

Proteomic MS analysis of FASP-processed FFPE leptomeningeal amyloid brain tissue identifies transthyretin where immunohistochemical staining fails

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Overview

Immunohistochemical staining (IHC) is a routine procedure performed by pathologists for disease diagnosis. Inconclusive results can occur however, due to increased background from non-specific reactivity or epitope loss in the physical structure of the protein.

Using Filter Aided Sample Preparation (FASP) of Formalin Fixed Paraffin Embedded (FFPE) tissue, we were able to identify proteins constitutive of amyloid plaques, specifically transthyretin, which was overlooked by IHC staining. In addition, we semi-quantified a point mutation (Tyr69His) in transthyretin.

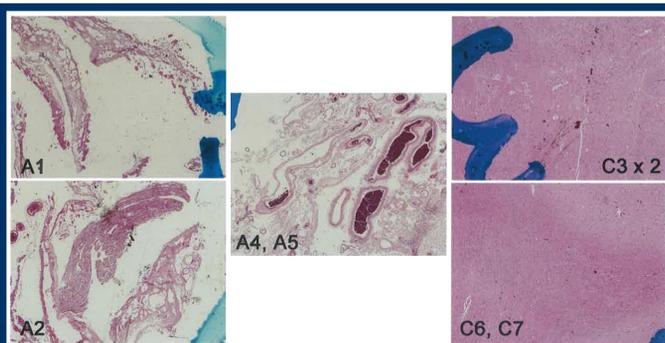


FIGURE 1.
A1-A2: leptomeningeal amyloid from pre-pontine cistern
A4-A5: leptomeningeal amyloid from cerebral leptomeninges
C3: pontine tegmentum (control)
C6-C7: cerebral white matter (control)

Introduction

In this study, we obtained rare post mortem FFPE tissue from a patient who suffered periods of memory loss/dementia, seizures, and schizophrenia, and was under extensive clinical care. Pathologists rely on IHC staining to readily identify and diagnose disease from FFPE tissue. In this case, IHC staining of this tissue was positive for amyloid proteins, but negative for transthyretin, a specific amyloid protein associated with the symptoms the patient exhibited.

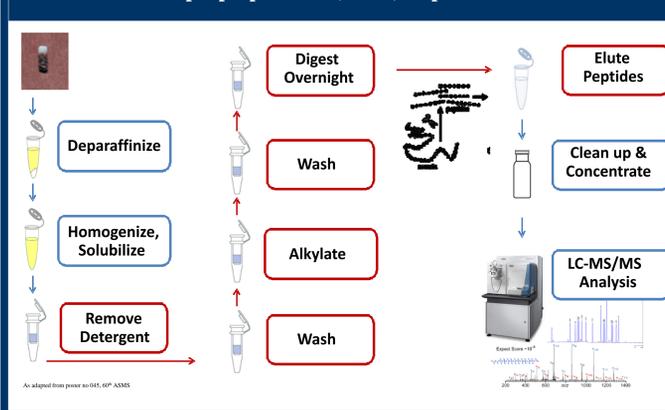
FFPE treated tissue is typically not amenable to proteomic analysis; FASP circumvents this problem. FASP MS of carefully excised amyloid plaque regions identified transthyretin as the most prominent amyloid protein in the plaques; transthyretin was absent in control (healthy) brain regions from the same patient.

When employed correctly, MS based proteomics can go far beyond identifying the proteins specific to particular regions of the brain. It can aid in disease diagnosis. It can also provide semi-quantitative information about particular point mutations, which were initially found by gene sequencing of this patient's DNA.

Methods

Tissue processing. Core samples of patient brain tissue measured 3 mm in diameter. The total weight of each core ranged from 0.8 mg to 1.7 mg. The samples were first deparaffinized in xylene and absolute ethanol. The samples were then placed in a solution of 4% SDS and 100 mM Tris pH 8.5 and processed using a Precellys 24-Dual tissue homogenizer. In order to maximize tissue solubilization, samples were incubated in a heating block at 99°C for 60 minutes.

SCHEME 1. Overview of FFPE tissue proteomic workflow.
Filter aided sample preparation (FASP) steps are outlined in red.



FASP. Following protein solubilization, disulfide bonds were reduced via the addition of DTT to 50 mM, and heating at 55°C for 20 min. Approximately 20% (30 uL) of each sample was then transferred to the FASP filter and washed three times by centrifugation with 8 M Urea in 0.1 M Tris pH 8.5, to remove the SDS. Proteins were alkylated by adding iodoacetamide solution to the FASP filter and incubating in the dark for 20 min, followed by centrifugation. This was followed with another three washes of 8 M Urea, 0.1 M Tris pH 8.5, then three washes of 50 mM ammonium bicarbonate. Trypsin was added to the filter for overnight digestion of the proteins on the filter. The peptides were eluted by centrifugation with 50 mM ammonium bicarbonate and 0.5 M sodium chloride. The samples were then acidified with 50% formic acid in water, and peptides were extracted and concentrated by C18 stage tip.

Mass Spectrometry. The resulting peptides were loaded onto a Waters nanoAcquity UPLC; the analytical column was pulled and packed in-house using 3µm C18 material. An LTQ Orbitrap Velos MS in DDA mode fragmented the 15 most intense multiply charged precursor ions via CID. Data were searched against the Uniprot Human database using Sequest, and results were filtered to 1% FDR at both peptide and protein levels.

Results

Implementing FASP to identify proteins from FFPE tissue that normally is analyzed with IHC staining gave conclusive results. Specifically, in samples "A" (amyloid regions, N=3), 490 proteins and 2763 peptides were identified, while in the control tissues "C" (N=3), 1045 proteins and 6063 peptides were identified. The proteome overlap of the two sample types was only 25% (311 proteins in common), indicating very different proteomes of the two brain regions.

A particular protein of interest, transthyretin, was only found in sample set "A", and it was identified as the most abundant protein after hemoglobin (165-357 spectral counts). The sequence coverage of transthyretin was 80% (Figure 3). Other key proteins found in sample set "A" include serum amyloid P-component (SAP) and serum amyloid A (SAA). SAP prevents fibrillar breakdown by enzymes so is considered key in maintaining the stability of amyloid deposits. In sample set C, SPTA/B2, CN37, MAP1B, AINX and TNF4 were identified as highly abundant proteins that were absent in the "A" samples.

Following transthyretin identification, gene sequencing of the patient's DNA was performed, revealing a point mutation within transthyretin at Tyr69His. We carried out a semi-quantitative analysis using extracted ion chromatograms (EIC) and measuring area under the curve (AUC) to suggest that approximately 85% - 90% of this patient's transthyretin, as sampled within the amyloid region, was of the mutated form (His69, Figure 4).

Protein Identification

FIGURE 2.
Venn diagrams of the number of proteins and peptides identified (at <1% FDR) for amyloid-containing (A) and control (C) tissue.

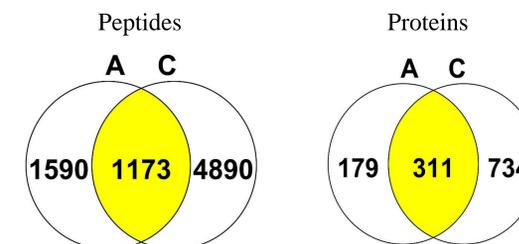
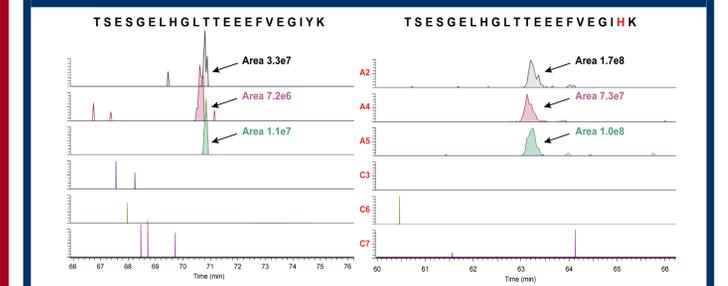


FIGURE 3.
Transthyretin was identified with 80% sequence coverage (14 peptides), and numerous modifications (highlighted in green)

MASHRLLLLC LAGLVFVSEA GPTGTGESKC PLVWVLDAY RGSPTAINVAV
HVFRKADDT WEPFASGKTS EGGELHGLTT EEEFVEGIIYM VEIDTKKQYWK
ALGISPPHEH AEVVFTANDS GPRRYTIALA LSPYSYSTIA VVTNPKK

Semi-quantitation of the Tyr69His mutation

FIGURE 4.
Extracted ion chromatograms (EIC) of the normal and mutated transthyretin peptide T49-K70 for the amyloid (A) and control (C) samples. Gene sequencing confirmed a point mutation at Y69H. Chromatographic peak areas suggest that approximately 85% - 90% of the transthyretin protein in the amyloid regions exists in the mutated form.



Conclusions

Applying FASP to FFPE post mortem brain tissue, it became clear that transthyretin is implicated in the amyloid formation as was visually identified and suspected by the pathologists. In this case where IHC failed, proteomics is a superior analytical approach.

Additionally, as a result of the gene sequencing, the Tyr69His point mutation within transthyretin was identified; MS analysis suggested that over 80% of transthyretin content within the plaques of this patient are of the mutated form. Structural studies are currently being performed to evaluate the propensity of the point mutation for aggregate formation.

Our findings also suggest that the proteome content of the regions of the brain containing the amyloid is uniquely different to that of the cerebral white matter (control). These proteome content and the quantities therein could lead to potential biomarkers. As a follow up analysis we are currently embarking on a complete PTM scan (phospho, methyl, acetyl, ubq) for the amyloid plaque samples.

Moving forward, the proteomic approach was applied recently when it was uncertain whether amyloid plaques were present in the ventricular cavity of the brain in the same patient; the resulting data showed substantial overlap with that of the control, and did not find transthyretin. This finding was consistent with the clinicians' hypothesis.

References

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- 2 J. Wiśniewski et al, *Nature Methods* **6**, 359 - 362 (2009)

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