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Apolipoprotein E and other cerebrospinal fluid proteins differentiate *ante mortem* variant Creutzfeldt-Jakob disease from *ante mortem* sporadic Creutzfeldt-Jakob disease

The ability to perform an *ante mortem* differential diagnosis of Creutzfeld-Jakob disease (CJD) is aided by several clinical and molecular tests. There is a need for molecular tests which can reliably distinguish *ante mortem* variant CJD (vCJD) from *ante mortem* sporadic CJD (spCJD). A proteomics approach employing two-dimensional protein electrophoresis is applied to the study of *ante mortem* CSF samples obtained in collaboration with the CJD Surveillance Unit and the National Hospital for Neurology and Neurosurgery. The sample set includes two cases of vCJD, three cases of spCJD and three neurologic controls. Preliminary data using a panel of seven molecular markers is able to distinguish vCJD from spCJD using a heuristic clustering algorithm. One of the molecular markers has been identified as apolipoprotein E which appears to be upregulated in the cerebrospiral fluid (CSF) of patients with vCJD as compared to spCJD. Analysis of *ante mortem* CSF may help to differentiate patients with vCJD from those patients with spCJD.

Keywords: Cerebrospinal fluid / Creutzfeldt-Jakob disease / Prion / Proteomics EL 5018

1 Introduction

There is a need to reliably diagnose Creutzfeldt-Jakob disease (CJD) patients *ante mortem.* The ability to make an *ante mortem* diagnosis is aided by different clinical and molecular tests including a test for the appearance of the 14-3-3 protein in cerebrospinal fluid (CSF) [1]. The highly conserved nature of the 14-3-3 amino acid sequence makes the 14–3-3 test useful also in the *ante mortem* diagnosis of animals affected with transmissible spongiform encephalopathies [2] as well as in the diagnosis of cases of new variant CJD (vCJD) [3]. Other CSF protein markers which may be useful in distinguishing CJD from other neurological conditions are Tau [4], neuron-specific enolase (NSE) [5], S-100 [6] and amyloid beta 1-42 [7]. It has also been shown that apolipoprotein E (ApoE) levels in bovine spongiform encephalopathy-affected cattle appear to be higher than in control animals [8] although an initial study of ApoE CSF levels indicated no significant difference between patients with sporadic CJD (spCJD) and neurologic controls as measured with an ELISA assay [9].

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Abbreviations: CJD, Creutzfeldt-Jakob disease; **CSF**, cerebrospinal fluid; **NSE**, neuron-specific enolase; **spCJD**, sporadic Creutzfeldt-Jakob disease; **vCJD**, variant Creutzfeldt-Jakob disease

The recent diagnosis of vCJD in an elderly patient [11] and the need to identify accurately the number of cases of vCJD for epidemiological and statistical analyses [12] has motivated the present study to identify CSF molecular markers which can distinguish vCJD from spCJD. At the United Kingdom CJD surveillance unit, the most important differential diagnosis of vCJD is spCJD [13]. In this report, we describe preliminary findings using a proteomics two-dimensional protein electrophoresis approach [14] to study CJD CSF. We find that a simultaneous measure of the expression of seven CSF protein spots is able to distinguish *ante mortem* vCJD from *ante mortem* sporadic CJD using a heuristic clustering analysis. One of these seven proteins has been identified as ApoE using a combination of matching to on-line databases and immunostaining with a monoclonal antibody.

2 Materials and methods

2.1 Samples

CSF samples were obtained in collaboration with the CJD Surveillance Unit and the National Hospital for Neurology and Neurosurgery (Queen Square, London). The diagnosis of spCJD [15], vCJD [13], Alzheimer's disease [16] and Pick's disease [17] were made according to clinically established criteria. Data from eight CSF samples are presented (Table 1). These include two cases of confirmed vCJD, three cases of confirmed spCJD and three neurologic controls (two Alzheimer's disease and one Pick's

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Table 1. CJD and control cases used

Diagnosis	Gender	Age	CSF NSE ng/mL	CSF S-100b ng/mL	CSF Tau pg/mL	CSF 14-3-3
VCJD	M	20	11	0.97	961	Negative
VCJD	M	30	89	2.71	1565	Positive
SpCJD	F	46	10	0.47	101	Negative
SpCJD	M	58	84	0.68	1467	Positive
SpCJD	F	76	N/D	1.16	6407	Positive
Alzheimer's	M	75	7	0.75	559	Negative
Alzheimer's	M	54	24	0.54	750	Negative
Pick's	F	50	32	0.53	1488	Negative

disease). Data from one other case of vCJD are not presented because the CSF sample was contaminated with serum.

2.2 Procedures

The detailed protocols for performing two-dimensional protein electrophoresis (2-DE) and ammoniacal silver staining of CSF proteins which were used in this study and have been described elsewhere [18, 19]. Briefly, a 250 μL aliquot of CSF was subject to an overnight ice-cold ethanol protein precipitation. The protein pellet was resuspended in a mixture containing 9 M urea, 2% 2-mercaptoethanol, 2% IGEPAL, and 0.8% carrier ampholytes. This sample was then hydrated directly into commercially available pH 3–10 nonlinear immobilized pH gradient (IPG) isoelectric focusing gels (Amersham Biosciences) with 8 M urea, 2% CHAPS, 20 mM dithiothreitol, 2% carrier ampholytes and a trace of bromophenol blue. Isoelectric focusing was performed for a total of 71 750 Vh at 20°C. The IPG gels were equilibrated in solutions containing dithiothreitol and iodoacetamide before polyacrylamide gel electrophoresis in 10–16%T vertical gradient slab gels. Proteins were detected using an ammoniacal silver stain and scanned using a Molecular Dynamics laser densitometer. The resulting gel image files were imported into the commercially available Melanie 3 gel analysis software package (GeneBio, Geneva, Switzerland). Spot detection was performed using default parameter values and features were manually edited to remove technical artifacts. The percent integrated optical density (% volume) was calculated for each of the protein spots detected with this procedure. The integrated optical density of any given feature is the optical density at each pixel of that feature integrated over the area of the feature. The % volume takes the integrated optical density and scales this value by the total integrated optical density of the gel which normalizes for gel-to-gel staining variability, different sample loads, *etc.* Heuristic clustering [20, 21] was performed using the Melanie 3 default parameter values. Two class levels were set to differentiate vCJD and spCJD. Immunostaining of 2-DE blots was done with a monoclonal antibody against ApoE (Santa Cruz Biotechnology SC-13521). A set of two 2-DE gels were run in parallel. One of the gels was silver stained as a reference. The other gel was blotted to a PVDF membrane (Millipore Immobilon P) at 150 mA for 6 h. The resulting blot was first stained with Colloidal Gold Total Protein Stain (Bio-Rad Laboratories 170–6527) and washed thoroughly in deionized water. The membrane was blocked in TBS with Tween (TBST) containing 5% dry powdered milk. The primary antibody was used at a 1:1000 dilution and a peroxidase-linked secondary goat anti mouse antibody (Sigma A9917) was used at 1:40 000 dilution prior to detection with an ECL Plus chemiluminescence kit (Amersham Biosciences RPN2132). The blot was exposed to a chemiluminescence screen and scanned on a Bio-Rad GS525 imaging system.

3 Results

A typical silver-stained CSF protein profile is shown in Fig. 1 which comes from a patient with vCJD. Proteins are separated in the horizontal axis by charge (p*I*) and in the vertical direction by size. Several reference marks for molecular weight and for isoelectric point are given in the

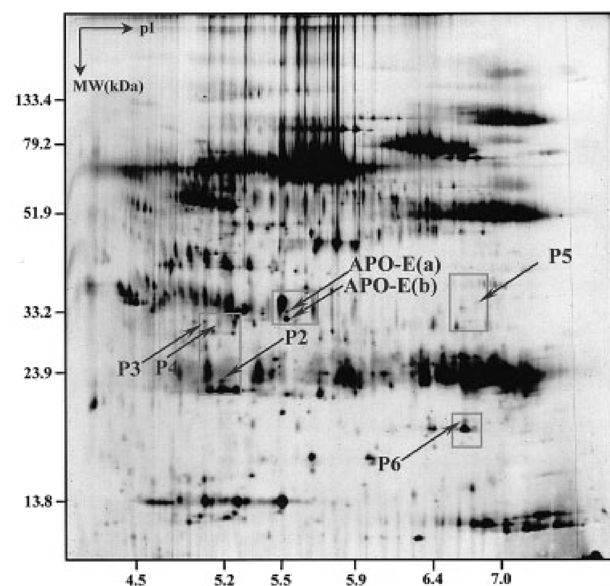

Figure 1. Silver-stained 2-DE gel of vCJD CSF. Proteins are separated by charge and by size. The location of the seven proposed markers for vCJD and spCJD are labeled and red boxes correspond to regions of the gel depicted in Figs. 2–5.

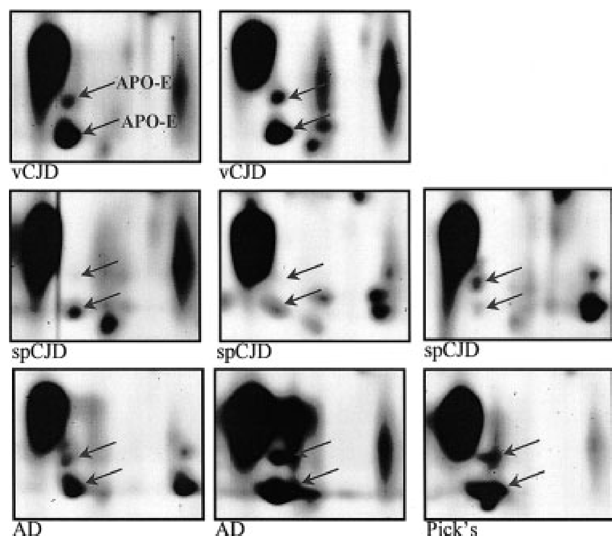

Figure 2. Region of silver-stained 2-DE gel from each of the eight samples studied. The location of ApoE (both isoforms) is given.

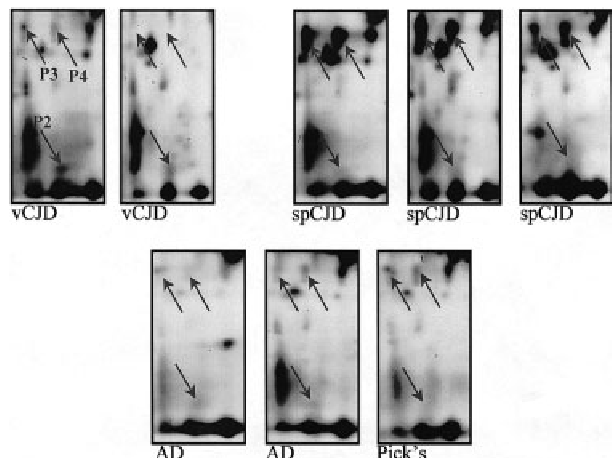

Figure 3. Region of silver-stained 2-DE gel from each of the eight samples studied. The location of P2, P3 and P4 is given.

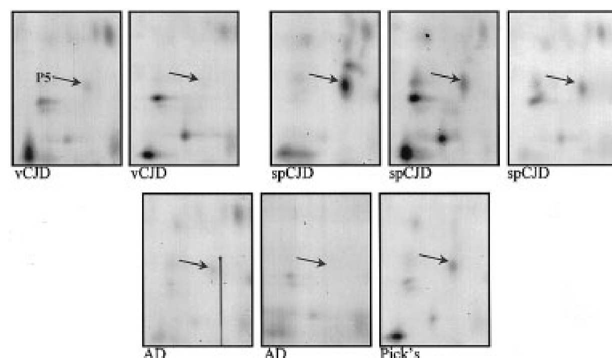

Figure 4. Region of silver-stained 2-DE gel from each of the eight samples studied. The location of P5 is given.

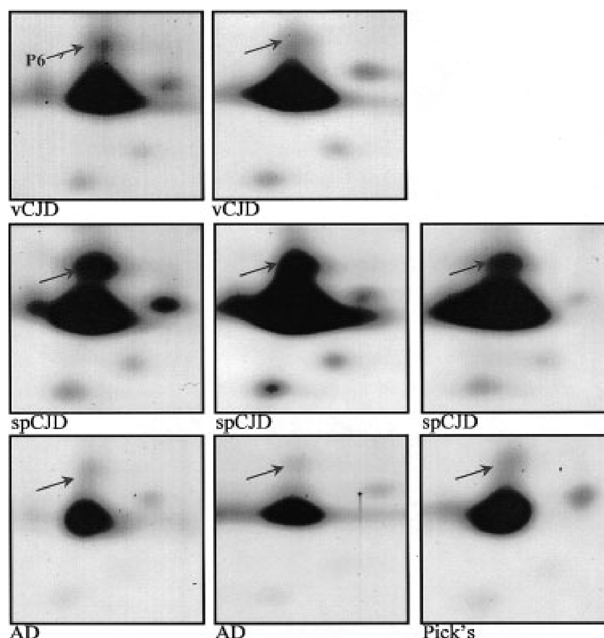

Figure 5. Region of silver-stained 2-DE gel from each of the eight samples studied. The location of P6 is given.

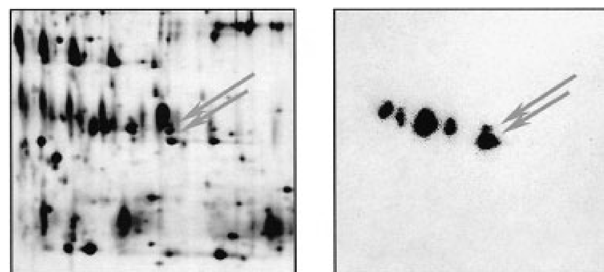

Figure 6. Region of silver-stained 2-DE gel and corresponding region of Western-stained 2-DE gel illustrating ApoE immunoreactive isoforms.

figure. There are four regions of the gel which appear to be most relevant to distinguish vCJD from spCJD using this molecular method. These regions are highlighted in red boxes in Fig. 1 and are presented in Figs. 2–5. We observe that seven of the protein spots appear to be differentially expressed in vCJD from spCJD. We have designated five of these protein spots as P2–P6. The other two spots, which migrate at approximately 32 kDa and p*I* 5.5, have been identified as ApoE using direct comparison with existing CSF protein databases [22] and using Western detection (Fig. 6). ApoE is known to migrate as a series of charge isoforms consistent with the pattern detected in Fig. 6.

Among these seven spots, ApoE appears to be expressed more highly in vCJD and control cases as compared to spCJD. P2 is expressed more highly in

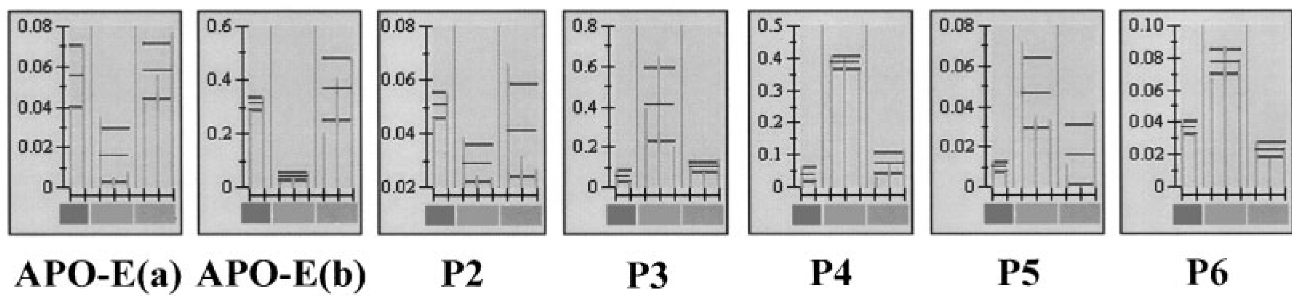

Figure 7. Histogram of % volume values for each of the seven markers over each of the eight samples. The red bar indicates values from vCJD cases. Blue bar indicates values from spCJD cases. The gray bar indicates values for the neurologic controls. The mean and standard deviation are depicted on each histogram.

vCJD than in spCJD and control cases. P3, P4, P5 and P6 appear to be upregulated in spCJD as compared to vCJD and control cases. Here, we use the % volume measurement which is output from the Melanie 3 analysis software to measure the relative abundance of these silver stained protein spots (Fig. 7). In Fig. 7, the blue line represents the mean of the measured values while the red lines indicate the standard deviation of the measurements. The vCJD cases are presented to the left of the spCJD cases which are shown to the left of the neurologic controls. We observe the same pattern of expression if we use any of the other quantitative measures which are available in the Melanie 3 software including % optical density, optical density, area or volume.

Although a measure of any of these markers alone might provide important information useful to distinguish vCJD from spCJD, an arguably better approach is to use a panel of all seven markers. An effective panel of markers can potentially provide information on different aspects of neurodegenerative diseases and potentially simplify the multiple test, Bayesian diagnostic approach currently employed in favor of a single multiple marker-based test. To this end, we use a heuristic clustering analysis of these data to permit an automated separation of these data into distinct classes. Heuristic clustering is a machine learning algorithm from the artificial intelligence community that is used to describe the characteristic spots of 2-DE gels while using heuristics to speed up the search. It is an alternative approach to classify blindly similar gels into two or more classes. When we apply a two-class heuristic clustering analysis of these data we observe the dendrogram depicted in Fig. 8. This dendrogram clearly demonstrates that vCJD and spCJD cases cluster on two distinct branches. These data further reinforce the notion that vCJD and spCJD are distinct diseases. Interestingly, when we use the seven spots to classify CSF images, we find that the cases of Alzheimer's disease and Pick's disease also fall on separate branches and we cannot offer a reasonable explanation for this data at this time.

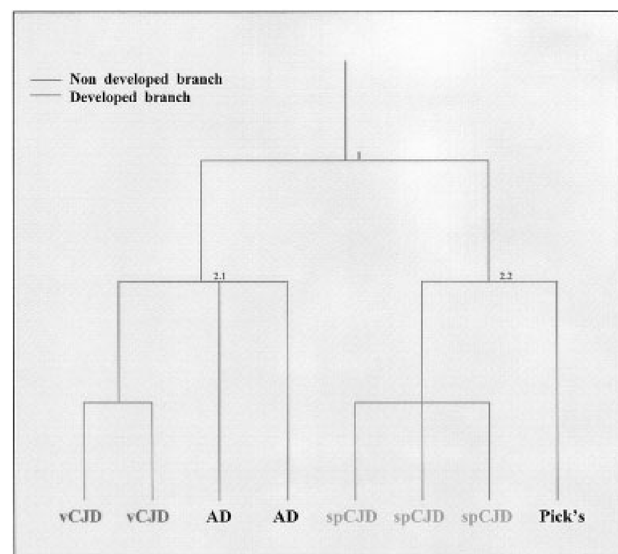

Figure 8. Heuristic clustering analysis dendrogram which indicates that vCJD and spCJD cluster on two distinct branches. Alzheimer's and Pick's samples also segregate on independent branches.

4 Discussion

We have demonstrated the utility of the proteomics approach to classify *ante mortem* vCJD CSF from *ante mortem* spCJD CSF using a panel of seven molecular markers. One of the molecular markers has been identified, by comparison to available on-line databases, as ApoE. Another group has reported that ApoE is not a useful marker in the differential diagnosis of sporadic CJD [9]; however, this prior study did not attempt to distinguish

vCJD from spCJD. We intend to increase the size and scope of this study to include a larger number of vCJD, spCJD and neurologic controls. Access to appropriate samples and better age-matched controls can be limiting as lumbar puncture is not always indicated. We recognize that the sample size places limits on the statistics which can be performed with this preliminary analysis. We are currently attempting to characterize each of the six other molecular markers as well as perform an even more comprehensive analysis of the data.

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