Expression of Protein 4.1R as the distinguishing feature of hyperplastic thymus tissue with myasthenia gravis

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Abstract: Objective: This study was designed to investigate the expressions of protein 4.1R in three different pathological category of thymus in patients with myasthenia gravis and discuss the relevance between the expression of protein 4.1R and the pathogenesis of MG. **Methods:** The expressions of protein 4.1R were detected by RT-PCR and immunohistochemical method in 33 thymus tissues of myasthenia gravis (20 cases of hyperplastic thymus, 7 cases of thymoma, 6 cases of atrophic thymus) and 10 thymus tissues of control. **Results:** It's showed by immunohistochemical staining that protein 4.1R was expressed in thymus tissues and more widely distributed in hyperplastic thymus with the feature of most in the cytoplasm and few in the nucleus of thymocytes. The expression of protein 4.1R in hyperplastic thymus of MG patients was significantly increased compared to the control ($1.55\times10^6\pm4.60\times10^5$ versus $9.79\times10^5\pm1.79\times10^5$, $P<0.05$). RT-PCR showed that 4.1R mRNA was significantly increased in MG thymus tissues contrast to the control (0.76 ± 0.33 versus 0.43 ± 0.39, $P< 0.05$), while no significant difference between the thymoma group and thymic atrophy group (0.84 ± 0.46 versus 0.67 ± 0.10, $P> 0.05$). **Conclusion:** Protein 4.1R can be constitutively expressed in thymus tissue, significantly in hyperplastic thymus. It's suggested that protein 4.1R may act as the distinguishing feature of hyperplastic thymus tissue with MG.

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Introduction

Myasthenia gravis (MG) is one of autoimmune diseases mainly involving acetylcholine receptor (AChR) on the neuromuscular postsynaptic membrane at the neuromuscular junction, mediated by acetylcholine receptor antibody (AChRab), depended on cell-mediated immunity, and with complement participating. It's confirmed not only that the thymus tissue of MG patients is closely related to occurrence of MG[1], but also that different pathogenesis of MG may caused by different thymic pathological patterns[2].

Protein 4.1R was isolated and identified as the first one in 4.1 protein family, found primarily in red blood cells. It's a basic component of spectrin-based membrane skeleton in the red blood cells[3], has an important biological role in the maintenance of normal cell morphology and physical properties**,** relevant to mitosis and the formation of synapses[4]. Protein 4.1R is composed of four domains, that're FERM which is located N-terminal domain (MW30 KD), 16 KD area, spectrin- actin binding domain(SABD, MW10 KD) and conserved carboxy-terminal domain(CTD, MW22 -24 KD)[5]. Kang, et al[6] had found that protein 4.1R could regulate the activation of T lymphocyte negativly by inhibiting LAT protein phosphorylation. In general, protein 4.1R has played a role in immune regulation.

Until now it is not known whether protein 4.1R plays a role in the pathogenesis of MG. Therefore, this study was designed to determine the expression of protein 4.1R in three kinds of pathological types of thymus tissues in patients with MG, and further investigate the relevance between them.

Materials and Methods

Materials

1. Specimen source and group Thymus specimens were taken from inpatients that were confirmed cases in the Second Affiliated Hospital of Zhengzhou University from September 2007 to July 2009. Diagnostic criterias for MG were: ① all patients on admission had typical symptoms of myasthenia gravis and showed volatility, fatigability; ② neostigmine test was positive; ③ the concentration of AChRab in serum should be more than 0.5 nmol/L; ④amplitude attenuation of LF nerve repetitive motion showed greater than 15 % or a single fiber EMG exerted abnormal tremors and blocks; ⑤ preoperative sufferers accepted only daily oral 150 mg ~ 450 mg pyridostigmine bromide. Exclusion criteria were: ① MG patients were also associated with other

autoimmune diseases such as hyperthyroidism, rheumatoid arthritis, etc., or HIV antibody positive; ② Disabled adrenal corticosteroids no longer than 1 month. Enrolled 33 MG patients, whose thymuses were resected by the second intercostal space by transverse sternotomy incision surgery[7], including 13 males and 20 females, aged 10 ~ 39 (27.5 ± 19.8) years. According to the pathological types were divided into: 20 cases of thymic follicular hyperplasia, 6 cases of thymic atrophy, 7 cases of B1 thymoma. 7 cases of Control group, part of whose thymus(pathological examination revealed a normal thymus) was resected in correction of congenital heart disease surgery to exposed to the surgical field, included 3 males, 4 females, mean age(21. 5 ± 19. 5 years), without MG and other autoimmune diseases. By the *t* test and χ^2 test, age and sex among the groups was no significant difference (P>0.05). Above research had been agreed by patients and ethics commission in Zhengzhou University.

2. Main reagents and instruments Trizol Reagent was purchased from Invitrogen Life Technologies Co., Ltd. United States and Prime-ScriptTM RT-PCR Kit and 2 × Taq PCR MasterMix purchased from TaKaRa Biotechnology (Dalian) Co., Ltd.. rabbit anti-mouse 4.1R polyclonal antibodies were gifted from Dr. An Xiuli in New York Blood Center.

Detection expression of protein 4.1R mRNA in thymus by RT-PCR

1. Primer According to reports in GeneBank about nucleotide sequences of protein 4.1 R and *β*-actin, using the Premier 5.0 and Oligo 6.0 software designed primer. The primer sequences of the gene encoding protein 4.1R were designed as follows: forward: 5' – TGCATTACCCTGTCTTTAC - 3' (1148-1167 bp), reverse: 5' – TCTGCTTTCTGAAGAATCTC - 3' (1312-1293 bp), and product size was 463 bp. *β*-actin was selected as the control, and the primer sequences of the genewere designed as follows: forward: 5' – ACACTGTGCCCATCTACGAGG - 3', reverse: 5' – CTTTGCGGATGTCCACGTC - 3', and product size was 409 bp.

2. Detection and quantitative analysis Total RNA in the thymus tissue of MG patients and controls was extracted by Trizol Reagent. The purity and concentration were detected by UV spectrophotometry, and its integrity was checked by 2 % agarose formaldehyde denaturing gel electrophoresis. cDNA was synthesized by reverse transcriptase. Reaction system was always 20 μL, 42 ℃ water bath for 1 h. 25 μL PCR reaction: Protein 4.1R mRNA 25 μL reaction system was make up of cDNA 2 μL, 2Taq PCR MasterMix 12.5 μL, upstream and downstream primer (10 μM) each of the 1 μL, and deionized water used complementally to 25 μL. They were placed on Biometra Tgradient gradient PCR instrument, and the reaction conditions were denaturation at 94 ℃ for 3 min, 94 ℃ for 30 s, 56 ℃ for 1 min, and 72 ℃for 1 min for 35 cycles, 72 ℃ for 5 min. Internal reference *β*-actin primers amplified the program according to above.

3. Semi-quantitative analysis of PCR products After electrophoresis, PCR products were placed in GelDoc 2000TM gel imaging scan image analysis system to measure the absorption peak area of the product, by calculating specimens 4.1R and *β*-actin protein absorption peak area ratio, which was as the relative mRNA expression of protein 4.1R of the specimens.

Detection expression of protein 4.1R in thymus by immunocytochemical staining

1. Sections and staining All samples were fixed in 10 % paraformaldehyde, embedded in paraffin, and thick of slices was 4μm. Sections were strictly operated according to immunohistochemical SP method. Rabbit anti-mouse protein 4.1R polyclonal antibody at concentration of 1: 100 was used as the primary antibody. PBS was used as blank control instead. HE staining was taken as histological control.

2. Method to determine results Image analysis in the unity magnification by light microscopy (10 × 20 times) was performed using Image-Pro Plus 6.0 image analysis software produced by the Media Cybernetics company in U.S. Five horizons in each slice selected by the principle of random sampling, that were entered into the computer as measure fields. The tissue localization of protein 4.1R were observed and integrated optical density (IOD) of each specimen should be calculated[8].

Statistical analysis

Each value was expressed as mean± standard deviation, and a set of two samples was conducted by *t* test using SPSS 11.5 software with test level was that α equaled to 0.05.

Results

The quantification of protein 4.1R mRNA in thymus tissues

As shown in Fig.1, the expression of 4.1R mRNA was significantly increased in thymus tissues of MG group (0.76 ± 0.33) compared to the control group (0.43 ± 0.39, $P < 0.05$). There was no significant difference for expression of 4.1R mRNA between the thymoma group (0.84 ± 0.46) and thymic atrophy group (0.67 ± 0.10, $P > 0.05$) (Tab. 1).

Expression of protein 4.1R in thymus tissue

As shown in Fig. 2，protein 4.1R was widely distributed in thymocytes of cortex and medulla of thymus tissue. Protein 4.1R was expressed mainly in the cytoplasm, fewly in the nucleus of thymocytes.

There was no significant difference for expression of protein 4.1R in thymus between the MG group (2067.5 ±2280.9) and the control group ($9.79 \times 10^5 \pm 1.79 \times 10^5$, $P>0.05$), and the same between the thymoma group (1395.9 ± 2651.9) and the thymic atrophy group (4403.0 ± 3629.3, $P>0.05$). But the expression of protein 4.1R was different between thymic hyperplasia group ($1.55 \times 10^6 \pm 4.60 \times 10^5$) and the control group ($9.79 \times 10^5 \pm 1.79 \times 10^5$, $P<0.05$) (Tab. 1).

Discussion

Myasthenia gravis is one of organ-specific autoimmune diseases characterized by repeated contraction fatigue of striated muscle, mainly involving acetylcholine receptor on postsynaptic membrane of nerve-muscle joints. MG was caused directly by AChRab attacking the endplate membrane, while cell-mediated immunity was also involved[9]. It's unclear that how MG was caused, but it's a consensus that it's related to thymic abnormalities. Thymoma and thymic hyperplasia were two common types of MG thymus pathology. So far, the same clinical symptoms could be caused by different pathologic basis, and it has always been received greater attention that whether there're common factors among them[10,11].

Based on our preliminary studies[12], expression of protein 4.1R in 20 cases of hyperplastic thymus tissue was significantly increased compared to 10 cases of the control group. We reviewed the literature and found no relative reports about protein 4.1R and MG. In this paper, protein levels of protein 4.1R expressed in thymoma group, thymic atrophy group, and control group had no difference, but gene levels were significantly different. It's remarkable that mRNA level of protein 4.1R expressed in MG non-hyperplastic thymus was inconsistent with its protein level, with gene level increased and protein level decreased. It's supposed that protein 4.1R was not involved in the process of MG non-hyperplastic thymus histopathological abnormalities, and its regulation at the post-transcriptional level. In addition, the process of translation of 4.1R mRNA was regulated by several signaling pathway. Breig, et al[13] stated that MEK/ERK signaling pathway did not alter 4.1R pre-mRNA splicing, while activation of MAPK p38 signaling triggered 4.1R pre-mRNA splicing. PI3K, not p38, could regulate 4.1R exon 16 splicing in a promoter-dependent manner. The relationship between protein 4.1R and non-hyperplastic thymus of MG mainly was that it's one of the proteins in the membrane skeleton and had basic functions in the maintenance of normal cell morphology, physical properties and so on. It's suggested that the protein was independent of non-hyperplastic thymus of MG patients.

On the other side, the protein expression level of protein 4.1R in MG hyperplasia thymus groups was obviously higher than the control group, and protein 4.1R was widely distributed in thymocytes of cortex and medulla of thymus tissue. It's pointed that protein 4.1R involved in the whole process of development, differentiation and maturation of thymocyte. Protein 4.1R was expressed mostly in the cytoplasm and fewly in the nucleus of thymocytes, and this kept in accordance with the report of Perez-Ferreiro, et al[14] that protein 4.1R is a key protein in the formation of microtubule structure in T lymphocyte interphase. We had found that the expression of protein 4.1R of thymic hyperplasia group was different from the control group, while no significant difference between the MG group and the control group, the thymoma group and the thymic atrophy group. It's suggested that protein 4.1R was not the common factor leading to the same symptoms of MG with different pathological types, only closely related to occurrence of MG hyperplastic thymus. We could speculate that abnormal hyperplastic thymus tissue may caused by thymocyte excessive proliferation probably on account of microtubule architecture during mitosis affected by high expression of protein 4.1R. Perhaps high expression of protein 4.1R could interfere with the stability of the membrane skeleton of normal thymus, affect the formation and function of immune synapse, lead to abnormal thymocytes, thus promote or maintain clinical symptom of MG.

So far, it's found that protein 4.1R could interact with multiple membrane signalling proteins, such as the membrane-associated guanylate kinase (MAGUK)[15], the Na/K-ATPase and the Na/Ca exchanger NCX1[16], G-Protein coupled receptors, voltage-gated and ligand-gated channels[17]. Indeed, the range of critical interactions of protein 4.1R was reflected in disease relationships that include hereditary anaemias, tumour suppression, control of heartbeat and nervous system function[18]. These studies provided us references for further studing the mechanism of protein 4.1R with MG. Therefore, protein 4.1R presumably plays an important role in the process of thymic dysplasia with MG, the machanism of it's function still remains to be confirmed.

In our research, expression of protein 4.1R could act as the distinguishing feature of hyperplastic thymus tissue with MG. However, we need more studies for signal transduction function of protein 4.1R in MG hyperplastic thymus tissue in order to understand the role in causing the thymus histological abnormalities and investigate the relevance between abnormality of the protein and occurrence of MG.

Table 1. Gene and protein expression of protein 4.1R in different pathological types of thymic tissue

Groups		*n*	Protein 4.1 R immunohistochemical stained integral	Protein 4.1 R mRNA
Control group		10	$9.79\times10^5 \pm 1.79\times10^5$	0.43 ± 0.39
Myasthenia gravis group		33	2067.5 ± 2280.9	$0.76 \pm 0.33^{**}$
	Thymic hyperplasia group	20	$1.55\times10^6 \pm 4.60\times10^5$ *	1.01 ± 0.37
	Thymoma group	7	1395.9 ± 2651.9	0.84 ± 0.46
	Thymic atrophy group	6	4403.0 ± 3629.3	0.67 ± 0.10

Note: "*" means $P = 0.016 < 0.01$, and "**" means $P = 0.033 < 0.05$.

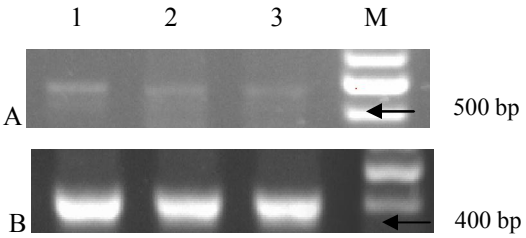

Fig. 1 The relative expression of protein 4.1mRNA in thymus. A: 463bp RT-PCR product of protein 4.1R by agarose gel electrophoresis; B: 409bp RT-PCR product of β-actin by agarose gel electrophoresis; 1: Thymoma group; 2: Thymic atrophy group; 3: Control group; M: DNA Ladder

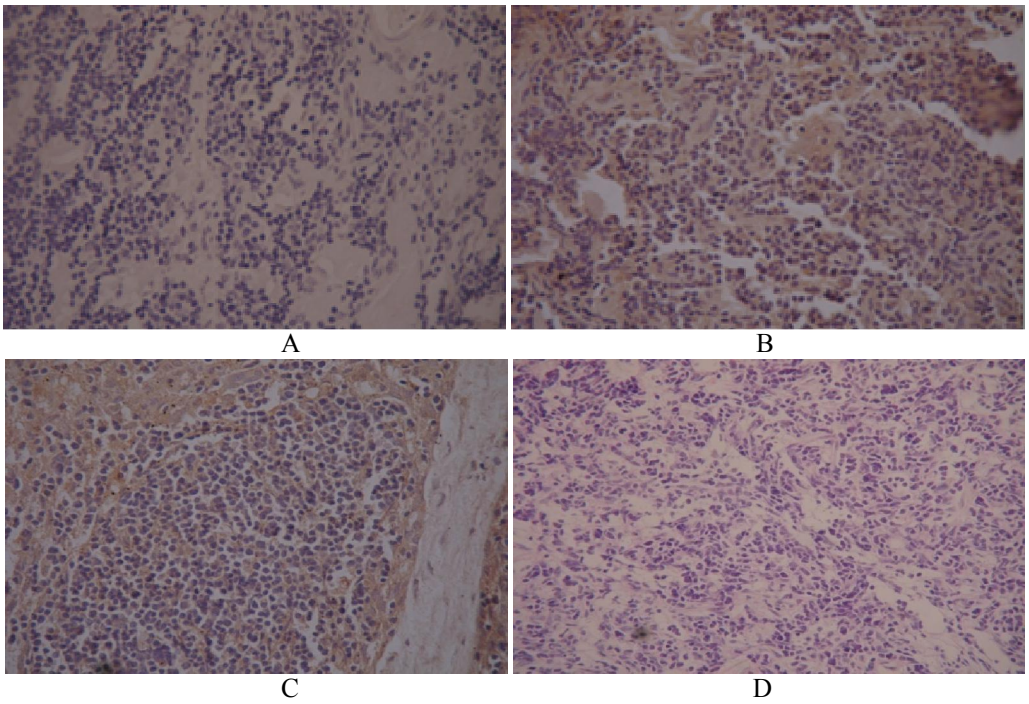

Fig. 2 Protein 4.1R expression in thymus tissue. A: Control group; B: Thymic hyperplasia group; C: Thymoma group; D: Thymic atrophy group

Note: The brown particles were seen in thymocyte cytoplasm, with yellow particles in thymocyte nucleus, when magnification was 10×20. So that protein 4.1R was expressed mainly in the cytoplasm, fewly in the nucleus of thymocytes.

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