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Targeted Next-generation Sequencing Reveals a Homozygous Nonsense Mutation in CAPN3 that Causes Limb-girdle Muscular Dystrophy Type 2A First in Vietnam

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Abstract

Limb-girdle muscular dystrophy (LGMD) is a genetically heterogeneous entity characterized by progressive wasting of the shoulder and pelvic-girdle muscles. Diagnosing particular types of LGMD is still challenging, especially in developing countries, with targeted next-generation sequencing (NGS) emerging as the most advanced diagnostic tool. Here, a 15-year-old Vietnamese girl with proximal muscle weakness was examined for genetic cause via targeted NGS using the AmpliSeq Inherited Disease Ready-to-Use Panel on the Ion Torrent Personal Genome Machine of the detected nucleotide changes, a mutation in exon 3 of CAPN3 was considered to be the responsible mutation. In the readpile-ups, only T was observed at the 424th nucleotide, while only C was observed in the normal sample. The c.424 C>Ttransition in CAPN3 shifted glutamine to a stop codon at the 142nd amino acid residue (p.Q142X). The homozygous c.424C>T genotype was confirmed via XspI restriction enzyme digestion using polymerase chain reaction-amplified product from the index case encompassing exon 3.The patient was concluded to be autosomal recessive LGMD type 2A. XsaIdigestion of 100 control Vietnamese genomes disclosed one carrier of this mutation. Thus, LGMD type 2Awas first diagnosed in Vietnam, a developing country, via targeted NGS, avoiding invasive muscle biopsy.

Keywords: Limb girdle muscular dystrophy; Next-generation sequencing; Calpain3; CAPN3 mutation

Introduction

Limb-girdle muscular dystrophy (LGMD) is clinically characterized by progressive involvement and wasting of the shoulder and pelvic-girdle muscles [1]. LGMD is a genetically heterogeneous disease entity that includes at least 23 distinct LGMDs; types 1A to 1H are autosomal dominant and types 2A to 2Q are autosomal recessive [2]. Diagnosis of LGMD requires information from clinical presentation and the results of various investigations, such as measurement of serum creatinine kinase levels and immunohistochemical examination of muscle biopsies. Diagnosing particular types of LGMD is challenging [3].

LGMD type 2A (LGMD2A) is characterized by symmetrical atrophy of the pelvic, scapular, and trunk muscles with involvement of the shoulder girdle, leading to scapular winging and elevated serum creatinine kinase levels. Most patients present in childhood, progress gradually, and are confined to wheelchairs 10-20 years after onset [4]. The key characteristics for discriminating between LGMD2A and other forms of autosomal recessive LGMD are preservation of respiratory muscle, scapular winging, and early Achilles tendon contracture [3]. LGMD2A is one of the most common forms of LGMD in several populations [5]. Mutations in CAPN3, which encodes the calpain-3 protein, are responsible for LGMD2A, but these mutations are scattered throughout the gene, and most mutations are individual variants [6]. Therefore, mutation analysis is long and costly [7].

The clinical application of next-generation sequencing (NGS) as a diagnostic tool has become increasingly evident [8-10]. The Ion Torrent Personal Genome Machine TM (PGM) from Life Technologies (Carlsbad, CA, USA) is a benchtop, high-throughput, semiconductor-based instrument for NGS that was the first technology to measure changes in pH rather than emitted light to register sequencing reactions. Coupling NGS technologies with genomic sequence enrichment methods has rendered feasible the sequencing of panels of target genes [11,12]. Recently, an Ion AmpliSeq Inherited Disease Ready-to-Use Panel (IDP) was released for the analysis of 300 genes that underlie more than 700 inherited diseases. However, the usefulness of the IDP with the PGM has rarely been evaluated [13,14].

In this study, we applied the IDP on a PGM to diagnose a Vietnamese female who presented with proximal muscle weakness. Using this strategy, we identified a nonsense mutation in CAPN3 responsible for LGDM2A. The carrier incidence of the mutation in Vietnam was 1/100, based on results from a restriction enzyme digestion study.

Case and Methods

Case

A 15 year-old Vietnamese girl (NTNQ) was followed in the National Hospital of Pediatrics, Hanoi, Vietnam. She was born as the first child to non-consanguineous parents. No particular family history was disclosed. Her developmental milestones at infancy were normal. The patient became symptomatic at 10 years of age because of walking difficulty. The disease course was progressive. At 13 years of age she could walk independently but exhibited Gowers' maneuver, symmetric muscular atrophy of the pelvic and trunk muscles, and difficulty in going up stairs. She was able to raise her arms above her head slowly, but displayed scapular winging. Laboratory tests revealed an elevated serum creatinine kinase level of 2.898IU/L (normal range <170 IU/L). At 15years of age, electrocardiography and echocardiography of the patient displayed no abnormal findings. A pulmonary function test demonstrated normal ventilation. She was clinically characterized by a progressive proximal weakness with elevated serum creatinine kinase levels. Her genomic DNA was sampled after obtaining informed consent from the parents. In addition, genomic DNA samples were obtained from one normal and 100healthy Vietnamese controls after obtaining informed consent.

Targeted semi-conductor NGS

A sample of genomic DNA from the patient was used for polymerase chain reaction (PCR)-based enrichment of target genes for the IDP (Life Technologies). The panel consisted of three separate PCR primer pools covering the coding exons of 300 genes associated with over 700 unique inherited diseases (http://lifetechnologies.com/ampliseqready). Amplicons were ligated to sequencing adaptors containing a unique barcode, purified, and quantified as recommended by the manufacturer. After clonal amplification of pooled barcoded libraries, samples were loaded onto an Ion 318™ chip v2 (1 Gb of sequence data output) (Life Technologies)and sequenced on the PGM with the 200-bp single-end run configuration, as recommended by the manufacturer (the full Ion Torrent AmpliSeq work flow can be found at http://ioncommunity.lifetechnologies.com). Data analysis, including alignment to the hg19 human reference genome and variant calling targeting the IDP target regions (4477686_IDP_designed, Life Technologies), was done using the Variant Caller Plugin of Torrent Suite Software v.4.0 (Life Technologies). Filtered variants were annotated using both Ion Reporter 4.0 (Life Technologies) and Ingenuity Variant Analysis Software (Qiagen, Redwood City, CA), and alignments were visualized with Integrative Genomics Viewer v.2.1 (http://www.broadinstitute.org/igv/Genomes).

Restriction enzyme digestion

The exon 3-encompassing region covering introns 2 to 3 of CAPN3 was PCR amplified from genomic DNA using the following primers: forward primerCAPN3-Ex3-Up-F: GGGGCTTTTTCTTCCCAGGAG and reverse primerCAPN3-Ex3-down-R: CCTTAAGGGGGTCCATGCCG. Amplified products were digested with XspI restriction enzyme as recommended by the manufacturer (Takara Bio Inc., Kyoto, Japan). Digested products were separated via high-resolution microcapillary electrophoresis with an Agilent 2100 Bioanalyzer on a DNA 1000 Labchip R (Agilent Technologies Inc., Santa Clara, CA, USA). Electropherogram analysis, PCR product size determination, and quantification were automatically performed using 2100 Expert software (version B.02.07)(Agilent Technologies, Inc.).

Results

The index case was clinically supposed to harbor one subtype of LGMD. To provide a proper diagnosis, immunohistochemistry of biopsied muscle sample was recommended as the next step [15]. To bypass this invasive step, her genomic DNA was examined via NGS using the IDP on a PGM. Among the detected nucleotide changes, only pathological mutations leading to LGMD types were evaluated.

We identified a nucleotide change of c.424 C>T located within exon 3 of CAPN3 and considered it to be the mutation responsible for the condition of the index case. In the read pile-ups, only T was observed at the 424th nucleotide (c.424T), while only C was observed in the normal sample (Figure 1). The nucleotide transition (c.424C>T) shifted the CAG codon for glutamine to a TAG stop codon at the 142nd amino acid residue (p.Q142X). This mutation was previously reported to cause LGMD2A in one patient [4]. We therefore concluded that the index case harbored LGMD2A caused by a homozygous c.424 C>T mutation.

To confirm the nucleotide change, we carried out restriction enzyme digestion of the patient's DNA. The c.424C>T mutation created an XspI restriction enzyme recognition site by changing the sequence from CCAG to CTAG. The exon 3-encompassing region was PCR amplified and digested with XspI. The amplified product from normal DNA remained undigested (323 bp; Figure2). In contrast, the amplified product from the patient was digested completely with Xsp1, yielding two products (213 and 110bp; Figure 2). This complete digestion indicated that the mutation was homozygous. Therefore, the patient was concluded to have LGMD2A caused by a homozygous nonsense mutation in CAPN3. Her parents were supposed to carry the mutation, but we could not obtain genomic DNA from them and no genetic evidence of the parents' carrier status was obtained.

In order to measure the prevalence of the c.424C>T mutation in the Vietnamese population, 100 control healthy Vietnamese genomic DNA samples were subjected to PCR amplification and XspI digestion. A single genomic DNA sample yielded three bands after XspI digestion, indicating that the donor was a carrier for the mutation (Figure 2). We concluded that the incidence of carriers of this mutation was1/100 people, and that the prevalence of LGMD2A in Vietnam was 1/40,000 people.

Discussion

This case is the first genetically confirmed LGMD2A patient in Vietnam. The index case was homozygous for the c.424 C>T mutation (p.Q142X) in exon 3 of CAPN3. IDP on the PGM effectively identified LGMD2A. NGS was previously reported to correct a misdiagnosis of facioscapulohumeral muscular diagnosis to LGMD2A [16]. The present investigation clearly showed the merit of analysis with targeted NGS, since the patient did not have to undergo invasive muscle biopsy. In addition to proper diagnosis of our patient, our results identified onecarrierin100 control Vietnamese samples (Figure 2), indicating that c.424C>T is a frequent mutation in the Vietnamese population.

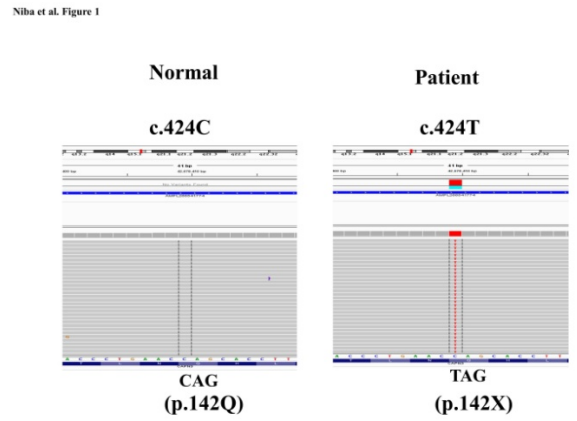

Figure 1: Read pile-ups derived from PGM sequencing. A segment of the read pile-ups for exon 3 of CAPN3. Sequencing results completely matched the reference sequence (Normal). However, a single nucleotide mismatch was detected in the patient at nucleotide 42, 678, 410 that replaced C with T (Patient). This nucleotide change corresponded to c.424 C>T of CAPN3 and changed CAG (glutamine) to TAG (stop codon) at the 142nd amino acid residue (p.Q142X). Thus, the index case is homozygous for this nonsense mutation

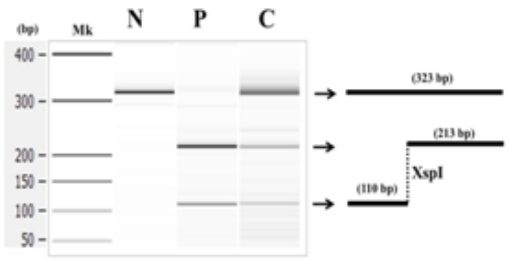

Figure 2: Restriction enzyme digestion. Microcapillary electrophoresis of digested products. The PCR-amplified product spanning the exon 3 region was subjected to digestion with Xsp1. Left, products were separated via electrophoresis. Mk, DNA size markers. Right, product structures are displayed schematically. XspI, Xsp1 recognition site. In the normal genotype (N), the amplified product remained intact. In the patient (P), two small bands were visualized: a larger 213-bp product and a smaller 110-bp product. In our survey of 100 genomic DNA samples, one sample displayed three bands, indicating carrier (C) status

Four hundred and sixty-five mutations in CAPN3 have been reported to cause LGMD2A worldwide [7]. Most mutations have been reported as individual variants, with the exception of a particular genetic isolate with a predominant founder effect [4]. In a recent study in the United Kingdom, 80 pathogenic mutations were identified in 57 families, and the authors concluded that the lack of recurring mutations implies that no role in genetic diagnosis can be given to allele-specific PCR [5]. In this study, we identified three alleles of this mutation (one patient with a homozygous nonsense mutation and one heterozygous carrier). In one extensive study on the genetic diagnosis of LGMD2A, a Vietnamese patient in Spain was described as heterozygous for this mutation [4]. To the best of our knowledge, therefore, four alleles from Vietnamese patients have been shown to encode this mutation. In China, which neighbors Vietnam, a variety of mutations in CAPN3have been reported, but not the c.424 C>T transition [17]. Further genetic studies are necessary to confirm the founder effect in the Vietnamese population.

Here, the prevalence of LGMD2A in Vietnam was estimated as at least 1/40,000 people. Considering the population of 88 million, the number of LGMD2A patients is predicted to be 2,200 in Vietnam. To our knowledge, no published report has described any case with LGMD2A in Vietnam, suggesting a lack of facilities for the extensive study of LGMD2A. The most important implication of our study is that undiagnosed LGMD2A can be diagnosed easily in Vietnam via restriction enzyme digestion of PCR products. Thus, every suspected LGMD case should be screened for c.424 C>T with digestion of the exon 3-encompassing region. This easy testing strategy will clarify the real prevalence of LGMD2A and facilitate the provision of proper genetic counseling and treatment for patients.

Currently, the induction of ribosomal read-through of premature termination codons is attracting much attention as a plausible treatment strategy for genetic disorders. In Duchenne muscular dystrophy, at aluren (PTC124), which enables ribosomal read-through of premature termination codons, has been shown to improve the expression of dystrophin protein [18]. It has been reported that homozygous LGMD2A patients harboring null mutations usually exhibit severe clinical features, rapid progression, and earlier onset [19]. The clinical phenotype of our patient was as severe as the case of two null mutations; disease onset occurred at the age of 10 years and she currently (at the age of 15 years) shows Gowers' maneuver. In the future, it is highly possible that many Vietnamese LGMD2A patients with a nonsense mutation will benefit from such treatment.

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