Original Article

CD4⁺ cells, macrophages, MHC-I and C5b-9 involve the pathogenesis of dysferlinopathy

Xi Yin1*, Qian Wang2*, Ting Chen1, Junwei Niu1, Rui Ban1, Jiexiao Liu1, Yanling Mao2, Chuanqiang Pu1

1Department of Neurology, Chinese PLA General Hospital, 28 Fuxing Road, Haidian District, Beijing 100853, China; 2Department of Emergency, General Hospital of Chinese People’s Armed Police Force, 69 Yongding Road, Haidian District, Beijing 100039, China. *Equal contributors.

Received December 15, 2014; Accepted February 20, 2015; Epub March 1, 2015; Published March 15, 2015

Abstract: Objective: Dysferlin is a sarcolemmal protein that plays an important role in membrane repair by regulating vesicle fusion with the sarcolemma. Mutations in the dysferlin gene (DYSF) lead to multiple clinical phenotypes, including Miyoshi myopathy (MM), limb girdle muscular dystrophy type 2B (LGMD 2B), and distal myopathy with anterior tibial onset (DMAT). Patients with dysferlinopathy also show muscle inflammation, which often leads to a misdiagnosis as inflammatory myopathy. In this study, we examined and analyzed the dyferlinopathy-associated immunological features. Methods: Comparative immunohistochemical analysis of inflammatory cell infiltration, and muscle expression of MHC-I and C5b-9 was performed using muscle biopsy samples from 14 patients with dysferlinopathy, 7 patients with polymyositis, and 8 patients with either Duchenne muscular dystrophy or Becker muscular dystrophy (DMD/BMD). Results: Immunohistochemical analysis revealed positive staining for immune response-related CD4⁺ cells, macrophages, MHC-I and C5b-9 in dysferlinopathy, which is in a different mode of polymyositis and DMD/BMD. Conclusion: These results demonstrated the involvement of immune factors in the pathogenesis of dysferlinopathy.

Keywords: Becker muscular dystrophy, Duchenne muscular dystrophy, dysferlinopathy, immunohistochemical analysis, muscle pathology, polymyositis

Introduction

Dysferlinopathy is an autosomal recessive neuromuscular disease caused by mutations in dysferlin (DYSF) gene, which is critical to membrane repair and vesicle trafficking. This disease comprises of Miyoshi myopathy (MM), limb girdle muscular dystrophy type 2B (LGMD 2B), and distal myopathy with anterior tibial onset (DMAT) [1]. Although rare, dysferlinopathy accounts for up to 30% of progressive recessive muscular dystrophies in certain geographical areas such as the Middle East and the Indian subcontinent [2].

It is possible that dysferlin interacts with significant immunological pathways [3] as the clinical manifestations of dysferlinopathy resemble the idiopathic inflammatory myopathy. In addition, inflammatory cell infiltrates are commonly observed in dysferlinopathy [4, 5], which often lead to a misdiagnosis of dysferlinopathy as inflammatory myopathy. In this study, we analyzed the clinical manifestation of immune factors in patients with dysferlinopathy, so that we may understand the role of immune factors in dysferlinopathy pathogenesis, and provide a differential diagnosis of dysferlinopathy.

Materials and methods

Patient data collection

This study consisted of 29 patients who underwent muscle biopsy from 2012 to 2013, and none of the patients received treatment during this period. Of these, 14 patients were diagnosed with dysferlinopathy based on their clinical manifestations and immunohistochemical analysis. Eight patients were diagnosed with Duchenne muscular dystrophy (DMD) or Becker muscular dystrophy (BMD) by histological and immunohistochemical analysis. Finally, seven patients with polymyositis (PM) were diagnosed according to clinical manifestations and histological analysis, which fulfilled the Bohan and
Immune factors and pathogenesis of dysferlinopathy

Peter’s criteria. Complete clinical and laboratory data were collected, including detailed personal history, clinical features, dysmorphic features, and muscle power test. The DMD/BMD and PM patient samples were used as a control. The IRB of Chinese PLA General Hospital approved this study.

Muscle biopsy and specimen processing

The patients underwent open biopsy in quadriceps femoris, gastrocnemius, or biceps brachii under local anesthesia after obtaining a written informed consent. The muscle specimens were immediately frozen by immersing in isopentane prechilled in liquid nitrogen, and stored at -80°C until processed. Transverse serial 8-μm frozen muscle sections were routinely stained for haematoxylin and eosin (H&E) to determine muscle morphology, and additional serial sections (5-μm thick) were prepared separately for immunohistochemical staining.

Immunohistochemical analysis

To characterize inflammatory infiltrates, serial frozen muscle sections from all patients were stained with rabbit monoclonal antibodies against CD4+ T lymphocytes, CD8+ T lymphocytes, CD68+ macrophages, CD20+ B cells, major histocompatibility complex class I (MHC-I), and membrane attack complex (C5b-9; all from Abcam, Cambridge, UK). Approximately 5-μm thick consecutive transverse muscle sections from each of the 14 dysferlinopathy, 7 classical PM, and 8 DMD/BMD patients were

<table>
<thead>
<tr>
<th>NO.</th>
<th>Sex/age</th>
<th>Age of onset (yrs)</th>
<th>Disease duration (yrs)</th>
<th>Symptoms at onset</th>
<th>Disease progression</th>
<th>CK value (2-200 u/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male/17</td>
<td>15</td>
<td>2</td>
<td>Lower limbs weakness</td>
<td>slow</td>
<td>8489.6</td>
</tr>
<tr>
<td>2</td>
<td>Male/22</td>
<td>20</td>
<td>2</td>
<td>Lower limbs weakness</td>
<td>slow</td>
<td>19619</td>
</tr>
<tr>
<td>3</td>
<td>Female/49</td>
<td>19</td>
<td>30</td>
<td>Lower limbs weakness</td>
<td>slow</td>
<td>1807.3</td>
</tr>
<tr>
<td>4</td>
<td>Male/8</td>
<td>3</td>
<td>5</td>
<td>Unsymptomatic hyperckemia</td>
<td>unprogressive</td>
<td>4822</td>
</tr>
<tr>
<td>5</td>
<td>Female/57</td>
<td>27</td>
<td>30</td>
<td>Upper and lower limbs weakness</td>
<td>slow</td>
<td>1542.6</td>
</tr>
<tr>
<td>6</td>
<td>Male/17</td>
<td>13</td>
<td>4</td>
<td>Lower limbs weakness</td>
<td>slow</td>
<td>13297</td>
</tr>
<tr>
<td>7</td>
<td>Male/33</td>
<td>30</td>
<td>3</td>
<td>Lower limbs weakness</td>
<td>slow</td>
<td>4791.5</td>
</tr>
<tr>
<td>8</td>
<td>Male/15</td>
<td>3</td>
<td>12</td>
<td>Delayed motor milestone</td>
<td>unprogressive</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Male/29</td>
<td>28</td>
<td>1</td>
<td>Upper and lower limbs weakness</td>
<td>unprogressive</td>
<td>1432.7</td>
</tr>
<tr>
<td>10</td>
<td>Female/16</td>
<td>10</td>
<td>6</td>
<td>Upper and lower limbs weakness; spine deformity</td>
<td>intermediate</td>
<td>96.3</td>
</tr>
<tr>
<td>11</td>
<td>Male/20</td>
<td>25</td>
<td>5</td>
<td>Lower limbs weakness</td>
<td>slow</td>
<td>1528</td>
</tr>
<tr>
<td>12</td>
<td>Male/26</td>
<td>16</td>
<td>10</td>
<td>Upper and lower limbs weakness</td>
<td>slow</td>
<td>4747</td>
</tr>
<tr>
<td>13</td>
<td>Female/45</td>
<td>43</td>
<td>2</td>
<td>Upper and lower limbs weakness</td>
<td>slow</td>
<td>7081</td>
</tr>
<tr>
<td>14</td>
<td>Male/46</td>
<td>26</td>
<td>20</td>
<td>Lower limb weakness</td>
<td>intermediate</td>
<td>77.4</td>
</tr>
</tbody>
</table>

1 Female/60 59 0.7 upper and lower limbs weakness; skin rash intermediate 22.3
2 Female/48 46 2 Muscle weakness; dysarthria intermediate 451.5
3 Male/11 10 1 Muscle weakness; dysphagia intermediate 461.5
4 Female/20 19 0.25 upper and lower limbs weakness intermediate 4218.1
5 Male/8 8 0.2 upper and lower limbs weakness; myalgia; skin rash rapid 4429
6 Female/73 73 0.7 upper and lower limbs weakness; skin rash intermediate 492.4
7 Female/65 65 0.8 upper and lower limbs weakness; myalgia intermediate 233.5

DMD/BMD

1 Male/20 5 15 Lower limb weakness; delayed motor milestone slow 2631.1
2 Male/12 2 10 Lower limb weakness; delayed motor milestone slow 6734.3
3 Male/16 2 14 Muscle fatigue; hyperckemia slow 1849.3
4 Female/12 2 10 delayed motor milestone slow 3827
5 Male/10 7 3 Lower limbs weakness intermediate 5811
6 Male/27 15 12 Lower limbs weakness slow 4709.4
7 Male/7 2 5 Lower limb weakness; delayed motor milestone slow 4763.1
8 Male/36 35 1 upper and lower limbs weakness intermediate 827
fixed in ice-cold acetone prior to the immunohistochemical analysis. Serial sections were air-dried in room temperature, blocked with 3% H₂O₂, washed with 1% phosphate buffered saline (PBS), and blocked with 5% goat serum for 15 min each. After 1% PBS wash, the samples were incubated with mentioned primary antibodies (1:200) for 1 h at 37°C. Then, samples were treated with secondary antibody (polymerized HRP mouse anti-rabbit IgG) for 30 min at 37°C, and 3',3'-diaminobenzidine (DAB) staining for 20 min. Finally, the slides were mounted with neutral balsam (all from Golden Bridge, Beijing) and observed under light microscope (BX51, Olympus, Japan). All incubation steps were performed in humidified chambers.

**Evaluation of immunohistochemical staining**

Because of high sample heterogeneity and low frequency of infiltrating inflammatory cells, quantification was performed with the hot spot method used for myositis and estimating new blood vessel formation in breast tumor [6]. This method allowed the quantification of CD4⁺, CD8⁺ and B cells in hot spots defined as muscle areas containing the highest density of infiltrating cells. Accordingly, two hot spots per muscle section were selected randomly. In each spot, positive cells were counted in 10 consecutive fields (100×). The average of the number of positive cells per two hot spots was taken and expressed as the number of positive cells per field (100×).

For MHC-I and C5b-9 evaluation, we applied a semiquantitative grading system mentioned by M. Fanin [7] as follows: negative staining (-), slightly positive staining (+), moderately positive staining (++), and strongly positive staining (+++). The immunohistochemistry data was observed and interpreted independently by two persons.

**Statistical analysis**

The independent t-test was used to compare the means of two samples for continuous variables. Quantitative results are expressed as the mean ± SD. Levels of MHC-1 and C5b-9 expression were compared using the non-parametric Kruskal-Wallis H test.

**Results**

**Demographic and clinical features**

In this study, of the 29 patients, 14, 8, and 7 patients were diagnosed with dysferlinopathy, DMD/BMD, and polymyositis respectively. The onset of dysferlinopathy in the 10 men and 4 women was from the age of 3-43 years (mean: 19.9 years), with disease duration of 1-20 years (mean: 9.4 years). The DMD/BMD (7 men and 1 woman) and polymyositis (2 men and 5 women) patients had disease onset between 2-35 years (mean: 8.8 years), and 8-73 years (mean: 40.0 years) respectively. The disease duration ranged from 1 to 15 years (mean: 8.8 years) and 0.2 to 2 years (mean: 0.8 years) for DMD/BMD and polymyositis, respectively. The demographic and clinical data of the patients are summarized in Table 1.

The most common disease onset symptom for all the three groups was weakness in the limbs or motor delay. The dysferlinopathy patients showed particular weakness in the lower limbs, with additional asymptomatic hyperCKemia. The clinical course was relatively benign as many patients were able to walk even years after the onset of the disease. The polymyositis patients exhibited skin rash or myalgia in addition to the weakened limbs with the clinical progression of the onset symptoms. Furthermore, elevated levels of creatine kinase (CK) were found in all the three groups. The CK values were the highest in the dysferlinopathy patients, who showed up to 10-100 times the normal range. The DMD/BMD patients showed slow
disease progression and had 10-30 times elevated CK values. However, not all the polymyositis patients showed increased levels of CK; those patients with elevated CK had 20 times CK than the normal. Patients from all the groups were confined to wheelchairs during muscle biopsy.

**Detection of CD4^+ cells, CD8^+ cells, B cells, and macrophages**

The detection of inflammatory cells in muscle biopsy samples from the 29 patients are shown in **Table 2** and **Figure 1**. Patients with polymyositis had the most severe inflammatory cell infiltration, specifically CD4^+ cells and macrophages. The frequency of CD4^+ cells infiltration was similar in dysferlinopathy and DMD/BMD patients, and both were fewer than that in the polymyositis patients (5.7 ± 4.4 vs. 12.3 ± 6.4, P = 0.009; 4.9 ± 5.7 vs. 12.3 ± 6.4, P = 0.009). Consistently, more macrophage infiltration was observed in the polymyositis patients than the DMD/BMD patients (10.8 ± 6.5 vs. 3.7 ± 3.1, P = 0.006) and dysferlinopathy patients. Unlike the CD4^+ cell infiltration, the number of macrophages infiltrating dysferlinopathy muscle sample was greater than that of DMD/BMD patients (7.8 ± 4.3 vs. 3.7 ± 3.1, P = 0.047). Thus, DMD/BMD patients showed the least amount of macrophage infiltration. The dysferlinopathy patients showed lesser CD8^+ cell infiltration than polymyositis patients did (1.3 ± 1.1 vs. 3.3 ± 1.8, P = 0.005). There were no other statistically significant differences in CD8^+ cell or B cell infiltration in the three groups.

**Expression of MHC-I and C5b-9 in muscle biopsy**

The staining data of MHC-I and C5b-9 expression in all the muscle biopsies is shown in **Table 3** and **Figure 2**. MHC-I expression was observed in 71.4% (10/14) of the dysferlinopathy patients and 100% of polymyositis patients. In contrast, only 25% (2/8) stained slightly positive for MHC-I in DMD/BMD patients. All the dysferlinopathy and polymyositis patient samples stained positive for muscle-specific C5b-9 expression, with 28% (4/14) of dysferlinopathy patients showing strongly positive fibers. The C5b-9 staining in the muscles of DMD/BMD patients was negative or slightly positive.

**Discussion**

Dysferlinopathy is an autosomal, recessive muscular dystrophy caused by the mutations in the DYSF gene, which encodes the dysferlin protein. Mutations in the dysferlin gene cause a
heterogeneous group of muscular dystrophies, including Miyoshi myopathy (MM), limb girdle muscular dystrophy Type 2B (LGMD2B), and distal myopathy with anterior tibial onset (DMAT). These diseases are collectively referred as dysferlinopathy [8-10], and MM and LGMD 2B are the most common types of dysferlinopathy. The patients share the same clinical manifestations such as adolescent onset, progressive muscle weakness, and elevated CK level. The MM patients show distal muscular weakness at onset, frequently involving the gastrocnemius muscles and LGMD 2B patients show shoulder and pelvic girdle weakness at onset [11]. Epigenetic interactions are suspected to underlie some of the clinical symptomatic variations; however, this remains to be confirmed.

In our study, the patients with dysferlinopathy, usually demonstrated disease onset at adult stage, relatively benign clinical course, and elevated CK values compared to the DMD/BMD or polymyositis patients. The deficient dysferlin protein results in defective membrane patching causing leakage of intracellular contents from the inside of the muscle fibers, leading to a significant increase in the CK level. We hypothesize that the damage caused by dysferlin is less progressive than dystrophin, which is supported by the relatively benign clinical course, the asymptomatic hyperCKemia, and the late symptom onset. According to the “danger” hypothesis proposed by Andrew et al [12], intracellular contents that leaked due to membrane disruption in dysferlinopathy can act as endogenous adjuvants that trigger an inflammatory response.

Muscle inflammation was observed in the dysferlinopathy patients, making the muscle pathophysiology indistinguishable from that of idiopathic inflammatory myopathy. Moreover, SL/J mice that are used as animal models for experimental autoimmune myopathy carry mutations in dysferlin [13]. Several studies have investigated the causes underlying inflammation in dysferlinopathy, and have reported the upregulation of inflammasome, and the involvement of macrophages [14, 15]. In fact, dysferlin-deficient monocytes display increased phagocytic activity [16]. In this study, we demonstrated that a significantly higher number of macrophages infiltrate muscles in dysferlinopathy patients than the DMD/BMD patients, implying a pivotal role for macrophages in dysferlinopathy pathogenesis.

Han et al. demonstrated that the complement system is important in muscle pathology in dysferlinopathy, and the genetic disruption of the central component (C3) of the complement system ameliorated muscle pathology in dysferlin-deficient mice [17]. Moreover, the decay-accelerating factor (CD55), the complement inhibitor, was downregulated in the skeletal muscle of dysferlin-deficient SL/J mice, indicating the involvement of complement system in the dysferlinopathy pathogenesis [18]. Supporting previous studies, C5b-9, a marker of membrane attack complex (MAC) displayed the strongest expression in the dysferlinopathy patients. In addition, overexpression of MHC-I was observed in the muscle tissue of dysferlinopathy patients, suggesting that both muscle inflammation and the complement system participate in the pathogenesis of dysferlinopathy.

SL/J (SJL) mice and A/J mice are two naturally occurring dysferlinopathy animal models [19]. In SL/J mice, a 171-bp in-frame deletion in the DYSF mRNA is predicted to remove 57 amino acids from the corresponding protein, while A/J mice carry a unique ETn retrotransposon insertion near the 5’ end (intron 4) of the DYSF gene [20]. Interestingly, the SJL (SJL) and A/J mice display divergent phenotypes. For example, A/J mice display a later onset, and a slower progression of the muscular disease compared to the SJL mice [21]; this difference may be due to varying genetic background. The SJL/J mouse model has been extensively used to study autoimmune diseases such as encephalomyelitis [22], myositis [23], and hypophysitis [24]. Hence, the immunological data obtained using these animal models can be used to understand the immune response pathways involved in dysferlinopathy.

Table 3. Immunohistochemical results of MHC-I and C5b-9

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
<th>++</th>
<th>+++</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysferlinopathy</td>
<td>4</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Polymyositis</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>DMD/BMD</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C5b-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysferlinopathy</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Polymyositis</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>DMD/BMD</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*P = 0.011; **P = 0.003 for MHC-I and C5b-9 staining respectively within the three groups.
In conclusion, this study demonstrated, at least in part, the immune factors expressed dysferlinopathy patients. Dysferlin protein plays a role in the membrane fusion and repair and the deficient function cause leakage of intracellular contents, which may be an attractant for immunological responses. Thus, it is important for future studies to focus on understanding the co-relation of the immune system and muscle dystrophy. The presence of MHC-I and C5b-9 might aid in the differential diagnosis of dysferlinopathy patients.

Acknowledgements

The National Natural Science Foundation of China under Grant No. 81271399 and the Natural Science Foundation of Beijing under Grant No. 7132216 supported this work.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chuan-Qiang Pu, Department of Neurology, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853 China. Tel: +86 10-66937757; Fax: +86 10-66939721; E-mail: pucq30128@sina.cn

References

Immune factors and pathogenesis of dysferlinopathy


