

# Genetic Variation Analysis and Treatment Strategy of 20 Patients with Dystrophin Gene Mutations

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## Research article

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# Abstract

## Introduction.

Dystrophin (*DMD*) gene mutations can affect muscular dystrophin isoform expression and result in progressive muscular dystrophy including Duchenne and Becker muscular dystrophies (DMD and BMD). To establish the correlation between phenotype and genotype and exemplify the current and future treatment for muscular dystrophy disorders, we investigated 20 patients suffering from a dystrophinopathy and summarized clinical manifestation and gene mutations of them.

## Methods.

The clinical manifestations, physical examination, laboratory work, and gene mutation results were collected in 20 patients with DMD or BMD diagnosed by clinical phenotype and genetic sequencing from July 2015 to December 2019. Multiplex ligation probe amplification (MLPA) and next-generation sequencing (NGS) were used to detect mutations in the *DMD* gene, and detected mutations were confirmed by Sanger sequencing.

## Results.

The clinical manifestation of patients was characterized by progressive symmetrical muscle degeneration, limb weakness, and pseudohypertrophy along with the elevated concentration of creatine kinase, alanine aminotransferase, and aspartate aminotransferase. We found 11 dystrophin gene deletions (55%) and 4 duplication mutations (20%) among the affected patients. However, we also found point mutations including 1 nonsense (20%), 3 frameshifts (60%), and 1 splice sites (20%) mutations in the rest 5 patients. Among the 15 cases of exon deletion or duplication mutations, 7 were inherited from the mother, 3 were de-novo, while the other 5 were not tested. Besides, all 5 point-mutation cases were inherited from the mother, among which 4 point mutations were identified for the first time and linked to the disease phenotype.

## Conclusions.

We provided clinical portraits, genetic results, and the molecular effects of mutations in patients. Four novel point mutations were identified for the first time and associated with the development of DMD and BMD. Further, we expanded knowledge of the *DMD* variant spectrum and exemplified the emerging therapies in muscular dystrophy.

## 1. Background

Duchenne muscular dystrophy (DMD, OMIM #310200) first described by the French neurologist in the 1860s<sup>1</sup> and Becker muscular dystrophy (BMD, OMIM #300376) discovered by Becker and Monaco<sup>2</sup>, are both X-linked recessive hereditary neuromuscular disorders due to the deficit of dystrophin protein that caused by the *Dystrophin* (*DMD*) gene mutations<sup>3</sup>. *DMD* gene (MIM #300377) is the largest gene identified in human beings, spanning 2.5 million bp, with 79 exons and 8 promoters, located on chromosome Xp21.2- Xp21.1. *DMD* gene encodes a 427-kDa dystrophin protein which is vital to the formation of a dystrophin-associated glycoprotein complex (DGC)<sup>4-6</sup>. Being the most common form of X-linked recessive hereditary disorders, the estimated prevalence of DMD is approximately 1/3500 in the general male population, characterized by progressive muscle degeneration and weakness. The disease has a poor prognosis and patients have a life expectancy of less than 20 years due to progressive respiratory or heart failure. BMD has mild symptoms, slow clinical progress, and late-onset compared with DMD. Previous work has shown that different dystrophin mutations were confirmed to cause DMD and BMD<sup>7-10</sup>. Because of the broad phenotype spectrum, it is difficult to use the traditional diagnostic methods to achieve an early accurate diagnosis of the disease.

Thus, it is of an urgent need to establish a library of genetic variations of DMD/BMD to facilitate early interventions. In this study, we investigated 20 male patients from separate families suffering from a dystrophinopathy, diagnosed by genetic testing and clinical manifestations. We provide clinical portraits and genetic results, particularly focusing on genotype-

phenotype correlations and the molecular effects of mutations in patients. Further, we expand knowledge of the *DMD* variant spectrum and exemplify the current and emerging therapies in muscular dystrophy.

## 2. Methods

### 2.1 Subjects

We investigated 20 children with the clinical diagnosis of DMD/BMD who were admitted to the Pediatric Department of the Affiliated Hospital of Qingdao University, Qingdao Women & Children Hospital, and Qilu Hospital of Shandong University (Qingdao), from July 2015 to December 2019. There is no kinship between patients and their parents denied consanguineous marriage. According to related literature and reports, the criteria for diagnosis were as follows: (1) Independent walking age is about 18 months and the development of the sport is lagging behind that of normal children at the same age; (2) Clinical manifestations are progressively aggravated: muscle weakness, waddling gait, the incapacity of running and using the stairs, Gower sign (+), gastrocnemius pseudohypertrophy and the most typical symptoms occur in 3–5 years old; The clinical phenotype of BMD is similar to DMD, with later onset age, milder symptoms, and longer duration; (3) Creatine kinase (CK) level in serum is significantly increased, tens to hundreds of times higher than normal reference value; (4) Electromyogram (EMG) shows myogenic lesion without peripheral nerve dysfunction and positive anti-dystrophin (dystrophin) immunostaining; (5) Other diseases such as peripheral neuropathy, myasthenia gravis, limb-girdle muscular dystrophy, polymyositis, dermatomyositis, periodic paralysis were excluded. Contents registered by each patient included basic information, clinical manifestations, physical examination, and laboratory assays results. The consents to participate were obtained from the parents of the children.

### 2.2 Sample collection

The genomic DNA specimens were extracted from a total of 2 ml of peripheral blood collected from patients and their parents using the FlexiGene DNA Kit (512206, Qiagen, Valencia, CA) according to the manufacturer's instructions.

### 2.3 Molecular genetic analysis

The *DMD* gene was first screened by Multiplex ligation probe amplification (MLPA) using the SALSA MLPA Probemix reagent kit (P034&P035, MRC-Holland, Amsterdam, the Netherlands) to detect large fragment deletions and duplications on patients. A total quantity of 50–250 ng of DNA in a 5  $\mu$ l volume was denatured and hybridized with the MLPA kit overnight. After ligation and PCR amplification, the data of MLPA that tested on DNA samples were examined using the ABI 3500 genetic Analyzer (Thermo Scientific Fisher, Waltham, MA) and analyzed by Coffalyser.Net software (<https://www.mrcholland.com/technology/coffalyser-net>).

We also selected a total of 258 candidate genes associated with inherited myopathy to detect point mutations in genes that could not be detected by the MLPA. We performed the gene panel to capture the coding regions from the unique-designed selected genes using target next-generation sequencing technology (NGS) which is a unique diagnostic method that drives the identification of copy number variations and single nucleotide variations at the same time. Variants with an allele frequency  $\geq 0.005$  in the East Asian population according to the 1000 Genomes Project (<http://browser.1000genomes.org>) were removed and several types of variants in the coding region, including frameshift, non-frameshift indel, stop-gain, and splicing mutations were retained; for missense mutation, the pathogenicity of amino acid alteration was evaluated and filtered by SIFT, Polyphen-2, and Mutation Taster programs. The known pathogenic mutations and reported polymorphisms were checked by searching the HGMD Professional database, the NCBI 1000 Genomes Browser, PubMed, and database SNP. The detected most likely disease-causing mutations were confirmed by Sanger sequencing.

## 3. Results

### 3.1 Clinical features and symptoms of study subjects

In 20 diagnosed cases of *DMD* gene mutations, we found that the patient under 1-year-old presented only increased serum creatine kinase level during physical examination and the mobility of the limb was grossly normal. Most of these children at the age of 4-7-year-old with *DMD* mutations showed poorer motor performance during squats, gait and stair walking, and mentally backward compared with their age-matched peers. Physical examination showed muscle atrophy, especially on the proximal muscles of both lower limbs, gastrocnemius muscle pseudohypertrophy, decreased muscle ton, and diminution or loss of tendon reflexes. After the assessment of intelligence, six children (30%) had mental retardation as shown in Table 1.

Table 1  
The clinical features and symptoms of male patients in different age groups

Age at onset (yrs)	Number	Low limb fatigue	Muscle weakness	Decreased muscle tone	Cramps /myalgia	GMP	Gower maneuver	Diminution or loss of tendon reflexes	CNS disorders
≤1	1	0	0	0	0	1	0	1	0
1-3	3	3	3	2	1	3	3	1	1
4-7	11	11	11	9	9	11	11	11	3
≥7	5	5	4	4	3	5	3	4	2

GMP: gastrocnemius muscle pseudohypertrophy; CNS: central nervous system

### 3.2 Laboratory examination

As shown in Table 2, serum creatine kinase (CK) among 20 patients were significantly increased compared to the normal reference value ( $6991.3 \pm 1805.1$  U/L vs.  $26 \sim 196$  U/L), accompanied by the elevated concentration of liver enzymes including AST ( $99.0 \pm 26.2$  U/L) and ALT ( $125.5 \pm 33.5$  U/L). The cardiac function performed on 8 patients was measured by echocardiography and 2 cases had left ventricular enlargement. The EMG was performed in 10 patients and showed myogenic damage.

Table 2  
Ancillary examination results

Test	Value	unites	reference interval
CK	$6991.3 \pm 1805.1$	U/L	$26 \sim 196$
CK-MB	$141.0 \pm 36.4$	U/L	$0 \sim 35$
AST	$99.0 \pm 26.2$	U/L	$15 \sim 40$
ALT	$125.5 \pm 33.5$	U/L	$9 \sim 50$
LDH	$530.7 \pm 177.0$	U/L	$80 \sim 220$
Abnormal Echocardiography	2/8	25%	-
EMG	10/10	100%	-

CK, creatine kinase; CK-MB, creatine kinase-myocardial isozyme; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; EMG, electromyogram.

### 3.3 Genetic analysis

The overall pattern of dystrophin gene exon deletion and duplication identified in our study detected by MLPA or NGS is given in Table 3 and a schematic diagram of the location of mutations identified in the *DMD* gene is shown in Fig. 1. There were 11 dystrophin gene deletions found among the affected male patients and 4 of 20 cases had the duplication

mutations. The most common of dystrophin gene deletions occurred mainly between exons 45 and 52 (54.5%), followed by deletions between exons 8 and 17. The patients with negative MLPA results were further investigated by NGS. We found 1 nonsense (20%), 3 frameshifts (60%), and 1 splice sites (20%) point mutations in the dystrophin gene in 5 patients. The maternal genes were sequenced using MLPA or NGS according to the types of the proband. In the 15 families with exon deletion or duplication mutations, 7 mothers were found to be carriers, while 3 mothers didn't carry the mutation and 5 were not tested. Whereas, in families with point mutations, five patients' mothers carried the mutation. Of all point mutations, four novel mutations (c.6913-1G > T, c.9014dupT, c.2702delG, c.8672delT) were identified for the first time that led to the premature termination codons which may contribute to truncated dystrophin protein were identified in unrelated families (Fig. 2).

Table 3  
The mutation spectrum of 20 patients

Patient number	Exon ID	Base change	Effect	Mutation type	Reading frame	Phenotype	Carrier status of the mother	Status
1	Exon 5	c.354G > A	p.Trp118Ter	Nonsense		DMD	Yes	Reported
2	Exon 48	c.6913-1G > T		Splice site		BMD	Yes	Novel
3	Exon 60	c.9014dupT	p.Leu3005PhefsTer9	Frameshift		DMD	Yes	Novel
4	Exon 21	c.2702delG	p.Gly901AspfsTer48	Frameshift		DMD	Yes	Novel
5	Exon 59	c.8672delT	p.Leu2891ArgfsTer28	Frameshift		DMD	Yes	Novel
6	Exon 3-5			Del	In-frame	BMD	Yes	Reported
7	Exon 48-52			Del	Out-of-frame	DMD	No	Reported
8	Exon 45-47			Del	In-frame	BMD	Yes	Reported
9	Exon 45-47			Del	In-frame	BMD	Yes	Reported
10	Exon 63-64			Dup	Out-of-frame	DMD	Yes	Reported
11	Exon 49-50			Del	Out-of-frame	DMD	Yes	Reported
12	Exon 51-54			Del	Out-of-frame	DMD	Yes	Reported
13	Exon 49-52			Del	Out-of-frame	DMD	N/A	Reported
14	Exon 8-17			Del	Out-of-frame	DMD	N/A	Reported
15	Exon 2-30			Dup	Out-of-frame	DMD	N/A	Reported
16	Exon 3-5			Dup	In-frame	BMD	No	Reported
17	Exon 48-54			Del	Out-of-frame	DMD	Yes	Reported

Patient number	Exon ID	Base change	Effect	Mutation type	Reading frame	Phenotype	Carrier status of the mother	Status
18	Exon 21			Del	Out-of-frame	DMD	No	Reported
19	Exon 2			Dup	Out-of-frame	DMD	N/A	Reported
20	Exon 8-43			Del	Out-of-frame	DMD	N/A	Reported

## 4. Discussion

Here we described patients with a history of DMD or BMD diagnosed by genetic examination and a total of 48 individuals derived from 20 families were included in the study. We analyzed the clinical manifestations, serum biochemical tests of 20 patients, and also detected gene mutations of 20 probands together with their parents and siblings. We found that the high prevalence of the first consultation among patients is in the age range of 4-7-year-old individuals with progressive irreversible muscle weakness, gastrocnemius muscle pseudohypertrophy, and elevated serum CK complicated with a rise of AST and ALT serum concentration. However, using liver and myocardium sensitive biochemical indicators, such as transaminase enzyme and myocardial enzyme, might lead to misdiagnosed as myocarditis, rhabdomyolysis, and hepatocellular injury<sup>11</sup>. Because of the diverse clinical manifestations, insufficient specificity, and difficulties in diagnosis of progressive muscular dystrophy (PMD), thus, we need to diagnose the disease with the help of EMG, muscle biopsy, and genetic testing to reduce the rate of clinical misdiagnosis. In our study, the clinical manifestations of patients conformed to the natural course of BMD/DMD, and several patients had the family history with the typical gastrocnemius muscle pseudohypertrophy, reduction or loss of tendon reflexes, positive Gower sign with the myogenic damage and abnormal echocardiography. All children's genetic testing results finally confirmed the clinical diagnosis.

### 4.1 Pathogenesis studies

Full-length dystrophin is a large rod-shaped protein in association with the formation of DGC which connects the subsarcolemmal F-actin cytoskeleton to the basal lamina of the extracellular matrix, stabilizing membrane during repeated cycles of contraction and relaxation<sup>12,13</sup>. Besides, The DGC also has an essential role in cell signaling apart from mechanical function. The dysfunction of dystrophin protein, which plays a crucial role in structural and functional maintenance, leads to the permeabilization of the cell membrane of skeletal muscle, the loss of sarcolemmal integrity, the leakage of intracellular creatine kinase, the degeneration and necrosis of muscle tissue, and the proliferation of adipose and fibrous tissue<sup>14-16</sup> which is consistent with the clinical manifestations (progressive muscle damage and weakness, gastrocnemius muscle pseudohypertrophy, decreased muscle ton) and laboratory examination (elevated serum CK) results in our patients. Besides, the protein also expresses in the central nervous system and localizes to postsynaptic neurons in the cerebral cortex and hippocampus which are critical for memory and cognitive functioning<sup>17,18</sup>. Dystrophin colocalizes with GABA<sub>A</sub> receptors subunit clusters and it plays an essential role in the stabilization of GABA<sub>A</sub> receptors in the subset of central inhibitory synapses<sup>19</sup>. According to recent reports, no severe abnormality has been reported in the brain of DMD patients. However, detailed microscope results indicate that abnormal dendritic development and arborization may contribute to intellectual impairment<sup>20</sup>. We can find mental retardation, movement disorders, language disorders, and panic attacks in some patients in our study which may be related to the abnormal organization of the structures and functions in the brain.

### 4.2 Molecular studies

As the largest gene in humans, *DMD* gene spans 2.5 million bases and consists of 79 exons, and a variety of mutations occur within the *DMD* gene. The large rearrangement including deletions and duplications were detected in 15 patients (11 deletions and 4 duplications), and point mutations were found in 5 patients including nonsense, frameshifts, and splice mutations. The result was consistent with the early reports that large deletions are the most common mutations in the *DMD* gene accounting for 55–65% and duplications represent up to 5–15%, while, point mutations account for 30%<sup>21,22</sup>. There are two hot spots in *DMD* gene deletion mutations, exons 1–22 located in the proximal region, and exons 43–55 in the distal region<sup>23–25</sup>. Deletions found in 11 patients in our study having its first and last exon within the proximal and distal hot spot made up 27% and 64% of the total deletions. Further, point mutations were identified in our patients using NGS and verified by Sanger Sequencing. This study reported five mutations (c.354G > A, c.6913-1G > T, c.2702delG, c.8672delT, c.9014dupT) detected in patients with a premature stop code in mRNA that eventually led to the production of truncated proteins.

A nonsense mutation was identified in the patient, c.354G > A, which was not found in the *DMD* gene of patient's father. The variant was predicted to lead to amino acid deletion p.Trp118Ter. The pathogenicity of this mutation has been reported in the literature and is related to DMD<sup>26</sup>. The splice sites point mutation, c.6913-1G > T, was identified in the *DMD* gene of our patient. The pathogenicity of this mutation has not been reported in the literature, but the pathogenicity of the mutation c.6913-1G > A in the same position as the mutation has been reported in the literature and is related to myopathy<sup>27</sup>. A nucleotide variation of c.2702delG was found in the *DMD* gene of the subject, resulting in translation termination of amino acids (p.Gly901AspfsTer48). This variation is classified as "likely pathogenic" according to rules for combining criteria to classify sequence variants published by the American College of Medical Genetics and Genomics (ACMG)<sup>28</sup>. The subject's father showed no abnormalities at this site, while his mother and her sister were heterozygous. The frameshift mutation which led to the No. 8672 nucleotide T deletion in coding region and caused to the production of truncated proteins (p.Leu2891ArgfsTer28) was identified in the patient and his mother was heterozygous. We also found another frameshift mutation of the *DMD* gene (c.9014dupT, p.Leu3005PhefsTer9) among the point mutations. The evidence of pathogenicity of this mutation was weighted as very strong (PVS1).

To our best knowledge, it is the first time that the four-point mutations (c.6913-1G > T, c.2702delG, c.8672delT, c.9014dupT) were reported and linked to DMD/BMD, which expand knowledge of the *DMD* variant spectrum. The carriers were proved to be DMD/BMD through pathological examination and clinical follow-up.

### 4.3 Current and future therapies

To date, no curative therapies are available for BMD or DMD patients. Corticosteroids, which was first suggested by Drachman<sup>29</sup>, is the gold standard therapy to improve children's muscle strength and slow down disease progression. Some new therapies are emerging as a future method to systematically treat dystrophin deficiency, such as using stem cells as the alternative approach for muscle regeneration, gene therapy for restoring the dystrophin protein, stop-codon read-through for expressing a full-length functional dystrophin protein, utrophin up-regulation to replace the mutated dystrophin and splicing therapy for skipping exons with mutations. Here, another emerging treatment for the disease focus on the receptor P2RX7. P2RX7 is an ATP-gated ion channel permeable to small cations, including Ca<sup>2+</sup>, and its expression and function are up-regulated by ATP which would release when tissue damage occurs. In DMD, due to the dysfunction of dystrophin protein, the cell membrane of skeletal muscle is damaged and the ATP in very large amounts is released into the extracellular space, creating an environment consistent with increased activation of P2RX7<sup>30</sup>. Over-activation of P2RX7 could contribute to damage both directly by causing the death of dystrophic muscles and indirectly by stimulating harmful inflammatory responses. Recent research has found that selective ablation or blockade of P2RX7 ameliorates the MDX dystrophic process both short and long term and does not cause detectable side effects in this DMD mouse model<sup>31</sup>. Therefore, the single treatment that takes P2RX7 as the therapeutic target for improving muscle function both short and long term, and also rescues cognitive impairment and bone loss could potentially be purposed for this lethal disease.

## 5. Conclusion

*DMD* mutations can lead to mobility and mortality in young adults as a result of the reduced expression and/or function of dystrophin. With the development of genetic sequencing, we are able to diagnose *DMD/BMD* more accurately and identify individuals with susceptible genetic variations, so that earlier intervention and treatment can be provided. However, the known genetic variations associated with *DMD/BMD* are still limited, case reports such as the present study can enrich the *DMD* variant spectrum and improve the clinical diagnosis, early reasonable treatment, genetic counseling, and avoidance of misdiagnosis and mistreatment of this disease.

## List Of Abbreviations

Abbreviations	Definition
DMD	Duchenne muscular dystrophy
BMD	Becker muscular dystrophy
DGC	Dystrophin-associated glycoprotein complex
GMP	Gastrocnemius muscle pseudohypertrophy
CNS	Central nervous system
CK	Creatine kinase
CK-MB	Creatine kinase-myocardial isozyme
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
LDH	Lactate dehydrogenase
MLPA	Multiplex ligation probe amplification
NGS	Next-generation sequencing technology
EMG	Electromyogram

## Declarations

### Ethics approval and consent to participate

All steps included in the study involving human participants obeyed the ethical standards of the Ethics Review Committee of the Affiliated Hospital of Qingdao University. Informed consent form was obtained from the patients' parents for publication in the journal.

### Consent for publication

The Ethics Review Committee, all the authors, and all the participants' caregivers agree to the publication.

### Availability of data and materials

All data generated or analysed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

YL and ZC conceived and designed research; YL, JC, MH, YZ and WW collected original data; YL analyzed and interpreted the patient data; YL, JC, MH, YZ drafted the manuscript; YL prepared figures; YL, JC, MH, YZ, YG, PL, FL, CY, JS and ZC edited and revised manuscript; YL, JC, MH, YZ, YG, PL, FL, CY, JS, WW and ZC approved the final manuscript.

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## Figures

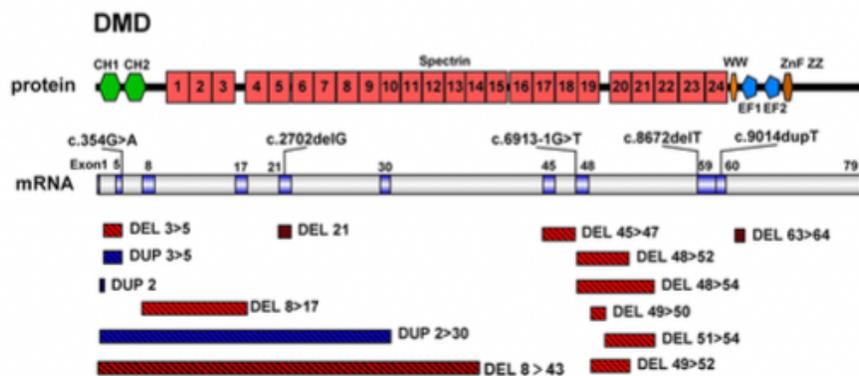


Figure 1

Schematic diagram of the location of mutations identified in the DMD gene.

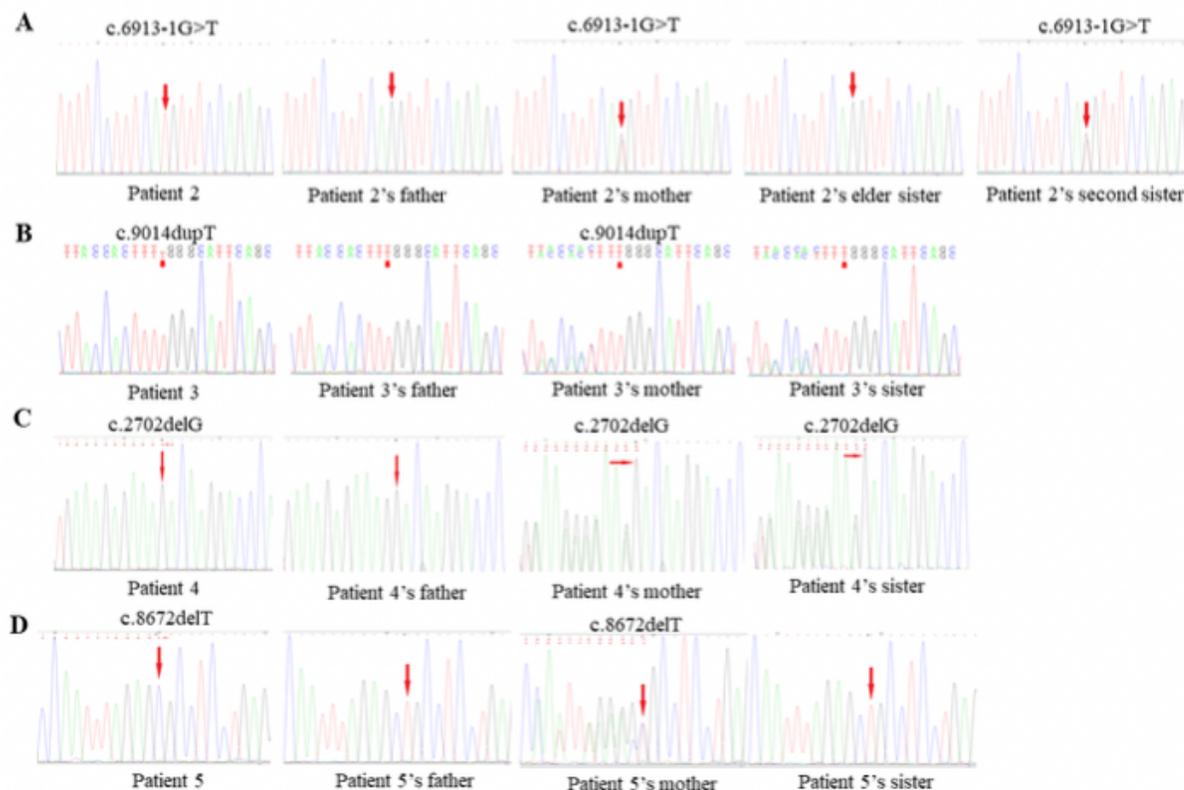


Figure 2

Novel mutations were detected using NGS and verified by Sanger Sequencing. (A) The novel mutation of c.6913-1G>T; (B) The novel mutation of c.9014dupT; (C) The novel mutation of c.2702delG; (D) The novel mutation of c.8672delT