

Identification of Biomarkers of TSE Pathogenesis Using A Mouse Model

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INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are fatal neurological diseases in humans and animals. Symptoms include changes in mental state, abnormalities of posture, movement and sensation. TSEs are characterised by: 1) spongiform (vacuolar) degeneration of synaptic regions of the grey matter, 2) severe astrocytic gliosis, and 3) the appearance of prion protein (PrP)-containing plaques.

Currently, biochemical diagnostic efforts primarily focus on the detection of the abnormal scrapie-associated isoform (PrP^{Sc}) in peripheral tissue or blood and, to date, there are no current effective *in vivo* tests for TSE infection. We have applied a proteomic platform to search for potential alternative biomarkers in plasma samples from mouse models that feature defined incubation periods after infection with the TSE agent.

BIOMARKER DISCOVERY

SAMPLE COLLECTION

Mice were injected intraperitoneally with 100 μ L 0.1% (w/v) brain homogenate from:

- non-infected control mice
- mice at terminal stage of infection with BSE (mouse-adapted strain 301C)

The incubation period of disease under these conditions was 320 \pm 7 days (mean \pm SEM)



- Sampling
- 120 days post-injection (half stage)
 - 255 days (three quarter)
 - 320 \pm 7 days (symptomatic end stage)



For each of the stages, 5 control and 5 infected mice were sacrificed. Heparinised whole blood was collected at each stage by exsanguination. Plasma was collected after centrifugation on standard Percoll gradients to separate out white blood cells.

SELDI: PLASMA PROFILING

Plasma samples, diluted 2:3 in SELDI solubilisation buffer, were arrayed in duplicate, and in a randomised manner, across pre-equilibrated Q10 and CM10 protein chip arrays using an in-house optimal protocol.

Following an incubation period, samples were carefully removed and chips were washed in the appropriate equilibration buffer conditions, followed by one wash with ddH₂O. Sannipic acid prepared in acetonitrile and trifluoroacetic acid was applied to each spot.

Data acquisition was performed using a PBS-11c reader (Ciphergen Biosystems). Baseline subtraction, normalisation to total ion count and peak detection were performed on all spectra using the Ciphergen ProteinChip Software (v3.2.0).

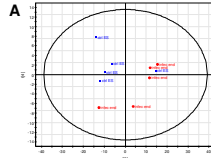
Peak information were exported in comma separated value (csv) format. All duplicate intensity readings were averaged and this data were imported into both SPSS for univariate (Mann-Whitney) and SIMCA-P for multivariate analysis. This included Principal Components Analysis (PCA) and partial least square discrimination (PLS-DA). Any skewed data were log transformed. Variables corresponding to masses between m/z 3000 and 100000 were centred to the mean value and Pareto scaled.



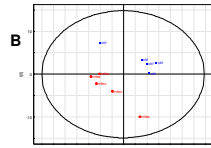
STATISTICAL COMPARISON

Multivariate and univariate analyses were performed between control mice and infected mice for each of the disease stages using the datasets obtained from the Q10 and CM10 protein chip arrays.

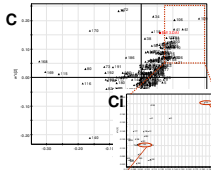
BIOINFORMATICS



PCA models were used to extract and display any differential profiling pattern that may occur between controls and infected mice for the different stages of the disease.



A PLS-DA observation scores (t) plot was used to discriminate any subtle clustering that may be present within the controls and infected samples.



A PLS-DA variable weight (w^c) plot indicates which peaks are influencing the clustering effects that can be seen in the observation scores plot.

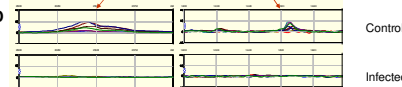


Figure 1: Selection of potential biomarkers for end stage (control vs infected) using the Q10 dataset. A) PCA scores plot displaying components 1 and 4. B) PLS-DA scores (t) plot. C) PLS-DA variable weight (w^c) plot. C) Zoomed in region of weights plot. D) Two overlaid spectra of peaks of interest.

Stage	CM10				Q10			
	VIP	COEFF	Sensitivity	Specificity	VIP	COEFF	Sensitivity	Specificity
Half	1.50	< -0.03, > + 0.02	40%	80%	2.50	< -0.035, > +0.035	80%	60%
Three Quarter	1.20	< -0.03, > + 0.025	60%	80%	1.56	< -0.05, > + 0.036	20%	20%
End	0.90	< -0.02, > + 0.03	100%	100%	2.90	< -0.03, > + 0.029	80%	80%

Table 1: PLS-DA model listing VIP, COEFF, Sensitivity and Specificity parameters used to select peaks of interest. The sensitivity / specificity of the model to correctly predict the class of samples was determined by dividing the dataset into training and a test set. A PLS-DA model was fitted to the training portion of the dataset and subsequently used to predict the classes of the test portion of the datasets. The training and test datasets were then switched and the process repeated. The number of correct and incorrect classifications from both rounds of testing were recorded and used to calculate the sensitivities and specificities.

In each of the comparisons, approximately 100-200 putative peaks were detected. From these, 9 and 5 peaks were found to be of potential interest by multivariate and univariate methods from the CM10 and Q10 dataset respectively.

Stage	Peak Number	Substance Name	p value	Fold Change	Direction of fold change in infected	Cohen's D	B
CM10	End	35	4841	0.008	4.3	4.5	-1.84
	End	52	12749	0.008	2.5	2.1	-0.54
	End	64	20191	0.008	5.4	5.1	-0.47
	End	85	20341	0.008	9.2	4.9	-0.17
	End	59	7397	0.008	2.0	1.9	2.88
Q10	End	55	14735	0.032	2.3	2.6	18.67
	End	66	14623	0.032	2.1	3.0	18.26
	End	57	15156	0.016	3.8	2.6	7.64
	End	56	14574	0.016	4.3	7.9	6.65
	Half	78	3592	0.056	3.9	3.3	-0.1
Three Quarter	End	72	8074	0.032	1.3	1.2	-4.3
	End	140	17049	0.008	1.3	1.3	1.0
	End	109	12537	0.02	6.6	10.75	-1.5

Table 2: Potential peaks of interest from both the CM10 and Q10 datasets. The table lists details of fold change, p value and Cohen's D. The peaks in this table were also selected by VIP and COEFF. Peaks highlighted in red were subsequently correlated to proteins listed in table 3.

IDENTIFICATION

EXTRACTION

For protein identification, Laemmli buffer [1] was applied to spots on the protein chip with the strongest intensity signal. The extracted material were run on a 16% pre-cast Tris-Glycine gel (NuPAGE). Subsequently the gels were stained with Modified silver [2]

LC/MS/MS

In-gel reduction, alkylation, and digestion with trypsin were performed prior to subsequent analysis by mass spectrometry. Samples were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide. Protein digestion was carried out overnight. Peptides were

LC/MS/MS- continued

extracted from the gel pieces by a series of acetonitrile and aqueous washes, pooled with initial digest supernatant and lyophilised. Samples were resuspended with 50mM ammonium bicarbonate and analysed by LC/MS/MS. Chromatographic separations were performed on a 75 μ m, reversed-phase (C18) PepMap column using an Ultimate LC system (Dionex). Peptides were ionised by electrospray ionisation using a Z-spray source fitted to a Q-ToF micro (Waters).

Sample	Protein ID	Species	Accession No.	MW	pI	No. Peptides	% Coverage	Sequence Matched
44	Calpactin- β (Brain)	Human	P02670	3025	5.51	179	11%	LELEKPKR
45	Huaxin (HSA)	Human	P27272	1462	3.07	1	0%	AKLQPKR
10	Hemoglobin- β (Chain-BE) (Hemoglobin-beta chain)	Mouse	P02670	6289	7.26	7	37%	
10	Hemoglobin- α (Chain-AE) (Hemoglobin-alpha chain)	Mouse	P02672	1495	6.98	5	47%	
10	Transferrin (Transferrin)	Mouse	P02670	6746	5.77	1	0%	TSEDAKPKK
16	Apolipoprotein A1 precursor	Human	Q0823	3059	5.64	8	3%	
16	Transferrin (Transferrin)	Human	P02670	6646	5.75	7	12%	
16	Calpactin- β (Brain)	Human	P02670	3025	5.51	179	11%	LELEKPKR
16	Hemoglobin- β (Chain-BE) (Hemoglobin-beta chain)	Mouse	P02670	6289	7.26	1	0%	LEVVVYK
55-4	Hemoglobin- α (Chain-AE) (Hemoglobin-alpha chain)	Mouse	P02672	1495	6.98	2	2%	KREKQKELR TYRTEAKELR

Table 3: Potential peaks of interest identified from both the CM10 and Q10 datasets.

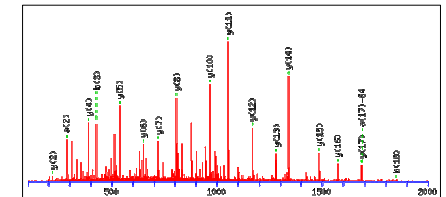
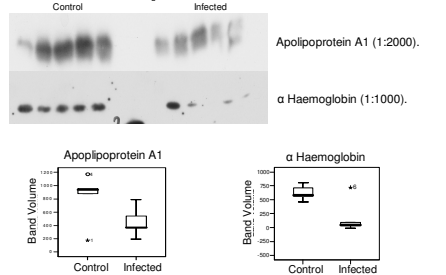


Figure 2: MS/MS Fragmentation spectra of the peptide YFDSFGDLSASAIMGNK. The sequence ions of this peptide originates from Haemoglobin beta 1 chain (B1). The methionine in this peptide is oxidised.

VALIDATION

Validation was performed by Western blotting. Equal volumes of plasma were run on a 16% pre-cast Tris-Glycine gel. Proteins were transferred onto nitrocellulose membrane and probed with the appropriate antibody. The bands of interest were quantified using Image Quant and SPSS.



CONCLUSIONS

SELDI-TOF profiling of plasma samples from the mouse model of BSE revealed several peaks of interest which exhibit changes between the study groups. These observations were followed up by extracting material from the chip surfaces and analyzing the resulting gel bands by LC/MS/MS. Using bioinformatics we were able to positively correlate m/z values of three of the peaks of interest to the theoretical molecular weights within the protein sequences represented in the LC/MS/MS data. Notably, we found that two peaks could be matched to Hemoglobin alpha chain (HbA) and a third peak could be explained by N-terminal truncation of APO-A1. Western blot experiments were subsequently performed to further evaluate the behaviour of both Hemoglobin alpha chain and APO-A1. In the latter case APO-A1 was found to show changes consistent with the initial SELDI experiments confirming that the concentration of this analyte differs between the study groups. In the case of Hemoglobin alpha chain however, the Western blot results indicate that the concentration of this protein does not change between the study groups. This suggests that although the MW correlations correctly predict HbA there is likely to be another, as yet unidentified component, at the same molecular weight. Further work to identify other components responsible and included within the 14kDa peak is required and experiments involving enrichment of the 14kDa species in mouse plasma are planned. In addition, the biological significance of changes in APO-A1 in this system and other TSE's remains to be further investigated.

References

- Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.
- Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH, Dunn MJA. A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption.