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ARTICLE

GNE myopathy in Chinese population: hotspot and novel mutations

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Abstract

GNE myopathy is a rare autosomal recessive distal myopathy caused by mutations in UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE), the bi-functional enzyme critical for sialic acid biosynthesis. In this study, we summarized the clinical features, pathological characteristics, and genetic profiles of 46 GNE patients. The clinical and mutational profile of 54 previously reported Chinese patients were also reviewed. A total of 21 novel mutations, including a gross deletion spanning exon 1–2 and a retrotransposon insertion were found in our cohort, enlarging the spectrum of *GNE* mutations. The most frequent mutation in Chinese population was D207V, which accounts for 25.5% of total alleles (51/200). The age of onset was much later in the patients carrying D207V compared to other patients, indicated the less deleterious effect of D207V on enzyme activity. GNE myopathy may be overlooked in China with a relatively milder phenotype due to the common mutation D207V.

Introduction

GNE myopathy, previously known as hereditary inclusion body myopathy (hIBM), distal myopathy with rimmed vacuoles (DMRV) or Nonaka myopathy, is caused by mutations of the *GNE* gene, which codes for a key enzyme of sialic acid biosynthesis pathway with both a UDP-GlcNAc 2-epimerase (GNE) domain and a ManNAc kinase domain (MNK) [1, 2]. It is a rare adulthood-onset autosomal recessive myopathy characterized by initial distal muscle weakness and subsequent proximal progression with relative sparing of the quadriceps. Pathological features of GNE myopathy include "rimmed" vacuoles, aggregation of various proteins and fiber size variation. Currently the diagnosis relies on the identification of bi-allelic *GNE* mutations [3].

To date, over 200 *GNE* mutations have been associated with GNE myopathy, with missense mutations comprising the majority of the spectrum [4]. Different common mutations between diverse ethnicities have been identified worldwide, such as M743T in Middle East, V603L and D207V in Japan, and V727M in South-East Asia [3]. A recent cohort indicated D207V to be the most common mutation in China, which contradicted the findings of previous northern China based study [5, 6]. Genotype–phenotype correlations were hampered by limited cases in earlier studies [5–12]. Herein, we reported the clinical and genetic profiles of 46 cases with GNE myopathy in East China. Twenty-one novel mutations were identified, including large deletion and retrotransposon

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insertion. We also provided a summary of 100 reported Chinese cases to review the mutation spectrum in China.

Material and methods

Patients

Forty-six cases from unrelated Chinese Han families were diagnosed as GNE myopathy in Huashan Hospital from February 2005 to December 2017. The diagnostic criteria included: (1) compatible clinical manifestations, such as distal weakness, myogenic changes on electromyography (EMG), and mildly to moderately elevated creatine kinase (CK) level; with/without rimmed vacuoles on muscle pathology; and (2) confirmed molecular diagnosis with *GNE* mutation identified on both alleles. All clinical information and biological materials used in this study were obtained with written informed consent and approved by the Ethics Committee of Huashan Hospital, Fudan University.

Clinical and pathological evaluation

Detailed clinical data were collected and reviewed retrospectively. Clinical information including onset age, initial symptoms, family history, EMG result, and maximum CK level were recorded using uniformed questionnaire. Open muscle biopsies were performed in 40 cases. Serial frozen sections of 8 μm thickness were used for routine histochemical studies, including hematoxylin and eosin, modified Gomori trichrome, nicotinamide adenine dinucleotidetetrazolium reductase, cytochrome C oxidase, succinate dehydrogenease, Oil red O, periodic acid Schiff, and adenosine triphosphatase staining.

Molecular analyses

Genomic DNA was extracted from the peripheral blood leukocytes using a genomic DNA extraction kit (Tiangen, China). Amplification of the whole coding sequence including intron/exon boundaries of *GNE* gene were performed according to previously described protocols in 24 cases [1] (Case 1–24, Supplementary Table S1). PCR products were purified and sequenced using automated sequencer (Applied Biosystems, 3730XL, USA). Targeted next-generation sequencing (NGS) covering 58 muscular disorder related genes and subsequent Sanger confirmation was performed in the other 22 patients (Case 25–46, Shanghai Amplicon-gene Bioscience Co., Ltd.). PCR products were analyzed by TA-cloning (Invitrogen, USA) when heterozygous insertion/deletions (indels) were encountered to allow clear illustration. The variants were named according to the reference sequence GenBank: NM_001128227 and NP_001121699, and *GNE* exon numbering is based on the latest expert recommendation [4]. Human Gene Mutation Database was used to address the novelty of variants. The predicted effect of novel missense mutations on protein function was analyzed using three prediction software programs: polymorphism phenotyping v2; http://genetics.bwh. harvard.edu/pph2/ [13], sort intolerant form tolerant human protein; http://sift.jcvi.org/ [14], and MutationTaster (http://mutationtaster.org) [15]. The novel variants were interpreted and classified according to the American College of Medical Genetics and Genomics (ACMG) recommendation [16].

In 2 cases (case 44 and case 45) that only one heterozygous missense mutation was identified by targeted NGS, integrative genomics viewer (IGV) [17] was used to check exon coverage and sequence depth, and a further copy-number variations analysis was performed according to previously reported methods [18]. Briefly, targeted NGS with enrichment primers placed in each exon as well as introns (average spacing 1 kb in the introns) and 5′- and 3′-untranslated region of *GNE* gene was performed on Ion PGM^{TM} (Thermo Scientific) according to the manufacturer's protocol, with an overall coverage of 97% and the mean depth over 2000. Depth of coverage (DOC) of each amplicon was determined by comparing the normalized depth of the patient with mean of three controls. Log_2DOC was determined in the case with deletion. In cases with splicing site mutation, cDNA analysis of muscle *GNE* transcripts was performed as previously reported [18].

Literature review

The following key words were used to search PubMed for previous reports in Chinese population: [distal myopathy with rimmed vacuoles/GNE/hereditary inclusion body myopathy] + [Chinese/China]. Reports with detailed information of age of onset were reviewed. Individuals with bi-allelic mutations in *GNE* gene were included for genotype–phenotype analysis.

Statistical analyses

Statistics analyses were performed using GraphPad Prism 5 software (La Jolla, USA) and comparison between groups was assessed with Student *t* test. All data were expressed as mean ± SD. Statistical significance was set at $p < 0.05$.

Results

Clinical features

The clinical data of 46 patients diagnosed in our hospital was listed in Supplementary Table S1. All patients were

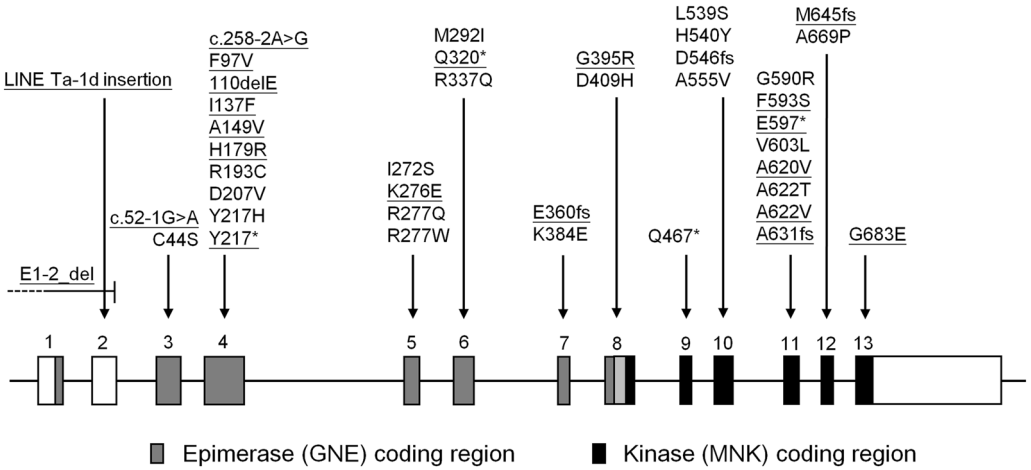

Fig. 1 Mutation spectrum of *GNE* gene in 46 patients in East China. White boxes indicate noncoding region. Gray boxes and black boxes represent epimerase and kinase encoding regions, respectively. Exon 8 encodes the last part of epimerase domain, junctional region (light gray) and initial part of kinase domain. Twenty-one novel mutations are underlined

from East China. The age of onset was 30.3 ± 9.2 years old on average (15–60 years). The ratio of gender was 20 male: 26 female. Six patients (13.0%) were offspring of consanguineous parents, and four (8.7%) had siblings with similar symptoms. Thirty-eight patients (82.6%) presented with unilateral/bilateral foot drop as initial symptoms. Two patients (4.3%) showed weakness and atrophy of hands at onset and five patients (10.9%) began with distal weakness in four limbs. One patient (2.2%) showed atrophy of lower limbs at onset. Most of the patients (45/46) could be classified as classical distal phenotype, with predominating weakness of distal muscles while sparing the quadriceps. Only one case presented with limb-girdle phenotype more severely affecting the quadriceps. The strength of neck flexor was affected in 14 patients (30.4%). Facial, extraocular, bulbar and masticatory muscles were spared. Maximum serum creatinine kinase (CK) levels ranged from 67 to 1836 U/L (normal range: 38–174 U/L). Myopathic changes were found in all 44 patients who had performed electromyography. No palpitation or chest tightness were reported, and the electrocardiogram, echocardiography and pulmonary function showed no significant abnormality.

Muscle pathology

The pathological findings of the patients were similar to those of previous reports. Muscle fiber size variation, atrophic fibers and small angular fibers could be seen in almost all patients. Necrotic fibers were rarely noted and inflammatory infiltration was absent. However, typical rimmed vacuoles were only shown in 21 biopsies (21/40, 52.5%), with 8 in biceps brachii (8/18, 44.4%), 3 in quadriceps femoris (3/5, 60%), 5 in gastrocnemius (5/13, 38.5%), and 4 in tibialis anterior (4/4, 100%), respectively. Unfortunately, electron microscopy was not available in most patients.

Table 1 Type and frequencies of *GNE* mutations in this study

Mutation Type	Alleles (%)
Missense	78 (84.9)
Small deletion	6 (6.5)
Nonsense	4 (4.3)
Splice site	2 (2.2)
Gross deletion	1 (1.1)
Retrotransposon insertion	1 (1.1)

Molecular studies

Forty-one different *GNE* mutations were identified from these 46 patients, including 28 missenses, 4 nonsenses, 5 frameshifts, 2 splice site mutations, 1 gross deletion, and 1 LINE Ta-1d insertion (Fig. 1). Missense mutations were the most common type of mutation in the cohort (Table 1). Twenty-one mutations (21/41, 51.2%) identified in this study were novel. Six variants were classified as "pathogenic" and 15 "likely pathogenic" according to ACMG recommendation (Table 2, Supplementary Table S2). In case 31, c.258-2 A > G was shown to cause aberrant splicing using the cryptic splicing site within exon 4 (Supplementary Figure 1A). The transcript with 48-bp deletion was predicted to result in a frame-shift and premature stop codon. Muscle cDNA analysis was unavailable in case 25 with c.52-1 G > A. A gross deletion spanning exon 1 and exon 2 was identified by coverage analysis in case 44, of which the left breakpoint residing in intron 2 and the right breakpoint extended into the intergene region (Supplementary Figure 1B). Only transcripts carrying the missense mutation on the other allele were detected in muscle cDNA, indicating the large deletion totally abolished the transcription. In case 45, only a heterozygous missense

Table 2 Novel *GNE* variants identified in this study

Mutation type	Ex/In	Nucleotide change NM_00001128227	A.A change NP_001121699	ACMG Classification	Patient no.
Nonsense	Ex4	c.651 T > G	p.Y217*	P	P10
	Ex6	c.958 C > T	p.Q320*	P	P26
	Ex11	c.1789G > T	p.E597*	P	P43
Frameshift	Ex7	c.1078_1081delinsATT	p.E360Ifs*43	LP	P5
	Ex11	c.1891delG	p.A631Qfs*43	LP	P2
	Ex12	c.1932delG	p.M645Cfs*29	LP	P37
In-frame deletion	Ex4	c.330_332delAGA	p.110delE	LP	P29
Missense	Ex4	c.289 T > G	p.F97V	LP	P18
	Ex4	c.409 A > T	p.I137F	LP	P13
	Ex4	c.446 C > T	p.A149V	LP	P30
	Ex4	c.536 A > G	p.H179R	LP	P38
	Ex5	c.826 A > G	p.K276E	LP	P9
	Ex8	c.1183 G > A	p.G395R	LP	P40
	Ex11	c.1778T > C	p.F593S	LP	P10
	Ex11	c.1859C > T	p.A620V	LP	P27
	Ex11	c.1865C > T	p.A622V	LP	P21
	Ex13	c.2048 G > A	p.G683E	LP	P13
Splice site	In2	c.52-1 G > A	–	P	P25
	In3	c.258-2 A > G	p.G85fs*	P	P31
Gross deletion	Ex1–2	Exon1-2_del	–	P	P44
Retrotransposon insertion	Ex3	LINE Ta-1d insertion	–	LP	P45

P pathogenic, *LP* likely pathogenic

mutation was identified during SNP filtering. IGV revealed a potential breakpoint in exon 3. Raw reads spanning the potential breakpoint were extracted from mapping data. The breakpoint position was found to be at chr9:36249242 (hg19/GRCh37) in human genome and a LINE Ta-1d insertion was identified in spanning read sequence by BLAST (Supplementary Figure 1C).

The mutational profile of each individual was listed in Supplementary Table S1. Ten (21.7%) patients harbored a homozygous mutation and 36 (78.3%) carried compound heterozygous mutations. The most frequent mutation in the 46 patients from East China was D207V, with an allelic frequency of 25.0% (23/92). Half of the patients (23/46) carried one allele with this mutation. However, no patient with homozygous D207V mutation was identified.

GNE myopathy in China

Eight previous reports fulfilling the criteria were included in this review [5–12]. A total of 100 genetically confirmed Chinese cases with GNE myopathy were analyzed in the cohort, including 46 cases from our study and 54 cases from previous reports (Supplementary Table S3).

In these 100 cases, 20 carried homozygous *GNE* mutations and 80 harbored compound heterozygous mutations. A total of 76 *GNE* mutations were detected, spanning both epimerase and kinase domains, and missense mutation was the most common type of mutation (88.5%, 177/200 alleles). D207V was also found to be the hotspot mutation, with an allelic frequency of 25.5% (Supplementary Table S4). Nearly half of the patients (49/100) carried at least one allele with D207V, including two homozygotes. The frequencies of common mutations in other parts of Asia, such as V603L, A662V, and V727M, were relatively rare (2.0%, 3.5%, and 0.5%, respectively). The common mutation in Israel and Middle East, M743T, was not observed in Chinese population.

The average onset age of all 100 cases was 28.9 ± 8.6 years, ranging from 11 to 60 years. As D207V is the most common mutation observed in nearly half of the patients, we compared the age of onset between those carrying D207V or not to investigate the genotype–phenotype correlations. The patients carrying at least one D207V manifested at an elder age than the others (31.9 ± 10.0 vs. 26.1 ± 5.7, $p < 0.001$). We further compared the group carrying D207V with those carrying variants other than D207V in epimerase domain. The patients were stratified by the domain in which the other variant in trans resided (GNE domain, MNK domain, or null mutations) (Fig. 2). In each group the patients carrying D207V tended to show a later

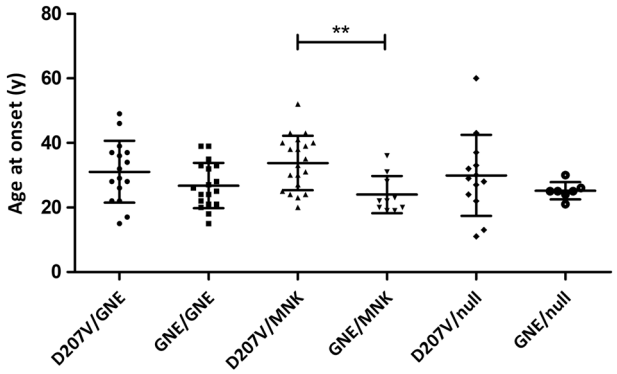

Fig. 2 The age at onset between different group of patients. GNE stands for missense amino acid changes other than D207V in UDP-GlcNAc 2-epimerase domain. MNK indicates a missense variant residing in the ManNAc kinase domain. Null includes nonsense mutations, frameshifts, canonical ± 1 or 2 splice sites, initiation codon or multiexon deletions

onset compared to patients carrying other GNE variant. The difference was of significance between D207V/MNK and GNE/MNK group (33.8 ± 1.9 vs. 24.0 ± 1.8, $p < 0.01$).

Discussion

Currently, over 200 *GNE* mutations have been reported in patients of different ethnic backgrounds, with missense mutations comprising the majority of the mutation spectrum [3, 4]. In our cohort, 21 novel mutations detected in our cohort further expanded the mutation spectrum of *GNE* gene, among which four novel insertions and deletions (indels) mutations were identified. Two deletions, c.1932delG and c.330_332delAGA, are both found in repeat regions and belong to the class of homopolymer run and tandem repeats, respectively. According to the indels formation mechanisms suggested by Kloosterman et al. [19], they most likely arise through polymerase slippage. Another deletion, c.1891delG occurs in nonrepeat region and it likely results from imperfect double-stranded DNA break repairs by nonhomologous end joining. The indel c.1078_1081delinsATT is a complex one and the formation mechanism is still unknown. Large deletion/duplications of *GNE* gene have long been neglected until recently the *Alu*-mediated genomic recombination was reported [18, 20]. A novel gross deletion spanning exon 1 and exon 2 was identified in our study. Although we did not further investigate the exact sequence of the breakpoint junction, the *Alu–Alu* recombination mechanism is speculated since the left breakpoint reside in intron 2, a region of condensed *Alus* with a peak level of 68.7% [18]. The LINE element insertion was firstly reported in GNE myopathy, of which the mechanism was left to be elucidated.

During 2005–2017, 102 patients were diagnosed as distal myopathies in our clinic (data not shown), 46 of which were genetically confirmed to be GNE myopathy. Taken together, over 100 patients with GNE myopathy have been reported in Chinese population in literature [5–12]. To our knowledge, this is the most common type of distal myopathy in China. D207V (the second most common mutation in Japan) was the most common mutation in Chinese patients. The common mutations identified in other ethnicities, such as V727M in South Asia and V603L in Japan [3], were quite rare in Chinese. The common mutation in Jewish, M743T, was not found in our cohort [3, 21, 22]. According to data from Exome Aggregation Consortium (http://exac.broadinstitute.org/), the allele frequency of D207V (rs139425890) is 4/8654 in East Asian, and 5/121398 in total. However, the allele frequency in Chinese population is 3/1342 (BGI, China, unpublished data), much higher than in East Asian. Data from 1000 Genomes Browser (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/) indicates the frequency of this mutation is 2/208 in Japanese. The relatively high frequency of D207V may explain its prevalence in Chinese and Japanese patients with GNE myopathy. However, previous haplotype analysis failed to suggest a founder effect of D207V in Chinese patients [6]. Analysis based on a larger cohort may further elucidate this hypothesis.

Earlier studies suggested that in Japanese patients, D207V predisposes to later onset and milder phenotype as opposed to V603L [23, 24]. And according to the first report from the GNE Myopathy Disease Monitoring Program (GNEM-DMP), A662V might be associated with a more severe phenotype, compared to V727M [25]. But previous reports on Chinese patients were insufficient for genotype–phenotype analysis due to limited patient number [4–12]. As half of the patient harbor at least one allele of D207V and the phenotype of autosomal recessive disease relies more on the milder allele, we compared the age of onset between patients with/without D207V. The onset of age was significantly later in patients carrying at least one allele of D207V, which is in concordance with previous reports. Based on the frequency data of D207V in China, the prevalence of GNE myopathy with homozygous D207V mutation is estimated to be 50:1,000,000. However, till now there were only 2 D207V homozygotes identified in Chinese population. A D207V homozygote was even reported to remain asymptomatic in his seventies in Japan [2, 23, 24]. It is very much likely that D207V homozygotes are in the mild extreme of the disease spectrum and are largely left undiagnosed. However, there was no significant reduction of GNE activity in D207V mutant compared to other missense mutants within epimerase domain in the in vitro recombinant enzyme-cell expression system [26]. The reason why it leads to milder phenotype still need further elucidation.

In conclusion, our study is till now the largest cohort of Chinese patients with GNE myopathy. Twenty-one novel mutations including 1 gross deletion and 1 retrotransposon

insertion were identified in our study, further expanding the mutation spectrum of *GNE* gene. We confirmed that D207V is the hotspot mutation for *GNE* gene in China. As it is associated with a milder phenotype, the population of patients with GNE myopathy may still be overlooked in China.

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Compliance with ethical standards

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

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