

Blood serum 48 kDa form of unconventional myosin 1c characterizes the early stage of multiple sclerosis

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In accordance with the modern ideas of multiple sclerosis (MS) B-lymphocytes play a significant role in the pathological process development. In this regard, it is relevant to search for new biomarkers of B-lymphocytic origin, which can reflect the clinical features of this disease.

Aim of study – to assess the relationships between the level of blood serum 48 kDa form of unconventional myosin 1c (48 kDa Myo1c) in patients with multiple sclerosis and stage of this disease, its severity and type.

Materials and methods. 1 ml of blood serum was diluted 2-fold with phosphate buffer saline and then trichloroacetic acid (TCA) was added to 10 % of final concentration. The supernatant containing TCA-soluble compounds was isolated and mixed with acetone. Then, centrifugation and electrophoresis in the presence of sodium dodecylsulphate were performed. The 48 kDa Myo1c was identified by its molecular weight comparing after Coomassie Brilliant Blue G staining of gel and Western blot analysis using polyclonal anti-Myo1c rabbit antibodies.

Results. The level of the 48 kDa Myo1c was significantly higher in the MS patients compared with that in healthy controls. The disease duration was shorter in patients with high level of the 48 kDa Myo1c, compared to patients with low level of the 48 kDa Myo1c. High level of the 48 kDa Myo1c was associated with a relapsing-remitting MS, while low level – with secondary progressive type of the disease. In the group with the low 48 kDa Myo1c level a disability rate was significantly higher, unlike in patients with the medium level of 48 kDa Myo1c.

Conclusions. An increased blood serum level of the 48 kDa Myo 1c in MS patients is combined with the early stage of the MS when its diagnostics is the most complicated.

Ключові слова:

розсіяний склероз, біомаркери, неконвенційний міозин 1с, клінічний перебіг, рання стадія.

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48 кДа форма неконвенційного міозину 1с у сироватці крові характеризує ранню стадію розсіяного склерозу

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Відповідно до сучасних уявлень про розсіяний склероз (РС), істотну роль у розвитку патологічного процесу відіграють В-лімфоцити. У зв'язку з цим важливо шукати нові біомаркери В-лімфоцитарного походження, що можуть показувати клінічні особливості цього захворювання.

Мета роботи – оцінити співвідношення між рівнем 48 кДа форми неконвенційного міозину 1с (48 кДа Myo 1c) у сироватці крові хворих на розсіяний склероз і стадією цього захворювання, його тяжкістю та типом перебігу.

Матеріали та методи. 1 мл сироватки крові розводили у 2 рази фосфатним буферним розчином, після чого додавали трихлороцтову кислоту (ТХО) до 10 % кінцевої концентрації. Надосадову рідину, що містила ТХО-розчинну фракцію, виділяли та змішували з ацетоном. Виконували центрифугування та електрофорез за наявності додецилсульфату натрію. 48 кДа Myo 1c ідентифікували за його молекулярною масою після фарбування гелю Coomassie Brilliant Blue G та Вестерн-блот аналізу з використанням поліклональних анти-Myo 1c антитіл кролика.

Результати. Рівень 48 кДа Myo 1c був значно вищим у хворих на РС порівняно зі здоровими донорами. Тривалість захворювання була меншою в пацієнтів із високим рівнем 48 кДа Myo 1c порівняно з низьким рівнем 48 кДа Myo 1c. Високий рівень 48 кДа Myo 1c асоціювався з рецидивно-ремітуючим РС, а низький – із вторинно прогресуючим типом перебігу захворювання. У групі з низьким рівнем 48 кДа Myo 1c інвалідність була значно вищою, на відміну від пацієнтів із середнім рівнем 48 кДа Myo 1c.

Висновки. Підвищений рівень 48 кДа Myo 1c у сироватці крові хворих на РС поєднується з ранньою стадією захворювання, коли діагностика РС є найскладнішою.

Ключевые слова:

рассеянный склероз, биомаркеры, неконвенционный миозин 1с, клиника, ранняя стадия.

48 кДа форма неконвенционного миозина 1с в сыворотке крови характеризует раннюю стадию рассеянного склероза

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В соответствии с современными представлениями о рассеянном склерозе (РС), значительную роль в развитии патологического процесса играют В-лимфоциты. В связи с этим имеет значение поиск новых биомаркеров В-лимфоцитарного происхождения, которые могут отражать клинические особенности этого заболевания.

Цель работы – оценить соотношение между уровнем 48 кДа формы неконвенционного миозина 1с (48 кДа Myo 1c) в сыворотке крови больных рассеянным склерозом и стадией этого заболевания, его тяжестью и типом течения.

Материалы и методы. 1 мл сыворотки крови разводили в 2 раза фосфатным буферным раствором, а затем добавляли трихлоруксусную кислоту (ТХУ) до 10 % конечной концентрации. Надосадочную жидкость, содержащую ТХУ-растворимые соединения, выделяли и смешивали с ацетоном. Затем выполняли центрифугирование и электрофорез в присутствии додецилсульфата натрия. 48 кДа Myo 1c идентифицировали по его молекулярной массе после окрашивания геля Coomassie Brilliant Blue G и Вестерн-блот анализа с использованием поликлональных анти-Myo 1c антител кролика.

Результаты. Уровень 48 кДа Myo 1c был значительно выше у пациентов с РС по сравнению со здоровыми донорами. Длительность заболевания была короче у пациентов с высоким уровнем 48 кДа Myo 1c по сравнению с пациентами с низким уровнем 48 кДа Myo 1c. Высокий уровень 48 кДа Myo 1c ассоциировался с рецидивирующе-ремиттирующим РС, а низкий уровень – со вторично прогрессирующим типом заболевания. В группе с низким 48 кДа Myo 1c уровень инвалидности был значительно выше, в отличие от пациентов со средним уровнем 48 кДа Myo 1c.

Выводы. Повышенный уровень 48 кДа Myo 1c в сыворотке крови больных РС сочетается с ранней стадией заболевания, когда диагностика РС является наиболее сложной.

Introduction

Multiple sclerosis (MS) is an inflammatory neurodegenerative disease that is strongly dependent on the immune effects, particularly targeting the myelin coatings [1]. Its development can lead to the neurological disability, especially in young people. MS is a significant personal and social issue responsible for major outlays in the public health [1]. The MS etiology stays poorly understood and both genetic and non-genetic factors (for example, viral infection or vitamin D deficiency) are presumed.

At MS autoantibodies to certain components of central nervous system (CNS) are formed. The potential antibody targets include the myelin autoantigens (including myelin basic protein, myelin oligodendrocyte glycoprotein, proteolipid proteins, myelin-associated glycoprotein), oligodendrocytes (cyclic nucleotide phosphodiesterase, transketolase, transaldolase), neurons and axons (neurofilaments, tubulin, neurofascin), astrocytes (potassium channel KIR4.1), miscellaneous cellular ion channels (anoctamin 2), ubiquitous autoantigens (nuclear proteins, heat-shock proteins), and microbial (e.g., viral) antigens [2].

The inflammation, demyelination, re-myelination and scar formation in glia affect both white and grey matter of brain and spinal cord, and they are the main causes for neurodegeneration in MS [2]. Time-dependent scenario of the MS development starts from the immune cells of the peripheral blood that cross the blood-brain barrier (BBB) in order to reach the CNS where the neural injuries appear in sites of the acute inflammation, finally leading to disability symptoms [1]. The MS-related autoimmunity is switched on mostly by T-lymphocytes, causing the inflammatory processes in the CNS and initiating impairments there [3]. It has recently been proved that not only the myelin targeting Th1 and Th17 CD4+ T cells, but also B cells, CD8+ T cells, macrophages and natural killers participate in the MS pathogenesis [3].

The MS diagnostics is rather complicated and quite lengthy. MS is a disease with varying clinical manifestations, radiological features, disease course and therapeutic response. Therefore, the individualized management of patients with MS is problematic. In routine clinical practice it is also difficult to obtain cerebrospinal fluid (CSF) samples for the diagnostic purposes. This is due to the procedure invasive character and thus, the probability of adverse effects such as post-lumbar puncture headache, back discomfort or pain, bleeding or brainstem herniation. In consequence, there is an urgent need for identification of clinically useful blood serum biomarkers which assist the MS

diagnosis, predicting prognosis, monitoring disease course and evaluating individual treatment response.

Recently, we used the trichloroacetic acid (TCA)-induced precipitation/extraction and the MALDI-TOF/TOF mass-spectrometry that allowed us to identify earlier unknown blood serum 48 kDa form of the human unconventional myosin 1c isoform b (48 kDa Myo 1c) in MS patients [4]. The myosin 1c (molecular mass 121.7 kDa with basal isoelectric point at pH 9.5) belongs to the unconventional class I myosins and these proteins can make a link between the plasma membrane and the microfilaments in vertebrates [5,6]. In addition to the function of cytoskeleton organizer that is important for cell motility via spreading, the unconventional myosins can assist in the glucose transporter recycling, endocytosis and transcription processes in the nucleus. In murine B-lymphocytes the myosin 1c is located at the plasma membrane, namely in microvilli, participating in the peripheral processes that are important for the activated B cells mobility [5]. It is supposed that its domains can interact with the inter-cellular adhesion molecule 1 (ICAM-1) and Major Histocompatibility Complex class II (MHC-II) that plays a critical role in cell-cell interactions in the immune response [6]. Thus, the Myo1c may be a part of the molecular motor whose principal elements are specific membrane receptors that interact with the adhesion molecules and signaling proteins of cellular microvilli [5]. Due to the interaction with the MHC-II via lipid rafts and associated molecules, the Myo1c can also participate in the antigen presentation. So, the Myo1c induces the immunologic synapse formation between T-cells and antigen-presenting B-cells [5].

In our previous work [4] we focused on the methodology of the 48 kDa Myo1c identification in blood serum of patients with autoimmune diseases.

The aim

The aim of this work was to assess the relationships between the 48 kDa Myo1c level in blood serum of the multiple sclerosis patients and stage of this disease, its severity and type.

Material and methods

All samples were collected under the approval of the Bio-Ethics Committee of Danylo Halatsky Lviv National Medical University, Lviv, Ukraine (Protocol № 2 dated 15 February 2016) in accordance with the regulations of the Ministry of Health Protection of Ukraine. A documented consent

was obtained from each patient included in the study and the informed consent form was approved by the Bio-Ethics Committee of Danylo Halytsky Lviv National Medical University.

61 patients (mainly women, 70.5 %, $n = 43$) with MS diagnosed according to the 2010 revised McDonald diagnostic criteria for MS participated in this examination. Control group consisted of 20 clinically healthy people with the mean age of 33.2 ± 2.2 years. The inclusion criteria for patient enrollment in the study were: age from 18 to 60 years, the presence of clinically definite MS according to the 2010 McDonald criteria and patient's consent to participate in the study. The exclusion criteria were: age less than 18 and more than 60 years, presence of comorbidities and administration of disease-modifying treatment (DMT) (cytostatics/steroids) last 6 months, pregnancy.

Patients' age varied from 19 to 57 years with the mean age of 36.6 ± 1.4 years. The majority of patients (82.0 %, $n = 50$) had a relapsing-remitting MS and the other (1.6 %, $n = 1$) had primarily progressive MS; 4.9 % patients ($n = 3$) had the MS debut and 11.5 % ($n = 7$) patients had secondary progressive MS. The mean age of the disease debut was 29.0 ± 1.2 years. The average disease duration was 7.6 ± 0.9 years. Patient disability rate varied from 1.5 to 6.5 points with the mean level of 3.7 ± 1.4 points.

The following methods of investigation were used: clinical – analysis of complaints, life and disease history, neurological examination and neurological impairment evaluation with the Expanded Disability Status Scale (EDSS); laboratory – the immune-biological blood test (determination of the Myo1c level).

Blood sampling from peripheral vein was performed and serum was prepared according to the stipulated diagnostic protocol [4]. 1ml of blood serum was diluted 2-fold with the phosphate buffer saline (PBS), and then 100 % TCA was added to 10 % of final concentration. After 30 min of incubation on wet ice (0°C) the sample was centrifuged at 10.000 g for 15 min.

The supernatant containing TCA-soluble compounds was isolated and mixed with cooled acetone in 1:6 ratio followed by 18 h incubation at -20°C . The precipitate was pelleted by centrifugation (10 min at 10.000 g) and TCA-extracted proteins of blood serum underwent electrophoresis in 12% polyacrylamide gel supplemented with 0.1 % sodium dodecylsulfate (SDS). The presence of the 48 kDa Myo1c was identified by its molecular weight comparing after 0.05 % Coomassie Brilliant Blue G staining of gel and detection by Western-blotting using polyclonal anti-Myo1c (N-terminal region) rabbit antibodies (AVIVA SYSTEM BIOLOGY, product number ARP56292) as described earlier [4]. To measure the amount of 48 kDa Myo1c Coomassie-stained electrophoregrams were scanned and digitalized by special computer programs Gel-Pro Analyzer and Media Cybernetics (L.P.). The level of 48 kDa Myo1c was quantified by the calibration curve that was obtained after digitalization of stained strips of bovine serum albumin with known amount of protein used as a standard. The 48 kDa Myo1c level of $0.1 \mu\text{g/ml}$ was the minimum reliable detection limit in our methodology.

All MS patients were divided into 3 groups depending on the 48 kDa Myo1c blood serum level. The division of patients into groups by the 48 kDa Myo1c level was performed first

and foremost to analyze the relationship between very high and low levels of this protein and clinical characteristics of MS. Therefore, in the first and the largest group of patients the 48 kDa Myo1c level was $<0.1 \mu\text{g/ml}$ (low level; Group 1). This group included individuals with 48 kDa Myo1c level below the error of determination. The investigated protein concentration in the 2nd group was $0.1\text{--}8.0 \mu\text{g/ml}$ (medium level; Group 2), and in the 3rd group – $>8 \mu\text{g/ml}$ (high level; Group 3).

Statistical analysis. The normality of the distribution was tested using the Shapiro-Francia test which showed a normal (Gaussian) character of data distribution. Differences between groups were evaluated using the Student's test. P values <0.05 were considered as statistically significant. Data were statistically processed using the StatsDirect 3, Ver. 3.1.7 software package (StatsDirect Ltd, UK, № 04399867).

Results and discussion

The 48 kDa Myo1c level was found to be significantly higher in the MS patients ($3.4 \pm 1.6 \mu\text{g/ml}$; Group MS) when compared with healthy controls ($0.3 \pm 0.1 \mu\text{g/ml}$; Group CT) ($P < 0.05$) (Fig. 1).

In the MS patients with high level of the 48 kDa Myo1c ($>8 \mu\text{g/ml}$; Group 3) the disease duration was significantly shorter (3.6 ± 1.1 years) compared to patients with low level of the 48 kDa Myo1c ($<0.1 \mu\text{g/ml}$; Group 1) – 8.1 ± 1.2 years ($P < 0.05$) (Fig. 2).

Besides, the patients of the Group 3 with high level of the 48 kDa Myo1c more often demonstrated a relapsing-remitting MS than the patients of the Group 1 with low level of the 48 kDa Myo1c – $100.0 \pm 0\%$ versus $78.6 \pm 6.3\%$ ($P < 0.1$). At the same time, in the patients of the Group 1 with low level of the 48 kDa Myo1c, the secondary progressive type of MS was diagnosed significantly more often ($p < 0.05$) than in patients with high level of the 48 kDa Myo1c ($14.3 \pm 5.4\%$ versus $0 \pm 0\%$) (Fig. 3).

In the patients of the Group 2 with medium level of the 48 kDa Myo1c ($0.1\text{--}8 \mu\text{g/ml}$) a disability rate was significantly ($P < 0.05$) lower (3.2 ± 0.3 EDSS points) comparing with MS patients of the Group 1 with low level of the 48 kDa Myo1c (4.0 ± 0.2 EDSS points) (Fig. 4).

There were no significant differences in the level of 48 kDa Myo1c in different groups of the MS patients divided by gender, the age of disease onset, the total number of relapses and the number of relapses during the last 3 years (data not shown).

Although the exact origin of the 48 kDa Myo1c stays poorly understood, we suggest that its high level in blood serum indicates the intensified process of autoimmune cells elimination, particularly the activation-induced cell death (AICD). The activated lymphocytes that are known to produce cytokines and perform other effector functions die by the mechanism of apoptosis in which Fas (death receptor) and its ligand (FasL) participate [7]. While the quiescent lymphocytes express only Fas, after stimulation these cells also express FasL, which interacts with the Fas, that induces apoptosis via the receptor-dependent caspase-8 activation [7]. This statement accords with the MS pathogenesis at the early stage when the immune pathological processes launch in the peripheral blood [8]. The activated autoreactive

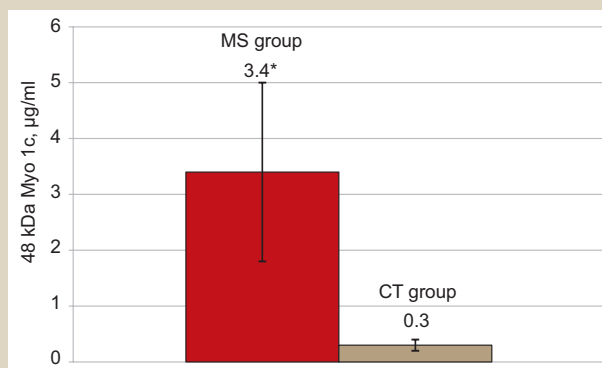


Fig. 1. Comparison of the 48 kDa Myo1c levels in TCA-extracted proteins of blood serum in MS Group (n = 61; MS patients) and CT Group (n = 20; healthy human controls).

*: P < 0.05

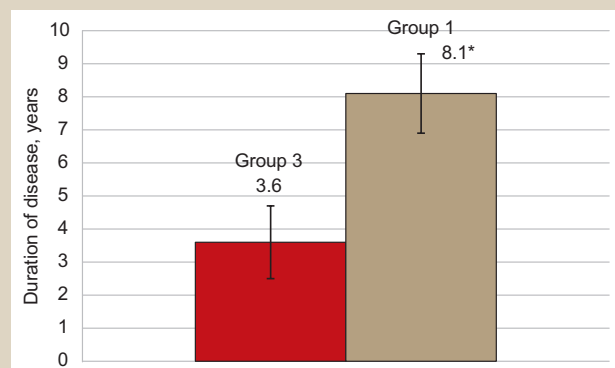


Fig. 2. Comparison of MS duration in patients of the Group 3 (48 kDa Myo1c >8 µg/ml, n = 6) and patients of the Group 1 (48 kDa Myo1c <0.1 µg/ml, n = 42).

*: P < 0.05

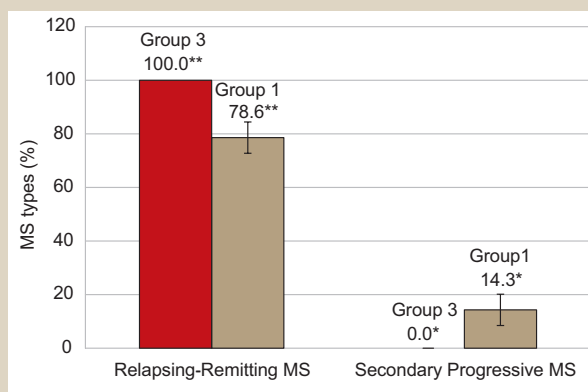


Fig. 3. The expression of MS types in patients of the Group 3 (48 kDa Myo1c >8 µg/ml, n = 6) and patients of the Group 1 (48 kDa Myo1c <0.1 µg/ml, n = 42).

*: P < 0.05; **: P < 0.1.

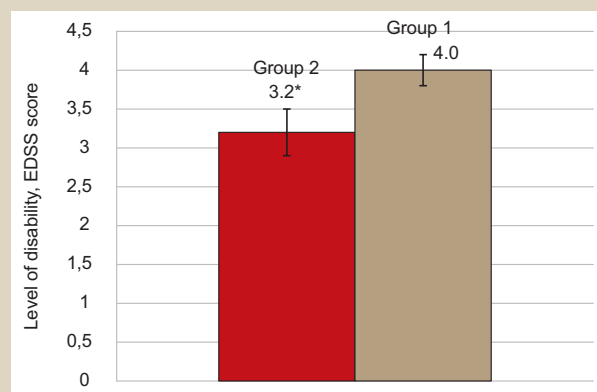


Fig. 4. Comparison of disability level in MS patients of the Group 2 (48 kDa Myo1c 0.1-8 µg/ml, n = 13) and patients of the Group 1 (48 kDa Myo1c <0.1 µg/ml, n = 42).

*: P < 0.05

immune cells damage the blood-brain barrier and get into the central nervous system [8]. Simultaneously, the anti-inflammatory processes including the AICD are initiated [8].

We suppose that the 48 kDa Myo1c is the product of peripheral blood autoreactive immune cells, mainly lymphocytes [5,6]. This hypothesis might be confirmed by the fact that high level of the 48 kDa Myo1c is detected exclusively at the early stage of the disease when so-called "peripheral blood period" takes place. On the contrary, when the pathological process starts to dominate in the CNS and moderates in the peripheral blood, the disease obtains a progressive course that is displayed by a decrease in blood serum 48 kDa Myo1c in patients. We assume that low 48 kDa Myo1c level at the late stage of the disease reflects a reduced death of activated lymphocytes – the inhibition of protective apoptosis over the time in MS [9].

A significant correlation between the 48 kDa Myo1c increased level in blood serum of MS patients and the early stages signs of disease (short disease duration, relapsing-remitting MS type and patients' disability low level) proves a possibility of existing link between the 48 kDa Myo1c level and the MS development. The advantages of blood serum 48 kDa Myo1c determining in MS patients is an opportunity to diagnose the early stage of disease when its diagnostics is the most difficult, but at the same time, the disease-modifying treatment is the most effective. Besides, this

test using eliminates the requirement for lumbar puncture.

The mechanism of some modern disease-modifying treatment action is based on targeting B-cells [10]. In particular, these drugs can selectively deplete the immune cells [10]. Therefore, the 48 kDa Myo1c could be an indicator of this treatment effectiveness.

The MS is also called a "chameleon disease" due to a unique diversity of its clinical symptoms that can imitate a variety of other illnesses [10]. That is why, a search for new MS biomarkers is extremely important. It is particularly reasonable at the early stage of MS when the disease diagnosis is the most difficult [10].

Conclusions

1. The 48 kDa Myo1c, a probable degradation product of activated B-lymphocytes, may reflect the apoptosis activity in these lymphocytes and therefore, to determine the clinical course of MS.

2. An increased level of the 48 kDa Myo1c in blood serum of MS patients was combined with the MS early stage, when its diagnostics is the most complicated, but treatment is the most effective.

3. The reduction of the 48 kDa Myo1c level was connected with the course of disease transition to a secondary progression stage, that characterized by

the dominance of neurodegeneration processes over demyelination.

Further investigation of the 48 kDa Myo1c role in the MS pathogenesis is highly relevant. "Peripheral blood stage" is the first one in the process of disease development and subsequent studies of the activation-induced cell death are on the way. We concluded that the AICD acceleration in the autoimmune cells could slow or even stop the pathological process at the MS peripheral stage before it severely affects the CNS.

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