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ORIGINAL ARTICLE

Clinical,biochemical and molecular analysis of five Chinese patients with Sandhoff disease

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Abstract Sandhoff disease (SD) is a rare autosomal recessive lysosomal storage disorder of sphingolipid metabolism resulting from the deficiency of β-hexosaminidase (HEX). Mutations of the *HEXB* gene cause Sandhoff disease. In order to improve the diagnosis and expand the knowledge of the disease, we collected and analyzed relevant data of clinical diagnosis, biochemical investigation, and molecular mutational analysis in five Chinese patients with SD. The patients presented with heterogenous symptoms of neurologic deterioration. HEX activity in leukocytes was severely deficient. We identified seven different mutations, including three known mutations: IVS12-26G > A, p.T209I, p.I207V, and four novel mutations: p.P468PfsX62, p.L223P, p.Y463X, p.G549R. We also detected two different heterozygous mutations c.-122delC and c.-126C > T in the promoter which were suspected to be deleterious mutations. We attempted to correlate these mutations with the clinical presentation of the patients. Our study indicates that the mutation p.T209I and p.P468PfsX62 may link to the infantile form of SD. Our study expands the spectrum of genotype of SD in China, provides new insights into the molecular mechanism of SD and helps to the diagnosis and treatment of this disease.

Keywords GM2 gangliosidosis · Sandhoff disease · HEXB gene · β-hexosaminidase

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Introduction

Sandhoff disease (SD) is a rare autosomal recessive lysosomal storage disorder of sphingolipid metabolism resulting from the deficiency of β-hexosaminidase (HEX). HEX is a catalytic enzyme responsible for the degradation of GM2 ganglioside. Its deficiency results in the accumulation of GM2 ganglioside in cells of the central nervous system and leads to neurologic deterioration. HEX consists of two major isoenzymes: HEXA, a heterodimer of α and β subunits, and HEXB, a homodimer of β subunits (Beutler et al. 1975). Mutations of the *HEXB* gene, encoding the β subunits present in both isoenzymes, result in deficiency of HEXA and HEXB, causing SD (Mahuran 1999; Gort et al. 2012). SD displays a wide range of clinical expression and has three clinical subtypes: infantile, juvenile and adult forms. Infantile SD is characterized by an early onset of symptoms with progressive neurologic impairment, hyperacusis, hypotonia, bilateral cherry-red spots with macular region of the retina and seizures Aryan et al. (2012); Gomez-Lira et al. 1998). The disease onset of juvenile and adult forms occurs in adolescence or adulthood and they belong to the late-onset form. The late-onset SD is rare and presents with less severe manifestations such as cerebellar ataxia, mental retardation, spinal muscular atrophy, and local panatrophy (Hendriksz et al. 2004; Santoro et al.2007). Patients with late-onset SD have a more prolonged survival (Hendriksz et al. 2004; Santoro et al. 2007).

The *HEXB* gene is located on 5q13 and includes 14 exons (Proia 1988). Around 50 different mutations have been detected in *HEXB* gene so far in patients with SD (Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff: *HEXB* Gene: http://www.hgmd.cf.ac.uk), including missense, nonsense, small deletions, small insertions, small indels, gross deletions, or splicing mutations.

In this study we described clinical,biochemical and molecular characteristics of five Chinese patients with SD.

Material and methods

Patients

Five patients (three females and two males) from five unrelated families with Sandhoff disease were included in this study. The diagnosis was confirmed by reduced HEXA&B activity in leucocytes at Guangzhou Women and Children's Medical Center from May 2012 to April 2015. Except patient 1, the other four proband's parents were studied to investigate inheritance of mutations.

Informed consent was obtained from all patients' parents. The study was approved by the Institutional Review Board of Guangzhou Women and Children's Medical Center.

Biochemical analysis

Total HEXA&B and HEXA activities were measured on peripheral blood leukocytes using fluorogenic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO, USA) and 4-methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside (Glycosynth, Warrington, UK), respectively. The assays were performed according to the protocol (Inui and Wenger 1984; Blau et al. 2008) at the Department of Genetics and Endocrinology, Guangzhou Women and Children's Medical Center. Normal HEXA&B activity is within the range of 600.0–3500.0 nmol/mg/h (Huang et al. 2014). Normal HEXA activity is within the range of 150.0–365.0 nmol/mg/h (Huang et al. 2014).

HEXB gene sequencing analysis

Genomic DNA was extracted from peripheral blood leukocytes of the patients and their parents by standard procedure using 250 μL of peripheral blood using a Blood gDNA Magentic Beads Purification Kit (Innovogen Inc., Brookline, USA). Based on the sequencing chromatograms of reference sequences (NCBI: NG_009770.1), PCR primer pairs *HEXB* 1–14 were designed by Primer 5 software (Biosoft International, Palo Alto, USA) to amplify each exon and exon–intron boundaries of *HEXB* gene. All primers were synthesized by Invitrogen (Shanghai, China). These primers and annealing temperature were listed in Table 1.

PCR was carried out in 25 μL volume containing 2.5 μL 10 × ExTaq buffer, 2.5 μL 5 mmol/L dNTPs, 2.5 μL 3.5 mmol/L bovine serum albumin, 0.15 U Ex Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan), and 0.3 μL 10 mmol/L of each primer for the amplification of each exon. All PCR amplification reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). The PCR amplification conditions started with 5 min of initial denaturation at 95 °C, followed by 35 cycles for 30 s at 95 °C, 30 s at annealing temperature, and 40s at 72 °C followed by a final extension step for 7 min at 72 °C.

5 μL of PCR product and 1 μL of 6 × loading buffer (TaKaRa Bio Inc.) were mixed. PCR products were checked on a 2 % TAE-agarose gel in 1 × TAE buffer (TaKaRa Bio Inc.) at 100 V for 30 min at room temperature. After purification of the PCR product, exons were sequenced by the fluorescent dideoxy cycle sequencing method with both forward and reverse primers using an ABI 3730 DNA Analyzer (Life Technologies Corporation, California, USA). Sequencing chromatograms were aligned with reference sequences (NG_009770.1) by Sequencher software DNAMAN (Lynnon Biosoft, Inc., Quebec, Canada). In case of mutation detection, the PCR and sequencing on the corresponding exons were repeated to verify reliability.

Protein function prediction for novel mutations

To predict the effect of amino acid substitutions, we performed in silico analysis using the SIFT/PROVEAN (http://sift.jcvi.org) and Polyphen-2 (http://genetics.bwh.harvard.edu/pp2) web software.

Results

Clinical and biochemical manifestations of the patients

Patient 1 was diagnosed at the age of nine years. He was the first child of a non-consanguineous couple from Guangdong province, China. He had one unaffected sister. He first experienced gait problems at age six years. Difficulties with speech, writing and swallowing, hand trembling, a balance disorder, and occasionally defecation incontinence gradually developed. Neurological examination showed cerebellar dysarthria, gait ataxia, ataxia of the extremities,mildly decreased muscular tone, normal sensory examination and normal tendon reflexes. MRI of the brain demonstrated bilateral cerebellar atrophy and MRI spectroscopy showed no abnormal sign. He did not take an examination of the fundus. The enzyme assay showed that total HEXA&B level was reduced in

Table 1 PCR primers for amplication of HEXB gene sequences

Exons	Forward primers(5′-3′)	Reverse primers(5′-3′)	Product size (bp)	Tm(°C)
1	GGAGGAAATTCTCGAGGTGAC	AGGTGTCCCTTAAGAAGCAGTAG	699	60
2	GGGTGAGAATCTCTAGTTGGACT	AGGAATCATAAACTCACTGGTTG	400	57
3	CATGTGCTTGGGAGAAAATAATA	CAATGGAAATCATTTTGGAACTA	326	57
4–5	ATTTGCCTTACCTGGTTATGAGT	CCCTGTTCCAAACTACACAATAG	611	57
6	TTAAAGGAAGCAGACATATTGGA	ATTTACACTTCCCCAAGATTGTT	443	57
7	TGGCTATCATCCTTTGAGTATGT	AGTGAGCCGAGATTGTACTACTG	473	58
8	AATGACGTAGTAAAATCATGTGGA	TTTAGTAGAGATGGGGTTTCACC	432	59
9	AAGCCATTTTTAAAGGAAATCAG	ATTGCCTCCTTTAGTGATTTTTC	432	57
10	ATCAAGTGCTAAAAAGGAGGAAC	CAAACCTAGCTTGGAACTATGAG	407	57
11	TCCCTAAAATGAGTATCACATGG	CTTGAATTTAGGCAACTGTATGG	582	57
12–13	AGGATAAAGATGGAGGAAACAAA	AAATTAAAGCAACTCAAGATGGA	641	57
14	TGCTGTGATTTAGTGATTCTAATTT	ATGTTTAAAGCCACTGTACCTGA	459	57

leucocytes to 119.0 nmol/mg/h; HEXA level was reduced to 69.5 nmol/mg/h.

Patient 2 was diagnosed at the age of 15 months. She was the second child of a non-consanguineous couple from Henan province, China. She had one unaffected sisiter. From the age of 6 months, motor and mental retardation were observed. From the age of 12 months, she presented with severe mental regression. She underwent recurrent seizures from age 15 months. Neurological examination showed hypertonia and brisk tendon reflexes without pathological plantar responses. At the age of 8 months, MRI of the brain demonstrated bilateral symmetric caudate nucleus T2 hyperintensities and T1 isointensities with delayed myelination. At the age of 12 months, MRI of the brain demonstrated the previous lesion along with diffuse white matter T2 hyperintensities and T1 hypointensities. At the age of 15 months, MRI of the brain demonstrated basal ganglia region T2 hyperintensities and T1 hypointensities along with diffuse white matter T2 hyperintensities and T1 hypointensities while MRI spectroscopy revealed diminished N-acetylaspartate with normal choline and lactic acid, suggestive of neuronal loss or dysfunction. An examination of the fundus revealed cherry-red spots at the macula with a pale optic disc. The enzyme assay showed that total HEXA&B level was reduced in leucocytes to 155.5 nmol/mg/h; HEXA level was reduced to 115.5 nmol/mg/h.

Patient 3 was diagnosed at the age of 12 months. She was the first child of a consanguineous couple from Guangdong province, China. From the age of 10 months, she presented with progressive neurodegeneration. She underwent recurrent seizures from the age of 12 months. Neurological examination showed hypertonia, brisk tendon reflexes without pathological plantar responses and extensive Mongolian spots. MRI of the brain demonstrated bilateral symmetric diffuse white matter, basal ganglia region T2 hyperintensities and T1 hypointensities along with cerebral sulci swelling. An examination of the fundus showed cherry-red spots at the macula. The enzyme assay showed that total HEXA&B level was reduced in leucocytes to 224.0 nmol/mg/h; HEXA level was reduced to 114.0 nmol/mg/h.

Patient 4 was diagnosed at the age of 8 months. He was the first child of a non-consanguineous couple from Guangdong province, China. From the age of 1 month, he manifested first with exaggerated startle response. Gradually, progressive muscular weakness, hypotonia, loss of motor skills and mental retardation were presented. He underwent recurrent seizures from the age of 10 months. Neurological examination showed hypotonia, quadriparesis and brisk tendon reflexes without pathological plantar responses. At the age of 8 months, MRI of the brain demonstrated delayed myelination while MRI spectroscopy revealed diminished N-acetylaspartate with slight enhanced choline and lactic acid, suggestive of neuronal loss or dysfunction and demyelination. At age 11 months, MRI of the brain demonstrated bilateral symmetric diffuse white matter and thalamus T2 hyperintensities and T1 hypointensities. An examination of the fundus did not find cherry-red spots at the macula. The enzyme assay showed that total HEXA&B level was reduced in leucocytes to 38.8 nmol/mg/h; HEXA level was reduced to 34.0 nmol/mg/h.

Patient 5 was diagnosed at the age of 15 months. She was the first child of a non-consanguineous couple from Guangdong province, China. From the age of 8 months, she manifested with progressive neurodegeneration. She underwent recurrent seizures from the age of 15 months. Neurological examination showed hypertonia and brisk tendon reflexes without pathological plantar responses. At the age of 15 months, MRI of the brain demonstrated bilateral symmetric diffuse white matter and basal ganglia T2 hyperintensities and T1 hypointensities. An examination of the fundus showed cherry-red spots at the macula. The enzyme assay showed that total HEXA&B level was reduced

in leucocytes to 98.0 nmol/mg/h; HEXA level was reduced to 65.9 nmol/mg/h.

HEXB gene mutation analysis

In Patient 1, gene mutation analysis revealed two heterozygous mutations. One was a known splicing mutation c.1509-26G > A (IVS12-26G > A) in Exon 12–13. The other was a novel frameshift mutation c.1404delT (p.P468PfsX62) in Exon 11.

Patient 2 had two different known homozygous missense mutations: c.619 A > G (p.I207V) and c.626C > T (p.T209I) both in Exon 5. The two mutations were found in the heterozygous state in the proband's father and her mother had two normal alleles of this gene.

Patient 3 had a homozygous frameshift novel mutation c.1404delT (p.P468PfsX62) in Exon 11 inherited from her parents.

Patient 4 had two novel heterozygous mutations. One was a missense mutation c.668 T > C (p.L223P) in Exon 5 which was not found in his parents. The other was a nonsense mutation c.1389C > G (p.Y463X) in Exon 11 inherited from his mother (Figs. 1 and 2).

In Patient 5, gene mutation analysis revealed three heterozygous mutations. One was a novel heterozygous mutation c.1645G > A (p.G549R) in Exon 14 inherited from her mother. The other two were different heterozygous mutations c.-122delC and c.-126C > T in the promoter. Her father has the same mutations in the promoter.

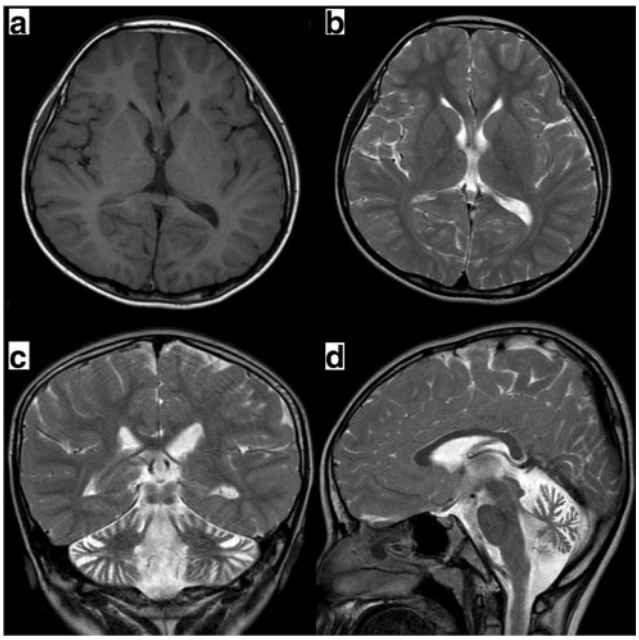

Fig. 1 MRI of patient 1 with juvenile SD. Axial T1-(**a**) and T2-(**b**) weighted, coronal T2-weighted(**c**) and sagittal T2-weighted(**d**) images demonstrate bilateral cerebellar atrophy

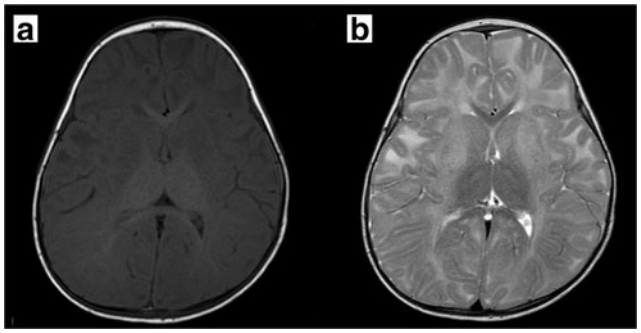

Fig. 2 MRI of patient 3 with infantile SD. Axial T1-(**a**) and T2-(**b**) weighted images demonstrate T2 hyperintensities and T1 hypointensities in bilateral symmetric diffuse white matter and basal ganglia with cerebral sulci swelling

Mutations identified in the study are summarized in Table 2 and Fig. 3.

Protein function prediction of novel mutation

The novel missense mutations c.668 T > C (p.L223P) and c.1645G > A (p.G549R) were predicted damaging by the SIFT/PROVEAN and Polyphen-2 web software. The frameshift mutation c.1404delT (p.P468PfsX62) was predicted damaging by the SIFT/PROVEAN web software.

Discussion

In this study we report the clinical and molecular characterization of a group of unrelated Chinese patients with SD. The group included one patient with juvenile form and four patients with infantile form. Infantile SD is more severe. It is characterized by an early onset and relatively rapid progression of disease symptoms with motor weakness and psychomotor retardation. It usually begins before the age of six months and culminates in death before 3–5 years of age (Bley et al. 2011; Smith et al. 2012). Juvenile form is less severe with the manifestation of cerebellar ataxia, mental retardation, spinal muscular atrophy, and local panatrophy. It begins in late childhood and shows slow progression. The affected patients may survive into adulthood (Maegawa et al. 2006; O'Dowd et al. 1986). Our study also showed the manifestation of juvenile SD was less severe than the infantile SD. There was significant difference in MRI feature between the two forms. The mechanism of the difference is still not elucidated. The juvenile SD has more residual enzyme activity of HEXA than the infantile SD. It suggests that the residual enzyme activity may be retard onset and provide a milder progression of the disease.

So far, around 50 *HEXB* gene mutations have been documented (Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff: *HEXB* Gene: http://www.hgmd.

Table 2 HEXB gene mutations of the patients with SD

Patient	Genotype	Type of mutation	Location	Base change	Amino acid change	Described
1	Compound heterozygous	Splicing	Exon 12–13	c.1509-26G > A	—	Previously
		Frameshift	Exon 11	c.1404delT	p.P468PfsX62	This study
2	homozygous	Missense	Exon 5	c.619 A > G	p.I207V	Previously
	homozygous	Missense	Exon 5	c.626C > T	p.T209I	Previously
3	homozygous	Frameshift	Exon 11	c.1404delT	p.P468PfsX62	This study
4	Compound heterozygous	Missense	Exon 5	c.668 T > C	p.L223P	This study
		Nonsense	Exon 11	c.1389C > G	p.Y463X	This study
5	Compound heterozygous	Missense	Exon 14	c.1645G > A	p.G549R	This study
		—	Promotor	c.-122delC	—	This study
		—	Promotor	c.-126C > T	—	This study

cf.ac.uk), including missense, nonsense, small deletions, small insertions, gross deletions, or splicing mutations. In this study, we found seven different mutations, including three known mutations: IVS12-26G > A, p.T209I, p.I207V, and four novel mutations: p.P468PfsX62, p.L223P, p.Y463X, p. G549R.

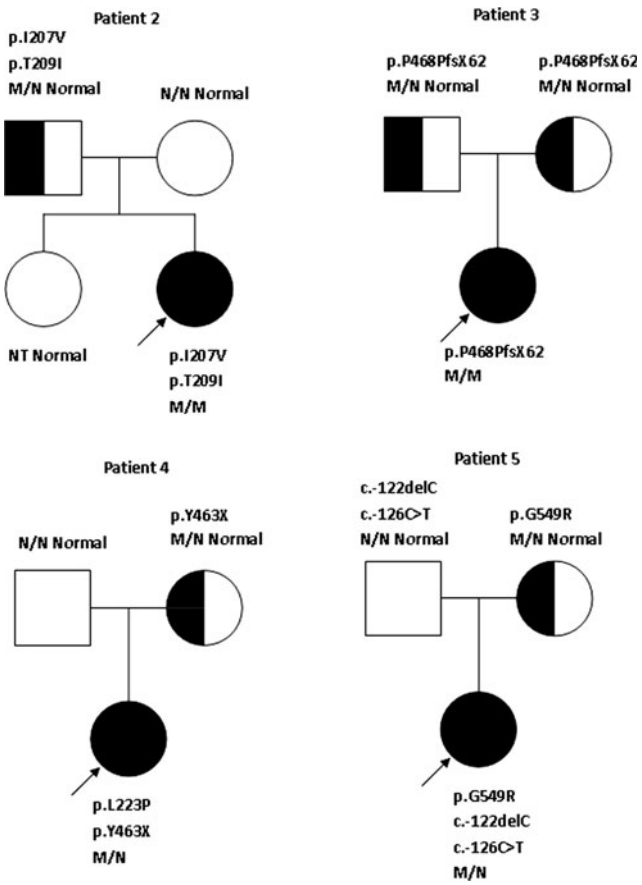

Fig. 3 Pedigress of patients of SD with HEXB gene mutation. Pedigress of the families showing genetic and phenotypic status of each member. M/M (homozygous mutant), M/N (heterozygous), N/N (homozygous normal), NT (not tested)

Patient 1 had two heterozygous mutations: IVS12-26G > A and p.P468PfsX62. In the first mutation IVS12-26G > A, the abnormality was a single nucleotide transition from the normal G to A at 26 bases from the 3′ terminus of Intron 12 (Delnooz et al. 2010). The mutation generates a new splice junction resulting in a 24-base insertion between Exons 12 and 13 in the processed mRNA and consequently an 8-amino acid insertion in the translation product (Nakano and Suzuki 1989. The inserted sequence maintains the downstream reading frame and contains no termination codon (Nakano and Suzuki 1989). The mutant enzyme protein therefore should be larger than the normal enzyme by 8 amino acids (Nakano et al.1989). The 8-amino acid insertion occurs in a region of the enzyme protein highly conserved between the HEX α and β subunits (Nakano et al.1989). But functional analysis of this mutation is not known in detail, except that the end result is the defective catalytic activity of HEX. The mutation IVS12-26G > A has been found in two juvenile SD and four adult SD (Delnooz et al.2010; Nakano et al.1989; Mitsuo et al. 1990). Therefore, The mutation IVS12-26G > A may link to late-onset SD. The second mutation p.P468PfsX62 was a novel mutation. At cDNA number 1404, the deletion of T leaded to the shift in the reading frame and the generation of a premature stop codon. The mutation caused the structure of the enzyme protein was incomplete and influenced the function of the enzyme.

Patient 2 had two known homozygous missense mutations: p.I207V and p.T209I. The mutation p.I207V was reported by Banerjee et al. (1991) in a patient with adult SD. The patient carried two different heterozygous mutations of p.I207V and p.Y456S (Banerjee et al. 1991). The mutation p.I207V results in a conversion of I at codon 207 to V. The difference due to this conversion is the loss of a methyl group on the side chain of the amino acid (Banerjee et al. 1991). This region was considered to be highly conserved associating with catalytic activity and activator protein binding (Banerjee et al.1991; Fitterer et al. 2014. In the following study, the mutation

p.I207V associated other mutant HEXB allele has been found both in infantile and late-onset SD (Redonnet-Vernhet et al. 1996; Hara et al. 1998; Zhang et al. 1995). Banerjee et al. (1994) found that the mutation resulted in the capability of α/β subunit dimerization to produce enough active HEXA. Meanwhile, it results in the incapability of β/β subunit dimerization to influence the production of HEXB only when the β-chain is expressed at low concentration by a second mutant *HEXB* allele (Banerjee et al. 1994). It is predicted that the mutation p.I207V is a silent polymorphism or an effector of the exacerbation for a second mutant *HEXB* allele (Zampieri et al. 2012). It is probably a neutral mutation that has little or no affect on the function of HEXB without the other mutant *HEXB* allele. Isolated the mutation p.I207V in heterozygous or homozygous state usually do not lead symptomatic SD. The viewpoint is compatible with the report of Redonnet-Vernhet et al. (1996) describing that the mutation p.I207V is frequently found (heterozygote: 14 %, homozygote: 6.7 %) among normal adult Portuguese. The mutation p.T209I was reported by Zampieri et al. (2012) in a patient with juvenile SD. The patient carried a compound heterozygous mutation of p.T209I and c.299 + 1471_408del2406 (Zampieri et al. 2012). In Zampieri et al.'s (2012) report, in vitro functional analysis of the mutant p.T209I protein, showing a very low HEX activity, clearly demonstrated its pathogenic nature. The analysis of the possible effect of the residue change on protein structure showed that the amino acid replacement would cause a severe impairment of protein structure and function (Zampieri et al.2012). The structural analysis of the mutation predicted that the replacement of a hydrophilic residue (T) with the hydrophobic residue (I), occurring in the proximity of a residue (R211) know to be involved in substrate binding, may lead to an impairment of the enzyme activity (Zampieri et al.2012). Through the above findings, we predict the mutation p.I207V may be a neutral mutation and the mutation p.T209I was probably the predominant mutation. The mutation p.T209I may link to the phenotype of infantile SD. Patient 2 was homozygous for the two mutations which were found in the heterozygous state in her father, but were absent in her mother. It is not yet clear how p.T209I and p.I207V mutations became homozygous in Patient 2. It may result from de novo mutation. However, it seems impossible that two de novo mutations would occur that are the same as the mutations inherited from the proband's father. We hypothesized that paternal uniparental disomy may be involved. Uniparental disomy denotes a situation when an individual has inherited two copies of a specific chromosome from a single parent. It is rare in recessively inherited diseases. Paternal uniparental disomy of chromosome 5 was reported in patients with recessive multiple epiphyseal dysplasia, childhood-onset schizophrenia, or spinal muscular atrophy et al. (Garcia et al. 2014; Seal et al. 2006; Brzustowicz et al. 1994). We plan to use microsatellite analysis on chromosome 5 to prove our hypothesis.

Patient 3 had a homozygous frameshift novel mutation: p.P468PfsX62. The mutation was inherited from her parents. Patient 1 carried the same heterozygous mutation p.P468PfsX62 and another mutation IVS12-26G > A. Function analysis of the novel mutation was not made. The mutation IVS12-26G > A may link to late-onset SD. Therefore, the mutation p.P468PfsX62 may be link to infantile SD.

Patient 4 had two novel heterozygous mutations: p.L223P and p.Y463X. Protein function prediction of the missense novel mutation p.L223P in silico was damaging. The mutation p.Y463X was a novel nonsense mutation. At cDNA number 1389, the C-to-G substitution leaded to the appearance of a premature stop codon. The mutation caused the synthesis of enzyme protein was disturbed and influenced the function of the enzyme. Function analysis of the mutation was not made. The relationship between the mutation and phenotype is not clear.

Patient 5 had one novel heterozygous mutation: p.G549R. In the mutation p.G549R, the neutral glycine was replaced by the basic arginine. Protein function prediction of the missense novel mutation p.G549R in silico was damaging. There were two different heterozygous mutations c.-122delC and c.-126C > T in the promoter. No report about HEXB gene mutation in the promoter was found. The effect of the mutations in the promoter is unkown. Function analysis of the mutation need to be made.

In summary, we reported five Chinese patients with SD including one juvenile form and four infantile forms. Seven different mutations were identified including three known mutations: IVS12-26G > A, p.T209I, p.I207V, and four novel mutations: p.P468PfsX62, p.L223P, p.Y463X, p.G549R. We also detected two different heterozygous mutations c.-122delC and c.-126C > T in the promoter which were suspected to be deleterious mutations. The mutation IVS12-26G > A may link to late-onset SD. The mutation p.T209I and p.P468PfsX62 may link to infantile SD. The mutation p.I207V may be a neutral mutation that is an effector of the exacerbation for a second mutant. The function of the other three novel mutations, p.L223P, p.Y463X, p.G549R is unknown. Our study expands the spectrum of genotype of SD in China, provides new insights into the molecular mechanism of SD and helps to the diagnosis and treatment of this disease.

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