Laser microdissection and mass spectrometry–based proteomics aids the diagnosis and typing of renal amyloidosis

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Accurate diagnosis and typing of renal amyloidosis is critical for prognosis, genetic counseling, and treatment. Laser microdissection and mass spectrometry are emerging techniques for the analysis and diagnosis of many renal diseases. Here we present the results of laser microdissection and mass spectrometry performed on 127 cases of renal amyloidosis during 2008–2010. We found the following proteins in the amyloid deposits: immunoglobulin light and heavy chains, secondary reactive serum amyloid A protein, leukocyte cell–derived chemotaxin-2, fibrinogen-α chain, transthyretin, apolipoprotein A-I and A-IV, gelsolin, and β-2 microglobulin. Thus, laser microdissection of affected areas within the kidney followed by mass spectrometry provides a direct test of the composition of the deposit and forms a useful ancillary technique for the accurate diagnosis and typing of renal amyloidosis in a single procedure.

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Amyloidosis is caused by extracellular deposition of proteins in an insoluble beta-pleated physical conformation. Amyloid deposits are identified based on their apple-green birefringence under a polarized light microscope on Congo red stains and the presence of rigid, non-branching fibrils 7.5–10 nm in diameter on electron microscopy.[1,2] The most common forms of systemic amyloidosis are immunoglobulin light-chain (AL) amyloidosis and reactive secondary amyloidosis (AA) due to chronic inflammatory diseases (e.g., rheumatoid arthritis and chronic infections). Hereditary or familial amyloidosis is another group of amyloidoses that is being diagnosed with increasing frequency and includes the amyloid derived from leukocyte cell–derived chemotaxin-2 (LECT2), fibrinogen-α chain, transthyretin (TTR), lysozyme, and apolipoproteins.[3,4]

Accurate typing of amyloidosis has implications for prognosis, genetic counseling, and treatment. Although renal biopsy can type AL and AA amyloidosis with the use of immunofluorescence and immunohistochemistry studies, some cases pose problems in accurate typing of amyloid, and ancillary tests including genetic analysis are often required. Heavy-chain amyloidosis and many forms of hereditary amyloidosis are particularly difficult to type on routine renal biopsy studies.[5,6] We have recently reported on the technique of laser microdissection (LMD) and tandem mass spectrometry (MS)–based proteomic analysis as a sensitive and specific tool for the diagnosis of amyloidosis.[7,8] In this report, we describe in detail the LMD/MS results of 127 cases of renal amyloidosis performed over a 2-year period (2008–2010). We also describe and discuss the value of LMD/MS as an important ancillary test for both the diagnosis and typing of renal amyloidosis.

RESULTS

LMD/MS was performed on 127 renal biopsy and/or nephrectomy specimens for diagnosis and typing of renal amyloidosis. The results are summarized in Table 1. Glomeruli, interstitium, or vessels were microdissected for MS studies (Figure 1).

AL amyloidosis, lambda light chain

Thirty-four cases of AL amyloidosis associated with immunoglobulin (Ig) lambda light chain deposition were diagnosed based on LMD/MS results. This was by far the largest group. Ig lambda light-chain constant region (I, II, and/or III) was present in all cases, with >95% probability (Figure 2a). The spectra for lambda light-chain constant region varied from 2 to 55, with an average of 8.5 spectra (±8.7; Figure 3a). Eighteen cases also showed spectra for Ig lambda light-chain variable (V) region (with >95% probability) in addition to the Ig lambda light-chain constant (C) region: 11 cases showed the Ig lambda light-chain V–III region, 6 cases showed the V–I region (Figure 3b), 6 cases showed the V–II region, and 6 cases showed the V–VI region. In 15 cases, a small number of spectra for IgG1, IgG2, or IgG3 was present with probability >95%. In all cases, large spectra

Table 1 | Renal amyloidosis typing based on mass spectrometry results

Amyloid type	Number of cases (%)
AL, lambda light-chain amyloidosis	34 (26.77)
AL, kappa light-chain amyloidosis	20 (15.74)
Heavy-chain amyloidosis and heavy+light-chain amyloidosis	13 (10.02)
AA amyloidosis	16 (12.5)
Fibrinogen A-α amyloidosis	7 (5.5)
Lect-2 amyloidosis	26 (20.4)
Apolipoprotein amyloidosis	2 (1.57)
Gelsolin amyloidosis	2 (1.57)
β-2 Microglobulin	1 (0.8)
TTR amyloidosis	1 (0.8)
Indeterminate	5 (3.9)[a]

Abbreviations: AA, reactive secondary; AL, immunoglobulin light-chain amyloidosis; TTR, transthyretin.
[a]On retrospective review of the mass spectrometry data, three of the five cases qualify as apolipoprotein A-IV amyloidosis.
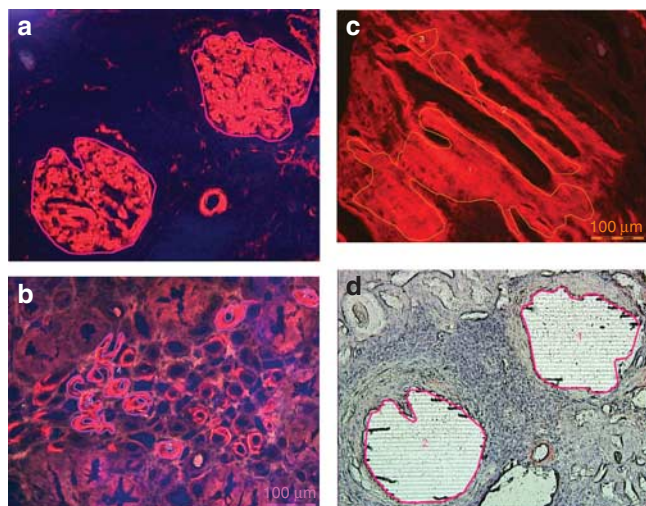

Figure 1 | Laser microdissection. Congo red–positive (**a**) glomeruli, (**b**) vessels, and (**c**) interstitium marked for microdissection. (**d**) Empty space following microdissection of glomeruli from **a**.

of apolipoprotein E protein were present (with probability >95%), whereas serum amyloid P component (SAP) was present in 31 cases with probability >95% and in 2 cases with probability >80–94%. In the one SAP-negative case, the sample size was suboptimal even though MS/MS clearly showed only Ig lambda light-chain constant region with >95% probability and the absence of Ig kappa light chains or other heavy chains. In all cases, MS failed to detect peptides representing LECT2, serum amyloid A (SAA), fibrinogen-α, lysozyme, TTR, or gelsolin.

AL amyloidosis, kappa light chain

Twenty cases of AL amyloidosis associated with Ig kappa light chain deposition were diagnosed based on LMD/MS results. Ig kappa light-chain C region was present in all cases with >95% probability (Figure 2). The spectra for the Ig kappa light-chain C region varied from 5 to 37, with an average of 21.5 spectra (±16.8; Figure 3c). Seven cases also showed spectra for Ig kappa light-chain V region (with >95% probability) in addition to the Ig kappa light-chain C region: five cases showed the Ig kappa light-chain V–I region, one case showed the V–III region, and one case showed the V–IV region. In 13 cases, a small number of spectra for IgG1, IgG2, or IgG4 were also present, with a probability ranging from 80 to >95%. In all cases, large spectra (with probability >95%) of apolipoprotein E protein and SAP were present. In all cases, MS failed to detect peptides representing LECT2, SAA, fibrinogen-α, lysozyme, TTR, or gelsolin.

## Heavy-chain amyloidosis and heavy + light-chain amyloidosis

Thirteen cases of Ig heavy-chain (AH) amyloidosis or mixed AH + AL amyloidosis were diagnosed based on LMD/MS results. Of these, six cases showed only AH amyloid (Figure 2) and seven cases showed AH + AL amyloidosis. The following spectra were noted in the six cases that showed AH amyloid: three cases showed spectra for the IgG1 C region, one case showed spectra for the IgG1 C region and the Ig heavy-chain V region, one case showed spectra for IgG-1 and IgG-3 (Figure 3d and e), and one case showed spectra for only Ig heavy-chain V region (in all cases, probability was >95%). The following spectra were noted in the seven cases that showed AH + AL amyloid: three cases showed the IgG1 C region + Ig lambda light-chain C region, two cases showed the IgG1 C region + Ig kappa light-chain C region, and two cases showed the IgA C region, one with Ig lambda light-chain C region and the other with Ig kappa light-chain C region (in all cases, the probability was >95%). In all cases, large spectra (probability >95%) of apolipoprotein E protein, as well as SAP, were present. MS failed to detect peptides representing LECT2, SAA, fibrinogen-α, lysozyme, TTR, or gelsolin in any of the cases.

AA amyloidosis

Sixteen cases of AA amyloidosis were diagnosed based on LMD/MS results. LMD/MS of AA amyloid cases showed a

large number of spectra of SAA protein in all cases (Figure 2). The range was from 15 to 79 spectra, with an average of 46.14 spectra (± 19.4) with >95% probability in all cases (Figure 3f). In all cases, large spectra (probability >95%) of apolipoprotein E protein were also present. SAP was also present in all cases; the spectra ranged from 5 to 32, with an
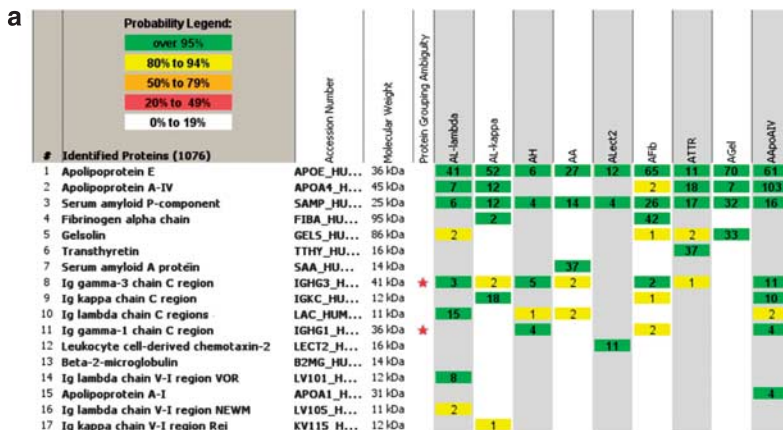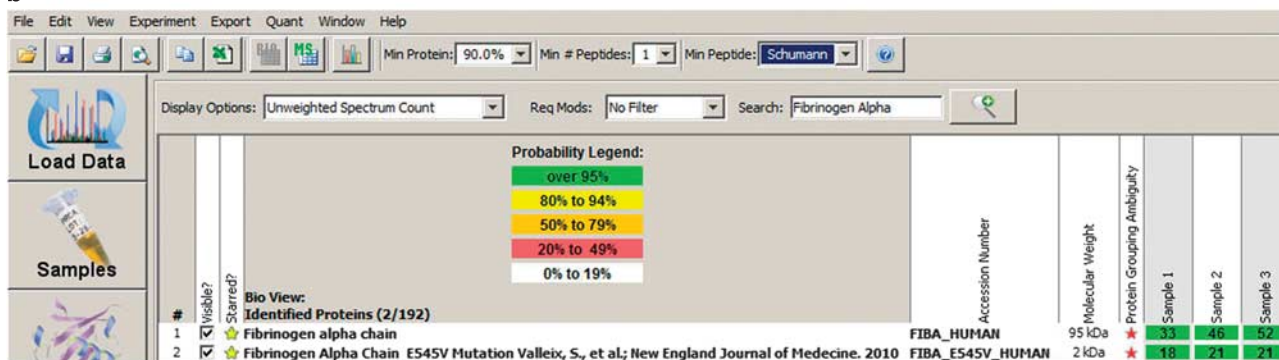
Figure 3 | Percentage sequence (peptide) coverage. Representative sequence (peptide) coverage of the protein detected in the microdissected sample showing: (**a**) 15 total spectra, 6 unique spectra, and 5 unique peptides for 64% coverage of Ig lambda light-chain constant region with 100% probabili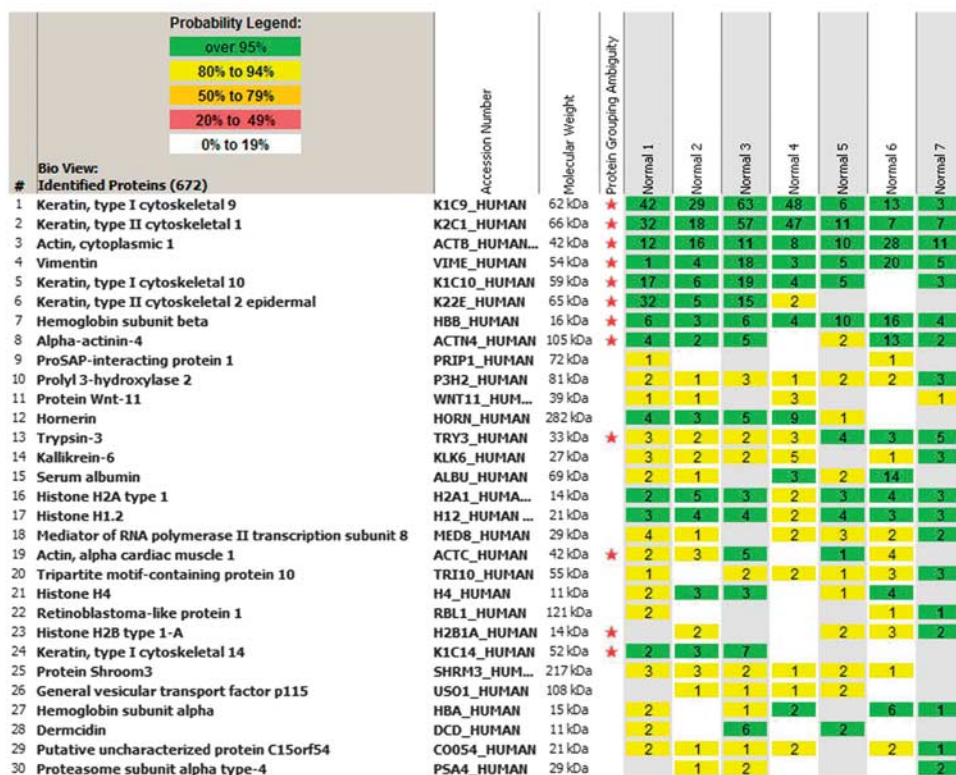ty; (**b**) 8 total spectra, 2 unique spectra, and 2 unique peptides for 15% coverage of Ig lambda light chain variable I region with 100% probability; (**c**) 18 total spectra, 6 unique spectra, and 4 unique peptides for 64% coverage of Ig kappa light-chain constant region with 100% probability; (**d**) 4 total spectra, 2 unique spectra, and 2 unique peptides for 19% coverage of IgG1 heavy constant chain with 100% probability; (**e**) 5 total spectra, 3 unique spectra, and 3 unique peptides for 8% coverage of IgG3 heavy constant chain with 100% probability; (**f**) 37 total spectra, 7 unique spectra, and 6 unique peptides for 35% coverage of serum amyloid A (SAA) protein with 100% probability; (**g**) 42 total spectra, 11 unique spectra, and 10 unique peptides for 10% coverage of fibrinogen-α protein with 100% probability; (**h**) 11 total spectra, 4 unique spectra, and 4 unique peptides for 23% coverage of LECT2 with 100% probability; (**i**) 33 total spectra, 16 unique spectra, and 16 unique peptides for 17% coverage of gelsolin protein with 100% probability; (**j**) 11 total spectra, 7 unique spectra, and 5 unique peptides for 32% coverage of β-2 microglobulin with 100% probability; and (**k**) 37 total spectra, 16 unique spectra, and 14 unique peptides for 63% coverage of transthyretin (TTR) with 100% probability. The yellow highlighted areas show the actual peptides detected by the mass spectrometry, and the green highlighted areas show oxidized or methylated amino acids.

Figure 2 | Mass spectrometry by spectra. (**a**) Representative mass spectrometry data by spectral analyses from a case of AL lambda light-chain amyloidosis (AL-lambda), AL kappa light-chain amyloidosis (AL-kappa), heavy-chain amyloidosis (AH), AA amyloidosis (AA), fibrinogen-α amyloidosis (AFib), LECT-2 amyloidosis (ALect2), transthyretin (TTR) amyloidosis (ATTR), gelsolin amyloidosis (AGel), and apolipoprotein A-IV amyloidosis (ApoAIV). The probability number (>95% is highlighted in green, 80–94% in yellow) indicates essentially the percent homology between peptides detected in the specimens and the published amino-acid sequences of their corresponding proteins. VOR, NEWM, and Rei are names that have been given to specific sequences of variable regions. (**b**) Representative mass spectrometry data from a case of fibrinogen-α amyloidosis showing both the fibrinogen-α chain and the mutated fibrinogen-α peptide. (**c**) Representative mass spectrometry data from day 0 protocol allograft biopsies in seven cases. Red star indicates shared peptides between different proteins. Ig, immunoglobulin.
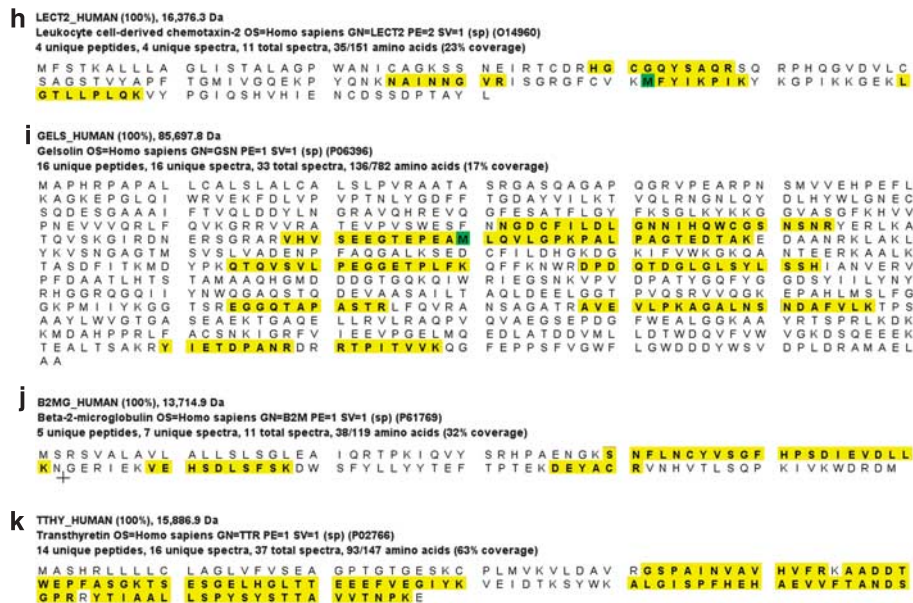
h LECT2_HUMAN (100%), 16,376.3 Da
Leukocyte cell-derived chemotaxin-2 OS=Homo sapiens GN=LECT2 PE=2 SV=1 (sp) (O14960)
4 unique peptides, 4 unique spectra, 11 total spectra, 35/151 amino acids (23% coverage)

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MFSTKALLLA  GLISTALAGP  WANICAGKSS  NEIRTCDRHG  CGQYSAQRSQ  RPHQGVDVLC
SAGSTVYAPF  TGMIVGQEKP  YQNKNAINNG  VRISGRGFCV  KMFYIKPIKY  KGPIKKGEKL
GTLLPLQKVY  PGIQSHVHIE  NCDSSDPTAY  L
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i GELS_HUMAN (100%), 85,697.8 Da
Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1 (sp) (P06396)
16 unique peptides, 16 unique spectra, 33 total spectra, 136/782 amino acids (17% coverage)

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MAPHRPAPAL  LCALSLALCA  LSLPVRAATA  SRGASQAGAP  QGRVPEARPN  SMVVEHPEFL
KAGKEPGLQI  WRVEKFDLVP  VPTNLYGDFF  TGDAYVILKT  VQLRNGNLQY  DLHYWLGNEC
SQDESGAAAI  FTVQLDDYLN  GRAVQHREVQ  GFESATFLGY  FKSGLKYKKG  GVASGFKHVV
PNEVVVQRLF  QVKGRRVVRA  TEVPVSWESF  NNGDCFILDL  GNNIHQWCGS  NSNRYERLKA
TQVSKGIRDN  ERSGRARVHV  SEEGTEPEAM  LQVLGPKPAL  PAGTEDTAKE  DAANRKLAKL
YKVSNGAGTM  SVSLVADENP  FAQGALKSED  CFILDHGKDG  KIFVWKGKQA  NTEERKAALK
TASDFITKMD  YPKQTQVSVL  PEGGETPLFK  QFFKNWRDPD  QTDGLGLSYL  SSHIANVERV
PFDAATLHTS  TAMAAQHGMD  DDGTGQKQIW  RIEGSNKVPV  DPATYGQFYG  GDSYIILYNY
RHGGRQGQII  YNWQGAQSTQ  DEVAASAILT  AQLDEELGGT  PVQSRVVQGK  EPAHLMSLFG
GKPMIIYKGG  TSREGGQTAP  ASTRLFQVRA  NSAGATRAVE  VLPKAGALNS  NDAFVLKTPS
AAYLWVGTGA  SEAEKTGAQE  LLRVLRAQPV  QVAEGGEPDG  FWEALGGKAA  YRTSPRLKDK
KMDAHPPRLF  ACSNKIGRFV  IEEVPGELMQ  EDLATDDVML  LDTWDQVFVW  VGKDSQEEEK
TEALTSAKRY  IETDPANRDR  RTPITVVKQG  FEPPSFVGWF  LGWDDDYWSV  DPLDRAMAEL
AA
```

j B2MG_HUMAN (100%), 13,714.9 Da
Beta-2-microglobulin OS=Homo sapiens GN=B2M PE=1 SV=1 (sp) (P61769)
5 unique peptides, 7 unique spectra, 11 total spectra, 38/119 amino acids (32% coverage)

```
MSRSVALAVL  ALLSLSGLEA  IQRTPKIQVY  SRHPAENGKS  NFLNCYVSGF  HPSDIEVDLL
KNGERIEKVE  HSDLSFSKDW  SFYLLYYTEF  TPTEKDEYAC  RVNHVTLSQP  KIVKWDRDM
```

k TTHY_HUMAN (100%), 15,886.9 Da
Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1 (sp) (P02766)
14 unique peptides, 16 unique spectra, 37 total spectra, 93/147 amino acids (63% coverage)

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MASHRLLLLC  LAGLVFVSEA  GPTGTGESKC  PLMVKVLDAV  RGSPAINVAV  HVFRKAADDT
WEPFASGKTS  ESGELHGLTT  EEEFVEGIYK  VEIDTKSYWK  ALGISPFHEH  AEVVFTANDS
GPRRYTIAAL  LSPYSYSTTA  VVTNPKE
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Figure 3 | Continued.

average of 16.6 spectra ($\pm$7.5). In addition, in one case, MS/MS also detected a variant SAA protein (SAA W71R) similar to that described by Møyner K et al.[9,10] Small amounts of IgG with polytypic Ig lambda and kappa light chains were noted in almost half the cases. MS failed to detect peptides representing LECT2, fibrinogen-α, lysozyme, TTR, apolipoprotein A-I, or gelsolin in any of the cases.

Fibrinogen-α amyloidosis

Seven cases of fibrinogen-α amyloidosis were diagnosed based on LMD/MS results. LMD/MS showed a large number of spectra of fibrinogen-α chain in all cases, with >95% probability (Figure 2). The range was from 18 to 109 spectra, with an average of 55.2 spectra ($\pm$21.51; Figure 3g). In addition, MS also identified the mutated fibrinogen-α chain with >95% probability in all cases (Figure 2b). In all cases, large spectra (with >95% probability) of apolipoprotein E protein were also present. SAP was also present in all cases; the spectra ranged from 7 to 40, with an average of 20.2 spectra ($\pm$7.5). Small amounts of IgG with polytypic Ig lambda and kappa light chains were also noted in half the cases. MS failed to detect peptides representing LECT2, SAA, lysozyme, TTR, apolipoprotein A-I, or gelsolin in any of the cases.

LECT2 amyloidosis

Twenty-six cases of LECT2 amyloidosis were diagnosed on LMD/MS results. LMD/MS of LECT2 amyloid cases showed a large number of spectra of LECT2 protein in 25 of 26 cases, with >95% probability in these cases (Figure 2). In one case, repeat LMD/MS showed only one unique LECT2 spectrum (with a probability of 80–94%). The range was from 2 to 16 spectra, with an average of 7.6 spectra ($\pm$3.8) with >95% probability in all cases (Figure 3h), except for the one

case mentioned above. Large spectra of apolipoprotein E protein and SAP (>95% probability) were present in all cases. Small amounts of IgG with polytypic Ig lambda and kappa light chains were also noted. IgG included the constant region of IgG1, IgG2, IgG3, and IgG4. In all cases, MS failed to detect peptides representing fibrinogen-α, SAA, lysozyme, TTR, apolipoprotein A-I, or gelsolin.

Gelsolin amyloidosis

Two cases of gelsolin amyloidosis were diagnosed based on LMD/MS results. LMD/MS of gelsolin amyloidosis cases showed a large number of spectra of gelsolin in both cases (Figure 2). The range was from 23 to 35 spectra, with an average of 33.3 spectra ($\pm$6.0), and >95% probability in all cases (Figure 3i). In all cases, large spectra (>95% probability) of apolipoprotein E protein were also present. SAP was also present in all cases; the spectra ranged from 24 to 30, with an average of 26.7 spectra ($\pm$3.4). Small amounts of IgG, IgA, and apolipoprotein A-IV were also noted. In all cases, MS failed to detect peptides representing LECT2, fibrinogen-α, SAA, lysozyme, TTR, or apolipoprotein A-I.

Apolipoprotein amyloidosis

One case of apolipoprotein A-IV amyloidosis, restricted to the renal medulla, was diagnosed based on LMD/MS results.[11] The most abundant peptides detected represented apolipoprotein A-IV protein (Figure 2). LMD/MS showed a large number of spectra of apolipoprotein A-IV ranging from 73 to 103 (>95% probability), with an average of 89.6 ($\pm$14.5). SAP was also present; the spectra ranged from 16 to 20, with an average of 17.3 spectra ($\pm$2.3). Small amounts of apolipoprotein E and apolipoprotein A-I were also detected. LMD/MS failed to detect peptides representing TTR, LECT2,

fibrinogen-α, SAA, apolipoprotein A-II, or significant immunoglobulin heavy or light chains.

One case of apolipoprotein A-I amyloidosis was diagnosed based on LMD/MS results. The most abundant peptides detected represented apolipoprotein A-I protein. LMD/MS showed a large number of spectra of apolipoprotein A-I ranging from 28 to 34 (>95% probability), with an average of 30.5 (±2.1). SAP was also present; the spectra ranged from 13 to 23, with an average of 19.25 spectra (±4.2). Small amounts of apolipoprotein E and apolipoprotein A-IV were also detected. MS failed to detect peptides representing TTR, LECT2, fibrinogen-α, SAA, apolipoprotein A-II, or significant immunoglobulin heavy or light chains.

β-2 Microglobulin

A single case of a nephrectomy specimen with multiple simple cysts consistent with polycystic kidney disease showed amyloid within tubular casts. Congo red stain was positive and electron microscopy confirmed the amyloid fibrils within the casts. LMD/MS detected a peptide profile consistent with β-2 microglobulin–type amyloid deposition. LMD/MS showed a large number of spectra of β-2 microglobulin ranging from 8 to 14, with an average of 11 (±2.4; Figure 3j). MS failed to detect peptides representing TTR, LECT2, fibrinogen-α, SAA, apolipoprotein A-II, or significant immunoglobulin heavy or light chains.

TTR amyloidosis

A single case of biopsy of the renal pelvis showed TTR amyloidosis based on LMD/MS results (Figure 2). Subsequent workup also showed cardiac involvement. LMD/MS showed a large number of spectra of TTR ranging from 18 to 32 (>95% probability), with an average of 27.25 (±5.4; Figure 3k). SAP was also present; the spectra ranged from 9 to 20, with an average of 15.6 spectra (±4.5). Small amounts of IgG, IgA, apolipoprotein A-IV, and apolipoprotein E were also noted. MS failed to detect peptides representing LECT-2, fibrinogen-α, SAA, β-2 microglobulin, gelsolin, or apolipoprotein A-I.

Indeterminate group

We were unable to type five cases of amyloidosis. Of these, two cases did not show or showed very weak spectra for SAP, thus questioning the diagnosis of amyloidosis. In the remaining three cases, LMD/MS showed a large number of SAP spectra, confirming the diagnosis of amyloidosis. The SAP spectra ranged from 14 to 42, with an average of 23.3 (±9.1). However, on retrospective review of the MS data for this manuscript, all three cases showed large spectra for apolipoprotein A-IV ranging from 29 to 64 (with >95% probability in all cases), with an average of 45.9 (±10.5). In addition, the amyloid was restricted to the interstitium, with no glomerular or vascular involvement. This raises the possibility of apolipoprotein A-IV amyloidosis. Apolipoprotein A-IV amyloidosis has only been recently described[11] and was not a defined entity at the time when LMD/MS was performed in these cases.

Control cases

For comparison, we also performed LMD/MS on glomeruli of day 0 protocol biopsies of the allograft kidney. We conducted these studies in 25 cases. The renal biopsy results were as close to those of normal kidneys on light microscopy examination. LMD/MS showed large spectra for keratin, actin, vimentin, actinin, hemoglobin, and smaller spectra for albumin, histones, hornerin, trypsin (used for digestion process), etc. The protein spectra of seven representative cases are shown in Figure 2c. Importantly, none of the amyloidogenic proteins was found in the day 0 protocol biopsies.

DISCUSSION

LMD/MS is a relatively new technique used for diagnosis and typing of amyloidosis. We have described the usefulness of this technique in unique cases of renal amyloidosis in native and transplant kidney biopsies.[5,10–13] This manuscript is a comprehensive review of LMD/MS results of 127 cases of renal amyloidosis. These include all cases of LMD/MS performed on renal biopsy or nephrectomy specimens from 2008 to 2010. LMD/MS was performed on most cases when immunofluorescence studies were equivocal or inconclusive and/or no material was available for immunofluorescence studies.

The diagnosis of amyloidosis at the proteomic level using LMD/MS is based on the presence of SAP (Table 2). In all cases, large spectra of apolipoprotein E are found as well, suggesting that it may have a role in fibrillogenesis. It should be pointed out that apolipoprotein E in the amyloid is likely not due to high serum lipid levels associated with nephrotic syndrome, as apolipoprotein E is also noted in amyloidosis

Table 2 | Criteria for diagnosis and typing of amyloidosis by LMD/MS

	Peptides on MS (>95% probability)
Diagnosis of amyloidosis	Serum amyloid protein Apolipoprotein E also present in 100% of cases
AL lambda light chain	Lambda light-chain C region, with or without V region; absence of significant kappa light chains
AL kappa light chain	Kappa light-chain C region, with or without V region; absence of significant lambda light chains
AH heavy chain	Heavy chain: γ, μ, α, with large number of spectra, in comparison with light-chain spectra
AA	SAA
LECT2	Leukocyte cell–derived chemotaxin-2 (LECT-2)
Fibrinogen-α	Fibrinogen-α
Apolipoprotein A-I or IV	Apolipoprotein A-I or IV, large and predominant spectra compared with few small spectra often found in other types of amyloid
TTR	Transthyretin (TTR)
Gelsolin	Gelsolin
β-2 Microglobulin	β-2 Microglobulin
Lysozyme	Lysozyme

Abbreviations: AA, reactive secondary amyloidosis; AH, immunoglobulin heavy-chain amyloidosis; AL, immunoglobulin light-chain amyloidosis; C, constant; LMD, laser microdissection; MS, mass spectrometry; SAA, serum amyloid A; V, variable.

not associated with nephrotic syndrome and high serum lipid levels, such as amyloid restricted to the renal interstitium or vessels, and in amyloidosis of other organs such as heart, liver, etc. Few methylated and oxidized amino acids were also detected, and it is possible that they were generated during processing of the sample. The typing of amyloid is then based on the presence of the spectra that correspond to the specific type of amyloid. For example, AL lambda light-chain amyloid contains large spectra of Ig lambda light-chain C region with or without lambda light-chain V region. Typically, Ig kappa light chains are absent or very small spectra of Ig kappa light chain along with heavy chain may be detected, representing small amounts of polyclonal reabsorption protein granules and/or entrapment of immuno-globulins in areas of amyloid. Similarly, AL kappa light-chain amyloid contains large spectra of kappa light-chain C region with or without kappa light-chain V region. It is uncommon to find the hypervariable region in light-chain amyloid. In the case of AH amyloid, there are large spectra of the heavy chain with or without an accompanying light-chain C or V region. In the remaining types of amyloidosis, the criteria for amyloid typing are even more straightforward and are based on identification of the specific peptide spectra belonging to the amyloid type, as these peptides are not found in the normal proteome of the glomerulus. For example, AA amyloidosis shows spectra of SAA protein, LECT2 amyloidosis shows spectra of LECT2 protein, fibrinogen-α amyloidosis shows spectra for fibrinogen-α including the mutated fibrinogen A peptide, TTR amyloidosis shows spectra for TTR, and so on. LMD/MS of normal glomeruli (day 0 protocol kidney biopsies) showed that these glomeruli do not contain spectra of any of these amyloidogenic peptides.[14]

The advantages of LMD/MS over conventional methods of amyloid typing are multifold: (1) LMD/MS is a single test that can identify the amyloid protein in question vs. testing the renal biopsy for individual amyloid proteins via immunohistochemistry or other ancillary studies. (2) LMD is performed directly on the involved tissue glomeruli, vessels, or interstitium, i.e., Congo red–positive areas are dissected. Thus, LMD/MS is performed on the tissue involved by the amyloid and not on the entire renal biopsy specimen. This is in contrast to other tests such as western blot analyses, which use large amounts of uninvolved tissue as well and typically screen for a single amyloid protein at a time. There is also a lack of specificity because the tissue contains non-amyloid tissue and serum, which may be a rich source of amyloidogenic proteins. (3) LMD/MS is performed on paraffin-embedded material and does not require frozen material. Thus, studies can be conducted on archival material. We have performed LMD/MS-based diagnosis and typing of amyloid on 30-year-old paraffin-embedded materi-al. Inadequate biopsy specimen, especially when there is limited tissue for immunofluorescence microscopy, is indeed a common indication for LMD/MS studies for amyloid typing. (4) Problematic amyloid cases that pose difficulty in

amyloid typing on immunofluorescence studies such as heavy-chain or heavy- and light-chain amyloidosis are another important reason for conducting LMD/MS studies.[5] We are becoming increasingly aware that many cases of AL amyloidosis have an AH component based on LMD/MS studies. Further studies are required to determine whether the presence of AH component has any effect on treatment, prognosis, and outcome of immunoglobulin-associated amyloidosis. (5) Familial and hereditary amyloidosis are difficult to diagnose on routine immunofluorescence and immunohistochemistry studies, e.g., LECT2, fibrinogen-α, and apolipoprotein amyloid. LMD/MS is extremely useful and sensitive in the diagnosis and typing of cases in which elaborate and time-consuming genetic analyses are often required.[13] (6) With the current software and database, it is possible to identify the genetic variants of amyloidogenic proteins, such as variants of SAA or fibrinogen-α protein.[10] (7) LMD/MS can detect and type early amyloid even before Congo red stain is positive, thus questioning the definition of amyloid, i.e., amyloid is Congo red positive. Early detection is based on the presence of SAP protein and the identification of unique amyloidogenic protein, such as fibrinogen-α or SAA.[12] (8) Monoclonal gammopathy may be present in patients with non-AL or AH amyloidosis. Some of these cases are presumed to be AL amyloid. The most common missed diagnosis is fibrinogen-α amyloid.[4] LMD/MS is extremely useful in such cases to confirm hereditary or other types of amyloid and rule out AL amyloid in the setting of a monoclonal gammopathy.

LMD/MS is also helpful in differentiating other causes of fibrillary deposits from amyloidosis. Fibrillary glomerulone-phritis shows glomerular fibrillary deposits that are positive for IgG and light chains that are sometimes monoclonal.[15] Such cases of fibrillary glomerulonephritis can be difficult to differentiate from AH or AH + AL amyloidosis, particularly when the Congo red staining is equivocal. In such cases, LMD/MS is extremely useful and will detect spectra of SAP and heavy and light chains, thus confirming the heavy-chain amyloid. On the other hand, SAP is typically not detected in fibrillary glomerulonephritis, and typically large spectra of heavy chains are present.[16] Another potential use of LMD/MS is in the setting of two or more types of amyloidosis coexisting together. However, in our practice, we have not come across such cases. On the other hand, LMD/MS in AA amyloidosis (diagnosis based on immunohistochemistry performed at a different institutions), in the setting of monoclonal gammopathy, confirmed spectra for AL amyloidosis and ruled out AA amyloidosis even though SAA was positive on immunohistochemistry studies. Similarly, on LMD/MS, few cases of presumed AL amyloidosis (based on the presence of a monoclonal gammopathy, as no immuno-fluorescence studies were conducted or were inadequate), in fact, revealed fibrinogen-α, LECT2, or apolipoprotein A-IV amyloidosis.

The common indications for LMD/MS studies included confirmation of amyloid type, inadequate sample for

immunofluorescence studies, difficult cases on routine renal biopsy studies such as heavy-chain amyloidosis, and familial and hereditary amyloidosis. In many cases, clinicians directly ordered LMD/MS studies at the time of renal biopsy for confirmation and typing. Thus, it should be noted that the amyloid types detected by LMD/MS in this study represent the challenging cases and are not representative of the incidence of amyloid types in the population. In our practice, LMD/MS studies were conducted in ~14% of the renal biopsy specimens for confirmation and typing of amyloidosis.

This study does not address the LMD/MS performed on fat-pad aspirate, cardiac tissue, bone marrow tissue, lymph node, gastrointestinal tract, brain, etc. Obviously, these are other sites of amyloid deposition, and paraffin-embedded material from these sites can also be used for amyloid diagnosis and typing by LMD/MS. In particular, LMD/MS on the fat-pad aspirate is more appealing, as it is a less invasive procedure for the diagnosis and typing of amyloidosis.

To summarize, we present the results of LMD/MS performed on 127 renal biopsies and nephrectomy specimens for diagnosis and typing of renal amyloidosis over 2-year period. LMD/MS studies are useful because it uses formalin-fixed paraffin-embedded tissue instead of fresh, frozen, or other specially stored tissue samples. Problematic and challenging biopsies, such as those with equivocal Congo red staining, equivocal or negative light-chain staining on immunofluorescence studies, heavy-chain component, and less common forms such as fibrinogen Aα, TTR, LECT2, apolipoprotein A-I and A-IV, gelsolin, lysozyme and β-2 microglobulin amyloidosis fall in a group of amyloidosis that can be accurately typed by LMD/MS.

MATERIALS AND METHODS
Specimen, specimen preparation, LMD, and MS-based proteomic analysis

MS data of renal biopsies received from 2008 to 2010 in the Department of Laboratory Medicine and Pathology, Mayo Clinic for diagnosis and typing of amyloid were analyzed. The Institutional Review Board at the Mayo Clinic approved the study.

The methods have previously been published.[7,8,14] The licensed Patent Rights to perform protein extraction from paraffin-embedded tissue for the MS-based amyloid testing have been granted by Expression Pathology. Briefly, for each case, 10-μm-thick sections of formalin-fixed paraffin-embedded tissues were stained with Congo red. Congo red–positive glomeruli viewed under fluorescent light source appeared bright red. The glomeruli with Congo red–positive deposits were identified under fluorescent light and microdissected with LMD using the Leica dissector (Leica DM 600 B). For controls, we used glomeruli from 25 cases of day 0 protocol biopsies of the kidney allograft. The glomeruli were selected for LMD as shown by the red line. Following LMD, there is a vacant space on the slide (Figure 1). Each microdissection contained an area of 50,000–60,000 μm², and 2–4 microdissections (typically two glomeruli per slide, and two slides per case) were analyzed for each case. Congo red–positive vessels and interstitium were also selected for microdissection if glomeruli were not available for microdissection, or if the sample size was felt to be limited. The microdissected

material was collected into 0.5-ml microcentrifuge tube caps containing 35 μl Tris/EDTA/0.002% Zwittergent buffer. Microdissected fragments were digested into tryptic peptides overnight and analyzed by liquid chromatography electrospray tandem MS. MS raw data files were queried using three different algorithms (Sequest, Mascot and X!Tandem), and the results were combined and assigned peptide and protein probability scores in Scaffold (Proteome Software, Portland, OR). For each case, a list of proteins based on peptides identified by MS was generated. Peptide identifications were accepted if they could be established at >90.0% probability as specified by the Peptide Prophet algorithm.[17–19] The MS data show peptides and spectra that match a particular protein based on the amino-acid sequence available in the database. Some of the peptides (and spectra) from different proteins can be common and be shared depending upon the homology of their amino-acid sequence. On the other hand, unique peptides and spectra are distinctive to the particular protein. The 'Spectra' value indicates the total number of mass spectra collected on the mass spectrometer and matched to the protein using the proteomics software. A higher number of mass spectra is indicative of greater abundance and will typically yield greater amino-acid sequence coverage. A higher mass spectra value also indicates a higher confidence in the protein identification. Our clinical amyloid testing requires a minimum number of four spectra in all samples before the protein identification will be deemed clinically valid.

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