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Editorial Comment

EDITORIAL COMMENT

Why laser microdissection and mass spectrometry is the method of choice for detection of membranous nephropathy antigens

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Membranous nephropathy is characterized by accumulation of antigen–antibody immune complexes along the subepithelial region of the glomerular basement membranes (GBM). The specific antigen in the immune complexes defines the type of MN. Until 2014, only two antigens—PLA2R and THSD7A—had been discovered. Most laboratories employ immunofluorescence microscopy or immunohistochemical methodology to detect these two antigens on the kidney biopsy tissue. However, since 2019, at least 11 more antigens have been discovered. The MN antigens now include EXT1 and EXT2, NELL1, SEMA3B, NCAM1, CNTN1, HTRA1, FAT1, PCDH7, NTNG1, PCSK6 and NDNF [1–16]. Many of the new antigens associated with MN have distinctive clinical, pathologic, treatment and prognostic findings. Thus, it has become increasingly important to identify the antigen associated with the MN.

EXT1/EXT2 and NELL1 are more common than the other antigens, and a few specialized laboratories employ immunohistochemical methods on the kidney biopsy specimen to detect these additional antigens. It would be impractical and difficult to perform immunohistochemistry/immunofluorescence (IHC/IF) for the remaining antigens due to many reasons that include availability of specific antibodies, availability of control samples, expertise in interpretation of IHC/IF stains, requirement of protease digestion for staining of certain antigens and the risk of exhausting the kidney biopsy sample due to large number of sections required to stain each antigen. Thus, clearly a different methodology for detection of the MN antigen is needed.

Laser microdissection and mass spectrometry (LMD/MS) was the essential methodology used for discovery of many of the new MN antigens. We have recently adapted the LMD/MS methodology that was used for discovery of MN antigens for routine clinical use [17]. The major differences from a discovery to clinical LMD/MS platform include thinner sections to conserve the biopsy sample, quicker mass spectrometry times with shorter columns and settings to detect already known antigens. Our initial validation studies show that LMD/MS is reliable and sensitive method for detection of MN antigens.

WHY LMD/MS METHODOLOGY WORKS

(i) The MN antigens are unique in that most of the antigens are not expressed in the normal glomerulus, except PLA2R, THSD7A and FAT1 which have very low baseline expression (spectral counts <10).
(ii) In MN, there is massive accumulation of the antigen–antibody immune complexes along the capillary walls, i.e. large amounts of antigen are present.
(iii) LMD of MN glomeruli followed by MS typically reveals high spectral counts of a known antigen, thus identifying the specific antigen in the glomerular digest. Dual antigens are very rare.
(iv) The list of antigens can be easily expanded to detect 'newer' antigens as they are discovered.
(v) Tissue is preserved as glomeruli are microdissected from two to four sections on a single slide, compared with the

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A.

ProbabilityLegend:
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

#	Visible?	Starred?	BioView: Identified Proteins (3197) Including 1119 Decoys	Accession Number	Molecular Weight	#1	#2
1			Proprotein convertase subtilisin/...	PCSK6_HU...	106 kDa	76	62
2			Protocadherin Fat 1	FAT1_HUM...	506 kDa		2
3			Protein kinase C-binding protein ...	NELL1_HUM..	90 kDa		1
4			Actin, cytoplasmic 2	ACTG_HUM...	42 kDa	1364	1356
5			Vimentin	VIME_HUM...	54 kDa	1012	1009
6			Laminin subunit beta-2	LAMB2_HU...	196 kDa	601	623
7			Complement C3	CO3_HUMAN	187 kDa	588	543
8			Actin, alpha cardiac muscle 1	ACTC_HUM...	42 kDa	98	82
9			Actin, cytoplasmic 1	ACTB_HUM...	42 kDa	87	94
10			(ENZYME) Trypsin precursor	ENZYME_TR..	24 kDa	648	623
11			Ig Gamma-1 heavy chain	IGG1_HUM...	49 kDa	401	347
12			Ig Gamma-4 heavy chain Consta...	IGHG4_HU...	36 kDa	206	211

B.

ProbabilityLegend:
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

#	Visible?	Starred?	BioView: Identified Proteins (827) Including 109 Decoys	Accession Number	Molecular Weight	# 1	# 2
1			Secretory phospholipase A2 rece...	PLA2R_HU...	169 kDa	99	57
2			Complement C3	CO3_HUMAN	187 kDa	1718	1591
3			Actin, cytoplasmic 2	ACTG_HUM...	42 kDa	1061	1048
4			Vimentin	VIME_HUM...	54 kDa	749	766
5			Laminin subunit beta-2	LAMB2_HU...	196 kDa	673	665
6			Actin, cytoplasmic 1	ACTB_HUM...	42 kDa	89	75
7			Actin, alpha cardiac muscle 1	ACTC_HUM...	42 kDa	57	59
8			(ENZYME) Trypsin precursor	ENZYME_TR..	24 kDa	745	737
9			Ig Gamma-1 heavy chain	IGG1_HUM...	49 kDa	225	214
10			Ig Gamma-4 heavy chain Consta...	IGHG4_HU...	36 kDa	67	59

Figure 1: LMD/MS studies showing high spectral counts of (**A**) PSCK6 in Case 1 and (**B**) PLA2R in Case 2. Samples were run in duplicate (#1 and #2). Also shown are housekeeping proteins such as actin, vimentin and laminin, C3 and IgG subytpes.

large number of sections and slides required to stain for each individual antigen using IHC/IF methodology.

(vi) Biopsy samples with no immunofluorescence material is not a limitation, as LMD/MS uses paraffin-embedded tissue.

(vii) Expertise in the interpretation of IHC/IF for MN antigens is required, whereas LMD/MS is a read-out that requires less expertise.

(viii) IHC/IF for many of the rare antigens does not exist even in specialized laboratories.

SUGGESTED APPROACH TO USE LMD/MS TO DETECT THE MN ANTIGEN

There are three suggested approaches to detect the MN antigen.

(i) In the first approach, we use IHC/IF methodology for detection of PLA2R on the kidney biopsy tissue. PLA2R positivity will account for 50%–60% of all MN. We then perform LMD/MS in the remaining 40%–50% PLA2R-negative MN.

(ii) In the second approach, IHC/IF is used to detect PLA2R, THSD7A, NELL1 and EXT1/EXT2. These antigens will account for 60%–70% of all MN. We then perform LMD/MS in the remaining 30%–40% cases of MN.

(iii) In the third approach, LMD/MS is performed upfront without any IHC/IF studies. This saves an additional step of performing IHC/IF studies.

Currently, at the Mayo Clinic we are using the first approach.

OTHER ASPECTS AND LIMITATIONS OF LMD/MS FOR MN ANTIGEN DETECTION

(i) If we use the first approach in which the MN is enriched for PLA2R negative MN, no known antigen is found in approximately 20%–30% of these cases.

(ii) If we include any case of immune complex glomerulonephritis, with subepithelial electron dense deposits, the sensitivity of the test to detect an antigen drops further.

(iii) It is critical to review the biopsy findings of MN prior to performing LMD/MS to detect the MN antigen.

(iv) Surprisingly, using LMD/MS we have detected PLA2R-positive MN in a small proportion (~3%–4%) of PLA2R-negative MN by IHC/IF (manuscript in preparation).

CASES TO ILLUSTRATE THE VALUE OF LMD/MS IN DETECTING THE MN ANTIGEN

Case 1

A young 15-year-old female presented with proteinuria, microscopic hematuria, lymphadenopathy and seizures. The urinary protein was 3.8 g/day, serum albumin 3.1 g/dL and serum creatinine 0.6 mg/dL. Serologies were positive for anti-nuclear antibody, anti-double-stranded DNA and SSA/SSB. She carried a diagnosis of undifferentiated mixed connective tissue disease. The kidney biopsy showed a membranous nephropathy. On light microscopy, 17 glomeruli were present, none of which were globally sclerotic. The glomeruli did not show crescents, fibrinoid necrosis or endocapillary hypercellularity. Glomerular basement membranes were mildly thickened and on trichrome stain, red minute subepithelial deposits were present. The interstitium was well preserved. Arteries and arterioles were unremarkable. Immunofluorescence microscopy showed granular glomerular capillary wall and mesangial staining for IgG (2–3+), C3 (1+), kappa (3+) and lambda (3+). Glomeruli were negative for IgM, C1q and IgA. Electron microscopy showed diffuse small subepithelial electron dense deposits, some of which were associated with short glomerular basement membrane spikes. No endothelial tubuloreticular inclusions were seen. Mesangial deposits were not present. The kidney biopsy diagnosis was membranous nephropathy, related to the autoimmune disease. PLA2R staining was negative.

LMD/MS was performed to detect the MN antigen. We expected to find EXT1/EXT2 which is the target antigen associated with autoimmune disease. On the other hand, LMD/MS found high spectral counts of PCSK6 (Fig. 1A), which is associated with non-steroidal anti-inflammatory drug use [15]. Thus, a diagnosis

of PCSK6-associated MN was made, likely altering the management of the MN.

Case 2

A 26-year-old woman with history of a biopsy-proven membranous nephropathy 7 years ago presented with nephrotic syndrome. PLA2R studies were negative on immunofluorescence studies on the biopsy tissue. Three years later, she developed serology consistent with syphilis. Patient now presented with nephrotic syndrome with positive low anti-PLA2R-positive titers as well as serologies consistent with active syphilis. A kidney biopsy was performed. There were 23 glomeruli, of which 2 were globally sclerosed. The glomeruli did not show any crescents, fibrinoid necrosis or endocapillary hypercellularity. The GBM was thickened, and basement membrane spikes and pinholes were noted along the GBM. Foam cells were present in the interstitium and there was mild (20%) tubular atrophy and interstitial fibrosis present. Arteries and arterioles are unremarkable. The material for immunofluorescence microscopy was suboptimal, however a glomerulus showed granular staining for IgG along the GBM. PLA2R staining could not be performed. Electron microcopy showed thickening of the GBM with diffuse accumulation of subepithelial electron dense deposits. Basement membrane spikes are noted between the electron dense deposits.

LMD/MS was performed to detect the MN antigen. LMD/MS showed high spectral counts for PLA2R (Fig. 1B), confirming a diagnosis of PLA2R-associated MN. On the other hand, LMD/MS was negative for NDNF, which is the target antigen in syphilis-associated MN [14]. Thus, LMD/MS was helpful in excluding syphilis-associated MN while at same time confirming a diagnosis of PLA2R-associated MN.

SUMMARY

LMD/MS is new methodology that can be used to detect the target antigen in MN. It is a one-stop test to detect all the known MN antigens and can expanded to include other antigens when they are discovered. We anticipate that LMD/MS methodology will become the gold standard for detection of MN antigens.

CONFLICT OF INTEREST STATEMENT

None declared.

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