

Original Article

Complement membrane attack complex is related with immune-mediated necrotizing myopathy

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Abstract: This study is to investigate the expression of complement membrane attack complex (C5b-9) in the skeletal muscle of patients with necrotizing myopathy (NM), and to investigate the relationship between C5b-9 and NM. Thirteen patients with NM and control patients with polymyositis and muscular dystrophy were enrolled in this study. Examinations including creatine kinase (CK) and L-lactate dehydrogenase (LDH) in the serum, electromyogram and muscle pathological examination were performed. C5b-9 expression in the skeletal muscle was determined by immunohistochemistry and analyzed by Image Plus Pro 6.0. C5b-9 expression was particularly prominent in necrotic muscle fibers, and also positive in blood vessels. C5b-9 diffusely expressed in vascular endothelial cells and smooth muscle layer. But the intensity was not related with the elevated level of serum CK. So, C5b-9 is strongly expressed in the necrotic muscle fiber and blood vessels, and may contribute to the pathogenesis of NM.

Keywords: Necrotizing myopathy, complement membrane attack complex, C5b-9, muscle fiber, endothelial cell

Introduction

Necrotizing myopathy (NM) is defined as the necrosis of skeletal muscle fiber caused by immunological mediation, malignant tumor, virus infection, statins [1] or connective tissue diseases. It usually consists of immune-mediated and secondary NM. The pathology features include diffused or focal muscle fiber necrosis, inflammatory cells infiltration, significant thinning of vascular and remnant wall. Most of the patients presented with progressive limb inability, muscular atrophy, and high creatine kinase (CK) in the serum. The severity of the disease was various. Respiratory muscle could be involved, which could lead to death. The pathogenesis of NM has not yet been clarified. NM is believed to be autoimmune with upregulated MHC-1, which could be triggered by unidentified endogenous or exogenous myotoxic factors [2, 3]. NM is often misdiagnosed as toxic myopathy, metabolic myopathy, or other inflammatory myopathies such as polymyositis, though there is no T cell infiltrates or MHC-I expression as seen in polymyositis [4]. Muscular dystrophy, a general term for a group of inherited disorders which are characterized

by progressive degeneration of skeletal muscles, also should be paid attention in the differential diagnosis of NM.

C5b-9, also called membrane attack complex (MAC), is of great importance in many inflammatory reactions. It is the common terminal effect production of complement system activated by some potentially destructive complement constituents, including anaphylotoxins and opsonins in classical, alternative and lectin ways [5]. C5b-9 has been associated with kidney disease, nervous system disease, blood disease, atherosclerosis and pregnancy hypertension disease. C5b-9 is obviously expressed in glomerular endothelial and epithelial cells in glomerulonephritis, and it plays a crucial role in the process of cell necrotizing. Hence, it was hypothesized that C5b-9 was involved in the pathogenesis of NM. The role of C5b-9 in NM has been rarely reported. Our previous study demonstrated the phenomenon that C5b-9 deposited in vascular wall of NM [6].

Up-regulation of C5b-9 in capillary wall of muscle tissue in NM, but there was still no appropriate explanation for this phenomenon. Therefore,

C5b-9 effect on necrotizing myopathy

Table 1. Clinical characteristics of patients with necrotizing myopathy, polymyositis, and muscular dystrophy

Case	Case Number	Gender	Age of onset (years)	Duration of disorder (months)	CK (U/L)	LDH (U/L)	GPT (U/L)	GOT (U/L)
Necrotizing myopathy	1	M	14	2	3897.6	829.1	156.5	62.5
	2	M	72	12	129.4	292.8	17.7	24.4
	3	F	31	12	1746.2	339.8	114.3	117.2
	4	F	64	6	4486.1	991.3	138.4	136.9
	5	F	52	3	4678.3	1353.1	406.2	206.9
	6	M	53	2	2508.5	540.2	290.1	183.3
	7	F	32	15	12619.9	2240.3	98.6	181.7
	8	M	29	2	8462.8	1254	338	255
	9	F	63	5	7297.3	1064	202.5	141.3
	10	M	56	4	132	492	209	179
	11	M	57	24	6252	964.9	194.5	227.8
	12	M	53	4	4770.3	999.8	537.9	314.9
	13	M	59	36	3840	618	185	130
Polymyositis	1	F	34	72	8502	897.7	89.7	69.5
	2	F	13	96	939.9	598.6	57.1	142.6
	3	F	30	24	3412	450	66	81
	4	M	7	84	2140	295	50	76
	5	F	15	36	251	247	27	26
	6	M	42	60	291	268	82	49
Muscular dystrophy	1	F	44	6	5997	1399	286.6	353.8
	2	M	56	7	9452.8	1649	187.3	218.3
	3	F	46	24	451.5	444.8	58.6	53.1
	4	F	19	3	4218.1	944	82	140.5
	5	F	22	1	1932.6	530.5	84.9	86

in this study we focus on the expression of C5b-9 in muscular tissues of patients with NM, polymyositis, and muscular dystrophy, in order to explore the difference of C5b-9 expression among the three diseases and demonstrate the potential role of C5b-9 in the mechanism of NM.

Methods

Patients and clinical data collection

Open muscle biopsy was performed after participants or their guardians provided their written informed consent, and this study has been reviewed and approved by the Ethic Committee of PLA General Hospital. Totally 32 patients, including 13 NM, 6 polymyositis and 5 muscular dystrophy, who admitted at neurology department of PLA general hospital from January 2010 to October 2011, were enrolled in this study. Diagnosis of NM, polymyositis, and muscular dystrophy was based on the com-

bination of clinical data, laboratory examinations and the diagnosis criteria by open skeletal muscle biopsies. General data including age, sex, time of onset, and duration was collected.

Laboratory and pathological examination

Creatine kinase (CK), creatine phosphokinase (CPK), L-lactate dehydrogenase (LDH), glutamic-pyruvic transaminase (GPT) and glutamic-oxalacetic transaminase (GOT) in the serum of the patients were analyzed by automatic biochemical analyzer. Biopsies of the involved muscle of NM patients were performed. Muscle tissues were taken from biceps brachii, deltoids, quadriceps and gastrocnemius with grade 4/5 myodynamia after evaluation by Medical Research Council (MRC). The sites which received EMG check or drug injections in the last 4-6 weeks were avoided. The samples were flash-frozen in precooled isopentane in liquid nitrogen and transferred to cryostat.

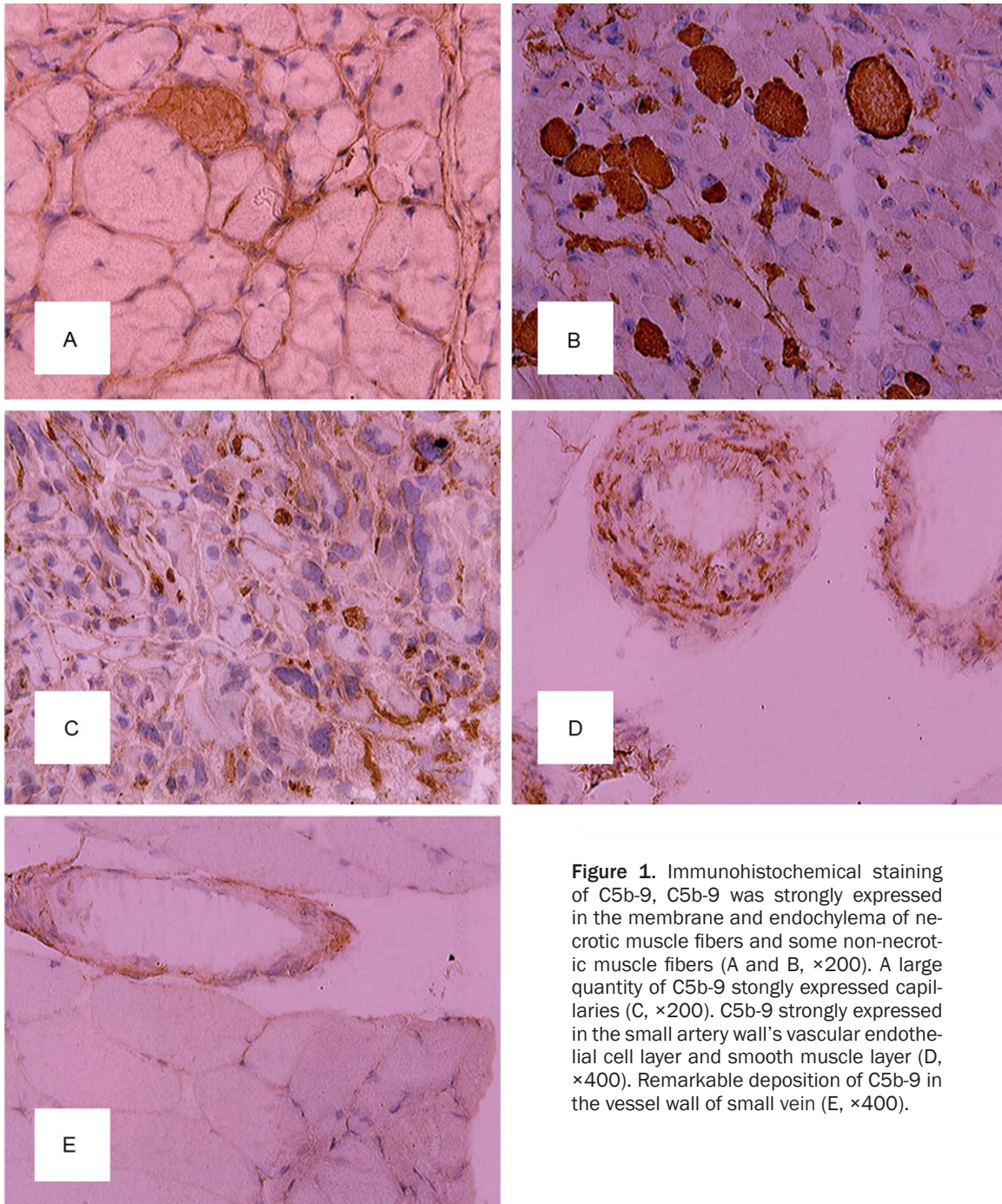


Figure 1. Immunohistochemical staining of C5b-9, C5b-9 was strongly expressed in the membrane and endochylema of necrotic muscle fibers and some non-necrotic muscle fibers (A and B, $\times 200$). A large quantity of C5b-9 strongly expressed capillaries (C, $\times 200$). C5b-9 strongly expressed in the small artery wall's vascular endothelial cell layer and smooth muscle layer (D, $\times 400$). Remarkable deposition of C5b-9 in the vessel wall of small vein (E, $\times 400$).

Immunohistochemical staining of C5b-9

C5b-9 protein in the all of the muscle samples was detected by immunohistochemical staining. Rabbit anti-human C5b-9 polyclonal antibody (Abcam, America, 1:100) was used as primary antibody. Sections of the muscles were dried at room temperature for 20 minutes and incubated with primary antibody for 1 hour. After three times of wash, the sections were

incubated with polymer reinforcing agents and secondary antibody (polymerized HRP anti rabbit IgG) for 30 min. The reaction was then visualized with a 3,30-diaminobenzidine (DAB) kit. Slides were counterstained with hematoxylin, dehydrated with gradient alcohol, and transparented with xylene. After mounting with neutral balsam, the slides were observed under light microscopy (Olympus, Japan). Criteria: Muscular cells containing yellow granulation in the cell

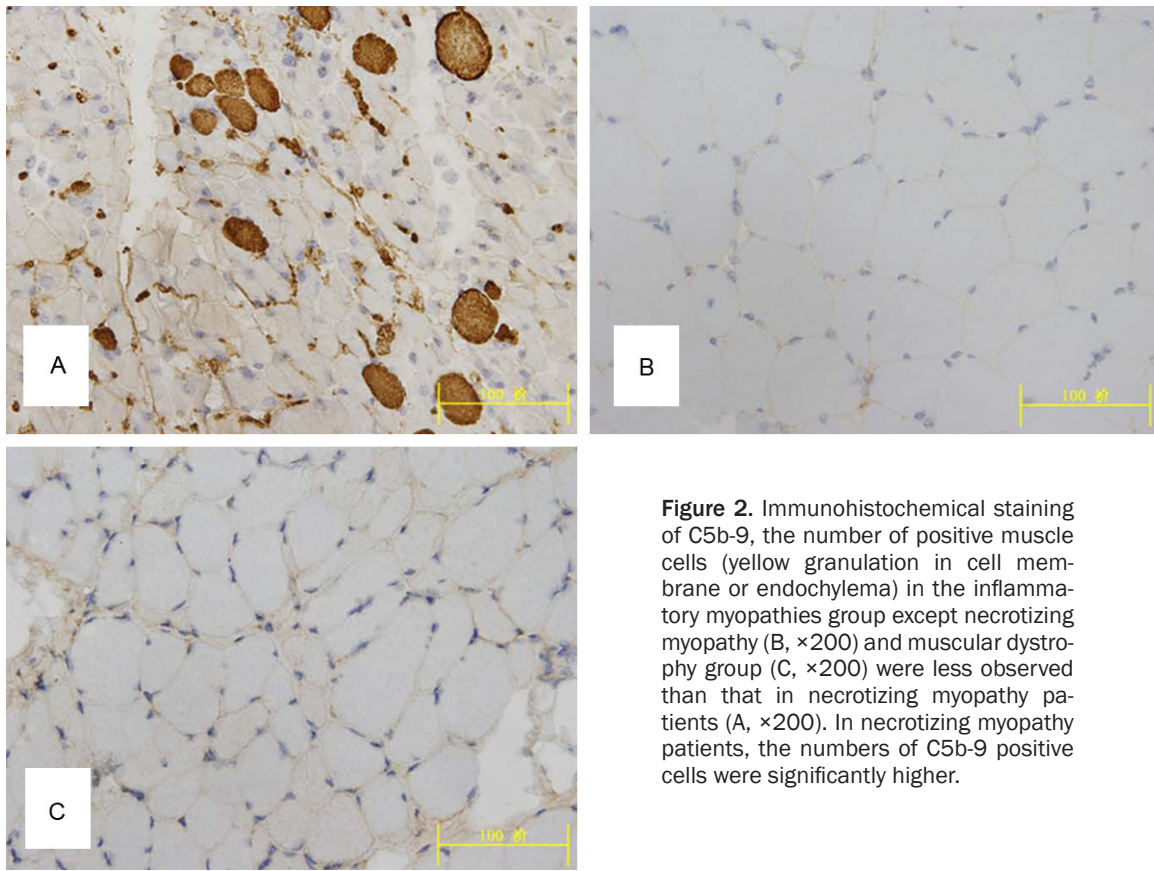


Figure 2. Immunohistochemical staining of C5b-9, the number of positive muscle cells (yellow granulation in cell membrane or endochylema) in the inflammatory myopathies group except necrotizing myopathy (B, $\times 200$) and muscular dystrophy group (C, $\times 200$) were less observed than that in necrotizing myopathy patients (A, $\times 200$). In necrotizing myopathy patients, the numbers of C5b-9 positive cells were significantly higher.

membrane and endochylema, or yellow color of vascular intima were considered as positive reactions [7]. Average optical density (integrated optical density/area) of positive reactions was analyzed with Image-Pro Plus 6.0 software.

Data analysis

SPSS 13.0 statistical software (SPSS Inc., Chicago IL, USA) was used for statistical analysis. Measurement data were expressed as mean \pm SD, and count data were expressed as frequency counts and percentages. After the Bartlett homogeneity of variance, comparison of measurement data was performed by T-test or nonparametric test. All tests were two-tailed and $P < 0.05$ was considered to be statistically significant.

Results

Clinical characteristics of NM patients

Age, sex, time of onset, duration, and level of biochemical enzymes (CK, LDH, GPT, GOT) in the serum of patients with NM, polymyositis,

and muscular dystrophy were displayed in **Table 1**. The 13 NM patients included 8 male (61.5%) and 5 female (38.5%), with the ratio of male to female of 1.6:1. There was no significant difference on onset age between male and female ($t = 1.94$, $P = 0.053 > 0.05$). The 13 NM patients showed symptom of weakness of limbs (84.6%, 11/13), bulbar muscle involvement (23.1%, 3/13), cervical muscle weakness (61.5%, 8/13), and respiratory muscle involvement (23.1%, 3/13). The major symptoms were displayed as weakness of four limbs. Weakness of distal limbs was more obvious than that of proximal limbs. Complain of muscle pain or tenderness and muscle atrophy was recorded in 53.8% (7/13) and 46.2% (6/13) of the patients. The level of CK in the serum ranged from 45 IU/L to 12619.9 IU/L (average: 4678.5 ± 3449.05 IU/L).

C5b-9 expression in the muscle from patients with NM

Muscle specimens of all the 13 NM patients showed scattered cell membrane of the necrotic muscle fibers and strongly expressed C5b-9 in the endochylema (**Figure 1A** and **1B**). The

C5b-9 effect on necrotizing myopathy

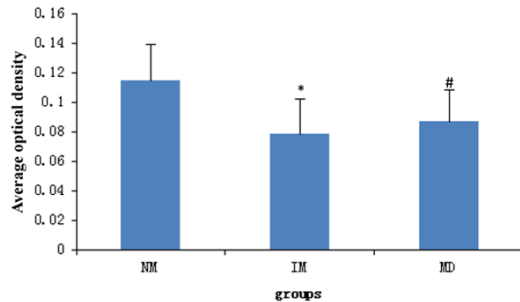


Figure 3. Immunohistochemical staining of C5b-9 of muscle tissues. NM: necrotizing myopathy group (n = 13), IM: inflammatory myopathies group, excluding necrotizing myopathy (n = 6), MD: muscular dystrophy group (n = 5). Muscular cells containing yellow granulation (arrow showing) in the cell membrane and endochylema, or yellow color of vascular intima were considered as C5b-9 positive reactions. The C5b-9 positive areas increased in the NM group in comparison to IM group and MD group. Data are expressed as means \pm SD. $P = 0.038 < 0.05$ in comparison to IM group, $P = 0.013 < 0.05$ in comparison to MD group.

staining intensity of C5b-9 ranged from positive to strongly positive even in the broken particles of muscle fibers. A few non-necrotic fibers also showed weakly positive expression of C5b-9. Immunoreactivity of C5b-9 could be found in blood vessels of muscle tissue of all the NM patients. C5b-9 expressed in vascular endothelial cells and muscular layer of small arteries, veins and capillaries, with different intensity. No expression of C5b-9 was observed in adventitia of the vascular wall and perivascular tissue (Figure 1C-E).

Difference of C5b-9 expression in NM, polymyositis and muscular dystrophy

Positive staining (brown granules) was found in cell membrane and endochylema of muscle tissues from the NM group (Figure 2A), but relatively less in other two groups (Figure 2B and 2C). The average intensity of C5b-9 in positive cells was significantly higher in NM patients compared with those in the polymyositis group ($P = 0.013$, Figure 3, and the muscular dystrophy group ($P = 0.038$, Figure 3). These results suggest that C5b-9 was overexpressed in NM patients.

Discussion

NM is a pathological diagnosis for a group of disorders. The pathogenesis of non-secondary

NM may be associated with immune-mediation. The main clinical features of NM are acute or subacute onset and progressive limb weakness. The main pathological characteristics are a large number of necrotic muscle fibers without significant inflammatory cell infiltration [8]. Serum creatine kinase (CK) is elevated to different degrees. In recent years, many studies have focused on the relationship between elevated serum sign recognition particle antibody (SRP) and NM [9]. To date, the pathogenesis of NM has not been fully elucidated, which may be resulted from a variety of reasons, such as immune-mediation, tumor, infections [10, 11], and application of statin [12]. Both the antigen-antibody reaction and positive expression of cytokines (such as TNF, IL-2) were considered as the evidences to support the opinion that immune abnormalities played a crucial role in the pathogenesis of NM. NM caused by unexplained reasons is more sensitive to immunosuppressant, so it is also called the steroid reactive NM or immune-mediated NM. Maybe the clinical and pathological features are different in secondary and non-secondary NM. However, the pathological change of muscle fiber necrosis is alike. Therefore, it is speculated that there may be a common pathway that involved in muscle fiber necrosis, but until now, the precise mechanism has not yet been reported.

C5b-9, as well as the membrane attack complex, plays an important role in a variety of physiological and pathological process. Soane's investigation released that sub C5b-9 can increase survival ability of oligodendrocytes by up-regulating Bcl-2 protein and inhibiting caspase-3 activation in the demyelination process mediated by inflammation and immune system [13, 14]. Sub C5b-9 shows a positive effect in the antiapoptotic process and promotes the formation of myelin sheath. After sedimentation in the vascular endothelial cells, C5b-9 can induce intracellular exocytosis secretion of vWF, which can mediate the combination of platelet and exposed GBM, as well as play a hemostatic function after depositing in the damaged vessel wall. C5b-9 also participated in the antiviral mechanism (enveloped virus as influenza virus, reverse transcribing virus as HIV) by combining with phosphatide to entirely disintegrate lipid double membrane. This effect is a very important mechanism in lysing envel-

oped virus. C5b-9 may also cause membrane irreversible damage in some specific pathological process. It increases permeability by inserting into the membrane, leading to internal flow of water, ions, and small molecules while macromolecules and proteins are incapable of escaping the cytoplasm. The osmotic pressure of internal and external become unbalanced, activating intracellular signal transduction pathways to promote cell synthesis and the release of certain inflammatory mediators and cytokines, leading to the cells secondary injury even necrosis, which plays a role in the pathogenesis and progression of many diseases. As we all know, the damage to the cell membrane caused by C5b-9 is an important factor which acts as a trigger for a variety of kidney diseases such as glomerulonephritis. Cybulsky's study shows that C5b-9 can form transmembrane ion channels when inserting Glomerular epithelial cell membrane (GEC) [15]. After that, the channel causes Ca^{2+} -inhalation and induces cPLA2-activation. Free arachidonic acid (AA) can be released via deacylation after the combination of cPLA2 and intracellular membranes. Prostaglandin (PG) and thromboxane A2 (TXA2), which can be transformed by AA, play a variety of pathophysiological functions in the occurrence and development of membranous nephropathy, but there was no report about the influence of C5b-9 on the occurrence of NM.

Our study showed that C5b-9 positively expressed in necrotic muscle fibers, atrophic muscle fibers, small arteries, veins and capillaries of muscle tissue of patients with NM. This is consistent with other studies. In 1991, Emslie-Smith firstly proposed the the concept of NM [16]. Immunohistochemical detection of muscle specimens found muscular fibers degeneration, necrosis, and deposition of membrane attack complex. In 2002, Miller reported 7 cases of NM with decreased muscle tissue capillaries and C5b-9 deposition in which [17]. In 2004, Bleeker *et al.* further confirmed C5b-9 deposition in some endomysial capillaries and arteriolar wall around muscle bundles [18]. Previous studies have demonstrated the phenomenon of membrane attack complex deposition in vascular wall of NM, but there is still much controversy and discussion regarding the role of C5b-9 plays in the mechanism of NM. It was speculated that muscle fiber necrosis may be related to antibody-dependent complement-

mediated immune reactions caused by the dissolution of the sarcolemma [19, 20].

Our findings suggest that C5b-9 may play an important role in the pathogenesis of NM. C5b-9 may function as follows: (1) C5b-9 accumulates in the vascular endothelium. Then the permeability of wall is increased and some immune substances ooze out of the wall, leading to stenosis or occlusion of the blood vessel. Finally it results in ischaemia, even the ischemic necrosis of the muscle fibers of the perivascular region. (2) C5b-9 directly goes around or seeps into the muscle fibers, causing destruction of muscle fibers.

This study offers a new vision for the treatment of NM, that is, the purpose of therapy can be achieved by disturbing and preventing the destructive effects of C5b-9, which contributes to muscle fibers and blood vessels. Our observations have led us to make a conclusion that there was no significant correlation between C5b-9 expression intensity and the level of serum CK. This result may not be reliable due to the limited number of cases and the inaccurate quantitation of C5b-9. Further study is needed to explore the relationship between C5b-9 and clinical index by expanding the number of cases and using a quantitative method.

In conclusion, C5b-9 is strongly expressed in the necrotic muscle fiber and blood vessels, and may contribute to the pathogenesis of necrotizing myopathy.

Disclosure of conflict of interest

None.

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