediction tool to find the potential pathogenicity of the mutation. Interestingly, the mutation site was highly conserved across species (Fig. 3A), suggesting this mutation may have an important effect. Furthermore, bioinformatics software was performed to illustrate this mutation may be harmful. Since *GLRB* expression and distribution were unchanged, we hypothesized this mutation may disturb the glycine sensitivity.

CONCLUSIONS

In our case, the patient had symptoms of exaggerated startle responses and slightly delayed motor development. Our case suggests that for unexplained abnormal gait and frequent falls in adults, it is also necessary to evaluate the possibility of potential undiagnosed hyperekplexia. We found a homozygous recessive *GLRB* mutation (c.T296A/p.M99K) in our patient. And this novel mutation did not alter *GLRB* expression and distribution in HEK293T cells. However, bioinformatics revealed this

mutation to be probably deleterious. To date, it is unclear how the mutation detected in our patient altered the function of glycine receptors to cause hyperekplexia. The exact mechanism of GLRB mutation needs to be explored in the future.

DECLARATIONS

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Ethical approval

This study was approved by the Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine. Consent was obtained from all participants. The patient and both of his parents were examined in the study.

Statement of conflicts of interest

The authors have declared no conflict of interest.

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