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Longitudinal Analysis of Novel Alzheimer's Disease Proteomic Cerebrospinal Fluid Biomarkers During Intravenous Immunoglobulin Therapy

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Abstract

Intravenous immunoglobulin (IVIg) therapy has shown promising results in treating Alzheimer's disease (AD). In this study, a Random Forest classification model was used to identify possible effects of IVIg on a group of eight subjects who underwent immunotherapy. Cerebrospinal fluid (CSF) samples from eight AD subjects who underwent IVIg therapy were collected before the therapy, after six months of therapy, and after a three-month drug washout period. Samples were analyzed using two-dimensional gel electrophoresis and further studied using a Random Forest classification model to identify effects of IVIg on a panel of 23 putative diagnostic AD biomarkers previously identified. Six of the eight subjects showed improvements with respect to the 23 AD diagnostic biomarkers after six months of therapy compared to the samples taken at the outset of the trial. All subjects reverted back to baseline during drug washout. These results are also consistent with clinical observations. The observed improvements in subjects during six months of IVIg therapy and the reversion back to baseline during drug washout provides preliminary evidence regarding the potential use of IVIg as an AD immunotherapy.

Keywords

Cerebrospinal fluid; IVIg; Random Forest; proteomics

1 INTRODUCTION

According to recent estimates, as many as 5.3 million Americans are living with Alzheimer's disease (AD) [1]. AD is a progressive, neurodegenerative disorder affecting more than five percent of people over the age of 65 and is the leading cause of dementia in the elderly [2]. AD is characterized by a progressive cognitive decline associated with

neurovascular dysfunction, accumulation of neurotoxic amyloid beta (A β) on blood vessels and in the brain parenchyma, intraneuronal lesions, and neurofibrillary tangles [2].

Active immunization against A β in preclinical studies using transgenic animal models of AD resulted both in reduction of A β in the cerebrospinal fluid (CSF) and plaque burden [3–4]. The change in A β plaque burden was also associated with restored cognitive function in some of these transgenic animals [5–6]. Similar results were obtained in animal studies with passive immunization using monoclonal antibodies against A β [7].

There is significant interest in the use of passive immunotherapy to treat AD. Intravenous immunoglobulin (IVIg) is a pool of human immunoglobulins obtained from the blood of thousands of healthy donors that has been approved for more than two decades by the United States Food and Drug Administration for treatment of immune deficiency disorders and other indications, but not AD. However, an initial study of IVIg in patients with neurologic diseases but not AD suggested that IVIg increased blood anti- A β antibody levels and fostered A β clearance [8].

An open label dose-ranging study in moderate AD subjects involved IVIg therapy for six months, which was discontinued for three months (washout period), and then resumed for another nine months [9]. As a result of this therapy, CSF A β decreased significantly after six months of therapy, returned to baseline after IVIg washout and decreased again after IVIg was re-administered. Mini-Mental State Examination (MMSE) scores increased an average of 2.5 points after six months, returned to baseline during washout, and remained stable during subsequent IVIg therapy. An important question is whether other molecular changes could be identified in the longitudinal CSF samples that were collected as part of this study.

Previously, a panel of 23 putative diagnostic AD biomarkers was identified and validated using proteomic analysis [10]. The proteins were separated using two-dimensional gel electrophoresis and the resulting pattern of protein expression was analyzed using the Random Forest (RF) multivariate statistical method. Important extensions to that study include the need to develop validation methods using methods other than 2DE to quantify changes as well as continue testing those observations in new cohorts. In this work, we consider the measurement of those spots using 2DE in a longitudinal study with the goal of observing whether that panel of markers tracks clinical outcomes. Specifically, CSF samples collected from AD subjects undergoing IVIg immunotherapy [9] is analyzed to characterize the response of the 23 biomarkers. CSF samples taken before the therapy, after six months of therapy, and after a three months IVIg washout were used for this study. The gels were analyzed using a previously described classification model [10] to identify possible effects of IVIg therapy on the panel of 23 AD biomarkers. This work represents the first longitudinal study of a panel of proposed AD CSF proteomics biomarkers as part of an AD clinical trial.

2 MATERIALS AND METHODS

2.1 Cerebrospinal Fluid Samples

Lumbar CSF samples were obtained from eight subjects enrolled in a phase I clinical trial of IVIg therapy for the treatment of AD at Weill Cornell Medical College [9] with institutional review board approval. All subjects have been diagnosed with probable AD based on NINCDS-ADRDA criteria [11]. The subjects have been randomly assigned to one of four different dosing regimens: 0.4 g/kg every two weeks, 0.4 g/kg every week, 1 g/kg every two weeks, and 2 g/kg every month, as described previously [9]. Serial lumbar CSF samples taken before IVIg therapy, after six months of therapy, and after three months of IVIg washout were analyzed for this study. This information is summarized in Table 1. CSF samples were also collected at an interim time point within the first 6 months of therapy. For the washout period, a CSF sample from subject #5 was not available. The CSF samples and their corresponding 2DE gels appeared free from blood contamination. Samples were stored at -70°C until used. Clinical information about the subjects involved can be found in [9].

2.2 Two-Dimensional Gel Electrophoresis

The CSF samples were separated using 2DE. The details of the 2DE protocol have been previously published [12]. Briefly, 300 μL of CSF (containing approximately 120 μg of protein) were precipitated using ice-cold ethanol. The resulting protein pellet was dissolved in a solution of 9 M urea, 2% 2-mercaptoethanol, 2% IGEPAL, and 0.25% carrier ampholytes. The sample was then loaded directly into a 18 cm, 3–10 nonlinear immobilized pH gradient (IPG) isoelectric focusing gel (GE Healthcare, Piscataway, NJ). A Protean IEF unit (Bio-Rad, Hercules, CA) was used to perform isoelectric focusing at 20°C for a total of 100 kVh. The IPG gels were equilibrated in solutions containing dithiothreitol and subsequently iodoacetamide for reduction and alkylation of the focused proteins. Polyacrylamide gel electrophoresis was performed using 12–15% T gradient slab gels. The separated proteins were fixed, stained with SYPRO Ruby Protein Gel Stain (Molecular Probes, Eugene, OR), and de-stained for 24 hours in a solution of 10% methanol and 7% acetic acid. The gels were scanned on a FLA-3000 Fluorescent Image Analyzer (Fujifilm, Tokyo, Japan).

The resulting gel images were imported into the Melanie software package (Version 4.0, GeneBio, Geneva, Switzerland). The software was used to auto-detect spots that were subsequently manually edited to remove technical artifacts. Each sample gel was then matched to a master gel image created by combining the spots present in two gels from before the therapy, and two gels from the interim time point within the 6 months of therapy. Matching was initially performed using the software's automated matching tool and then checked and corrected manually.

2.3 Statistical Analysis

The gels were analyzed using a previously described classification model built using a set of AD and non-AD gels with the RF method [10]. The non-AD CSF samples included both normal and neurological control subjects. The classification model uses the percent volume data from 23 spots previously determined to be important in separating CSF 2DE gels from

AD and non-AD subjects. A forest was built in the R environment and then used to predict the classification (AD class or non-AD class) of the IVIg gels. The percent of trees classifying the gels as AD or non-AD was recorded. For an IVIg gel, the greater the percent of trees classifying the sample as AD, the more similar the percent volume data of the IVIg sample is to the percent volume data of the AD samples used to build the forest.

3 RESULTS

Detailed information on the 23 spots previously identified is available in a previous publication [10]. The percent volume data for the 23 proteins previously identified to be useful in differentiating AD and non-AD CSF 2DE gels were extracted from the IVIg gels and recorded and representative 2DE images from three time points are provided in Figure 1. This data (see Supplementary Information) was run through a classification forest built using 68 CSF 2DE gels (34 AD, 34 non-AD). Based on these 23 spots, all of the IVIg gels, corresponding to CSF taken before the therapy, after six months, and during washout were classified as AD. A classification of AD indicates that over 50% of the trees in the forest classified the sample in the AD category. These data are plotted in Figure 2. Six of the eight subjects (1-6) received a lower AD classification after six months of therapy compared to the samples taken before the trial. All of these subjects (except for subject #5, whose CSF sample was not available during washout) received a higher AD classification after the washout compared to the samples taken during and at 6 months of the IVIg therapy.

Relkin *et al.* [9] observed that after six months of IVIg immunotherapy, the group's MMSE scores increased on average by 2.5 points. After IVIg washout, MMSE scores declined towards baseline. In the current RF analysis, the lower AD predictions during IVIg immunotherapy and the reversion during drug washout was consistent with observed MMSE score changes. The correlation coefficient between changes in MMSE and AD classification during IVIg therapy and drug washout were 0.11 and 0.95, respectively. Figure 3 shows the AD classification normalized with baseline for all patients to monitor for possible dose dependent effects.

4 DISCUSSION

In this study, CSF samples from AD subjects undergoing IVIg therapy were analyzed using two-dimensional gel electrophoresis. The data was studied using the previously developed RF classification model to identify longitudinal effects of IVIg therapy on the panel of 23 AD diagnostic biomarkers. An important feature of the RF method is that it is an appropriate multivariate statistical approach to study underspecified datasets. Underspecified datasets are characterized by having many more measurements per sample than the number of samples, as is often the case in proteomics data. For any given sample studied by the RF method, the higher the AD prediction for one class (AD or non-AD), the more similar the sample is to that class. According to this analysis, six of the eight AD subjects received a lower AD classification after six months of IVIg therapy, and these predictions reverted to baseline after IVIg washout.

Relkin *et al.* [9] reported that the administration of IVIg to the eight subjects with moderate AD led to transient, reproducible, and dose dependent increases in serum anti-A β antibody titers and parallel increases in plasma A β 40 and A β 42 levels. It was also observed that after six months of IVIg immunotherapy, CSF A β 40 and A β 42 levels decreased and the group's MMSE scores increased on average by 2.5 points, following a decline towards baseline during the drug washout. The consistency between the RF results and the MMSE scores suggests the possibility that the panel of 23 previously proposed diagnostic biomarkers may reflect disease severity or cognition.

Here, subjects 1–6 showed a decline in fraction of AD classifications during IVIg therapy, while subjects 7–8 did not show a similar response. MMSE scores for subject 7 at baseline, after six months of treatment, and after IVIg washout were 25, 28, and 24 respectively corresponding to 80%, 84%, and 79% AD classification (Figure 2). MMSE scores for subject 8 at baseline, after 6 months of treatment, and after IVIg washout were 20, 20, and 18 respectively corresponding to 72%, 76%, and 84% AD classification (Figure 2). These MMSE scores are also consistent with the RF observations for these subjects.

The fraction of AD classifications after the washout period shows a significant increase from baseline in subjects 2 and 6. The MMSE scores for these two subjects, who also received the same dose of IVIg, improved significantly during therapy, and were unchanged during the washout period [9]. While the majority of the observations related to RF and MMSE scores are consistent, the washout results for these two subjects suggest the possibility that the 23 diagnostic biomarkers may not accurately reflect MMSE scores in all cases just as the MMSE score demonstrated a poor correlation coefficient between MMSE and AD classification changes during IVIg therapy. It is noteworthy that subjects 2 and 6 both followed the same dosing regimen (0.4 g/kg/wk). One possible reason for this observation is that the RF analysis measures changes in the 23 AD biomarkers, and it is possible that disease-modifying effects of IVIg goes beyond the panel of 23 AD biomarkers in such a way that the 23 markers do not fully capture all of the effects across the doses tested. Further analysis of these subjects might provide additional explanation for these observations.

By performing a proteomic and RF statistical analysis, we showed that six of the eight subjects who underwent IVIg therapy received lower AD classifications with respect to the 23 AD biomarkers after the therapy. These results are in agreement with the reported clinical outcomes (e.g. MMSE changescore), and also provide an example on how newly identified proteomic CSF biomarkers may be useful in assessing treatment paradigms for AD. However, additional studies with a larger cohort size are necessary to fully correlate the statistical results with clinical outcomes - something that may be feasible as part of an ongoing Phase 3 clinical trial. We believe that longitudinal AD CSF studies proposed in the context of clinical tools are critically needed and this report is the first such study using proteomic biomarkers in this way.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IVIg	Intravenous Immunoglobulin
AD	Alzheimer's disease
CSF	Cerebrospinal fluid
Aβ	Amyloid beta
RF	Random Forest
MMSE	Mini-Mental State Examination

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Figure 1. Representative 2DE images of longitudinal samples at 0 months, 6 months, and 9 months of IVIg treatment. Gels were Sypro Ruby stained and % volume was collected.

Figure 2. Fraction of votes for each CSF sample using a previously built Random Forest classification model. The patient numbers refer to the dosing schedules listed in Table 1.

Figure 3.

Fraction of AD classification normalized with baseline for each patient. Patients receiving 1g/kg/2wk and 0.4g/kg/1wk showed a lower AD classification during IVIg therapy. Relkin *et al.* [9] also observed optimum changes in MMSE scores in patients who received 1g/kg/2wk of IVIg.

Table 1

Dosing and sample collection information for the 8 AD subjects.

Subject	IVIg dose (g/kg)	# weeks between doses	Time of lumbar puncture (weeks since IVIg started)	
			6 months after therapy	Washout period
1	1.0	2	23.85	36.85
2	0.4	1	25.14	35.71
3	0.4	2	22.85	34.42
4	2.0	4	27	40.71
5	1.0	2	26.42	–
6	0.4	1	27.14	41.28
7	0.4	2	27.14	41.28
8	2.0	4	27	41.42