

The Reaction of Placental GRP78 Protein with Sera from Women with Multiple Sclerosis

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ABSTRACT

Background: Multiple Sclerosis (MS) with four different types is one of the well studied autoimmune diseases of the central nervous system. Generally, two-thirds of MS patients are females who are at risk of pregnancy-related complications. Inappropriate responses of mother's immune system, such as antibody production against placental proteins, may lead to pregnancy-related disorders. The association between pregnancy complications and some autoantibodies including anti-phospholipid and anti-angiotensin II type-1 receptor antibodies are clear examples in this regard.

Objective: To investigate the probable placental antigens that might be targeted by the antibodies in the sera of MS patients. **Methods:** Total placental proteins were extracted from normal fresh placentas and were separated using two-dimensional gel electrophoresis (2-DE) technique. The separated proteins were transferred onto a Polyvinylidene Fluoride (PVDF) membrane and blotted with the pooled sera of MS women or healthy controls (20 individuals in each group). The differentially blotted spot was identified by mass spectrometry and confirmed by western blot technique.

Results: The results indicated that the women afflicted with MS had an antibody against placental HSP70kDa protein 5 (GRP78). **Conclusion:** In the present study, a new placental autoantigen candidate, which was targeted by antibody present in MS women sera, was found. The clinical importance of this finding regarding pregnancy complications in MS patients should be investigated by further experiments.

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Keywords: Anti-GRP78 Antibody, Glucose-Regulated Protein 78kDa, Heat Shock 70kDa protein family, Multiple Sclerosis, Placenta, Pregnancy

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INTRODUCTION

Multiple Sclerosis (MS) is the most common autoimmune disease of the central nervous system. This disease has four different types that the relapsing-remitting MS (RRMS) is the most common one with the frequency of 85% (1). The incidence of the disease is 2.5 to 3.6 per 100,000 population globally each year and the prevalence of the disease is increasing worldwide (2,3). Many studies which were conducted in different provinces of Iran indicated that the disease prevalence was intermediate to high according to Kurtzke geographical distribution and followed an ascending trend (4,5). Although cell-mediated immunity is the main mechanism of the disease pathogenesis, humoral immunity, and antibody production by B cells can also play an important role (6,7). In line with the role of B cells, several autoantibodies against different proteins including myelin basic protein, myelin oligodendrocyte glycoprotein, proteolipid protein peptide, Heat Shock Protein (HSP) 60, and phosphoglycerate mutase-1 have been reported in MS patients, which can be used as biomarkers for the disease (8,9). Moreover, using interferon beta 1a and 1b, as a treatment, could induce production of different antibodies in MS patients, such as anti-mitosomal antibodies and anti-thyroglobulin antibodies (10). Interestingly, in case of pregnancy, Boskovic and his colleagues revealed that using interferon beta in the first trimester of pregnancy could lead to miscarriage (11). Several other studies also presented a two-sided relation between MS and pregnancy (12,13). During pregnancy, placental tissue develops as a unique temporal organ that expresses a diverse group of functional proteins, such as phosphoglycerate mutase-1 and Neurokinin B. These proteins are involved in different processes, such as angiogenesis, apoptosis, and immune system responses (14-16). Inappropriate responses of mother's immune system, such as autoantibody production, may lead to pregnancy-related complications (17). The best autoantibodies associated with pregnancy complications are Anti-Phospholipid Antibodies (APAs) and Angiotensin II Type 1 receptor Autoantibody (AT1-AA). These autoantibodies are significantly associated with the incidence of recurrent miscarriage and pre-eclampsia (18,19). Generally, two-thirds of MS patients are female and most of them are at childbearing ages (3,20). Despite finding of several autoantibodies in MS patients' sera and the relation between MS and poor outcomes of pregnancy, no study has investigated the probable placental antigens that might be targeted by these antibodies. Considering the production of several antibodies in MS patients mostly women at childbearing ages and the important role of placenta tissue in the pregnancy period, the present study aimed to investigate the probable placental antigens that might be targeted by antibodies in the sera of MS women using immunoproteomics technique.

MATERIALS AND METHODS

Serum Samples. The research protocol was approved by the local Ethics Committee of Shiraz University of Medical Sciences, and written informed consents were taken from all the cases and controls. Blood samples were taken from 20 non-pregnant women (mean age: 31 ± 8.2 years) diagnosed with RRMS based on the Macdonald criteria (21). For all of the patients, Expanded Disability Status Scale (EDSS score) was determined by the same neurologist. At the time of sampling, the patients' mean EDSS score was 1.23. All of the women were new MS cases without a history of previous and current

treatment for MS and they had at least one pregnancy history before the diagnosis of MS. Blood sampling was done in relapse and attack phase of the disease. Also, 20 age-matched healthy women (mean age: 30 ± 7.2 years) without any history of pregnancy complications or autoimmune diseases, but with a positive history of at least one successful delivery were enrolled into this study as the control group. It should be noted that none of the cases and controls was pregnant at the time of sampling and there were at least 6 months interval between the last delivery and sampling. Moreover, after sampling, the sera were separated from the blood samples and were kept at -70°C until the time of experiments.

Placenta Samples. In this study, 5 normal full-term placenta samples (39-40 weeks) were taken and pooled from the healthy women who had elective cesarean sections. The method, which previously described by Gharesti-Fard and his colleagues was used for placenta sampling (22). Briefly, 5 different areas of placenta were punched and pooled immediately after section. To reduce blood contamination, the tissues were washed with cold saline, transferred to liquid nitrogen, and kept at nitrogen tank until use.

Protein Extraction and 2D-PAGE. Frozen placenta samples were removed from nitrogen tank, homogenized, and lysed with lysis buffer, as described previously (22). The total protein concentration was measured by means of 2-D Quant kit (Amersham, Uppsala, Sweden). The extracted proteins were aliquoted and stored at -70°C . Linear precast 18 cm IPG strips (pH 3-10 and pH 4-7, Bio-Rad, USA) and IPGphor III isoelectric focusing system (Pharmacia, Uppsala, Sweden) were used for isoelectric focusing. Active rehydration of IPG strips was performed at 60 V for 16 h (100 μg protein, 2% IPG buffer, 2% DTT, 8 M urea, and 0.001% bromophenol blue). The strips were focused at the total voltage of 50–55 kV. Then, two steps of equilibration were performed using equilibration buffer. Finally, the strips were put on top of a 15% SDS-PAGE gel and the second dimension was done using a twin gel electrophoresis system (SCIE-PLAS, Cambridge, UK).

Immunoblotting. After performing 2-D electrophoresis, the proteins were transferred from gels to PVDF membranes by means of a semi-dry blotter system (Pharmacia, Uppsala, Sweden). Then, the membranes were blocked using 3% bovine serum albumin in Tris Buffered Saline (TBS buffer) containing 0.05% Tween 20 (pH=7.5). The blocked membranes were incubated with the pooled sera from 5 cases or controls (at room temperature for 2 hours). Subsequently, the membranes were washed with TBS buffer (five times each for five min). After that, anti-human whole immunoglobulin horseradish peroxidase-conjugated antibody (Abcam, AL-Ain, UAE) was added to the membranes and incubated for an additional one hour. Then, the membranes were re-washed for five times and developed using Sigma Fast Diaminobenzidine (DAB) tablets (Sigma, Steinheim, Germany). Based on the blotted pattern, only IPG strips with pH levels between 4-7 were used for further steps.

Identification of Blotted Spots on 2D-PAGE Gel. The location of the one differentially blotted spot that was only detected in all the four membranes from the MS patients, was compared to its location on the 2D gel stained with colloidal Coomassie Brilliant Blue (CBB) G-250 based on the method described by Neuhoff *et al.* (23). The targeted spot was manually excised from the gel and identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/TOF) technique. To ensure the result, identification of the spot was re-checked using another 2D stained gel. Mass analysis was performed in Sir Henry Wellcome Functional

Genomics Facility, University of Glasgow by using 4700 MALDI-TOF/TOF Proteomics Analyser instrument (Applied Biosystems, United Kingdom).

Western Blot Analysis for Confirming the Mass Result. To confirm the Mass results, western blot technique was done using a monoclonal antibody against HSP70 kDa protein5 (GRP78) (Abcam, AL-Ain, UAE). In doing so, two 2D gels (pH:4-7) were simultaneously run. The first one was stained with colloidal CBB (G-250), while the second one was transferred to the PVDF membrane and blotted with mAb to GRP78 protein. Finally, the location of the blotted spot on the membrane was compared with current 2D stained gel and the location of excising spot on gel explained in the previous section.

RESULTS

Immunoreactivity of MS Patients' Sera with Placental Antigen.

Several spots were observed in membranes blotted with sera from MS patients and healthy women. Comparing four PVDF membranes blotted with pooled sera of MS patients with four PVDF membranes blotted sera of healthy women, indicated that only one reproducible blotted spot was observed in all blotted membranes of MS patients, but not in any blotted membranes of healthy controls. The location of this spot was determined by comparing the blotted spot location on membranes, with two 2D gels stained with Coomassie blue. The corresponding spot was manually excised from one of the gels and sent for mass analysis (Fig. 1).

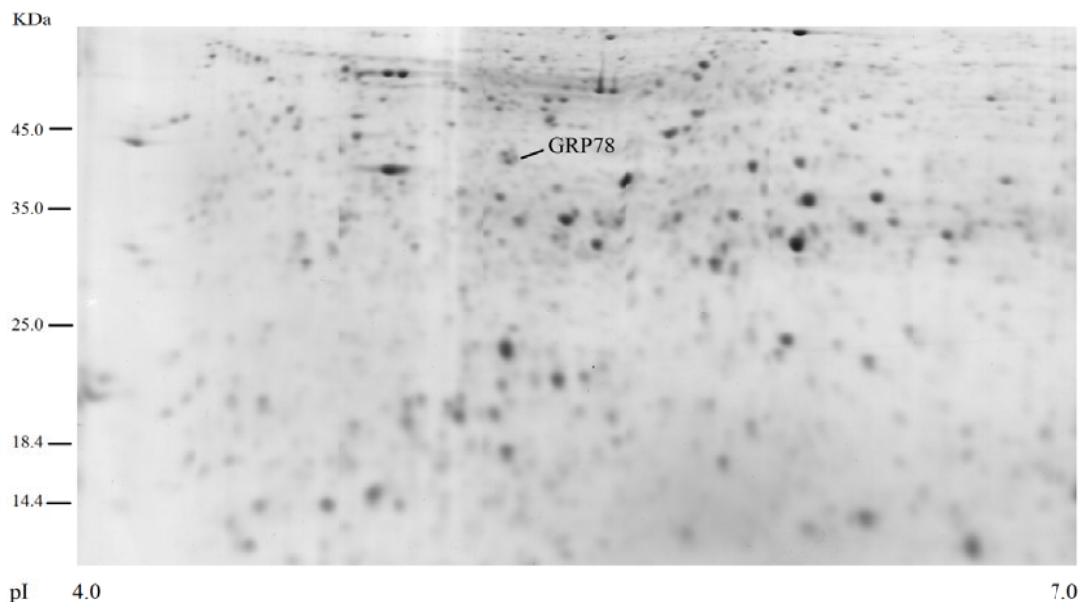


Figure 1. A 2 Dimensional Coomassie brilliant blue stained gel of placental proteins. Total human placental proteins were separated using 2 DE technique. The first dimension was performed using linear precast 18 cm IPG strips (pH: 4-7), while 15% SDS-PAGE gel was used for the second dimension. By comparing this stained gel to the PVDF membranes blotted with the patients' or controls' sera, the target spot was selected, excised from the gel, and sent for Mass analysis.

Characterization of Target Spot.

After identification of the target spot, MALDI-TOF/TOF mass analysis was performed to characterize it. The result indicated that the picked targeted spot in both samples was heat shock protein 70 kDa protein 5 (GRP78). Data of Mass analysis are shown in Table 1.

Table 1. Characteristics of the targeted spot picked from Coomassie blue stained gel and identified by MALDI-TOF/TOF mass technique.

Protein name	Mr ^a	pI ^a	Score ^b	Peptides ^c	Match peptides	Association no.
Human 70kDa Heat Shock Protein 5 ATPase domain (BipGRP78)	45.2	5.8	192	14	VMEHFIK	GI:259090349
					ALSSQHQAR	
					VEIANDQGNR	
					DAGTIAGLNVMR	
					TFAPEEISAMVLTK	
					NGRVEIANDQGNR	
					ITPSYVAFTPEGER	
					KSDIDEIVLVGGSTR	
					TKPYIQVDIGGGQTK	
					NQLTSNPENTVFDAK	
					IINEPTAAAIAAYGLDKR	
					NQLTSNPENTVFDAKR	
					VTHAVVTVPAYFNDAQR	
					KVTHAVVTVPAYFNDAQR	

a: Experimental/Mass (KDa) or pI, b: Protein scores greater than 86 were considered to be statistically significant ($p < 0.05$), c: Number of matched peptides.

Validation of Mass Result by Western Blot.

Western blot with mAb against GRP78 protein confirmed the mass result. Two 2D gels were simultaneously run; one was stained with colloidal CBB (G-250), while the second one transferred to PVDF membrane and blotted with mAb to GRP78 protein. The interaction between anti GRP78 mAb and placental GRP78 is represented in Fig. 2.

DISCUSSION

MS is the most common autoimmune disease of the central nervous system. Although cell-mediated immunity is the main mechanism of the disease pathogenesis, humoral immunity and B cells are also important players in the MS pathogenesis. The presence of several autoantibodies, such as anti-phosphoglyceratase 1, anti-neurokinin B, anti-thyroglobulin, and anti-mitochondrial antibodies, has been reported in MS patients (8-10). Sakurai and his colleagues revealed that MS patients had an antibody against a member of the HSP70 family (HSP70s) named HSP70-9 (7). HSPs are a family of proteins that are produced by various cells in response to stressful conditions. HSP70s are conserved molecules, which are found in both prokaryotes and eukaryotes. Different members of HSP70 family are expressed by eukaryotes in different cellular compartments and production of most of them is inducible. However, some family

members including heat shock cognate, have a constitutive expression (24). These proteins can be regularly found in the cytosol, but they have secretory and membrane bound forms, as well. Many cytosolic forms are considered to act as chaperones that 'proofread' structures and conformations of other proteins and inhibit apoptosis (25,26).

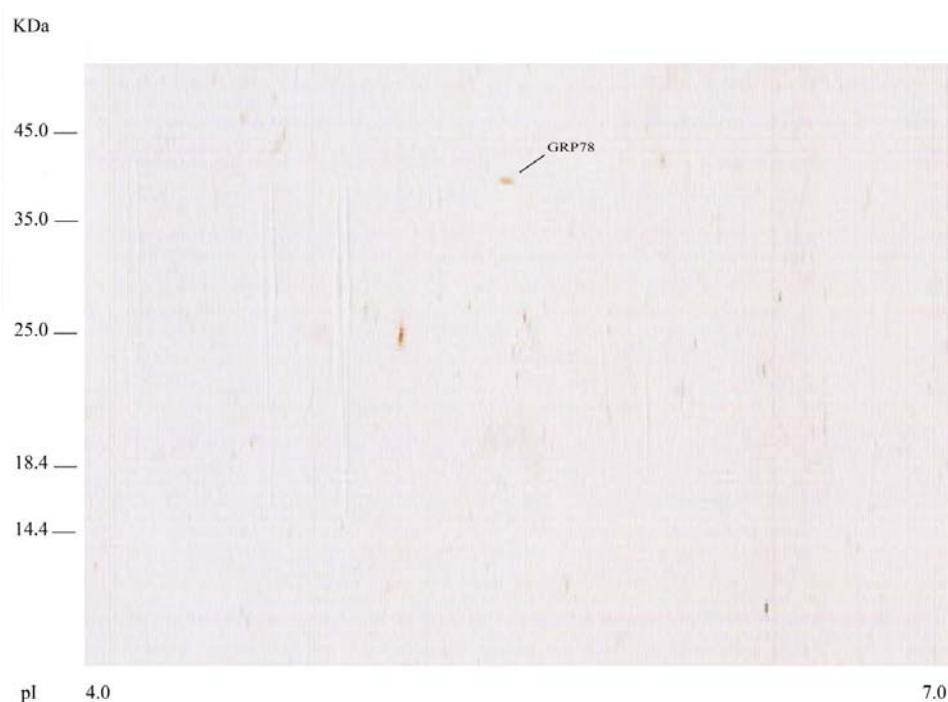


Figure 2. Western blot analysis of placental proteins using monoclonal antibody against GRP78. Total human placental proteins were separated using 2DE technique, transferred to the PVDF membrane, and blotted with mAb to GRP78 protein. The arrow indicates GRP78 location.

Besides, membrane-bound and secretory forms are believed to engage in immune system responses (26,27). Several experiments have revealed that extracellular HSP70s can shift Dendritic Cells (DCs), monocytes, and Myeloid-Derived Suppressor Cells (MDSCs) toward a tolerogenic phenotype. These tolerated cells can also affect the adaptive immune system, promote the production of anti-inflammatory cytokines, such as IL-10, and diminish inflammatory cytokines, such as TNF- α or IFN- γ (28-31). Heat shock 70 kD protein 5 (HSPA5) that known as Glucose Regulated Protein 78 (GRP78) or Binding immunoglobulin Protein (BiP), is a member of HSP70 family located in Endoplasmic Reticulum (ER) with chaperonic functions. Similar to other HSP70s, GRP78 also has secretory and membrane-bound forms with different roles (32,33). Previous experiments showed that GRP78 can resolve inflammation and render an anti-inflammatory profile to Peripheral Blood Mononuclear Cells (PBMCs), such as production of IL-10 and IL-1 receptor antagonists (34,35). Monocytes treatment with BiP results in lower expression of CD86 co-stimulatory molecule and down regulation of HLA-DR molecules (34). GRP78 can also modulate DCs differentiation, causing them to express Indoleamine 2, 3-dioxygenase (IDO) and develop tolerogenic

characteristics with low level of co-stimulatory molecules, which is in favor of development of regulatory T lymphocytes (36). Considering that all these anti-inflammatory and immune modulatory mechanisms are important in maintaining tolerance toward self-antigens, it can be hypothesized that presence of anti-GRP78 antibody may interfere with its normal immune modulatory functions and contribute to autoimmune diseases. Presence of GRP78 protein as an autoantigen and importance of anti-GRP78 antibody in the context of different autoimmune and inflammatory diseases, such as rheumatoid arthritis and type 1 diabetes, are documents for this hypothesis (37-39). Also, as different members of a protein family have high sequence homology, cross reactivity of the antibody to other members of HSP70 family members should be kept in mind. Additionally, identification of different antibodies present in sera of MS patients can be useful for designing a diagnostic antibody panel for characterizing the disease. For example, Quintana and his colleagues demonstrated that patients with RRMS had higher levels of antibodies against HSP60 and HSP70 compared to those with secondary progressive or primary progressive form of the disease (40).

On the other hand, the GRP78 molecule is expressed in the placenta tissue and on cytoplasmic membrane of trophoblast cells and confers an aggressive phenotype to trophoblast cells during pregnancy (41). Besides, the presence of anti-GRP78 antibody is important in some pregnancy complications including pre-eclampsia (42,43). Considering placental expression of the GRP78 protein and its functions, this issue comes to mind that the presence of anti-GRP78 antibody in women afflicted with MS may be important during their pregnancy and might be associated with some pregnancy complications. Interestingly, Chen *et al.* and Kelly *et al.* showed that MS disease was associated with higher rate of some pregnancy complications like Intrauterine Growth Retardation (IUGR) and preterm birth (12,13). Thus, further experiments are recommended to investigate the clinical significance of anti-GRP78 antibody in pregnant women suffering from MS. In addition, different studies have shown that GRP78 protein is expressed on the cell membranes of endothelial cells (44) and anti-GRP78 augments the pro-coagulant activity of tissue factors, causes thrombosis, and accelerates atherosclerotic plaque formation (45,46). It could be imagined that anti-GRP78 antibody in MS patients may cause some pregnancy complications, which are linked to abnormal placental vascularization if the GRP78 molecule is expressed on the cell membranes of placental endothelial cells. It is also worth noting that other experiments need to define the exact epitopes recognized by anti-GRP78 antibody.

In conclusion, the results of the present study indicated that the women afflicted with MS have an antibody, which reacts with placental GRP78. The clinical importance of this finding regarding MS disease and its probable association with pregnancy complications should be assessed by further studies.

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