

## ARTICLE

# Distinguishing the 4qA and 4qB variants is essential for the diagnosis of facioscapulohumeral muscular dystrophy in the Chinese population

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Facioscapulohumeral muscular dystrophy (FSHD) is the third most common inherited muscular dystrophy with markedly clinical variability and complex genetic cause. Several reports pertaining to the Caucasian population have confirmed that there are 4qA and 4qB variants of the 4qter subtelomere, and FSHD is uniquely associated with the 4qA variant. However, few data relevant to the Chinese population have been published. In present paper, detailed clinical and genetic re-evaluations were performed in members of four special families who had been initially diagnosed as atypical or asymptomatic FSHD based only on the D4Z4 repeat length analysis. The FSHD-sized D4Z4 repeats in the probands from families 1, 2 and 3 were identified as 4qB variants. These patients were further confirmed as limb-girdle muscular dystrophy (LGMD2) or myotonic dystrophy (DM1) by molecular analyses. Specifically, we identified a 4qB variant on chromosome 10 in the healthy members of the fourth FSHD family with complex D4Z4 rearrangements of two exchanged repeat arrays. For the first time, we demonstrated in the Chinese population that D4Z4 contractions on the 4qB variant do not cause FSHD and 4qB variant on chromosome 10 might also represent intermediate structures in the transition from 4q to 10q. Furthermore, our results emphasize that D4Z4 repeat length analysis alone is not sufficient for the diagnosis of FSHD, especially when used as an exclusion criterion. This analysis should be accompanied by 4qA/4qB variant determination and integrated chromosome assignments, especially in patients with obscure and unclassified myopathies similar to atypical forms of FSHD.

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## INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD, MIM 158900) is an autosomal dominant disorder that presents the third most common human muscular dystrophy, affecting skeletal muscles in a pattern that starts in the facial, shoulder and upper arm muscles and gradually progresses to the abdominal and foot-extensor muscles.<sup>1</sup> Although FSHD can be diagnosed using well-established diagnostic criteria,<sup>2</sup> there is extreme intrafamilial and interfamilial variability in age at onset, severity and progression of the disease. Moreover, other myopathies can present a clinical picture that resembles FSHD, resulting in misdiagnosis.

Because the genetic defect of FSHD has been mapped to the 4q35 subtelomere, it has been recommended that the clinical diagnosis be confirmed via the screening of contractions of D4Z4 repeat arrays located on a 4qter subtelomere.<sup>3–5</sup> Furthermore, several reports have confirmed that there are 4qA and 4qB variants of the 4q subtelomere, and FSHD is uniquely associated with the 4qA variant.<sup>6–10</sup> These findings add to the diagnostic challenge and pathogenetic complexity.<sup>11</sup> Therefore, the correct diagnosis of FSHD depends on the ability to distinguish the 4qA and 4qB variants. However, all published data regarding the identification of the 4qA and 4qB variants are limited to the Caucasian population, and few are related to the Chinese population.

In a previous study, we detected the 4qA and 4qB variants in a group of patients that included those who had been formerly diagnosed with FSHD based exclusively on the D4Z4 repeats length analysis.<sup>12,13</sup> Four families were identified as carriers of the 4qB variant, and one of them had a complex genetic constitution. To better characterize these patients and to confirm the correct diagnosis, we performed a detailed clinical and genetic re-evaluation. The results revealed that the individuals who carried the 4qB variant were previously misdiagnosed with FSHD. These results also confirmed that the 4qB variant does not cause FSHD in the Chinese population.

## MATERIALS AND METHODS

### Subjects and PFGE-based DNA analysis

PFGE-based analysis of D4Z4 repeats and the identification of 4qA and 4qB variants using probes p13E-11, 4qA and 4qB were performed as previously reported, in members of 55 clinically diagnosed Chinese FSHD families.<sup>6,12,13</sup> Four families were found to have a FSHD-sized 4qB-type D4Z4 repeat. These families were subjected to further clinical examinations and molecular analyses. A ten-grade clinical severity (CS) scale score<sup>14</sup> was used to evaluate the phenotypes of the affected individuals. To identify the chromosomal origin of these fragments, *NotI*-digested DNA was hybridized to the chromosome 4-specific probe B31, as previously reported.<sup>15</sup> Informed consent was obtained from each patient (if <18 years of age, consent

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was obtained from their legal guardians). The research protocol was approved by the local Ethics Committee for medical research.

### Western blot analysis

Western blot analysis of protein extracts from muscle biopsy specimens was performed using a calpain 3 rabbit polyclonal antibody (H-80; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), a dysferlin rabbit monoclonal antibody (Epitomics Inc., Burlingame, CA, USA), a  $\beta$ -sarcoglycan rabbit polyclonal antibody (H-98; Santa Cruz Biotechnology Inc.) and a GAPDH mouse monoclonal antibody (Beyotime Inc., Jiangsu, China). The procedure was performed as previously described.<sup>16,17</sup> The average density of the bands were visualized and analyzed by Quantity One 4.6.2 software (Bio-Rad Laboratories, Sundbyberg, Sweden).

### Analysis of the CTG repeat of *DMPK*

Expansion of the CTG repeat in the 3' untranslated region of *DMPK* was analyzed by standard polymerase chain reaction, denaturing polyacrylamide gel electrophoresis, silver staining and sequencing. The primers were designed based on the sequence of *DMPK* (Ensembl Gene ID: ENSG 0000104936): forward 5'-GCTCGAAGGGTCTTGTAGC-3' and reverse 5'-CTTCCCAGGC CTGCAGTTTG-3'. The copies of the CTG repeat were calculated from the length of the amplification product as follows: copies of the CTG repeat = (length of amplification product (bp) – flanking sequence 105 bp)/3 bp.

## RESULTS

### Family 1

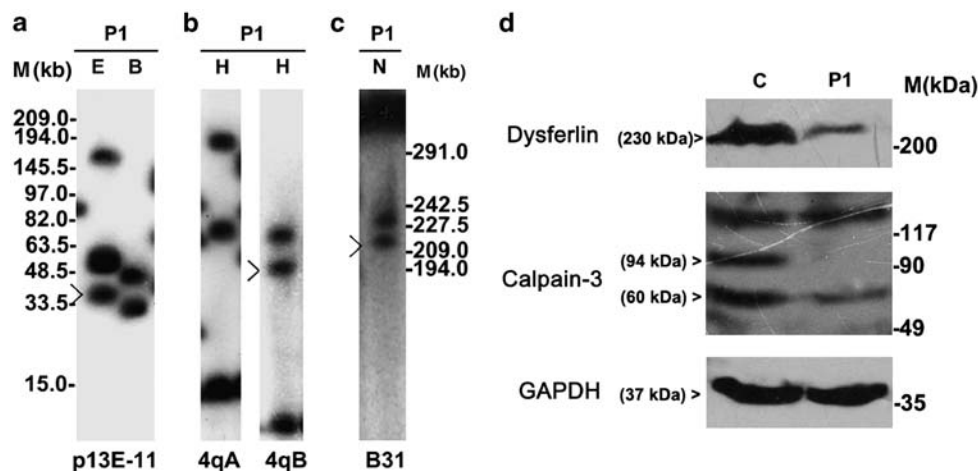
The proband was a 32-year-old female who developed lower limb weakness at age 10. Her family history was negative. Upon first examination at age 20, she presented proximal muscle weakness in the lower limbs and slightly affected facial muscles. CK was 97 U/l (reference range, <170 U/l), and electromyography (EMG) was myopathic. A muscle biopsy showed mild dystrophic changes. Muscle fiber necrosis or ragged red fibers were not observed. The presence of one 36-kb 4q-type D4Z4 repeat gave rise to a diagnostic consideration of mild atypical FSHD (Figure 1a).

Thereafter, the progression of weakness was very slow, and she was still able to rise from a squat by herself. Manual muscle testing revealed

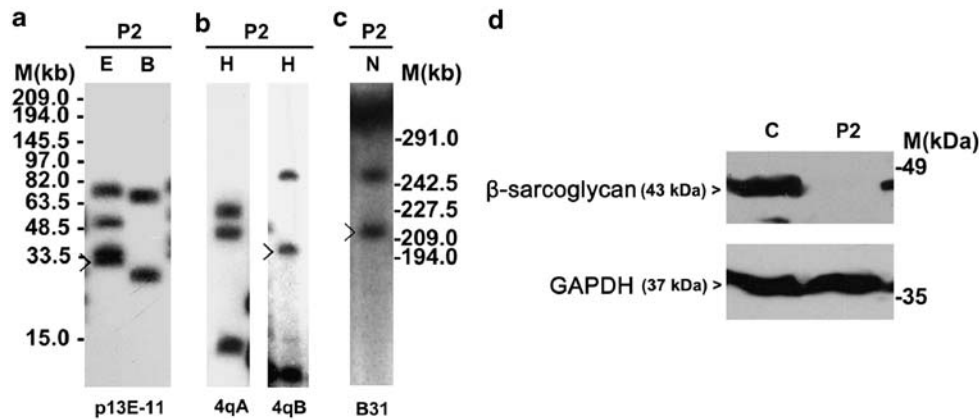
mild limb-girdle muscle weakness and a slight deltoid muscle paresis without scapular winging. Tendon reflexes were absent, and sensory examination was normal. The CS score was 3.5. The 36-kb 4q-type D4Z4 repeat was further identified as the 4qB variant (Figure 1b). Hybridization of probe B31 with *NotI*-digested DNA yielded a chromosome 4-derived fragment of 218 kb (36 kb+182 kb; Figure 1c), which confirmed that this 36-kb 4qB variant was from chromosome 4. Therefore, we had to re-assess the patient's clinical manifestation and took into consideration limb-girdle muscular dystrophy (LGMD). Western blot analysis of the patient's biopsy specimen revealed abnormal expression of calpain 3 and dysferlin (Figure 1d), suggesting that she suffered from primary calpain 3 deficiency and secondary dysferlinopathy (LGMD2A).

### Family 2

The proband was a 17-year-old female who developed body limb weakness at age 8. She had progressive symmetric girdle myopathy spreading from her shoulders to her hips. Neurological examination revealed that her stance was hyperlordotic and her abdomen was slightly protuberant. Bilateral weakness of the extensors of the shoulders, knees and hips interfered with activities such as raising arms above the head, climbing stairs or rising from a stooped posture. The lip had some looseness and protrusion. Bilateral calf hypertrophies were also observed. CK was markedly elevated at 5460 U/l, and EMG was myopathic. A muscle biopsy displayed moderately dystrophic features without obvious inflammatory infiltration. Her asymptomatic parents were consanguineous and her two elder sisters and one brother had died in their first decade of life. Her gender and myopathological features did not support Duchenne/Becker muscular dystrophy, and it was difficult to distinguish clinically whether the variable myopathic weakness was due to FSHD or LGMD. Thus, Southern blot analysis with probe p13E-11 was performed for this patient and the result revealed the presence of one 4q-type D4Z4 repeat of 31 kb (Figure 2a), initially confirming facial-sparing FSHD.



**Figure 1** (a–c) PFGE-based Southern blot analysis of the proband (P1) from family 1. (a) DNA was digested with *EcoRI* (E) and *EcoRI/BlnI* (B), subsequently hybridized with probe p13E-11. 4q-type alleles are resistant to *BlnI*, whereas 10q-type alleles are sensitive to *BlnI*. The proband carried two 4q-type alleles of 36 kb (arrow) and 50 kb, and two 10q-type alleles of 52 kb and 157 kb. (b) DNA was digested with *HindIII* (H) and hybridized with probes 4qA and 4qB. 4q-type alleles of 36 kb (arrow) and 50 kb were identified as 4qB variants. (c) DNA was digested with *NotI* (N) and hybridized with probe B31. The 4q-type alleles of 36 kb and 50 kb were visualized at 218 kb (36 kb+182 kb; arrow) and 232 kb (50 kb+182 kb), showing that the 36-kb 4qB variant resided on chromosome 4. The size marker was the MidRange PFG marker (M). (d) Western blot analysis of muscle proteins. GAPDH was used as the internal control. Dysferlin is normally visualized as a 230-kDa band, and calpain 3 as 94-kDa and 60-kDa bands. Expression of dysferlin showed a significant decrease (<30% of normal) in the muscle protein of the proband (P1) compared with that of the control (C). In the expression of calpain 3, the 94-kDa band was completely absent, and the 60-kDa band was obviously reduced in the patient.



**Figure 2** (a–c) PFGE-based Southern blot analysis of the proband (P2) from family 2. (a) The proband carried two 4q-type alleles of 31 kb (arrow) and 72 kb, and two 10q-type alleles of 36 kb and 51 kb. (b) Allelotyping identified one 4qA variant of 31 kb (arrow) and three 4qB variants of 72 kb, 36 kb and 51 kb. (c) Two 4q-type alleles of 31 kb and 72 kb were both located on chromosome 4, as they were visualized at 213 kb (31 kb+182 kb; arrow) and 254 kb (72 kb+182 kb). (d) Western blot analysis of muscle proteins.  $\beta$ -sarcoglycan is normally visualized as a 43-kDa band. Expression of  $\beta$ -sarcoglycan was absent in the proband (P2) compared with the control (C), suggesting that sarcoglycan deficiency caused LGMD2.

Recently, further haplotyping analysis showed that this 31-kb repeat was the 4qB variant (Figure 2b). After hybridization with probe B31, the chromosomal assignment revealed a *NotI* fragment of 213 kb (31 kb+182 kb), confirming that this 31-kb fragment was on chromosome 4 (Figure 2c). We therefore reconsidered the diagnosis and analyzed the patient's dystrophin-associated glycoproteins. A  $\beta$ -sarcoglycan deficiency was confirmed by muscle protein analysis (Figure 2d), and she was finally diagnosed with severe childhood LGMD2E.

### Family 3

The proband was a 38-year-old female who was referred for slowly progressing weakness of the arm and shoulder girdle in her second decade of life. On first neurological examination at age 35, ptosis of the eyelids, and thinness and slackness of the facial muscles were evident, along with mild weakness and atrophy of the shoulder girdle and pelvic girdle muscle without obvious scapular winging. Her parents both died in their sixties without signs of muscle problems. Her CK was slightly elevated at 370 U/l. EMG showed myopathic changes with a few myotonic discharges. A muscle biopsy performed in another hospital showed mild dystrophic changes. The initial molecular analysis showed one 36-kb 4q-type D4Z4 repeat compatible with the diagnosis of atypical FSHD.

Her symptoms continued to progress slowly, with gradual involvement of the proximal muscles of the lower limbs and the trunk. Specifically, she started to notice that strong clenching of the fist and closure of the eyelids were followed by a delay in relaxation. Moderate muscle weakness and myotonia of the hands and extensors of the forearms were also present on a neurological re-examination. Thus, we had to repeat the PFGE analysis with probes p13E-11, 4qA, 4qB and B31. The patient carried three *BlnI*-resistant D4Z4 repeats of 36 kb, 76 kb and 86 kb (Figure 3a), and the 36-kb repeat was identified as the 4qB variant (Figure 3b). Finally, the 36-kb 4qB variant was confirmed to reside on chromosome 4, and the 86-kb D4Z4 repeat was a mixture of 4q- and 10q-specific sequences resided on chromosome 10 (Figure 3c).

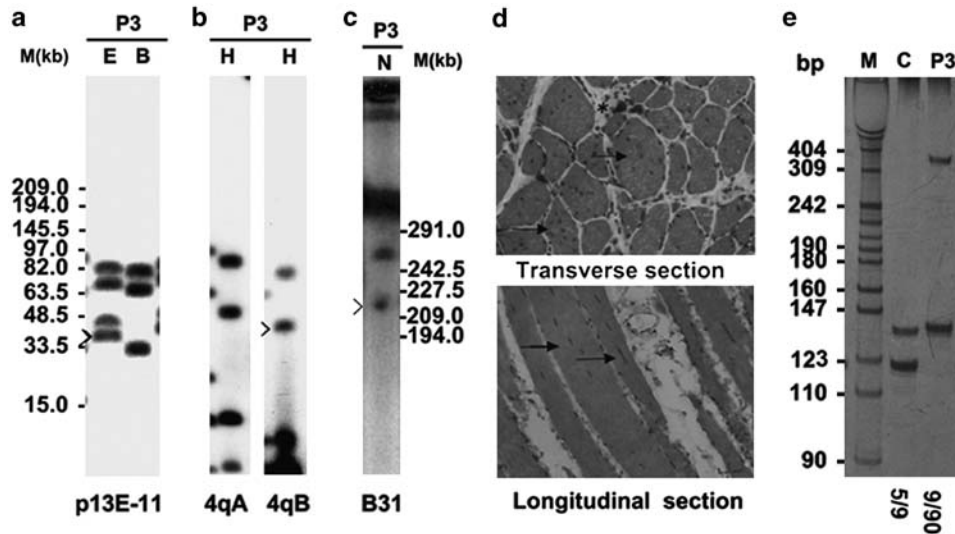
A new muscle biopsy was performed and several unusual myopathological features were observed: central nucleation was marked, and peripherally placed sarcoplasmic masses and circular bundles of myofibrils existed (Figure 3d). Molecular genetic analysis revealed that

the patient was heterozygous for the *DMPK* mutation; the copy number of CTG repeats in one chromosome was 90 (generally <50 copies in normal individuals; Figure 3e). The patient was re-diagnosed with DM1, and the moderate expansion of CTG copies was consistent with her relatively mild clinical features.

### Family 4

The proband (II<sub>2</sub>), a 34-year-old woman, presented onset of symptoms at age 16, with a progressive inability to raise both arms above shoulder level, close her eyes and puff up her cheeks or smile. On first examination at age 29, she presented marked bifacial weakness and mild proximal muscle weakness with scapular winging in her upper limbs. From then on, the disease progressed very slowly for several years. Her CS score was 2.0 when she was 34-years old. Her lower limb muscle bulk and strength were intact, and her gait was normal. Her CK was normal and the EMG showed myopathic changes in the proximal muscles of upper limbs. The left biceps muscle biopsy showed the presence of mildly degenerative and necrotic muscle fibers with secondary fibrosis, together with increased variation in myofiber size and the presence of inflammatory infiltrates of the vascular wall. She unequivocally fulfilled the clinical criteria of FSHD.<sup>2</sup> Her mother (I<sub>2</sub>) had a slowly progressing form of FSHD at age 20. Upon neurological examination at age 60, she had a CS score of 3.5. Her CK was slightly elevated (270 U/l). The proband's 36-year-old husband (II<sub>1</sub>) and two daughters (III<sub>1</sub> and III<sub>2</sub>, 10- and 8-years old, respectively) showed no clinical symptoms or signs of FSHD on physical and neurological examination. Their CK levels were normal.

Complementary allele typing and segregation analysis by PFGE revealed this pedigree with complex D4Z4 rearrangements (Figure 4a). The proband carried one 18-kb *BlnI*-resistant D4Z4 repeat and three *BlnI*-sensitive D4Z4 repeat arrays of 47 kb, 83 kb and 102 kb. The 18-kb and 102-kb arrays were inherited from her affected mother. Surprisingly, her husband carried four *BlnI*-resistant arrays of 24 kb, 57 kb, 57 kb and 78 kb, which indicated that two *BlnI*-resistant D4Z4 repeat arrays were mixtures of 4q- and 10q-specific sequences resided on chromosome 10. Both daughters inherited the *BlnI*-resistant arrays of 24 kb from their father (Figure 4b). Based on these findings, the father and the two daughters could not be excluded as an asymptomatic FSHD case. Further, the 18-kb array was identified as the 4qA variant, and the 24-kb array was identified as the 4qB variant



**Figure 3** (a–c) PFGE-based Southern blot analysis of the proband (P3) from family 3. (a) P3 carried three *BlnI*-resistant D4Z4 repeats of 36 kb (arrow), 76 kb, 86 kb and one *BlnI*-sensitive repeat of 43 kb (arrow) and 76 kb. (b) Allelotyping analysis identified two 4qA variants of 43 kb and 86 kb, and two 4qB variants of 36 kb and 76 kb resided on chromosome 4. (c) Two *NotI* fragments of 218 kb (36 kb+182 kb; arrow) and 258 kb (76 kb+182 kb) demonstrated that 4q-type alleles of 36 kb and 76 kb resided on chromosome 4. So the extra 86 kb D4Z4 repeat was a mixture of 4q- and 10q-specific sequences resided on chromosome 10. (d) Histopathological features of P3. Central nucleation is marked with an arrow (>50%). Peripherally placed sarcoplasmic masses and circular bundles of myofibrils were observed (asterisk). In addition, necrosis of single muscle fibers and many atrophic fibers was observed. HE,  $\times 200$ . (e) PAGE analysis of CTG repeats in the 3' untranslated region of *DMPK*. The copy number of CTG repeats in one chromosome of P3 was 90 ((385–105)/3), indicating heterozygosity for the *DMPK* mutation. The exact numbers of CTG repeats are indicated below each lane. C, normal control; M, the size marker.

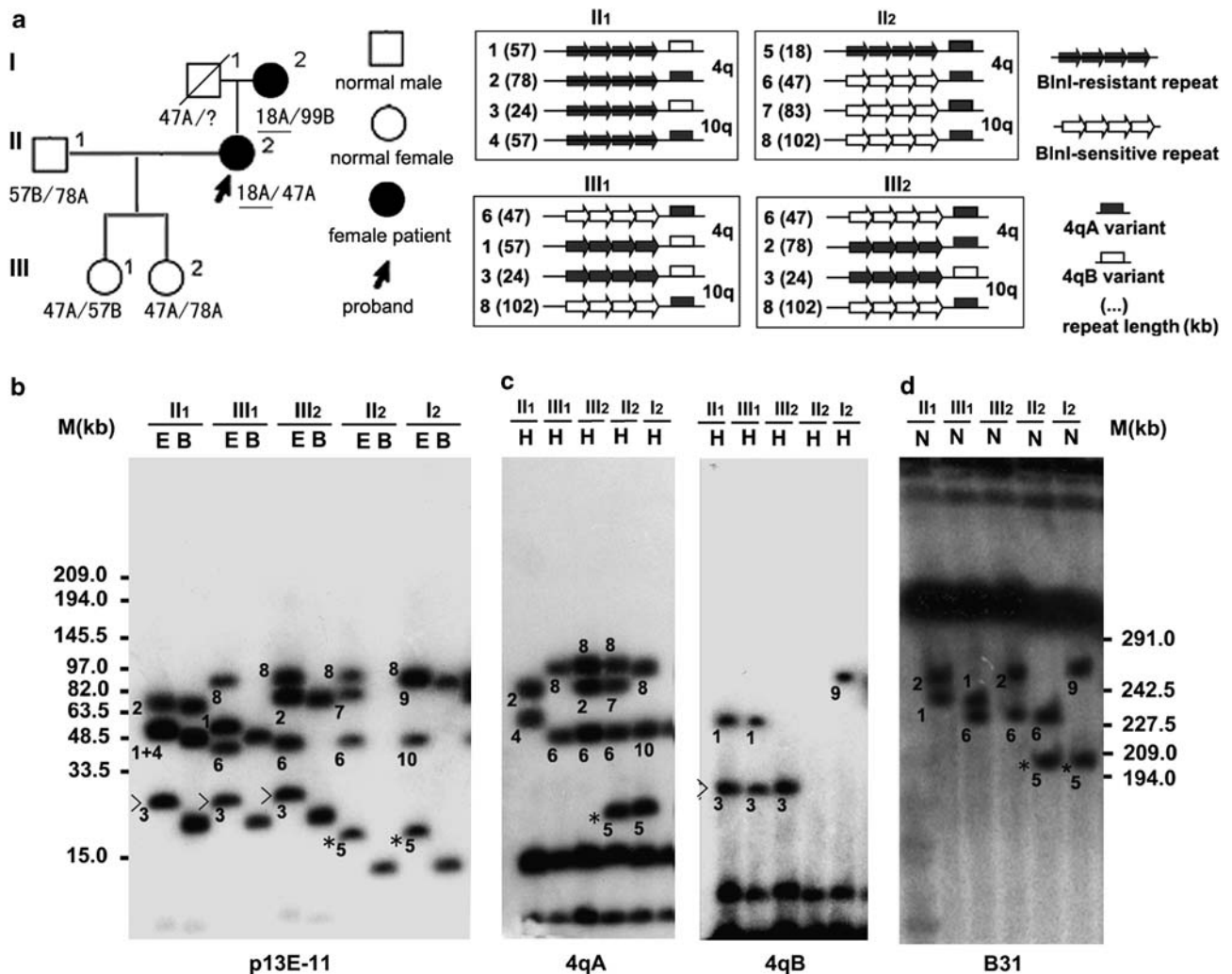
(Figure 4c). The chromosomal origin analysis revealed that the 18-kb 4qA variant resided on chromosome 4 and the 24-kb 4qB variant of 4q $\rightarrow$ 10q sequence mixture resided on chromosome 10. *NotI* fragments of 239 kb (57 kb+182 kb) and 260 kb (78 kb+182 kb) from  $\text{II}_1$  confirmed the presence of the 57-kb and 78-kb D4Z4 repeats on chromosome 4, and another 57-kb D4Z4 repeat was a 4q $\rightarrow$ 10q sequence mixture (Figure 4d). Both daughters inherited a 47-kb 10q $\rightarrow$ 4q sequence mixture from their mother, indicating that they were normal.

## DISCUSSION

Here, all three patients diagnosed with unrelated neuromuscular disorders had a 4qB variant smaller than 38 kb on chromosome 4, which would have caused substantial diagnostic confusion in the absence of the 4qA/4qB variant determination. Neuromuscular disorders that may be clinically and histopathologically similar to FSHD include the various limb-girdle and scapuloperoneal syndromes, which also may cause mild facial weakness. Ancillary investigations, such as the assessments of CK, EMG and muscle biopsy, are often insufficient for diagnosis, as was the case in the probands from families 1 and 2. However, subsequent DNA analysis of the non-pathogenic short 4qB variant excluded the diagnosis of FSHD, and western blot analysis further confirmed the diagnosis of LGMD2 in these patients. The proband from family 3 also presented some FSHD-like symptoms and signs based on clinical observation, such as prominent involvements of the facial muscles and mild shoulder girdle and pelvic girdle weakness. The facial-sparing scapular myopathy was described as the most common clinical variant of FSHD, and some atypical phenotypes including LGMD, distal myopathy, mild or partial forms have been described in several papers published before 2004.<sup>18–20</sup> However, the identification of the 4qA and 4qB variants had not been performed in these special cases. Here, the D4Z4 contraction in the proband from family 3 was confirmed as linked to the 4qB variant and considered as nonpathogenic.

We had previously demonstrated the presence of an FSHD-sized 4qB variant on chromosome 4 in healthy individuals.<sup>21</sup> In the present study, we provided further evidence of the nonpathogenic 4qB variant in other neuromuscular disorders. This may also explain the rare finding of FSHD-sized D4Z4 repeats that do not co-segregate with unrelated disorders.<sup>22</sup> Therefore, the extension of the clinical spectrum of FSHD needs to be carefully evaluated. Although some rare cases with 'double trouble' had been reported,<sup>11,23</sup> *NotI* digestions followed by hybridizations with B31 need to conclusively identify the correct chromosome 4, and some complex genetic constitutions with exchanged D4Z4 alleles of chromosome 4 and chromosome 10 origins are presented below.

There are two uncommon factors that made the genetic confirmation of the fourth pedigree confusing. First, both 4qA- and 4qB-type FSHD-sized D4Z4 alleles were found in the same family. Second, complex genetic constitutions were observed in the same family. Before complementary allelotyping analysis and integrated chromosome assignments, the members of the family who carried 4qB-type FSHD-sized alleles had been misdiagnosed as asymptomatic cases. Through PFGE-based DNA analysis with probes 4qA, 4qB and B31, we confirmed that the 18-kb 4qA variant correlated with FSHD in the proband and that the one carried by her mother resided on chromosome 4, whereas the 24-kb 4qB variant, which is considered as nonpathogenic, in the healthy husband and the two daughters resided on chromosome 10. Moreover, we were able to confirm the complex D4Z4 rearrangements of two exchanged repeat arrays, one on chromosome 4 and one on chromosome 10, inherited from the maternal 10q $\rightarrow$ 4q repeat and from the paternal 4q $\rightarrow$ 10q repeat. Although the haplotypes composed of a mixture of 4q and 10q sequences were detected at frequencies >15% in the Chinese FSHD,<sup>12</sup> such exchanged constitution cannot be identified by conventional DNA diagnosis and never been observed in the Chinese population. As reported, all 4q subtelomeres originate from only four discrete interchromosomal sequence transfers during human evolu-



**Figure 4** PFGE-based Southern blot analysis of the family 4 with complex genetic constitutions. (a) Schematic diagram showing the genetic arrangement of 4q and 10q alleles in this pedigree. (b) The proband (II<sub>2</sub>) carried one *BlnI*-resistant D4Z4 repeat of 18 kb (5) and three *BlnI*-sensitive D4Z4 repeat arrays of 47 kb (6), 83 kb (7) and 102 kb (8), indicating one 10q→4q sequence mixture resided on chromosome 4. The 18-kb fragment and 102-kb fragment were inherited from the patient's affected mother (I<sub>2</sub>) who carried another *BlnI*-resistant D4Z4 repeat of 99 kb (9) and *BlnI*-sensitive D4Z4 repeat of 49 kb (10). The husband (II<sub>1</sub>) carried four *BlnI*-resistant D4Z4 repeat arrays of 24 kb (3), 57 kb, 57 kb (1+4, comigrating alleles) and 78 kb (2), indicating that two *BlnI*-resistant D4Z4 repeat arrays were mixtures of 4q- and 10q-specific sequences resided on chromosome 10. Two daughters (III<sub>1</sub> and III<sub>2</sub>) inherited the FSHD-sized allele of 24 kb from their father. (c) The 18-kb fragment was identified as the 4qA variant, whereas the 24-kb fragment was identified as the 4qB variant. (d) The 18-kb 4qA variant resided on chromosome 4, whereas the 24-kb 4qB variant was a likely 4q→10q sequence mixture resided on chromosome 10 owing to its disappearance on *NotI*-digestion. Two *NotI* fragments of 239 kb (57 kb+182 kb) and 260 kb (78 kb+182 kb) were detected in the husband, confirming that the 57-kb and 78-kb fragments, respectively, resided on chromosome 4 and were inherited by his daughters. Thus, another *BlnI*-resistant D4Z4 repeat of 57 kb resided on chromosome 10. Both daughters inherited the 47 kb fragment (10q→4q) from their mother. The 83 kb *BlnI*-sensitive D4Z4 repeat resided on chromosome 10 of the proband was of paternal origin. The FSHD-causing 4qA variant (asterisk) is underlined, and the FSHD-sized 4qB variant is indicated by an arrow.

tion, and haplotypes with mixtures of 4q- and 10q-specific sequences represent intermediate structures in the transition from 4q to 10q subtelomeres.<sup>10</sup> We also found that in this family, a 4qB variant resided on chromosome 10, confirming the same detection in other populations,<sup>10</sup> and further suggesting that the breakpoint of the D4Z4 repeat was distal to the 4qA and 4qB variants. The pathophysiological pathway from 4q35 deletion together with the 4qA variant to the clinical picture of FSHD is unclear.

In summary, this is the first study to confirm that D4Z4 contractions on the 4qB variant do not cause FSHD in the Chinese population. Furthermore, our results emphasize that the D4Z4 repeat length analysis alone is insufficient for the diagnosis of FSHD, especially

when used as an exclusion criterion. This analysis should be accompanied by 4qA/4qB variant determination and integrated chromosome assignments, especially in patients with obscure and unclassified myopathies similar to atypical forms of FSHD.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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