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ARTICLE

Novel NGLY1 gene variants in Chinese children with global developmental delay, microcephaly, hypotonia, hypertransaminasemia, alacrimia, and feeding difficulty

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Abstract

NGLY1 deficiency is the first and only autosomal recessive congenital disorder of N-linked deglycosylation (NGLY1-CDDG). To date, no patients with NGLY1 deficiency has been reported from mainland China or East Asia in English literature. Here, we present six patients with a diagnosis of NGLY1-CDDG on the basis of clinical phenotype, genetic testing, and functional studies. We retrospectively analyzed clinical phenotypes and *NGLY1* genotypes of six cases from four families. Informed consent was obtained for diagnosis and treatment. In-silico tools and in vitro enzyme activity assays were used to determine pathogenicity of *NGLY1* varaints. All patients had typical features of NGLY1-CDDG, including global developmental delay, microcephaly, hypotonia, hypertransaminasemia, alacrimia, and feeding difficulty. Dysmorphic features found in our patients include flat nasal bridge, loose and hollow cheeks, short stature, malnutrition, and ptosis. Pachylosis could be a novel cutaneous feature that may be explained by lack of sweat. We found three novel variants, including one missense (c.982C > G/p.Arg328Gly), one splice site (c.1003+3A > G), and one frame-shift (c.1637-1652delCATCTTTTGCTTATAT/p.Ser546PhefsTer) variant. All mutations were predicted to be disease causing with in-silico prediction tools, and affected at least one feature of gene splicing. Protein modeling showed missense variants may affect covalent bonding within the protein structure, or interrupt active/binding amino-acid residues. In vitro studies indicated that proteins carrying missense variants (p.Arg328Gly and p.Tyr342Cys) lost the enzyme activity. We expanded clinical phenotype and genetic mutation spectrum of NGLY1-CDDG by reporting six cases, three novel variants, and novel clinical features from mainland China.

Introduction

NGLY1 gene (OMIM* 610661) encodes human N-glycanase (EC 3.5.1.52) that is responsible for delycosylation during the endoplasmic reticulum-associated degradation (ERAD) of misfolded proteins [1]. NGLY1 deficiency is the first and only autosomal recessive congenital disorder of N-linked deglycosylation (NGLY1-CDDG, OMIM# 615273) that first reported by Need et al. [2] in 2012. Clinical phenotypes include developmental delay, hypotonia, hypertransaminasemia, small feet, seizure, peripheral neuropathy, and absent/decreased tears [2–6]. Other features of NGLY1-CDDG may also include optic atrophy, retinal pigmentary changes, cone dystrophy, delayed bone age, joint hypermobility, lower than predicted resting energy expenditure, low-cerebral spinal fluid total protein and albumin, and unusually high-antibody titers toward rubella and/or rubeola following vaccination [7]. According to NGLY1 patient network (www.ngly1.org), >60 patients

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have been diagnosed worldwide. However, no patients with NGLY1 deficiency has been reported in English literature from mainland China. Here, we present six patients with NGLY1-CDDG on the basis of clinical phenotype and genetic testing results.

Materials and methods

Pubmed searches were conducted at 13 July 2019 using key words such as NGLY1, N-glycanase (species were filtered for humans), and no report from Mainland China or East Asia was found. We also conducted Chinese language literature search by using the CNKI (China Knowledge Resource Integrated Database) and Wanfang Database. We, retrospectively, analyzed clinical phenotype and NGLY1 genotype of six cases (P1–P6) from four families (F1—F4). Among them four cases from three families (patient ID: P1, P2, P3, and P6) were treated in the Department of Hepatology, Children's Hospital of Fudan University, and were asked to fill out questionnaire with updated phenotypes of NGLY1-CDDG [7]. Other two cases from a single family that were reported in the Chinese medical literature (patient IDs: P4 and P5) [8]. *NGLY1* variants were identified either through whole-exome sequencing or targeted screening after affected sibling is identified. Novelty of *NGLY1* variants were checked against dbSNP152, 1000 Genome Database, Exome Variant Server, and gnomAD. Pathogenicity of *NGLY1* variants were predicted by using in-silico tools such as Mutation Taster, SIFT&Provean, Polyphen-2, MutPred2, and M-CAP. Effect of variants on gene splicing were predicted with BDGP/NNSplice, ESE Finder 3.0, HSF3.1, Mutation Taster, ASSP, and SPiCE. We conducted protein modeling with SWISS-model using the most similar structure (2f4o.2.A, peptide N-glycanase, 88.85% sequence similarity with 43.4% coverage), and polar contacts of wild-type and mutated amino-acid residues were compared with Pymol software. Website addresses for online tools or software for pathogenicity prediction, splice site changes, and protein modeling were provided at the end of this article.

To determine the NGLY1 activity in vitro, the amplified PCR product with human NGLY1 gene was inserted between the NcoI and XhoI cloning sites on the pET-28a (+) plasmid for bacterial expression. The 1025A > G (specific oligonucleotides: 5′-gctgctgagaaggagaacagacttctgtccagaca-3′ and 5′-tgtctggacagaagtctgttctccttctcagcagc-3′) and 982C > G (specific oligonucleotides: 5′-aatcccaaacatagccagcttcaaaccctacagct-3′ and 5′-agctgtagggtttgaagctggctatgtttgggatt-3′) NGLY1 mutants were obtained by a PCR-based mutagenesis protocol [9]. All the constructed expression plasmids were confirmed by sequencing.

NGLY1 protein was expressed and purified with a standard reported method [9–12]. The expression plasmids were transformed into *E.coli* BL21 (DE3). The transformants were cultured at 37 °C for 12 h in Luria-Bertani medium containing 50 μg/ml kanamycin. IPTG (1.0 mM) was used to induce the expression of the protein at 28 °C for 12 h. The cells were harvested by centrifugation at 10,000 rpm 10 min and the pellet was resuspended in a lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0). The suspended cells were sonicated (program: 5 s of sonication followed by a pause (10 s) per cycle, 30% power) for 20 min on ice. The debris was removed by centrifugation (30 min, 10,000 rpm) at 4 °C. The supernatant was loaded on a HisTrapTM column (GE Healthcare). The expressed protein on loaded column was washed twice with a wash buffer (20 mM Tris, 500 mM NaCl, 20 m Mimidazole, pH 8.0), and the eluted in an elution buffer (20 mM Tris, 500 mM NaCl,200 mM imidazole, pH 8.0). NGLY1 R328G, NGLY1 Y342C, PNGase F, and PNGase F-II were expressed and purified respectively with the same method. To verify the N-glycosidase activity of NGLY1, bovine pancreatic ribonuclease B (RNase B) with N-linked high-mannose oligosaccharide (Sigma-Aldrich) was chosen as the standard. Glycoprotein was subjected to cleavage by PNGase F, PNGase F-II, NGLY1, NGLY1 R328G, and NGLY1 Y342C [10]. The assay was conducted in phosphate-buffered saline (PBS) buffer, pH 7.8, at 37 °C for 10 h.

Results

Case profiles

P1 and P2 were siblings born to healthy non-consanguineous parents. P1 is a full-term younger brother with uneventful pregnancy and normal birthweight. He presented to our hospital with global developmental delay, intellectual disability, movement disorders, seizure like movements during sleep, hypolacrimia, and hypertransaminasemia at the age of 11 months. Although liquid feeding was normal, feeding with solid food was difficult, and weight percentile dropped from 33rd by WHO standard at the age of 9 months to 7th at the age of 17 months. However, height percentile is grown from less than the 1st at the age of 11 months to 32nd at the age of 17 months. Head circumference was measured at the age of 17 months and is on the 2nd percentile. Severe hypertransaminasemia improved over time, but fluctuated during intercurrent infections. Slight coagulopathy was observed at the age of 8-months with slight elevation of APTT (42 s, normal 24–38 s) and TT (25 s, normal range 14–21 s), while having low fibroginin levels (1.1 g/L, normal range 2–4 g/L). Alphafetoprotein (AFP) was slightly elevated at 9 months (38 μg/L, normal range 0–25 μg/L). At 8.5 months of age, slight elevation of IgM (1.34 g/L, normal range 0.68–1.26)

and lower levels of ceruloplasmin (0.15 g/L, normal range 0.2–0.6 g/L) were detected. Normal findings include complete blood count, thyroid function test, total cholesterol, triglyceride, lymphocyte sub-population, TORCH antibodies, immunoglobulin, transferrin, ammonia, serologies for HBV/HCV/HIV/syphilis, fundoscopy, electrocardiography, abdominal ultrasound (liver, gallbladder, spleen, and pancrease), and chest X-rays.

P2 is an elder sister of P1 with full-term, uneventful pregnancy and normal birthweight (2900 g). At the age of 3.9 years, she presented with delay in motor and language development, intellectual disability, involuntary movements, hypotonia, feeding difficulty, malnutrition, and mild hypertransaminasemia. Parents recalled alacrimia during infancy, but little bit of tear started to appear during when crying after 1 year of age. At the age of 5 years, she still has difficulty chewing on solid foods, and oral muscles seems weak. Head circumference at 5 years of age was on the 2nd percentile. At 4.3 years of age, she was evaluated with the Adaptive Behavior Assessment System second edition (ABAS-II) [13]. Percentiles for general adaptive skill, conceptual skill, social skill, and practical skill were on the 1st, 0.5th, 1st, and 5th centile, respectively. Auditory brainstem response and brainstem auditory evoked response at the age of 4.3 years showed bilaterally lower amplitudes of Wave III, although wave I/wave V/inter-wave intervals were normal. Normal findings include complete blood count, thyroid function test, and bone density profiling (slightly abnormal at 1 year of age, normal at the age of 4 year).

P3 is a 25-month-old girl born to non-consanguineous parents after uneventful full-term pregnancy (41 weeks) and normal birthweight (2800 g). She presented with global development delay, movement disorder, hypotonia, alacrima, and mild hypertransaminasemia at the age of 25 months. She started walking with ataxia at the age of 21 months. Brief adventitious movements of the finger with irregular and random frequency were observed by parents. Feeding with solid food was difficult due to less chewing activity, difficulty of steadily holding foods with hands, and following verbal suggestions. Tears were rarely seen, although sweating was normal. However, she seems to have verbal understanding, loves watching television, and playing with electronics, such as cellphones and iPads. Gesell developmental evaluation (at the age of 19 months) showed fine motor and language skills were severely affected, adaptive and personal-social skills were moderately affected, and gross motor skills were slightly affected. Dysmorphic features include flat nasal bridge, hypertelorism, small palperbal fissures and earlobe, short philtrum, slightly long and pointed chin, loose hollow cheeks, and lower hairline. Adrenocorticotropic hormone (ACTH) level at 19 months was significantly elevated (359 pg/mL, normal range 7.2–63.3 pg/mL), while cortisol level was at the higher end of normal range (497 nmol/L, normal range 172–497 nmol/L). However, repeated ACTH level after 5 months was normal (40 pg/mL). Serum lactate was slightly elevated (2.2 mmol/L, normal range 0.5–1.8 mmol/L). Blood amino-acid and acylcarnitine profiling in P3 showed slightly elevated levels or ratios of C4DC + C5OH (0.55 umol/L, normal range 0.08–0.45 umol/L), C10:1 (0.17 umol/L, normal range 0.02–0.14 umol/L), (C5DC + C6OH)/C16 (0.22, normal range 0.01–0.16), C14:1/C16 (0.13, normal range 0.02–0.08), and Valine/Phenylalanine (4.78, normal range 1.2–4.6 umol/L). Blood glycine level was slightly lower than the normal range (227 umol/L, normal range 250–1500 umol/L). Urine organic acid levels showed slight elevations of 2-methyl-3-hydroxybutyric acid (1.58 μM, normal range 0–0 μM), uridine (7.47 nmol/mg creatinine, normal range 0–5.5 nmol/mg creatinine), 3-methyl glutaconic acid (3.71 nmol/mg creatinine, normal range 0–3.16 nmol/mg creatinine), and 3-(3-Hydroxyphenyl)-3-hydroxypropionic acid (75.45 nmol/mg creatinine, normal range 0–37.2 nmol/mg creatinine). Normal findings include thyroid function test, serum folic acid and vitamin B12 levels, blood ammonia, and brain MRI.

Patients P4 and P5 were full-term sisters with normal birthweight born to non-consanguineous parents [8]. Both pregnancies were uneventful except for low maternal serum uE3 levels in the second trimester, but karyotyping after amniocentesis ruled out Downs syndrome. Younger sister (P4) presented with hypotonia, developmental delay, hypertransaminasemia, and alacrimia at the age of 8 months. Dysmorphic features (small hand, small feet, broad forehead, deep nasal bridge, and chubby face), pachylosis, and ptosis were noted on physical examination. Her brain MRI was normal, but serum AFP level was slightly elevated (86.5 ng/mL). Her 4-year-old elder sister (P5) also had developmental delay with other features similar to her younger sister. She had normal AFP levels, but brain MRI showed small stroke like lesions at the vicinity of anterior horn of right lateral ventricle.

Patient P6 is a male patient born to healthy consanguineous parents (first generation cousins). He first presented with developmental delay, alacrima, and anhydrosis at the age of 10 months. Physical examinations at the age of 22 months were positive for low muscle tone, flat nasal bridge, loose facial muscle, hollow cheeks, depressed nipples, small penis (about 1 cm long), abnormal fat distribution around pubic area, inability of walking, small hands, and small feet. Feeding was difficult due to irregular movements of arm and inability to hold milk bottle still, or hold foods for longer than one bite. Chewing of solid food was also weak and slow. Although anxious around strangers, he loves playing with cell phone and would cry for a long time when cell phone was taken away. Electromyography (EMG) at the age of 11 months showed higher amplitudes of motor unit potential (MUP).

Nerve conduction velocity (NCV) of the common fibular nerve was normal, but the median nerve showed reduced amplitudes of maximal voluntary contraction (MCV) and compound muscle action potential (CMAP). Sensory nerve action potential (SNAPs) we absent on the right median nerve and right sural nerve. Right ulnar nerve was not responsive to repeated electric stimulation. EMG indicated peripheral neuropathy with both sensory and motor neurons affected, upper extremities affected more severely than the lower extremities. Brain MRI at the age of 10 months showed bilateral temporal lobe atrophy. Normal findings include ultrasound evaluation of hip dysplasia at the age of 45 days (Graf type 1), thyroid function test, blood amino-acid and acylcarnitine profiling, blood succinylacetone, urine organic acid, chromosome karyotyping, and TORCH antibodies.

Clinical characteristics of all six patients along with percentages of abnormalities in previous reports were summarized in Table 1.

Genetic testing, carrier frequencies, pathogenicity prediction, effects of genetic variants on splicing, and protein modeling

All patients were screened for genetic disorders by trio whole-exome sequencing, and siblings were by targeted sequencing after index cases were confirmed. Three novel variants of *NGLY1* gene, including one missense (c.982C > G/p.Arg328Gly), one splice site (c.1003 + 3A > G), and one frame-shift (c.1637-1652delCATCTTTTGCTTATAT/p.Ser546PhefsTer), were found. There were two additional rare variants (one missence c.1025A > G/p.Tyr342Cys and one nonsence c.1231C > T/p.Arg411Ter) that have been reported in EXAC and gnomAD databases, but never been reported to be associated with NGLY1-CDDG. All five variants were predicted to be damaging by various in-silico pathogenicity prediction tools, and predicted to affect gene splicing by at least one splice site prediction tool. *NGLY1* variants, parental origin, carrier frequency, and results of in-silico tools were provided in Table 2. Sanger sequencing results (for P1, P2, P3, and P6) and conservation status for missense, non-sense, and truncating amino-acid changes were provided in Fig. 1. Both missense variants carried by our patients (c.982C > G/p.Arg328Gly and c.1025A > G/p.Tyr342Cys) lead to change of fully or strongly conserved amino-acid residues. Sanger sequencing results for P4 and P5 were already provided within the Chinese language report [8]. Two missense variants lead to changes in the PNG core domain (Fig. 2a), and one missense variant (c.982C > G/p.Arg328Gly) caused changes in polar contacts within the protein structure (Fig. 2b). On the other hand, non-sense, frame-shift, and splicing site variants may affect protein function by non-sense-mediated messenger RNA (mRNA) decay or by skipping of exons during transcription. Missense variants such as c.982C > G/p.Arg328Gly and c.1025A > G/p.Tyr342 Cys are located within close proximity of PNGase active or binding sites (Fig. 2c).

In vitro functional studies

Both Y342C and R328G were defective in glycosidase activity. To determine the impact of detected mutations on NGLY1 function, wild-type NGLY1 and its mutants were expressed, purified, and subjected to an in vitro glycosidase assay, respectively. NGLY1 was first cloned into a pET-28a (+) expression vector to produce a fusion protein with an C-terminal His6 tag. The purified protein migrated as a dominated band with an apparent molecular mass of 75 kDa when subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown), in agreement with its calculated mass. Both R328G and Y342C of NGLY1 were obtained with same protocol. The result of in vitro assay was presented in Fig. 2d. Like PNGase F and PNGase F-II, wild-type NGLY1 could remove the conjugated oligosaccharides on RNase B, generating the new down shifted band. However, the glycosidase activities of NGLY1 were lost in both mutants. The results of this assay indicated that both Y342C and R328G missense variants could be loss-of-function mutants.

Discussion

NGLY1-CDDG is an autosomal recessive disorder caused by pathogenic variants in *NGLY1*. NGLY1 deficiency may cause clinical phenotype by failing to degrade misfolded proteins during endoplasmic reticulum-associated degradation (ERAD) process, leading to tissue accumulation and damage. Predominant neurological and muscle involvement might be due to FAF1 (Fas-Associated Factor 1) protein accumulation, since FAF1 is the only protein outside the ERAD machinery that strongly associated with NGLY1 [14] and FAF1-to-NGLY1 ratio is the highest in skeletal muscle and brain tissues [15]. In addition, NGLY1 enzyme might have essential functions other than deglycosylation such as cellular signaling [16], cellular polarity [17], and presentation of class I major histocompatibility complex antigen [18, 19] that may explain other organ/system abnormalities seen in NGLY1-CDDG.

Our patients not only have typical phenotypes that were previously reported to be associated with NGLY1-CDDG such as severe global developmental delay, microcephaly, hypotonia, hypertransaminasemia, alacrimia, and feeding difficulty [2–6], but also have some additional phenotypes that have been recently updated [7]. As patients grow, microcephaly improved in P3 (from 2nd centile at the age of

Table 1 Clinical characteristics of patients

Clinical phenotypes (number of patients with positive phenotype/ number of patients tested or with available information)	Patient number, gender (F, female; M, male) and age at diagnosis (m, month)						Percentage of abnormalities in previous reports [2–4, 6, 7, 20, 21, 26, 27]
	P1, M, 17 m	P2, F, 60 m	P3, F, 19 m	P4, F, 8 m	P5, F, 48 m	P6, M, 10 m	
Parental consanguinity (1/6)	No	No	No	No	No	Yes	50% (8/16)
Anthropometric percentiles by WHO standards							
Weight (malnutrition 1/6)	7th (33rd at 9 m)	3rd	87th	70th	6th	98th	38% (5/13)
Height (short stature 1/6)	32rd (< 1st at 11 m)	24th	96th	3rd	34th	49th	23% (3/13)
Head circumference (microcephaly 6/6)	2nd	2nd	2nd	2nd	2nd	2nd	52% (14/27)
Neuromotor developmental delay (6/6)	Yes	Yes	Yes	Yes	Yes	Yes	100% (29/29)
Hypotonia (6/6)	Yes	Yes	Yes	Yes	Yes	Yes	92% (12/13)
Hyperkinetc movement abnormalities (5/6)	Yes	Yes	Yes	No	Yes	Yes	96% (26/27)
Prenatal							
IUGR (4/6)	No	No	Yes	Yes	Yes	Yes	56% (9/16)
Second trimester low uE3 (4/4)	Yes	-	Yes	Yes	Yes	-	71% (5/7)
Head and neck							
Hearing abnormalities (2/3)	-	Yes	Yes	-	-	No	57% (12/21)
Alacrima/hypolacrima (6/6)	Yes	Yes	Yes	Yes	Yes	Yes	92% (24/26)
Corneal ulcerations/scarring (0/6)	No	No	No	No	No	No	54% (13/24)
Strabismus (1/6)	No	No	No	Yes	No	No	68% (15/22)
Ptosis (3/6)	No	No	No	Yes	Yes	Yes	37% (7/19)
Microcephaly (6/6)	Yes	Yes	Yes	Yes	Yes	Yes	52% (14/27)
Skeletal							
Scoliosis (1/5)	No	No	No	-	Yes	No	47% (27/57)
Fractures due to osteoporosis (0/4)	No	No	No	-	-	No	35% (14/40)
Small hands/feet (3/6)	No	No	No	Yes	Yes	Yes	70% (16/23)
Gastrointestinal							
Feeding difficulties (6/6)	Yes	Yes	Yes	Yes	Yes	Yes	100% (18/18)
Hypertransaminasemia (6/6)	Moderate to severe	Mild	Mild	Mild	Mild	Mild	84% (21/25)
Neonatal jaundice (3/4)	Yes	Yes	No	-	-	Yes	50% (6/12)
Vomitting (1/4)	No	No	No			Yes	13% (1/8)
Constipation (2/4)	No	Yes	No	-	-	Yes	88% (21/24)
Neuromuscular							
Peripheral neuropathy (1/1)	-	-	-	-	-	Yes	88% (14/16)
Seizures (3/6)	Yes	Yes	Yes	No	No	No	60% (18/30)
Ocular apraxia (0/6)	No	No	No	No	No	No	38% (5/13)
Diminished reflexes (2/4)	No	No	Yes	-	-	Yes	82% (9/11)
Speech impairment (6/6)	Yes	Yes	Yes	Yes	Yes	Yes	92% (22/24)
Decreased pain sensation (0/5)	No	No	No	-	No	No	100% (2/2)
Abnormal brain imaging (2/4)	-	-	No	No	Yes	Yes	60% (9/15)
Abnormal EEG (0/1)	-	-	-	-	-	No	70% (16/23)

Table 1 (continued)

Clinical phenotypes (number of patients with positive phenotype/ number of patients tested or with available information)	Patient number, gender (F, female; M, male) and age at diagnosis (m, month)						Percentage of abnormalities in previous reports [2–4, 6, 7, 20, 21, 26, 27]
	P1, M, 17 m	P2, F, 60 m	P3, F, 19 m	P4, F, 8 m	P5, F, 48 m	P6, M, 10 m	
Nerve conduction velocities (1/1)	-	-	-	-	-	Yes	88% (15/17)
Abnormal EMG (1/1)	-	-	-	-	-	Yes	60% (12/20)
Others							
Anhydrosis (3/6)	No	No	No	Yes	Yes	Yes	48% (10/21)
Dysmorphic features (4/6)	No	No	Yes	Yes	Yes	Yes	58% (7/12)
Undescendend testis (1/2)	No	-	-	-	-	Yes	25% (1/4)
Elevated alpha fetoprotein (2/3)	Yes	-	-	Yes	No	-	33% (3/9)
Elevated blood Lactate (1/2)	No	-	Yes	-	-	-	43% (9/21)
Died within two decades (0/6)	No	No	No	No	No	No	40% (4/10)
Other (3/6)	No	No	Transiently elevated ACTH	Pachylosis,	Pachylosis,	No	Chalazions (60%, 6/10), Liver fibrosis (33%, 3/9), Abnormal liver storage (88%, 7/8), Lagophthalmous (82%, 9/11), Nystagmus (25%, 3/12), elevated ACTH (%, 2/13)

- not available or not applicable, *ACTH* adrenocorticotropic hormone, *IUGR* intrauterine growth restriction, *uE3* estriol

19 months to 16th centile at the age of 25 months) and P6 (from 2nd centile at the age of 10 months to 6th centile at the age of 23 months). Five patients had movement abnormalities, three had seizures, and two had abnormal brain MRI. Among three patients with anhydrosis, two had pachylosis, which may be related to lack of sweat production in the skin. One of our patients had transiently elevated serum ACTH levels but normal cortisol levels. Since deaths could be explained by adrenal insufficiency in NGLY1-CDDG patients [20], we asked patients to test serum ACTH/cortisol levels and perform adrenal ultrasound during regular follow-up or emergency situations before receiving hormone replacement if needed. Skeletal phenotypes may include bone fracture, spinal deformity, joint contracture, hip dysplasia, and small hand or feet [21]. Three patients in our cohort had small feet with small hands, one patient had scoliosis, while none experienced fractures, joint contractures or hip dysplasia.

NGLY1 protein has three domains: The N-terminal PUB domain functions as a AAA ATPase binding domain [22]; The C-terminal PAW domain of PNGase binds to the mannose moieties of N-linked oligosaccharide chains [23]; The catalytic domain of TGase-superfamily domain (PNGase-core domain) binds to N, N-diacetylchitobiose-containing glycans [24]. As with previously reported truncating and splice site mutation, c.1003 + 3A > G, c.1637-1652del-CATCTTTTGCTTATAT/p.Ser546PhefsTer, and c.1231C > T/p.Arg411Ter variant in our report led to partial or complete loss of either PNG core domain or PAW domain (Fig. 2a). Similar to previously reported missense mutations, amino-acid changes in c.982C > G/p.Arg328Gly and c.1025A > G/p.Tyr342 Cys variants in our report are located within close proximity of PNGase active or binding sites (Fig. 2c). The c.1201A > T/p.Arg401Ter mutation is the most frequently occurred mutation in previous reports from western countries [7]. In comparison, three patients from two unrelated families (P1&P2 and P3) in our report carried the same compound heterozygous variants in the *NGLY1* gene (c.1231C > T/p.Arg411Ter of maternal origin, and c.1025A > G/p.Tyr342Cys of paternal origin). More patients need to be identified before determining if these mutations are the hotspot mutation among NGLY1-CDDG patients in mainland China.

Pathogenicity of two missense variants in our report (p.Arg328Gly and p.Tyr342Cys) was confirmed by an in vitro PNGase assay. In the previous studies, transcription and translation of NGLY1 were analyzed by quantitative PCR and western blot [5]. In addition, a cellular deglycosylation-dependent Venus fluorescence assay was applied to determine the glycosidase activity in a cell [5]. Comparing to previous methods, a unique feature of our assay is to directly check the impact of the mutant(s) on the glycosidase activity.

Previous attempts of using traditional methods (including transferrin isoelectric focusing, N-glycan, or O-glycan profiling) did not detect deglycosylation disorder in NGLY1 deficiency in already reported patients [6]. However, NGLY1-CDDG now could be confirmed by testing a

Table 2 Carrier status of *NGLY1* variants among family members, carrier frequency among different population groups, and in-silico pathogenicity prediction results (all variants were not reported in Clinvar)

Patients (parental origin)	P1&P2 (siblings), and P3		P4 and P5 (siblings)		P6	
	Paternal	Maternal	Paternal	Maternal	Paternal	Maternal
Physical location	chr3:25777619T > C	chr3:25775392G > A (rs146140738)	chr3:25761642_25761657delATATAAGCAAAAGATG	chr3:25778822T > C	chr3:25778846G > C	chr3:25778846G > C
Nucleotide change (NM_018297)	c.1025A > G	c.1231C > T	c.1637-1652delCATCTTTTGCTTATAT	c.1003 + 3A > G	c.982C > G	c.982C > G
Amino-acid change	p.Tyr342Cys	p.Arg411Ter	p.Ser546PhefsTer	NA	p.Arg328Gly	p.Arg328Gly
Carrier frequency (number of heterzozygous/homozygous carrier)						
1000G	0	(2/0)	0	0	0	0
EXAC	0.000008443 (1/0)	0.00002473 (3/0)	0	0	0	0
East Asians	0	0.0001157 (1/0)	0	0	0	0
South Asians	0.00006229 (1/0)	0	0	0	0	0
European	0	0.000015 (1/0)	0	0	0	0
African	0	0.00009615 (1/0)	0	0	0	0
gnomAD	0.00001225 (3/0)	0.00002441 (6/0)	0	0	0	0
East Asians	0	0.0001740 (3/0)	0	0	0	0
South Asians	0.00003264 (1/0)	0	0	0	0	0
European	0.00001805 (2/0)	0.0001740 (1/0)	0	0	0	0
African	0	0.0001308 (2/0)	0	0	0	0
In slico prediction of pathogenicity (Score)						
Mutation Taster	Disease causing (1)	Disease causing (1)	Disease causing (1)	Disease causing (1)	Disease causing (1)	Disease causing (1)
SIFT	Damaging (0.001)	NA	NA	NA	Damaging (0.000)	Damaging (0.000)
Provean	Deleterious (-7.64)	NA	NA	NA	Deleterious (–6.99)	Deleterious (–6.99)
Polyphen-2	Probably damaging (1)	NA	NA	NA	Probably damaging (1)	Probably damaging (1)
MutPred2	Pathogenic (0.621)	NA	NA	NA	Pathogenic (0.938)	Pathogenic (0.938)
M-CAP	Possibly pathogenic (0.134)	NA	NA	NA	NA	NA
Effect of variants on gene splicing						
BDGP/NNSplice	Acceptor site affected	None	None	Donor site affected	None	None
ESE Finder 3.0	None	ESE site affected	Loss of BranchSite	NA	ESE site affected	ESE site affected
HSF3.1	New Donor Site, New ESS Site	ESE Site Broken, New ESS Site	ESS Sites broken, New ESE Site	Broken donor site	None	None
Mutation Taster	Acc marginally increased	Donor marginally increased	Donor gained	Donor lost	Donor increased	Donor increased
ASSP	None	None	None	None	None	None
SPiCE	None	None	None	Affected	None	None

ASSP alternative splice site predictor, *BDGP/NNSplice* Berkeley Drosophila Genome Project/splice site prediction by neural network, *ESE* exonic splicing enhancer, *ESS* exonic splicing silencer, *HSF* human splicing finder, *NA* not available or not applicable, *SPiCE* Splicing Prediction in Consensus Element

Pathogenicity thresholds: MutationTaster (probability), SIFT (<0.05), Provean (≤–2.5), Polyphen-2 (>0.8), Mutpred2 (>0.5), M-CAP (>0.025)

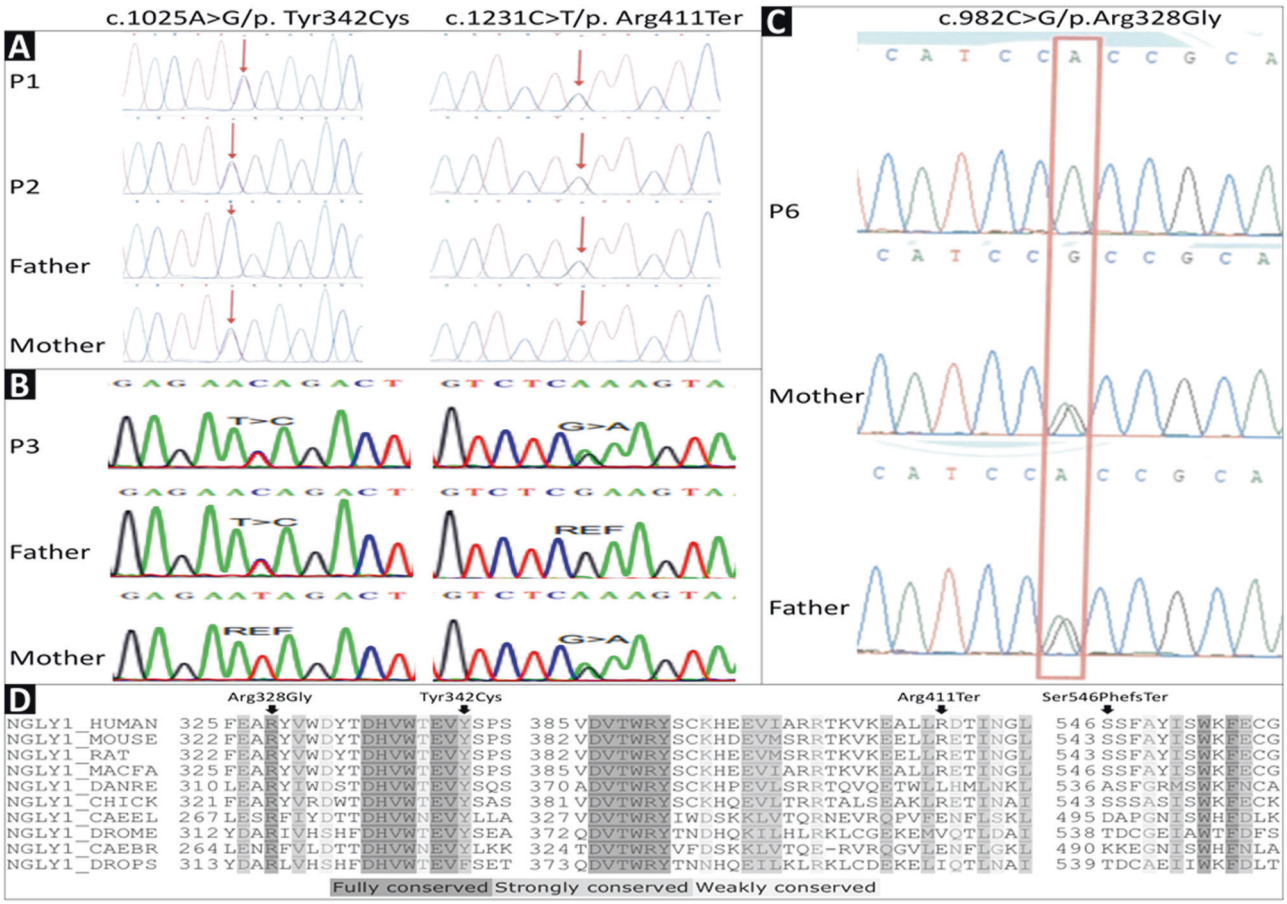

Fig. 1 Sanger sequencing results for P1, P2 (**a**), P3 (**b**), and P6 (**c**), and conservation status for missense, non-sense, and truncating amino-acid changes (**d**)

specific urine biomarker (Neu5Ac1Hex1GlcNAc1-Asn) with high sensitivity (92.3%) and specificity (99.6%) [25] providing evidence to interpret variants of uncertain significance. Recently, an untargeted metabolomics screening using dried blood spots (DBS) indicated aspartylglycosamine as a biomarker for NGLY1-CDDG [26]. Although we determined pathogenicity of NGLY1 variants in our patients using in-silico tools and in vitro enzyme activity assay, abovementioned biomarkers were not screened in our patient samples. Chang et al. [27] recently reported transient elevations of serum methionine, S-adenosylmethionine, and S-adenosylhomocysteine levels, we did not observe similar abnormalities in two patients who have been tested for serum amino-acid profiling. Rather, slight or non-specific abnormalities of blood amino-acid with acylcarnitine profiling and urine organic acid testing were observed in P3. In particular, serum 3-(3-Hydroxyphenyl)-3-hydroxypropionic acid, which was reported to be associated with autism spectrum disorders [28], is elevated. Whether or not NGLY1 deficiency is associated with autism spectrum disorders remained to be determined by future studies.

There is no disease-specific treatment for NGLY1-CDDG, and only supportive therapies were available. Endo-β-N-acetylglucosaminidase (ENGase) in NGLY1 knockout cellular model may be responsible for the production of partially deglycosylated proteins that could be harmful [29], and NGLY1/ENGase double knockout mouse model had less severe phenotype and mortality [30]. In vitro studies indicated that rabeprazole sodium, acting as a small molecule ENGase inhibitor [31], could be a potential treatment option for NGLY1-CDDG.

In summary, we reported six patients with NGLY1-CDDG, three novel NGLY1 gene variants from mainland China, analyzed phenotypic and genotypic features, and confirmed pathogenicity of two missense varaints with functional studies. However, some of our cases may still need confirmation by biomarker testing using blood and urine samples from patients.

Internet resources

1000 Genome Database (http://www.1000genomes.org/)

ASSP (http://wangcomputing.com/assp/)

BDGP/NNSplice (http://www.fruitfly.org/seq_tools/splice.html)

China Knowledge Resource Integrated Database (http://new.oversea.cnki.net/index/)

Clinvar (https://www.ncbi.nlm.nih.gov/clinvar)

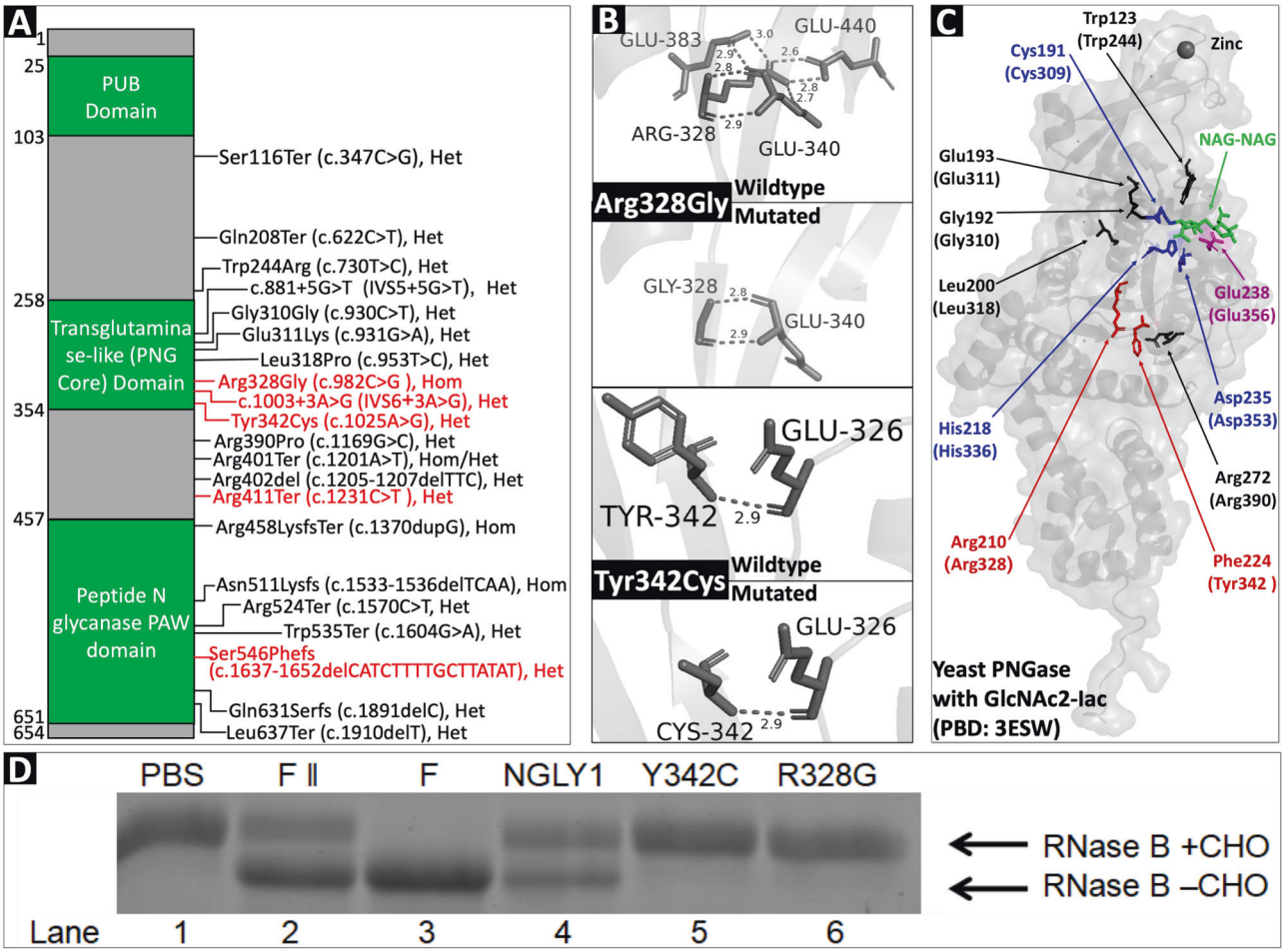

Fig. 2 Genetic variants, protein modeling, and enzyme activity. **a** Schematic Representation of Human N-glycanase showing the functionally conserved domains, previously reported mutations (black), and variants from our report (red). **b** Protein modeling with pymol and effects of missense variants on polar contacts with other atoms within the structure. **c** Crystal structure of yeast PNGase with GlcNac2 (NAG-NAG, green), demonstrating locations of amino-acid residues (corresponding human NGLY1 amino-acid residues in protein alignment within bracket), active sites (blue), the binding site (magenta), previously reported missense mutations (black), and missense variants from our report (red). NGLY1 protein domains were determined with the protein family database (Pfam). Het heterozygous, Hom homozygous, PUB peptide: N-glycanase/UBA or UBX-containing proteins domain, PAW present in PNGase and other worm proteins. **d** Results of in vitro PNGase assay by SDS-PAGE analysis. Denatured RNase B was first treated with the controls (PBS, PNGaseF-II, PNGase F, and NGLY1), and both mutants (Y342C and R328G) before being analyzed by SDS gel separation. Glycosylated and deglycosylated forms of the substrate were indicated by + CHO and –CHO, respectively. In addition to wild-type NGLY1, PNGase F-II (F-II), and PNGase F (F) two of the conventional enzymes used in glycomics were also included as positive controls

dbSNP152 (http://www.ncbi.nlm.nih.gov/snp)
ESE Finder 3.0 (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi)
ExAC Browser (http://exac.broadinstitute.org)
Pfam (http://pfam.xfam.org/protein/Q96IV0)
gnomAD (http://gnomad.broadinstitute.org)
Human Splicing Finder 3.1 (http://www.umd.be/HSF);
MutationTaster (http://www.mutationtaster.org)
Mutpred2 (http://mutpred.mutdb.org)
M-CAP (http://bejerano.stanford.edu/MCAP/)
NCBI (https://www.ncbi.nlm.nih.gov)
OMIM (https://omim.org/)
Poyphen-2 (http://genetics.bwh.harvard.edu/pph2/)
Pymol software (https://pymol.org/2/)
SIFT&Provean (http://provean.jcvi.org)
SPice software (https://sourceforge.net/projects/spicev2-1/)
SWISS-Model (https://www.swissmodel.expasy.org)
Wanfang Database (http://www.wanfangdata.com).

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Author contributions WJS and CL designed the study and approved the final submission; AK collected clinical and genetic data, performed protein modeling, conducted literature search, and summarized relevant information. Both WJS and AK clinically managed patients. ZL, WL, and CL performed functional studies. AK, ZL, and WL co-wrote the manuscript, and contributed equally for this study.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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