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Autoantibodies as diagnostic biomarkers for the detection and subtyping of multiple sclerosis

Cassandra DeMarshall[a,b], Eric L. Goldwaser[a,b], Abhirup Sarkar[a,b,c], George A. Godsey[a,b,c], Nimish K. Acharya[d], Umashanger Thayasivam[e], Benjamin A. Belinka[f], Robert G. Nagele[a,c,f,*]

[a] Biomarker Discovery Center, New Jersey Institute for Successful Aging, Rowan University School of Osteopathic Medicine, Stratford, NJ, USA
[b] Department of Geriatrics and Gerontology, Rowan University School of Osteopathic Medicine, Stratford, NJ, USA
[c] Graduate School of Biomedical Sciences, Rowan University, Stratford, NJ, USA
[d] Department of Neurosurgery, Penn Center for Brain Injury and Repair, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[e] Department of Mathematics, Rowan University, Glassboro, NJ, USA
[f] Durin Technologies, Inc., New Brunswick, NJ, USA

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ABSTRACT

The goal of this preliminary proof-of-concept study was to use human protein microarrays to identify blood-based autoantibody biomarkers capable of diagnosing multiple sclerosis (MS). Using sera from 112 subjects, including 51 MS subjects, autoantibody biomarkers effectively differentiated MS subjects from age- and gender-matched normal and breast cancer controls with 95.0% and 100% overall accuracy, but not from subjects with Parkinson's disease. Autoantibody biomarkers were also useful in distinguishing subjects with the relapsing-remitting form of MS from those with the secondary progressive subtype. These results demonstrate that autoantibodies can be used as noninvasive blood-based biomarkers for the detection and subtyping of MS.

1. Introduction

Multiple sclerosis (MS) is a neuroinflammatory autoimmune disease that primarily affects white matter of the central nervous system (CNS) (Kis et al., 2008; Reynolds et al., 2011; Sanai et al., 2016). Recent estimates show that between 250,000 and 350,000 people in the United States currently suffer from MS (Sanai et al., 2016; Miller and Hens, 1993). As is the case with many autoimmune conditions, women are disproportionately affected by MS, with a ratio of three women for every one man diagnosed (Sanai et al., 2016; Harbo et al., 2013). The reason for this discrepancy in gender is unknown, but it is speculated to be influenced by hormonal, genetic, or environmental differences (Harbo et al., 2013). Currently, MS is pathologically characterized by subcortical white matter lesions separated temporally and spatially, with microscopic structural defects accruing in the myelin sheaths that insulate axons for proper neuronal firing (Miller and Hens, 1993; Diaz-Sanchez et al., 2006; Bitsch et al., 2000). Demyelination occurs commonly in the white matter of the brain, including in the optic nerve and spinal cord, but later progresses to include gray matter lesions that are readily visualized in magnetic resonance images (MRIs). Common symptoms include lower extremity muscle weakness, paresthesias, vision changes, and later, cognitive decline as the disease progresses (Jurynczyk et al., 2015; Harris and Sadiq, 2014).

Presently, a diagnosis of MS involves a thorough patient history, imaging such as MRI to detect white matter lesions, an electrophysiological examination using evoked potential tests, and cerebrospinal fluid (CSF) analysis to detect the presence of increased immunoglobulin species (Birnbaum, 2006). While some success has been achieved to accurately diagnose and treat the symptoms of some patients, others succumb to progressively worsening disease symptoms and opportunistic conditions (Evlice et al., 2016; Noseworthy, 1994; Daumer et al., 2009). Due to the autoimmune nature of MS, much research attention has focused on specific components of the immune system to attempt to identify and diagnose patients at the earliest possible stage of their disease. For instance, autoantibodies targeting myelin surface proteins, such as myelin oligodendrocyte glycoprotein, myelin basic protein, myelin proteolipid protein, and myelin-associated glycoprotein, have demonstrated either associative or correlative links to MS, however, they currently lack utility as accurate diagnostic biomarkers (Harris and Sadiq, 2014; D'Ambrosio et al., 2015; Schirmer et al., 2014; Axelsson et al., 2011; Greeve et al., 2007; Tomassini et al., 2007). Other autoantibody targets with growing interest are glycans, and include

* Corresponding author at: Department of Geriatrics and Gerontology, New Jersey Institute for Successful Aging, Rowan University School of Osteopathic Medicine, 2 Medical Center Drive, Stratford, NJ 08084, USA.
E-mail address: nagelero@rowan.edu (R.G. Nagele).

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anti-GAGA4 or anti-glucose antibodies, as well as other cell surface ion channel proteins like KIR4.1 (Brettschneider et al., 2009; Freedman et al., 2009; Srivastava et al., 2012). Despite the abundance of potential biomarker candidates, thus far there is no definitive biofluid test capable of accurately diagnosing MS or monitoring its progression.

In several previous studies, we demonstrated the utility of blood-borne autoantibodies as sensitive and specific biomarkers capable of diagnosing and staging Alzheimer's and Parkinson's diseases at early stages with high overall accuracy, as well as successfully differentiating them from other neurodegenerative and non-neurodegenerative diseases (E. Nagele et al., 2011; Han et al., 2012; DeMarshall et al., 2016; C.A. DeMarshall et al., 2015). In the present study, our objective was to use this strategy to determine if autoantibodies can also be used as blood-based biomarkers to diagnose individuals with MS using sera from MS subjects afflicted with either the relapsing-remitting MS (RRMS) or secondary progressive MS (SPMS) subtype, the two most prevalent clinical courses of this disease. Roughly 80% of all MS patients are initially diagnosed with RRMS and, during the course of their disease, more than 60% of RRMS patients will transition to SPMS (Compston and Coles, 2002). Our results show that a panel of autoantibody biomarkers can be used to differentiate patients with MS from appropriate age- and gender-matched control subjects with an overall accuracy of 95.0%. We also identified additional autoantibody biomarker panels that are subtype-specific for RRMS or SPMS, and then used each of these panels to successfully differentiate these MS subtypes. Using RRMS-specific autoantibody biomarkers, we were able to differentiate RRMS patients from SPMS patients with 100.0% accuracy. Similarly, SPMS-specific autoantibody biomarkers were capable of differentiating SPMS patients from RRMS patients with 92.0% accuracy. These comparisons demonstrate the potential of autoantibody biomarker panels to effectively and sequentially stage the clinical course of MS, as well as possibly identify the point of transition between subtypes. Finally, MS subjects were also readily distinguished from those with breast cancer, a non-neurodegenerative disease control group, with comparable accuracy.

2. Methods

2.1. Ethics statement

Approval for the use of serum samples in this study was obtained from the Rowan-Stratford Institutional Review Board.

2.2. Study population

Thirty-one relapsing-remitting multiple sclerosis (RRMS) and twenty secondary progressive multiple sclerosis (SPMS) serum samples were obtained from *BioServe Biotechnologies, Ltd.* (Beltsville, MD). Fifteen early-stage PD samples were obtained from the Parkinson's Study Group (Boston, MA), and fifteen stage 3–4 breast cancer samples were obtained from *BioServe Biotechnologies, Ltd*. Healthy control samples were obtained from a variety of sources, including two from *Analytical Biological Systems, Inc.* (Wilmington, DE), twenty-eight from *BioServe Biotechnologies, Ltd.*, and one from *Asterand, Inc.* (Detroit, MI). All samples were handled using standard procedures and stored at − 80 °C until use, and freezer conditions were monitored using Sensaphone 1400 (Phonetics, Inc., Aston, PA). Demographic characteristics of the study population are listed in Table 1.

Table 1
Sample demographics. The number of individuals (n), age, range of age, gender, and ethnicity are listed for each disease and control group.

Group	n	Age		Gender	Ethnicity
		(Years)	(Range)	(% female)	(% Caucasian)
Multiple sclerosis	51	48.8 ± 10.7	25–67	75	96
-Relapsing-remitting	31	45.8 ± 11.1	25–67	81	94
-Secondary progressive	20	53.5 ± 8.0	36–67	65	100
Controls	31	53.7 ± 13.4	30–79	81	100
Early-stage Parkinson's disease	15	63.5 ± 6.8	51–73	80	100
Breast cancer	15	52.3 ± 6.6	45–63	100	87

2.3. Human protein microarrays

To identify autoantibodies in human sera, we used *Invitrogen*'s ProtoArray v5.1 Human Protein Microarrays (Cat. No. PAH0525020, *Invitrogen*, Carlsbad, CA, USA), each containing 9,486 unique human protein antigens (www.invitrogen.com/protoarray). All proteins were expressed as GST fusion proteins in insect cells, purified under native conditions, and spotted in duplicate onto nitrocellulose-coated glass slides. Arrays were probed with serum and scanned according to the manufacturer's instructions using commercially prepared reagents. Microarray slides were blocked (Blocking Buffer, Cat. No. PA055, *Invitrogen*) and then each was incubated with serum diluted to 1:500 in washing buffer. After washing, arrays were probed with anti-human IgG (H + L) conjugated to AlexaFluor 647 (Cat. No. A-21445, Invitrogen). Arrays were then washed, dried, and immediately scanned with a GenePix 4000B Fluorescence Scanner (*Molecular Devices*, Sunnyvale, CA, USA).

2.4. Microarray data analysis

Fluorescence data were acquired by aligning the Genepix Array List onto the microarray image using the Genepix Pro analysis software. The resulting Genepix results files were imported into Invitrogen's *Prospector 5.2* for analysis. The “group characterization” and “two-group comparison” features in the Immune Response Biomarker Profiling (IRBP) toolbox within *Prospector* then enabled M-statistical analysis of the differential autoantibody expression between the two groups being compared. Positive hits were determined by a Z-Factor > 0.4 and a minimum signal intensity of 1500 RFU, which allows for stringent biomarker selection and minimizes the number of false positives. Autoantibodies were first sorted into descending order by difference of prevalence between MS and control groups, and the top 50 most differentially expressed autoantibodies in the MS group were chosen as potential MS diagnostic biomarkers and tested further. Additionally, a second round of biomarker selection was carried out by sorting autoantibodies in descending order by difference of prevalence between control and MS groups. This time, the 50 most differentially expressed autoantibodies in the control group, putatively reflecting the selective depletion of these blood-borne autoantibodies in the MS group, were also chosen as potential diagnostic biomarkers and tested. All data are MIAME compliant and raw data from the microarrays have been deposited in a MIAME compliant database (GEO) under accession number (GSE95718).

Subjects were randomly split into Testing and Training Sets such that both sets included cases and controls matched by age and gender. The Training Set was used to rank candidate protein biomarkers by their predictive power and to establish the diagnostic logic. The initial Training Set for the MS group consisted of 26 MS and 16 control samples; the remaining samples were relegated to the independent Testing Set, containing 25 MS and 15 control subjects. The predictive classification accuracy of the selected biomarkers in the Training Set, Testing Set, and in both sets combined was tested with *R*'s *Random Forest* (*RF*; v 4.6–10), using the default settings (O'Bryant et al., 2014; Breiman, 2001). Selected biomarkers were tested with the *RF* model algorithm, and classification accuracy is reported in a confusion matrix and misclassifications as an out-of-bag (OOB) error score. Receiver