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## Identification of a 48 kDa form of unconventional myosin 1c in blood serum of patients with autoimmune diseases

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## ABSTRACT

We searched for protein markers present in blood serum of multiple sclerosis (MS), rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients in comparison to healthy human individuals. We used precipitation/extraction methods and MALDI TOF/TOF mass spectrometry, and identified a protein with Mr ~46 kDa as a fragment of human unconventional myosin IC isoform b (Myo1C). Western blotting with specific anti-human Myo1C antibodies confirmed the identity. Screening of blood serum samples from different autoimmune patients for the presence of Myo1c revealed its high level in MS and RA patients, relatively low level in SLE patients, and undetected in healthy donors. These data are suggesting that the level of p46 Myo1C in blood serum is a potential marker for testing of autoimmune diseases.

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## 1. Introduction

Blood serum has been extensively explored as a source of markers, as it may contain not only blood proteins *per se*, but also proteins originating from all tissues of the body [1,2]. It is estimated that up to 10,000 different proteins (and/or its fragments) may be present in the blood serum, and most of them are in very low concentrations [3]. Selection of a protein preparation, and especially enrichment procedures, may aid in successful search for markers [2,3].

Autoimmune diseases are characterized by auto destructive processes. Initiation of these processes may differ for different autoimmune diseases, but they may also include similarities related to execution mechanisms of cells and tissues degradation. Therefore, it is possible that different autoimmune diseases may share even presentation of markers. Multiple sclerosis (MS) is an

inflammatory disease in which the myelin sheaths around axons of brain and spinal cord are damaged, thus, leading to demyelination and scarring [4]. This results in a wide range of symptoms. Mechanisms of MS have autoimmune character, due to cellular and humoral immune reactions towards brain tissue self-antigens, mainly the myelin basic protein (MBP) [5]. The key feature of the MS patients is the formation of inflammatory sites in brain tissue, which may lead to presence of tissue proteins leaking into the blood [4,5]. RA and SLA also characterized by inflammation and tissue demerged which can elevate of amount of cellular proteins in blood serum [6,7]. For purification and concentration of proteins for MALDI TOF/TOF a 2,2,2-trichloroacetic acid (TCA)-induced protein precipitation is frequently used [8,9], although in our hands TCA-extracted proteins remain out of the study.

We attempted to identify the proteins in TCA-extracted fraction of blood serum of MS, RA and SLA patients versus normal human donors and to estimate their potential diagnostic value.

Here, we report identification in human blood serum of unconventional myosin Ic (Myo1C) that is ubiquitously expressed in vertebrates. We propose that this protein could serve as a potential marker for MS, RA and SLE autoimmune disorders.

Abbreviations: MS, multiple sclerosis; PA, rheumatoid arthritis; SLE, systemic lupus erythematosus; NHD, normal healthy donors

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## 2. Materials and methods

### 2.1. Human blood serum samples

Serum was obtained from the peripheral blood of 28 MS patients (diagnosed according to the McDonald diagnostic criteria for MS), 12 SLE patients (diagnosed according to ACR criteria for SLE), 12 RA patients (diagnosed according to ACR/EULAR Rheumatoid Arthritis Classification Criteria) and of 12 healthy volunteers. The samples were collected under the approval of Bio-Ethics Review Board of the Danylo Halytsky Lviv National Medical University in accordance with the regulations of the Ministry of Health of Ukraine. A documented consent was obtained from all patients included in the study, and the form of the informed consent was approved by the Bio-Ethics Review Board of the Danylo Halytsky Lviv National Medical University.

### 2.2. Preparation of TCA-soluble proteins

According to our protocol, 1 ml of blood serum was diluted in 2-fold with phosphate buffer saline (PBS), and then 100% TCA was added to 10% of final concentration. After 30-min incubation at  $-20^{\circ}\text{C}$ , the solution was centrifuged for 15 min at 10,000 g. The supernatant containing TCA-soluble compounds was isolated and mixed with acetone in 1:6 ratio, followed by incubation at  $-20^{\circ}\text{C}$  for 18 h. The precipitate was pelleted by centrifugation for 10 min at 10,000 g. The pellet was diluted in distilled water and protein concentration was measured at 280 nm using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, USA). The solution was stored at  $-20^{\circ}\text{C}$  until use.

### 2.3. SDS-electrophoresis and Western-blot analysis

SDS-electrophoresis in 12% PAG [10] of blood serum TCA-extracted proteins was followed by gel staining with Coomassie Brilliant Blue G. For Western blot analysis, proteins were transferred from the gel onto a nitrocellulose membrane. The membranes were blocked (1 h at  $20^{\circ}\text{C}$ ) with 5% non-fat milk in the PBS buffer containing 0.05% Tween-20. The blots were washed with PBS-Tween-20, three times for 5 min each, and then probed with the specific Abs. To identify 46 kDa polypeptide in a pool of TCA-extracted proteins of blood serum, polyclonal anti-MYO1C (N-terminal region) rabbit antibody (AVIVA SYSTEM BIOLOGY, product number ARP56292) in 1:1,000 dilution was incubated overnight at  $4^{\circ}\text{C}$ . Corresponding secondary antibodies were used for ECL detection. Generated images were scanned, digitalized and images were quantified using ImageJ software.

### 2.4. MALDI-TOF mass spectrometry

Individual electrophoretic protein bands were excised from the gel and subjected to the in-gel trypsin digestion as was described earlier [11]. To identify the proteins, their tryptic digests were subjected to peptide mass fingerprinting (PMF) followed by post-source decoy (PSD) fragmentation. MALDI TOF/TOF mass spectrometry was performed on an Ultraflex instrument (Bruker, Germany). Samples were mixed with alpha-cyano-4-hydroxy-cinnamic acid, and loaded on a metal target plate. Mass spectrum was collected in the positive mode, i.e. for  $\text{MH}^+$  ions. Internal calibration was performed with tryptic peptides generated upon auto-digestion of trypsin added to samples (842.51, 1045.56 and 2211.10 Da). Collected mass spectra were used for searches with Profound tool of NCBI nr database. Search criteria were as follow: no limitations of species and pI, mass selection as for 10–100 kDa,  $\text{MH}^+$ , complete alkylation of Cys, partial oxidation of Met, one missed cleavage by trypsin, and tolerance of 0.5 Da. Selected main

peaks were subjected to PSD by LIFT technology developed by Bruker and available for Ultraflex instruments. Fragmentation spectra were collected, and delta masses of observed fragments were used for calculation of the sequence.

### 2.5. Statistical analysis

Significance of the difference in a typical experiment was assessed by Student's *t*-test. The level of significance was set at 0.05. Three levels of significance were used  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ .

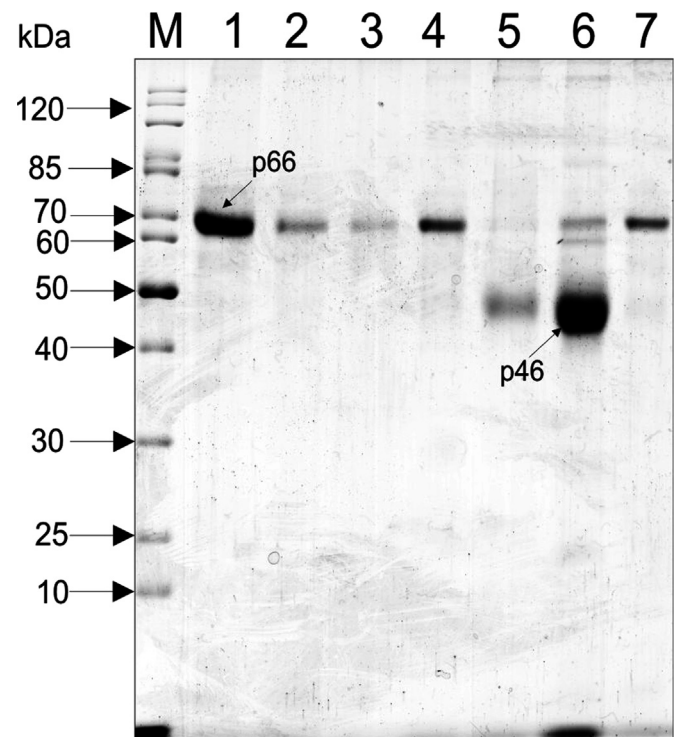
## 3. Results and discussion

### 3.1. TCA-extracted blood serum proteins and their characterization

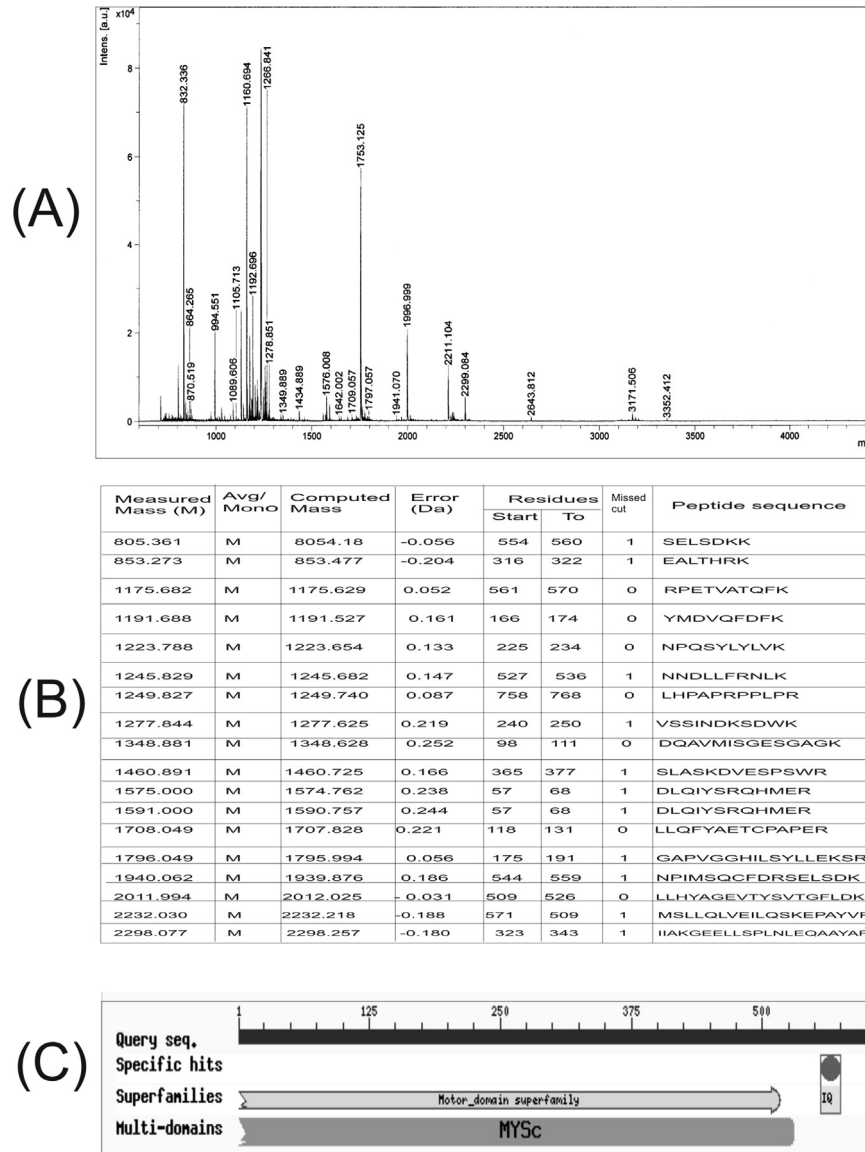
For isolation of the TCA-extracted proteins from blood serum, a procedure including treatment of blood serum by 10% trichloroacetic acid, pelleting of the insoluble debris by centrifugation and following precipitation of the soluble compounds with acetone in 1:6 ratio were used. Fig. 1 shows a typical SDS-PAG electrophoretic profile of the acetone precipitated proteins. We observed that the TCA-soluble fractions contained proteins with the molecular mass of 66 kDa (Fig. 1, lanes 1–7) and 46 kDa (Fig. 1, lanes 5, 6).

### 3.2. Identification of MyoC1-like protein in a pool of TCA-extracted blood serum proteins

We focused further on identification of detected proteins, which are shown in Fig. 1. To identify the polypeptides with molecular mass of 66 kDa and 48 kDa, MALDI TOF/TOF mass spectrometry analysis based on peptide mass fingerprinting was used.



**Fig. 1.** A typical SDS-electrophoresis in 12% PAG of TCA-extracted proteins isolated from blood serum of multiple sclerosis patients. M, molecular masses protein standards. Lanes 1–7, the samples of TCA-extracted proteins. Arrows point at the polypeptides which were subjected to MALDI-TOF mass spectrometry.

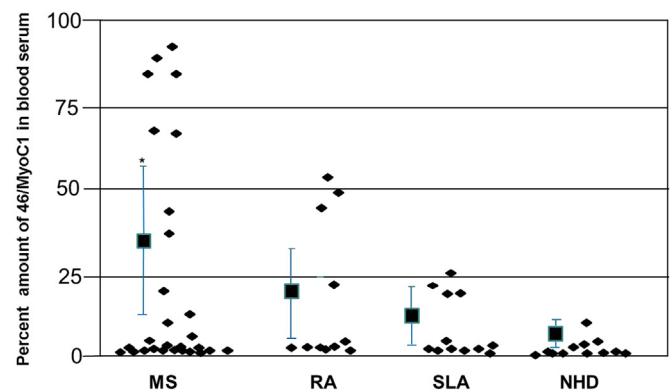


**Fig. 2.** A full scan of matrix-assisted laser desorption/ionization mass spectrometry of the 48 kDa polypeptide. A, Mass spectrum of the protein p46. The whole spectrum is shown. B, Results of search using mass list of p46 with ProFound search tool. Note that Z-value of 2.43 indicates highly significant identification. C, Homology search using results of identification of p46 with BLAST tool confirmed homology to myosin. BLAST search results image is shown.

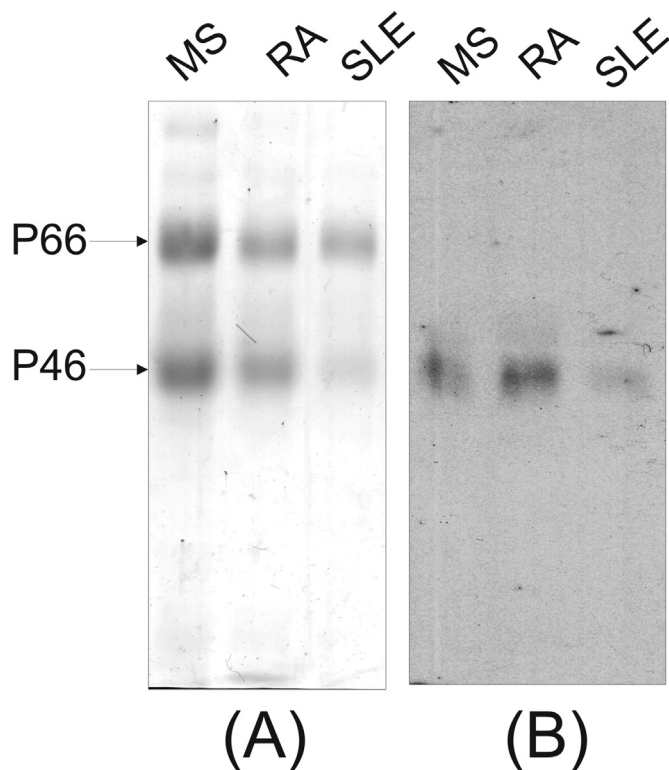
We found that a 66 kDa protein has a similarity with the blood serum albumin (data not shown), and therefore we did not continue its study. A result of 46 kDa protein identification is shown in Fig. 2. This protein showed high similarity to a non-conventional human myosin IC (accession number EAW90619.1).

### 3.3. Enhanced expression of p46/unconventional Myo1C in blood serum of MS, RA and SLE patients

To explore whether the identified fragment of unconventional Myo1C in blood serum of MS patients was indeed enriched in the blood serum of patients with other autoimmune disease, we additionally checked the TCA-extracted fractions isolated from blood serum of MS patient (27 species), RA patient (12 species), SLE patient (12 species) and 12 samples obtained from normal human donors. Quantification of data of electrophoretic analysis allowed estimating the relative amount of a 46 kDa protein in different blood serum species (Fig. 3). High level of detected Myo1C was observed in the blood serum of multiple sclerosis and rheumatoid arthritis patients, while relatively low concentration of that



**Fig. 3.** The distribution within different ranges of the levels of Myo1C in different human blood serum samples. MS, multiple sclerosis ( $n=27$ ); RA, rheumatoid arthritis ( $n=11$ ); SLE, systemic lupus erythematosus ( $n=12$ ); NHD, normal human donors ( $n=12$ ).



**Fig. 4.** Detection of Myo1C in the TCA-extracted proteins of samples isolated from blood serum of multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and normal human donors (NHD). (A), SDS electrophoresis in 12% PAG stained with Coomassie G. (B), Western-blotting using a rabbit polyclonal antibodies against N-terminal region of Myo1C (Aviva System Biology, product number ARP56292). Arrows in A, point to the positions of p66 and p46 proteins.

protein was found in blood serum of SLE patients and it was very low in healthy donors (Fig. 3).

To check whether the identified in blood serum 46 kDa protein indeed corresponds to Myo1C, we performed immunoblotting study. TCA-extracted samples of blood serum of MS, RA, and SLE patients containing highest amount of 46 kDa protein were subjected to SDS-electrophoresis, followed by western blotting with anti-human Myo1C antibody specific to synthetic peptide directed to N-terminal part of human Myo1C (Fig. 4). It was found that anti-human Myo 1C antibody interacts with a 46 kDa polypeptide of TCA-extracted fraction from blood serum of MS, RA, and SLE patients. This data additionally proved relationship between the 46 kDa polypeptide and the human Myo1c.

The results of proteome analysis of blood serum clinical samples are of potential diagnostic significance and can also lead to identification of drug targets [1–3]. To address a search of novel molecular markers of the Multiple sclerosis (MS), we have studied TCA-extracted proteins obtained from blood serum of 28 MS patients as well as 12 RA patients, 12 SLE patients and 12 healthy human donors. A procedure including separation of TCA-insoluble proteins from TCA-soluble proteins was applied. TCA-soluble proteins were pelleted with acetone and their characterization was performed by SDS-electrophoresis, mass-spectrometry and Western-blot analysis. It was found that TCA-soluble fraction of blood serum of the MS patients mostly contained blood serum albumin, and 46 kDa protein whose MS-sequence and Western blotting demonstrated strong structural similarity with human unconventional myosin 1c isoform b-Myo1C (NM 001080950.1).

High concentration of albumin in human blood serum could serve as the most likely cause of its appearance in the pool of TCA-extracted proteins. In contrast to this, our attempts to find any published data about the presence of Myo1C or its fragments in

human blood serum were not successful. Thus, we firstly detected a new protein, belonging to subclasses of cellular unconventional Myo1C in blood serum of the autoimmune patients (MS, RA and SLE), while blood serum of normal human donors was generally lacking such a protein marker.

It is known that Myo1C (molecular mass 121.7 kDa with basal isoelectric point 9.5) is a member of the unconventional class I myosins of vertebrates, which directly links plasma membrane with the microfilament cortical web [12,13]. This myosin is implicated in a panoply of cell functions such as cytoskeleton organization, cell motility, and nuclear transcription. Myo1C is abundantly expressed in murine B lymphocytes, and it is preferentially located at plasma membrane, e.g. microvilli. It was shown that the Myo1C is involved in the cytoskeleton dynamics and membrane protein anchoring or sorting in B lymphocytes [12]. High cationic charge of Myo c1 could serve of explanation for its presence in TCA-extracted pools extraction from Nets in blood serum with high acidic solution.

Origination of the 46 kDa form of Myo1c protein in human blood serum remains unknown. Here, we can offer two possible scenario of its appearance in blood serum. In first case, rising level of a Myo1C fragment in blood serum of patients with autoimmune disorders is result of apoptosis of different lymphoid cells in bloodstream during treatment procedure. Hence, the level of 46 kDa fragment could be tightly associated with blood cells degradation. In this scenario one could also expect the presence of significant amounts of Myo1C in the control blood samples which was not the case.

In second scenario, elevation of the 46 kDa fragment of Myo1C in blood serum of autoimmune patients could be caused by increase of NETosis. NETosis is a distinct form of active cell death of neutrophils, which is characterized by a release of decondensed chromatin and granular contents to the extracellular space [14]. NETs were shown to ensnare and kill microbes [15]. Experimental evidence suggests that NETs participate in pathogenesis of autoimmune and inflammatory disorders [16]. Exaggerated NETosis or diminished NET clearance likely increases risk of autoreactivity to NET components. Nets as are extracellular structures composed of chromatin, granule, and other proteins including cytoskeleton proteins [17]. Nets also contain protease 3 which can be engaged in restricted proteolysis of NETs component [17] including full form of unconventional Myosine. We suggest that 46 kDa proteins is a proteolytic fragment of Myo 1C, amount of which in blood serum could serve as a quantitative marker of NETosis in human bloodstream.

#### 4. Conclusions

For the first time, we isolated from human blood serum a 46 kDa protein identified as N-fragment of unconventional myosin 1C (isoform\_b). Elevation of the amount of this protein in human blood serum can be associated with the autoimmune disorders.

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#### Appendix A. Transparency document

Transparency document associated with this article can be

found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2015.12.001>.

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