

Clinical diagnosis and typing of systemic amyloidosis in subcutaneous fat aspirates by mass spectrometry-based proteomics

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Manuscript received on December 15, 2013. Manuscript accepted on April 14, 2014.

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Supplemental Methods

MS-based proteomic analysis SFA specimens

The SFA specimens were processed for MS analysis using a modification of a previously established protocol.²⁶ Briefly, the fat specimens were put into RPMI media containing 10 U/ml heparin sulfate and subsequently collected on a polyester mesh filter (Netwell; Corning). The fragments were washed with media and an erythrocyte lysis buffer (Buffer EL; Qiagen, Valencia, CA). The tissue was solubilized and dilapidated in a solution of hexafluoroisopropanol + 0.002% zwittergent 3-16. The solution was subsequently lyophilized and any remaining tissue disrupted by sonication in a 0.1 M TRIS pH 8.0 buffer. The samples were digested in trypsin (Promega, Madison WI) and used for protein identification by nano-flow liquid chromatography electrospray tandem MS using a ThermoFinnigan LTQ Orbitrap Hybrid Mass Spectrometer (ThermoElectron Bremen, Germany) coupled to a nanoLC-2D HPLC system (Eksigent, Dublin, CA). **For each injection 0.5 ug of total protein was loaded.** Peptide spectra present in the raw data files were matched against a composite protein sequence database using three different search engines (Sequest²⁷, X!Tandem²⁸ and Mascot²⁹). The composite database contains protein sequences obtained from the SwissProt database selected for human subspecies, known human immunoglobulin variant domains obtained from ImMunoGeneTics database³⁰, known amyloidogenic mutations collected from literature and common contaminants. Reversed protein sequences are appended to the database for estimating the false discovery rates of the identifications³¹. Resulting peptide identifications were combined, filtered, and assembled into protein sequences using Scaffold software (Proteome Software, Portland, OR). For each case, a list of proteins with at least two confident peptide identifications (probability > 0.95) was generated. Amyloid type was determined according to previously established criteria for paraffin embedded tissues.⁹ A known amyloidogenic protein (with exception of APOA1) with the largest number of peptide spectra in the sample was considered to be the causative protein as long as at least 2 peptide spectra were present. APOA1 was excluded from assessment as it is frequently present in the proteome of different amyloid types but is rarely a primary cause of systemic amyloidosis.

Total testing time per case was on average 24 hours. Preanalytical sample preparation time is 19 hr 30 min including the sonication and desiccation steps and overnight trypsin digestion. Analytical MS instrument time was a total of 4 hours/patient with 3 separate samples. Post-analytical computational processes ing time was 30 min.

As part of our clinical validation, the stability for clinical samples was checked up to 7 days storage at 4C. No deterioration of the signal was seen, therefore the clinical standard operating procedure accepts specimens stored at 4C up to 7 days.

Statistical Analysis

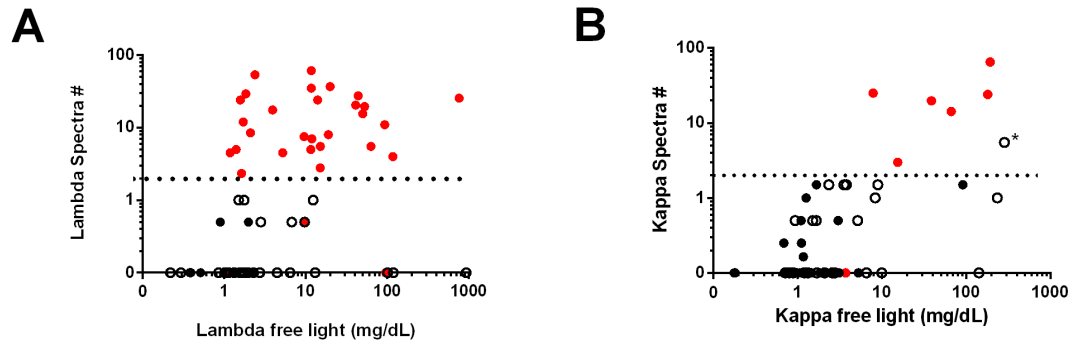
The relative abundances of the following four proteins between the CR negative/no amyloid (n=32) and CR positive/amyloid (n=43) cases were compared in the validation cohort:

Apolipoprotein E (APOE), Apolipoprotein A1 (APOA1), Apolipoprotein A4 (APOA4) and Serum Amyloid P-component (SAP). For this, MS spectral counts of the identified proteins were normalized on a per case basis in order to remove the inter-sample variability. A Mann-Whitney U test compared the distributions of the normalized spectral counts of each protein between the two groups.

Any association between the abundance of patient's FLC levels and immunoglobulin kappa or lambda spectra identified by MS-based proteomic assay was examined. The CR positive cases from the validation cohort were selected and the spectral counts of kappa or lambda proteins found in the matching amyloid deposits were log₂ transformed. Spearman rank correlation assessed the correlation between the abundances of serum light chains and the kappa or lambda proteins in the amyloid deposits.

To investigate relative levels of amyloidosis associated proteins, the spectral counts were normalized using the total number of identified spectra in each case. Normalized counts were scaled to a set value which allows different cases that could have different protein concentrations to be comparable.

Supplemental Figure 1



Supplemental Figure 1:

No correlation was seen between immunoglobulin light chain constant region spectra identified by MS-based proteomic analysis and serum immunoglobulin free light chain levels. (Spearman $r=0.45$; p value 0.0061).

A: Filled red circles are CR positive/AL-lambda specimens. Four of the AL-lambda specimens were inconclusive by MS. Filled black circles are CR positive/AL-kappa or ATTR specimens. Open circles are CR negative specimens. B: Filled red circles are CR positive/AL-kappa specimens. One of the AL-kappa specimens was inconclusive by MS. Filled black circles are CR positive/AL-lambda or ATTR specimens. Open circles are CR positive specimens. Asterisk indicates the one false positive myeloma specimen. One AL-lambda and one ATTR case did not have light chain values.