Kidney360

Transforming Growth Factor Beta Receptor 3 (TGFBR3)–Associated Membranous Nephropathy

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Key Points

- TGF-β receptor 3 (TGFBR3) staining identifies a novel type of membranous nephropathy (MN).
- TGFBR3-associated MN is enriched in patients with membranous lupus nephritis with similar characteristics as exostosin 1/2- and neural cell adhesion molecule 1–associated MN.
- Identification of TGFBR3-associated MN should alert the clinician to evaluate for underlying autoimmune disease.

Abstract

Background Membranous lupus nephritis (MLN) comprises 10%–15% of lupus nephritis and increases morbidity and mortality of patients with SLE through complications of nephrotic syndrome and chronic kidney failure. Identification of the target antigens in MLN may enable noninvasive monitoring of disease activity, inform treatment decisions, and aid in prognostication, as is now possible for idiopathic MN caused by antibodies against the phospholipase A2 receptor. Here, we show evidence for type III TGF-β receptor (TGFBR3) as a novel biomarker expressed in a subset of patients with MLN.

Methods Mass spectrometry was used for protein discovery through enrichment of glomerular proteins by laser capture microdissection and through elution of immune complexes within MLN biopsy specimens. Colocalization with IgG within glomerular immune deposits from patients and disease controls was evaluated by confocal microscopy. Immunostaining of consecutive case series was used to determine the overall frequency in MN and MLN.

Results TGFBR3 was found to be enriched in glomeruli and coimmunoprecipitated with IgG within a subset of MLN biopsy specimens by mass spectrometry. Staining of consecutive MN cases without clinical evidence of SLE did not show TGFBR3 expression (zero of 104), but showed a 6% prevalence in MLN (11 of 199 cases). TGFBR3 colocalized with IgG along the glomerular basement membranes in TGFBR3-associated MN, but not in controls.

Conclusions Positive staining for TGFBR3 within glomerular immune deposits represents a distinct form of MN, substantially enriched in MLN. A diagnosis of TGFBR3-associated MN can alert the clinician to search for an underlying autoimmune disease.

KIDNEY360 2: 1275–1286, 2021. doi: https://doi.org/10.34067/KID.0001492021

Introduction

Lupus nephritis is a common disease manifestation in SLE, affecting up to 60% of patients, and is associated with increased morbidity and mortality (1). Membranous lupus nephritis (MLN) affects 10%–15% of patients with SLE who have kidney involvement; they typically present with worsening proteinuria that often progresses to nephrotic syndrome (1). MLN is characterized by the presence of subepithelial Ig deposits within glomeruli, often accompanied by mesangial immune deposition. In the absence of disease remission, lupus nephritis progresses to ESKD in 50% of patients within 20 years (2). In addition, there are complications associated with nephrotic syndrome, such as hypercoagulability and exposure to long-term cytotoxic and nephrotoxic medications in its treatment (3).

Membranous nephropathy (MN) has historically been categorized as "secondary" in patients that have a known secondary etiology, and "primary" for those that do not. More recently, classification is shifting to identifying the underlying antigenic target of the autoantibodies driving disease. Perhaps a more meaningful classification would be to identify the MN by the antigenic target when known, and as "idiopathic" when the antigenic target is not known (4,5). Knowledge of the underlying autoantigen not only provides an etiologic classification scheme but, more importantly, provides the possibility of noninvasive monitoring of disease activity through serum-based immunoassays.

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Although the majority of the antigenic targets in MN are known (>80% of cases), most of the associated autoantigens in MLN are currently unknown. The autoantigens for idiopathic MN include the phospholipase A2 receptor (PLA2R; 70% of cases [6)], thrombospondin type 1 domain containing 7A (THSD7A; 3%–10% of cases [7,8]), neural EGF-like 1 (NELL1; 4%–6% of cases [9,10]), semaphorin 3B (1% of cases and up to 10% of pediatric cases [11]), serine protease HTRA1 (4% of cases [12]), and protocadherin 7 (6% of cases [13]). These proteins are not commonly expressed in MLN, which is thought to be due to different antigenic drivers and immune dysregulation. Currently, there are two biomarkers described in MLN, which include the exostosin 1/2 (EXT1/2) complex (implicated in 18% of MLN cases) and neural cell adhesion molecule 1 (NCAM1; comprising 7% of MLN cases [14]); the majority of MLN cases are of an unknown type.

In this study, we used mass spectrometry to identify antigenic targets of autoantibodies in MLN through elution of immune complexes from kidney biopsy tissue and through laser capture microdissection for enrichment of glomerular proteins. We found type III TGF-β receptor (TGFBR3) to be specifically present in a subset of MLN biopsy specimens, which may serve as a novel biomarker of disease.

Materials and Methods

Case Selection

Cases of MLN, with or without a concurrent proliferative component (classified under the International Society of Nephrology/Renal Pathology Society criteria [ISN/RPS] class V with or without III or IV), were identified within our database. In addition, PLA2R-negative, THSD7A-negative, NELL1-negative, and EXT1/2-negative "idiopathic" MN cases were examined. All of the data were collected according to a study protocol approved by the Solutions Institutional Review Board and all ethical principles and guidelines for the protection of human subjects in research were followed. Serum samples were obtained from the biorepository at Arkana Laboratories under the same institutional review board protocol.

Study Cohorts

The discovery cohort for mass spectrometry included 17 biopsy specimens for protein G immunoprecipitation to elute immune complexes from frozen tissue, and 18 biopsy specimens that were prepared by laser capture microdissection.

For the validation cohorts, two separate, consecutive biopsy sample series were screened by paraffin immunofluorescence staining. A total of 104 presumed idiopathic MN cases (which were PLA2R-negative, THSD7A-negative, EXT1-negative, and NELL1-negative) and 199 MLN cases were evaluated. The idiopathic MN series comprised 9 months of kidney biopsy specimens from patients with idiopathic MN, and the MLN cohort comprised 6 months of consecutive kidney biopsy specimens. For the idiopathic series, PLA2R-, THSD7A-, EXT1-, and NELL1-negative cases were stained for TGFBR3. The MLN series was screened for EXT1 and NCAM1.

The Arkana biorepository samples were also evaluated for TGFBR3 staining to identify serum samples from patients with positive TGFBR3 staining on their biopsy sample. Formalin-fixed, paraffin-embedded (FFPE) tissue sections from a total of 110 kidney biopsy specimens, including 75 idiopathic MN and 35 MLN cases, were stained in this analysis, and a total of four positive cases were identified.

To evaluate the specificity of the TGFBR3 stain, a separate analysis of 20 PLA2R-positive MN biopsy samples, 20 THSD7A-positive MN biopsy samples, 91 NELL1-associated MN biopsy samples, 102 EXT-associated MLN biopsy samples, and 12 NCAM1 biopsy samples were evaluated.

Clinical and Laboratory Data

Clinical data recorded included the age and sex of each patient, history of SLE and other diagnoses, follow-up interval, treatment(s), and whether or not clinical remission was achieved. This was defined as <300 mg proteinuria/d. Laboratory parameters included serum creatinine, serum albumin, proteinuria (urine protein-creatinine ratio or 24-hour urinary protein), serologic studies (anti-nuclear antibodies, anti–double-stranded DNA antibodies, and anti-nuclear antibody specificities when available).

Protein G Immunoprecipitation

Residual frozen kidney biopsy tissue was thawed and washed in PBS, followed by lysis with 300 µl Pierce IP lysis buffer (catalog number 87787; Thermo Fisher Scientific, Waltham, MA) with the addition of protease and phosphatase inhibitors (Halt, catalog number 78429; Thermo Fisher Scientific), and disaggregation by bead beating. PBS-washed Protein G Dynabeads (50 µl; catalog number 10004D; Thermo Fisher Scientific) were added to protein lysates and incubated for 1 hour at room temperature. Beads were then washed three times in PBS before peptide digestion and preparation for mass spectrometry.

Preparation of Laser Capture Microdissected Glomeruli for Mass Spectrometry

Kidney biopsy tissue from FFPE tissue was cut at a thickness of 10 µm onto Leica polyethylene terephthalate–membrane frame slides and stained with Mayer hematoxylin. The glomeruli were microdissected into microcentrifuge tubes containing PBS using a Leica DM6000B microscope. At least 40 glomeruli were dissected per biopsy sample. The microdissected glomeruli were lysed in 2% SDS and 0.1 M dithiothreitol at 99°C for 1 hour and processed by filter-assisted sample preparation (15). The clarified lysate was transferred onto Vivacon 500 concentrators (molecular mass cutoff of 30 kDa; catalog number VN01H22; Sartorius, Goettingen, Germany). SDS was removed by repeat washes with 8 M urea in 0.1 M Tris hydrochloride, pH 8.5, and the samples were then alkylated with 0.05 M iodoacetamide. Iodoacetamide was removed by three washes with 8 M urea/0.1 M Tris hydrochloride, pH 8.5, followed by three washes with 0.05 M ammonium bicarbonate. Proteins were digested with trypsin (sequencing grade; catalog number V5111; Promega) at a 40:1 wt/wt ratio at 37°C for 16 hours. Peptides were collected by centrifugation and desalted on C18 Stage tips (catalog number 87782; Thermo Scientific).

Mass Spectrometry

Digested peptides were analyzed by nanoscale liquid chromatography coupled to tandem mass spectrometry (MS/MS) using a Thermo Orbitrap Fusion Lumos mass spectrometer. The peptides were loaded onto a reverse-phase trap column (Integra-Frit, New Objective, MA) containing 2.5 μm Waters XSelect CSH resin coupled to a 150 × 0.075 mm analytical column containing the same reverse-phase resin as that used in the trap. A nanoAcquity UPLC System (Waters Corp, Milford, MA) was then used to generate a 60-minute gradient of the buffer A/B ratio (buffer A, 0.1% formic acid and 0.5% acetonitrile; buffer B, 0.1% formic acid and 99.9% acetonitrile) from 98:2 to 60:40. Peptides were eluted from the column with an integrated spray tip (PicoFrit, New Objective) and ionized by electrospray (2.0 kV), followed by MS/MS analysis using higher-energy collision-induced dissociation. Survey scans of peptide precursors were performed at a resolution of 240,000 (at 400 m/z), with a 5×10^5 ion count target. MS/MS was performed by isolation at 1.6 Th with the quadrupole, higher-energy collision-induced dissociation fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The obtained MS/MS data were searched against the most recent Uniprot human database containing both the Swiss-Prot and the TREMBL entries using MaxQuant. Visualization of data was done using Scaffold version 4.6. The false discovery rate was set at 1% for the peptide-to-spectrum matches. Normalized iBAQ values from MaxQuant were used for quantitation. Intensity-based absolute quantification (iBAQ) distributions for each sample were median adjusted to control for differences in loading. iBAQ values equal to zero were removed from the dataset. For statistical hypothesis testing, a two-sample Welch *t* test was performed for each protein using normalized iBAQ values for the two groups. If a protein was only detected in one group, a one-sample Welch *t* test was performed, using the smallest detected iBAQ value as the null hypothesis.

Staining and Confocal Microscopy

All samples were processed for light, immunofluorescence, and electron microscopy using standard techniques (16). TGFBR3 staining was performed on 3 μm FFPE tissue sections after deparaffinization. Antigen retrieval was performed at 99°C in high-pH citrate buffer (BOND Epitope Retrieval Solution, pH 9.0, catalog number PA5-21994). The sections were reacted with rabbit polyclonal anti-TGFBR3 (1:25; Thermo Fisher Scientific), followed by a Rhodamine Red X–conjugated goat anti-rabbit secondary antibody, which was solid-phase adsorbed to ensure minimal crossreaction with human IgG (1:100; catalog number 111-295-144, Jackson Immunoresearch). Negative controls were performed through omission of the primary antibody. The slides were coverslipped with aqueous mounting medium and then evaluated by standard immunofluorescence microscopy. The immunofluorescence staining was considered to be positive if there was granular capillary loop staining in the glomeruli, and negative if there was no capillary loop staining in glomeruli.

Anti-TGFBR3 (1:25 dilution)/Rhodamine Red X (1:100 dilution) and anti-human IgG-FITC were costained for colocalization studies. For this analysis, polyclonal (FITC-conjugated) rabbit anti-human IgG (1:40; Agilent, Santa Clara, CA) was reacted with the heat-retrieved tissue after staining for TGFBR3 as described above. Colocalization of IgG and TGFBR3 along the glomerular basement membranes was examined by confocal microscopy using a Leica SBA DMI8 confocal laser scanning microscope. A total of five cases positive for TGFBR3 were compared with nine control cases, including five PLA2R- and four EXT1-positive MN control cases. Confocal images were captured using sequential scanning of Z-stack images that were overlaid for maximal projection.

Primary antibodies used for staining membranous antigens include rabbit polyclonal anti-PLA2R (catalog number HPA012657; Sigma), mouse monoclonal anti-THSD7A (catalog number AMAB91234; Atlas Antibodies), rabbit polyclonal anti-EXT1 (catalog number PA5-60699; Invitrogen), rabbit polyclonal anti-NELL1 (catalog number PA5-27958; Invitrogen), and rabbit polyclonal anti-NCAM1 (catalog number HPA039835; Sigma). IgG subclass staining used direct FITC conjugates for IgG1 (catalog number 115-095-205; Jackson Immunoresearch), IgG2 (catalog number 115-095-207; Jackson Immunoresearch), IgG3 (catalog number F4641; Sigma), and IgG4 (catalog number F9889; Sigma).

Serologic Evaluation for TGFBR3 Antibodies

The methods attempted to evaluate seroreactivity of anti-TGFBR3 antibodies are detailed in the Supplemental Methods.

Results

Mass Spectrometry Identified TGFBR3 within Glomerular Immune Deposits in a Subset of MLN Biopsy Samples

To discover antigens bound to IgG within the glomerular immune deposits in MLN, IgG was eluted from frozen kidney biopsy tissue through protein G immunoprecipitation followed by mass spectrometry. This technique has been shown to be highly sensitive and specific for identification of antigenic targets in MN of known type (9,14). Mass spectrometry was performed on 31 kidney biopsy specimens, including four with PLA2R-positive MN, three with THSD7A-positive MN, four with EXT1/2-associated MN, four with diffuse lupus nephritis (without concurrent MLN), and 16 cases of MLN of unknown type. A total of 2024 unique proteins were identified in this dataset. TGFBR3 was exclusively present in two of the MLN biopsy specimens, with a total of 14 unique peptides identified (Figure 1A). Immunofluorescence staining confirmed the presence of TGFBR3 protein uniquely in the deposits of these two cases.

As an independent discovery method, mass spectrometric analysis of laser capture microdissected glomeruli from FFPE tissue was performed. This analysis included one of the TGFBR3-associated biopsy specimens identified by immunoprecipitation, in addition to 17 other cases (eight with PLA2R-positive MN, four with THSD7A-positive MN, two with EXT-positive MN, and three with diffuse lupus nephritis). A total of 3176 proteins were identified in this analysis. Fourteen unique TGFBR3 peptides were present at high levels from one sample identified as being associated with TGFBR3 by protein G immunoprecipitation. No significant TGFRB3 peptides were identified in the remaining cases (Figure 1B). This analysis represents an independent method that confirms glomerular enrichment of TGFBR3 in a sample that had previously been shown to

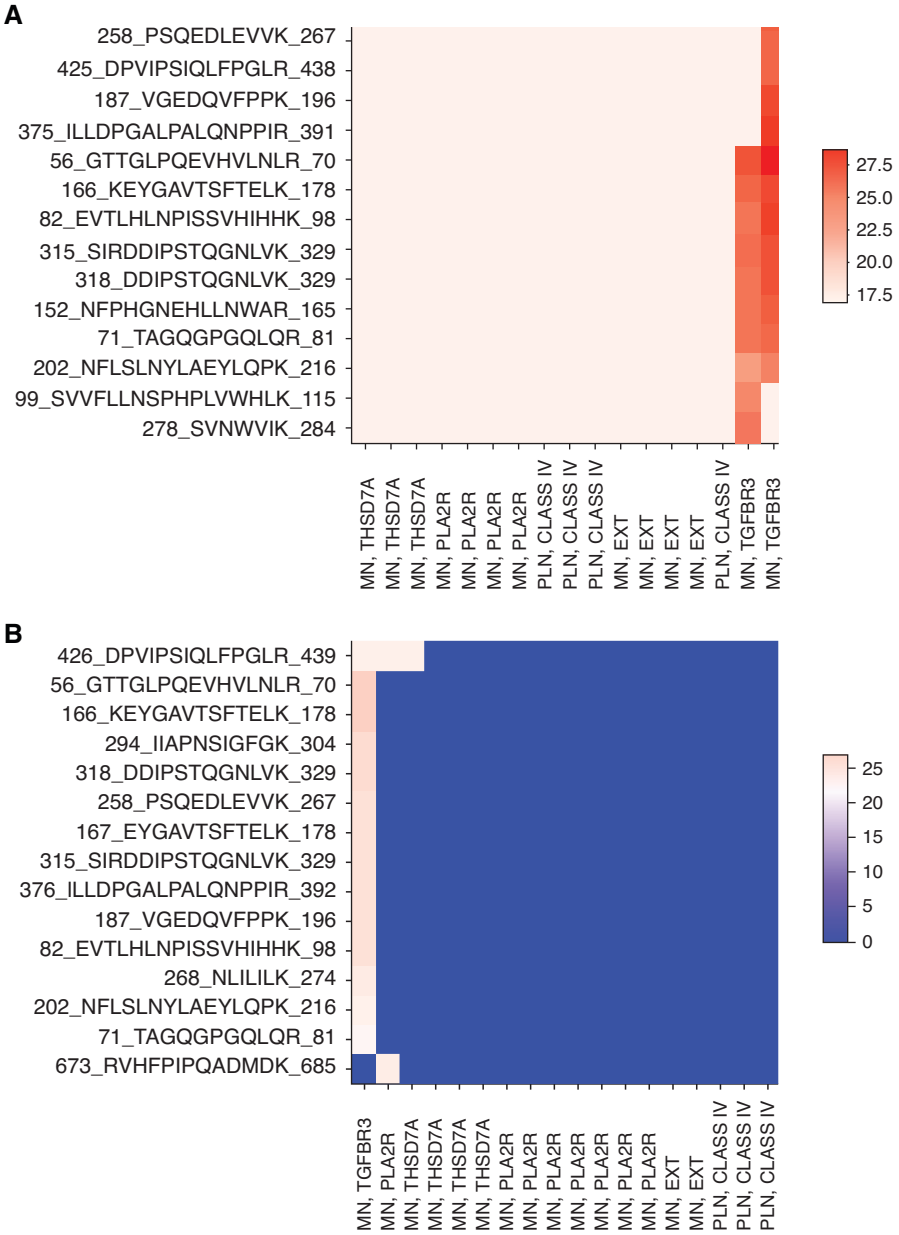

Figure 1. | **Mass spectrometry reveals type III TGF-beta receptor (TGFBR3) to be uniquely present in a subset of membranous nephropathy cases, as shown in the heat map below.** The heat maps show the sequence of the identified TGF-β receptor 3 (TGFBR3) peptides and the color intensity reflects the abundance of each peptide. (A) M ass spectrometric analysis shows that TGFBR3 is significantly enriched within protein G tissue immunoprecipitates (n=2) and (B) laser capture microdissected glomeruli (n=1) from patients with membranous lupus nephritis. TGFBR3 peptides were identified throughout the entire extracellular domain of the protein, including (A) nine peptides by protein G immunoprecipitation and (B) 14 peptides by laser capture microdissection. EXT, exostosin; MN, membranous nephropathy; PLA2R, phospholipase A2 receptor; THSD7A, thrombospondin type 1 domain containing 7A; PLN, proliferative lupus nephritis.

have IgG coimmunoprecipitation with TGFBR3 in total kidney biopsy tissue.

TGFBR3 Colocalizes with IgG Immune Deposits within Glomeruli

Immunofluorescence staining with a rabbit polyclonal antibody against TGFBR3 showed diffuse granular capillary loop staining in glomeruli of TGFBR3-positive cases identified by mass spectrometry, but not in PLA2R- (n=20), NELL1- (n=20), THSD7A- (n=20), NCAM1- (n=12), or EXT1/2-associated MN (n=33) controls (Supplemental Figure 1). Moreover, there was no TGFBR3 staining along the glomerular capillary loops from other kidney diseases, including minimal change disease (n=10), FSGS tip variant (n=10), diabetic glomerulopathy (n=10), and fibrillary GN (n=10; Supplemental Figure 2). TGFBR3 staining was also performed on 40 cases of pure diffuse lupus nephritis (ISN/RPS class IV), of which one case was positive (3%). This demonstrates TGFBR3 positivity to be specific in a subset of MN cases, of which all are classified as a type of lupus nephritis.

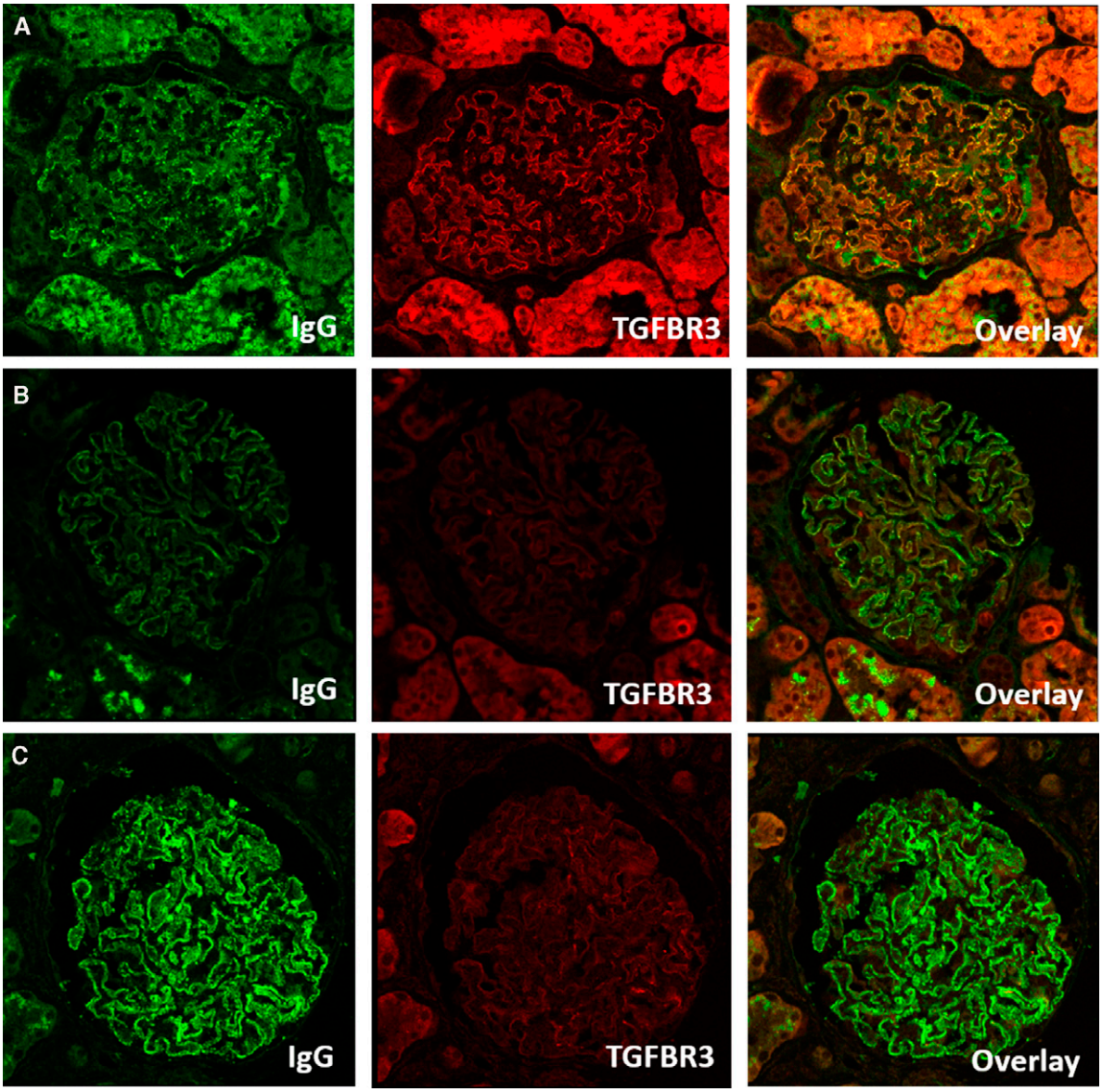

Figure 2. | **IgG and TGFBR3 colocalize within glomeruli of TGFBR3-associated membranous nephropathy.** Representative confocal microscopy images show IgG staining in green (FITC), TGFBR3 staining in red (Rhodamine Red X), and overlay images of the red and green channels in yellow. Staining is shown of a representative (A) TGFBR3-associated MN case (predicted by mass spectrometry), (B) an EXT1/EXT2-associated MN case, and (C) a PLA2R-associated MN case.

TGFBR3 was found to colocalize with IgG within glomerular immune deposits by confocal microscopy (n=5; Figure 2A), without significant colocalization within EXT-associated MN cases (n=4; Figure 2B), or within PLA2R-positive MN (n=5; Figure 2C). Although TGFBR3 has known expression in podocytes (17), we did not detect the granular subepithelial deposits within other membranous types.

The Frequency of TGFBR3-Associated MN Was Determined through Staining Consecutive Series of Kidney Biopsy Specimens

A series of consecutive of MLN cases were tested for the prevalence of TGFBR3 glomerular staining. A total of 222 consecutive cases of MLN from a 6-month time period were identified, of which 199 cases had sufficient tissue remaining for immunostaining. Of these 199 MLN cases, concurrent focal or diffuse (proliferative) lupus nephritis was present in 93 (47%) of the biopsy samples within this cohort. TGFBR3 was positive in 11 (6%) of the biopsy specimens, whereas 14 of 199 cases were NCAM1 positive (7%), 34 were EXT1/2 positive (17%), and 70% of cases were of an unknown antigen type (TGFBR3-, NCAM1-, and EXT1/2-negative cases, n=140; Supplemental Figure 3). TGFBR3 staining was not identified in a separate analysis of 152 consecutive idiopathic PLA2R-negative MN cases (Supplemental Figure 3).

To identify specimens with associated serum samples for subsequent analysis, paraffin immunofluorescence was performed on idiopathic MN (n=75) and MLN (n=35) biopsy specimens from our biorepository containing tissue with

Table 1. Demographic and laboratory data comparing TGFBR3-associated membranous nephropathy with membranous nephropathy of other antigen types

	Mean±SD					
Parameter	TGFBR3 Positive (n=17)	PLA2R Positive (n=183)	THSD7A Positive (n=10)	NELL1 Positive (n=91)	EXT1/2 Positive (n=102)	NCAM1 Positive (n=20)
Age (yr)	39.6±16.1	56.4±13.9 (P≤0.001)	45.1±16.3 (P>0.05)	66.8±10.8 (P≤0.001)	36.9±1.5 (P>0.05)	34.1±12.4 (P>0.05)
Creatinine (mg/dl)	1.6±1.3	1.9±2.3 (P>0.05)	1.9±2.3 (P>0.05)	1.3±0.9 (P>0.05)	1.5±1.9 (P>0.05)	1.3±0.9 (P>0.05)
Proteinuria (g/d)	8.4±7.3	8.0±5.1 (P>0.05)	6.7±4.8 (P>0.05)	6.2±3.5 (P>0.05)	5.4±5.4 (P>0.05)	7.6±10.3 (P>0.05)

TGFBR3, TGF-β receptor 3; PLA2R, phospholipase A2 receptor; THSD7A, thrombospondin type 1 domain containing 7A; NELL1, neural EGF-like 1; EXT1/2, exostosin 1/2; NCAM1, neural cell adhesion molecule 1.

matched sera (Supplemental Figure 3). Four TGFBR3-positive MN cases were identified, of which three were from patients with MLN and one was from a patient with idiopathic MN. Images of all TGFBR3-positive MN cases are shown in Supplemental Figure 4.

Demographics and Clinical Characteristics of Patients with TGFBR3-Associated MN Were Compared with Those with MLN and MN of Known Antigen Type

In total, we had a cohort of 17 patients with TGFBR3-associated MN for evaluation. These 17 patients comprised two patients from the mass spectrometry discovery cohort, 11 from the longitudinal cohort (to evaluate disease frequency), and four from the biorepository. Patients with TGFBR3-associated MN were younger than patients with PLA2R-positive or NELL1-positive MN (mean±SD age 39.6±16.1 years versus 56.4±13.9 years or 66.8±10.8 years, respectively; P<0.001). There was no significant difference in the mean age of patients with THSD7A-positive MN, EXT1/2-associated MN, or NCAM1-associated MN (Table 1).

Patients with TGFBR3-associated MN did not have a significant difference in renal function or proteinuria when compared with those with other forms of MN. Quantitative proteinuria (which included either 24-hour urinary protein or urine protein-creatinine ratio) at the time of biopsy was 8.4±7.3 g/d in patients with TGFBR3-associated MN, which was not significantly different from patients with MN of other antigenic types (Table 1).

A majority of patients with TGFBR3-associated MN had positive autoimmune serologies, with 16 of 17 having antinuclear antibodies. Of the 17 patients with TGFBR3-associated MN, 16 had a history of autoimmune disease before biopsy, with 14 of 17 with a diagnosis of SLE before biopsy, one with rheumatoid arthritis, and one with antiphospholipid antibody syndrome. All 15 of the patients with available follow-up data received immunosuppressive therapy, yet only two had achieved clinical remission at follow-up (defined as proteinuria of <300 mg/dl, mean follow-up of 10.8 months). Demographic and laboratory data of individual patients with TGFBR3 are shown in Table 2.

Histopathologic Characteristics of TGFBR3-Associated MN Biopsy Specimens Were Compared with MN Cases of Known Antigen Type

A concurrent proliferative lupus nephritis component was present in 29% of patients with TGFBR3-associated MN compared with 47% of total patients with MLN and concurrent proliferative lupus nephritis within our 6-month cohort. Of those with a proliferative component, the mean National Institutes of Health (NIH) activity index for patients who were TGFBR3 positive was 5.8 out of 24 and the chronicity index was 4.8 out of 12. Activity and chronicity were not significantly different when compared with EXT-associated MLN (27% with ISN/RPS class III/IV, mean NIH mean activity and chronicity indexes of 5.1 out of 24 and 4.1 out of 12, respectively) or NCAM1-associated MN (25% with ISN/RPS class III/IV, mean NIH activity and chronicity indexes of 5.4 out of 24 and 5.6 out of 12, respectively).

Glomeruli from patients with TGFBR3-associated MN showed mesangial expansion, mesangial hypercellularity, and prominent glomerular capillary loops, some of which contained "spikes" and "holes" on silver stains (Figure 3, A–D). Patients with a concurrent proliferative component also showed endocapillary hypercellularity and/or crescent formation (Figure 3, E–F). Patients with TGFBR3-associated MN have a high frequency of positivity for other immune reactants besides IgG, and a "full-house" immunofluorescence pattern was present in 47% of patients (Table 3). A full-house immunofluorescence pattern was significantly more frequent in TGFBR3-associated MN, compared with PLA2R-positive MN (P=0.0001), THSD7A-positive MN (P=0.01), NELL1-associated MN (P<0.001), and EXT-associated MN (P=0.0006). All P values represent Fisher exact tests (Table 4).

Glomerular IgG staining of TGFBR3 cases showed a diffuse and global granular mesangial and capillary loop pattern in a majority of patients (94%; Figure 3G, Table 4), with an identical staining pattern as TGFBR3 (Figure 3H). IgG subclass staining was performed for 13 of 17 TGFBR3-associated MN cases with tissue available for immunofluorescence staining (Figure 4). There was no consistent pattern of IgG subclass staining in TGFBR3-associated MN cases and none of the cases were IgG4 restricted. Two TGFBR3-associated MN cases (15%) had IgG4 dominant or

Table 2. Clinical and laboratory featu res of patients with TGFBR3-associated membranous nephropathy

Patient	Diagnosis	Age (yr)	Sex	Cr (mg/dl)	Albumin (g/dl)	Proteinuria (g/d)	Serologies (positive)	SLE Dx	Additional Diagnoses	Remission	Treatment
1	Lupus V	26	M	2.6	0.9	13.5	ANA+, dsDNA+	Yes	Pulmonary embolism	Unknown	Prednisone, MMF, HCQ
2	Lupus V	29	F	0.4	3.4	3	ANA+, dsDNA+	Yes	None	No	Prednisone, MMF, HCQ
3	Lupus V	25	F	0.6	Low	2.8	ANA+, dsDNA+	No	APLS	Deceased	Azathioprine, HCQ
4	Lupus V	26	F	1.8	1.4	2.7	ANA+	Yes	Deep venous thrombosis	No	Prednisone, MMF
5	Lupus V	53	F	0.8	4.2		ANA+, dsDNA+	Yes	None	None	MMF, HCQ
6	Lupus IV and V	33	F	3.23	Low		ANA+, dsDNA+	Yes	Anemia, thrombocytopenia	No	Prednisone, ACTHar, MMF
7	Lupus IV and V	35	F	2.7		Nephrotic		Yes	Diabetes, obesity	Unknown	Unknown
8	Lupus III and V	69	F	5.3		18.5	ANA+, dsDNA−,	Yes	HTN, diabetes, MGUS	No	Dexamethasone, cytophosphamide
9	Lupus IV and V	60	F	1.2	WNL	1	ANA+, dsDNA+, RNP+	Yes	None	Unknown	None
10	Lupus V	29	F	2.53	3.3	Nephrotic	ANA+, dsDNA−	Yes	Autoimmune hemolytic anemia	Yes	Prednisone, cyclophosphamide
11	Lupus V	39	F	1.05	1.9	20.4	ANA+, dsDNA+	Yes	HTN, prior lupus podocytopathy, pregnancy	No	Lisinopril
12	Lupus V	48	F	Normal		1.3	ANA+, dsDNA+	Yes	Raynaud phenomenon	Yes	MMF
13	Lupus III and V	16	F	Normal	2.3	Subnephrotic	ANA+, dsDNA+, RNP+, Smith+	Yes	None	No	Cyclophosphamide, MMF, rituximab
14	Lupus V	33	F	0.6	2.9	7.6	ANA+	Yes	None	No	MMF
15	Lupus V; plasma cell–rich interstitial nephritis	33	F	1.23	0.5	16	ANA+, dsDNA−, SS-A+	No	RA, Lyme disease, chronic EBV, autonomic dysfunction	No	Prednisone, MMF, rituximab, spironolactone
16	Lupus V	49	F	0.6	0.9	5.6	ANA+, dsDNA+, SS-A+, SS-B+	Yes	Antiphospholipid syndrome, HTN, MGUS	Unknown	Prednisone, MMF, lisinopril, HCQ
17	Membranous nephropathy, PLA2R and THSD7A negative	71	F	1.19	1.7	Nephrotic	Rheumatoid factor+	No	Hypothyroidism, asthma, chronic NSAID use	No	Prednisone, cyclosporine

TGFBR3, TGF-β receptor 3; Cr, creatinine; Dx, diagnosis; M, male; ANA, anti-nuclear antibody; dsDNA, double-stranded DNA (antibodies); MMF, mycophenolate mofetil; HCQ, hydroxychloroquine; F, female; APLS, antiphospholipid antibody syndrome; ACTHar, repository corticotropin injection; MGUS, monoclonal gammopathy of renal significance; WNL, within normal limits; RNP, ribonuclear protein; HTN, hypertension; RA, rheumatoid arthritis; EBV, Epstein–Barr virus; SS-A, anti-Ro antibody; SS-B, anti-La antibody; NSAID, nonsteroidal anti-inflammatory drug.

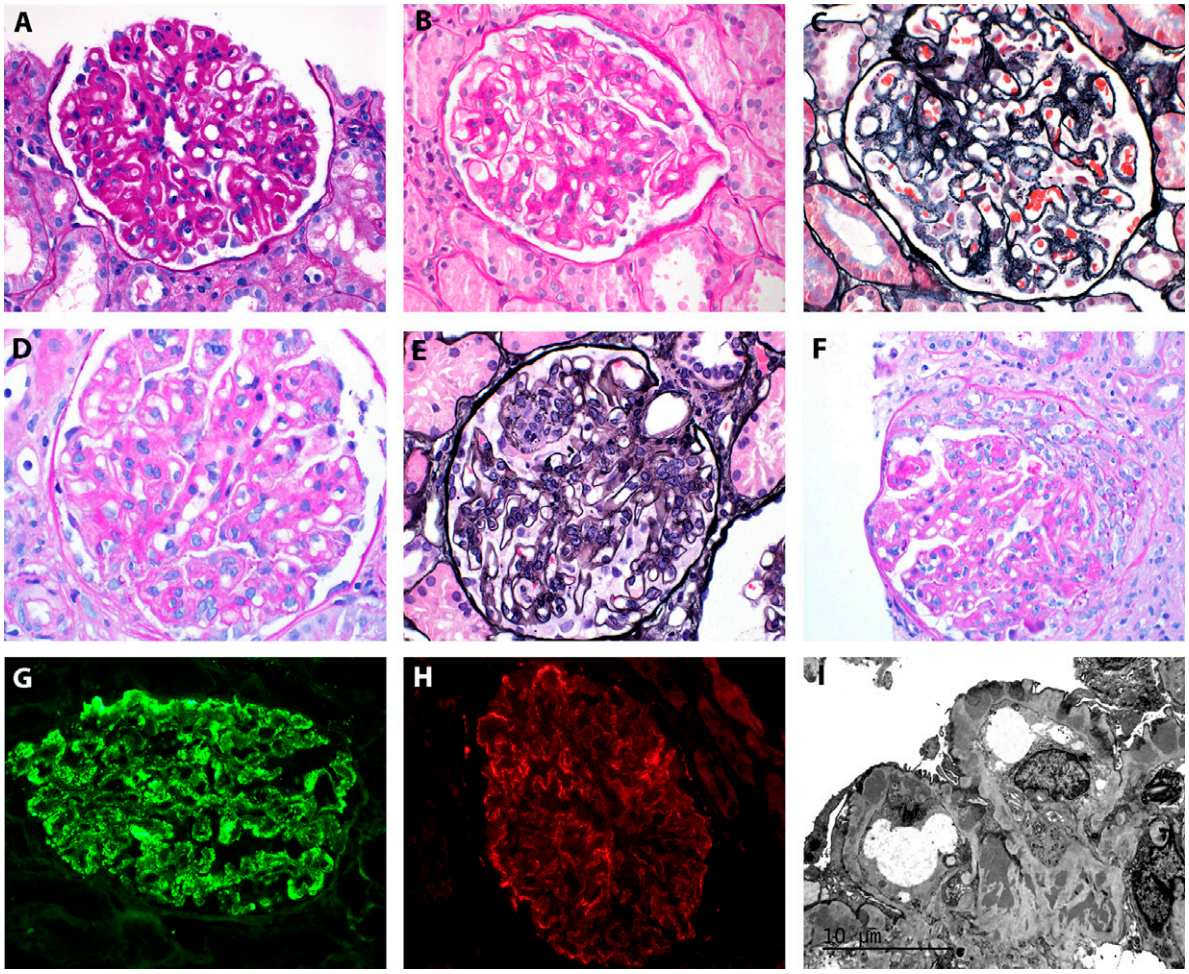

Figure 3. | **TGFBR3-associated membranous nephropathy can occur with or without a proliferative lupus nephritis component.** (A) Periodic acid–Schiff stain showing a glomerulus with prominent capillary loops. (B) Hematoxylin and eosin stain showing mesangial expansion within a glomerulus. (C) Jones methenamine silver stain highlighting spikes and holes along the glomerular capillary loops. (D) Periodic acid–Schiff stain showing mesangial hypercellularity. (E) Jones methenamine silver stain displaying endocapillary hypercellularity. (F) Periodic acid–Schiff stain showing cellular crescent formation within a glomerulus. (G) IgG immunofluorescence showing global granular mesangial and capillary loop staining within a glomerulus. (H) TGFBR3 immunofluorescence staining showing global granular capillary loop staining. (I) Subepithelial, intramembranous, and mesangial electron-dense deposits seen by electron microscopy. Original magnification, 400×.

codominant deposits (Supplemental Table 1). Tubular basement membrane deposits were present in seven of 17 TGFBR3-associated MN cases.

Electron microscopy showed a similar degree of subendothelial and mesangial deposits in TGFBR3-, EXT-, and NCAM1-associated MN (Table 4 and Figure 3I). TGFBR3-associated MN showed an Ehrenreich and Churg classification of stage I in one of 17 cases, stage II in five of 17, stage III in nine of 17, and stage IV in two of 17. Tubuloreticular inclusions were identified in three of 17 cases (Supplemental Figure 5).

Serologic Examination of Anti-TGFBR3 Antibodies

We pursued several methods to assess the presence of circulating anti-TGFBR3 antibodies in the four available serum samples, including Western blotting of recombinant TGFBR3 under reducing and nonreducing conditions and with deglycosylation (Supplemental Figure 6), Western blotting of native TGFBR3 within extracts of human glomeruli, and using a cell-based indirect immunofluorescence assay (Supplemental Figure 7, see Supplemental Methods). Despite the detection of recombinant and native glomerular TGFBR3 with commercial antibodies, the four sera did not appear to contain anti-TGFBR3 antibodies. Immunoprecipitation of glomerular protein extracts with the patient sera was also not able to bring down TGFBR3 (Supplemental Figure 8).

Discussion

We have identified TGFBR3 as a unique subtype of MN. Because TGFBR3 expression within immune deposits is rare in idiopathic MN, but enriched in MLN, identification of this form of MN may alert clinicians to pursue a thorough rheumatologic workup to identify a possible underlying

Table 3. Histopathologic features of patients w ith TGFBR3-associated membranous nephropathy

Patient	GGS (%)	IF/TA	Proliferative Component	IgA	IgG	IgM	C3	C1q	Global or Segmental	Full House	Mesangial Deposits	Subendo Deposits	TBM Deposits	FPE	EM Stage
1	0	Absent	No	2	3	2	1	3	Global	Yes	Yes	No	Yes	Severe	3
2	0	Mild	No	0	3	1	1	1	Global	No	Yes	No	No	Severe	2
3	25	Absent	No	2	3	1	3	0	Global	No	Yes	Yes	No	Mild	1
4	33	Severe	No	2	2	3	3	2	Global	Yes	Yes	Yes	Yes	Severe	3
5	21	Absent	No	0.5	3	0	1	3	Global	No	Yes	No	No	Moderate	4
6	48	Mild	No	2	2	0.5	0	0	Global	No	Yes	No	No	Severe	3
7	42	Moderate	Yes	1	2	3	3	1	Segmental	Yes	Yes	No	Yes	Severe	4
8	52	Mild	Yes	0	3	1	3	0	Global	No	Yes	Yes	Yes	Severe	2
9	10	Absent	Yes	2	3	3	3	3	Global	Yes	Yes	Yes	Yes	Moderate	2
10	24	Moderate	Yes	0	3	2	3	0	Global	No	Yes	No	Yes	Severe	3
11	26	Moderate	No	0	2	2	0.5	0	Global	No	Yes	No	No	Severe	3
12	0	Moderate	No	1	3	3	2	0.5	Global	Yes	Yes	No	No	Moderate	3
13	0	Mild	Yes	2	2	2	3	2	Global	Yes	Yes	Yes	No	Moderate	2
14	15	Moderate	No	0.5	2.5	2	1	0.5	Global	Yes	Yes	Yes	No	Severe	3
15	0	Severe	No	3	2.5	3	2	0.5	Global	Yes	Yes	No	Yes	Severe	3
16	7	Severe	No	1	3	2	0	0	Global	No	Yes	No	No	Severe	3
17	12.5	Mild	No	0	3	0	2	0	Global	No	No	No	No	Severe	2

TGFBR3, TGF-β receptor 3; GGS, global glomerulosclerosis; IF/TA, interstitial fibrosis/tubular atrophy; subendo, subendothelial; TBM, tubular basement membrane; FPE, foot process effacement; EM, electron microscopy.

Table 4. Histopathologic parameters of renal biops y specimens with TGFBR3-associated membranous nephropathy, compared with PLA2R-associated MN, THSD7A-associated MN, NELL1-associated MN, EXT1/2-associated MN, and NCAM1-associated MN

	n Positive/Total *N* (%)					
Parameter	TGFBR3 Positive (*N*=17)	PLA2R Positive (*N*=183)	THSD7A Positive (*N*=10)	NELL1 Positive (*N*=91)	EXT1/EXT2 Positive (*N*=102)	NCAM1 Positive (*N*=20)
IgA positivity	12/17 (71)	19/183 (10) *P*<0.0001	4/10 (40) *P*=0.22	7/91 (8) *P*<0.001	50/102 (49) *P*=0.12	13/20 (65) *P*>0.99
IgG positivity	17/17 (100)	183/183 (100) *P*>0.99	10/10 (100) *P*>0.99	91/91 (100) *P*>0.99	102/102 (100) *P*>0.99	20/20 (100) *P*>0.99
IgM positivity	15/17 (88)	28/183 (15) *P*<0.001	1/10 (10) *P*=0.0001	9/91 (10) *P*<0.001	47/102 (46) *P*=0.001	13/19[a] (68) *P*=0.24
C3 positivity	15/17 (88)	166/183 (91) *P*=0.67	8/10 (80) *P*=0.61	71/91 (78) *P*=0.52	91/102 (94) *P*>0.99	17/20 (85) *P*>0.99
C1q positivity	10/17 (59)	4/183 (2) *P*<0.001	1/10 (10) *P*=0.18	0/91 (0) *P*<0.001	49/102 (48) *P*=0.44	11/20 (55) *P*>0.99
"Full-house" immunofluorescence pattern	8/17 (47)	1/183 (0.5) *P*<0.001	0/10 (0) *P*=0.01	0/91 (0) *P*<0.001	10/102 (10) *P*=0.0006	8/20 (40) *P*=0.75
Mesangial deposits	16/17 (94)	19/183 (10) *P*<0.001	4/10 (40) *P*=0.004	21/88[b] (24) *P*<0.001	98/99[c] (99) *P*=0.27	19/20 (95) *P*>0.99
Subendothelial deposits	6/17 (35)	2/183 (1) *P*<0.001	1/10 (10) *P*=0.18	0/88[b] (0) *P*<0.001	20/99[c] (20) *P*=0.10	11/20 (55) *P*=0.50

TGFBR3, TGF-β receptor 3; PLA2R, phospholipase A2 receptor; MN, membranous nephropathy; THSD7A, thrombospondin type 1 domain containing 7A; NELL1, neural EGF-like 1; EXT1/2, exostosin 1/2; NCAM1, neural cell adhesion molecule 1.
[a]One of 20 NCAM1-positive patients had a limited immunofluorescence panel.
[b]88 of 91 NELL1-positive patients had electron microscopy available for evaluation.
[c]99 of 102 EXT1/2-positive patients had electron microscopy available for evaluation.

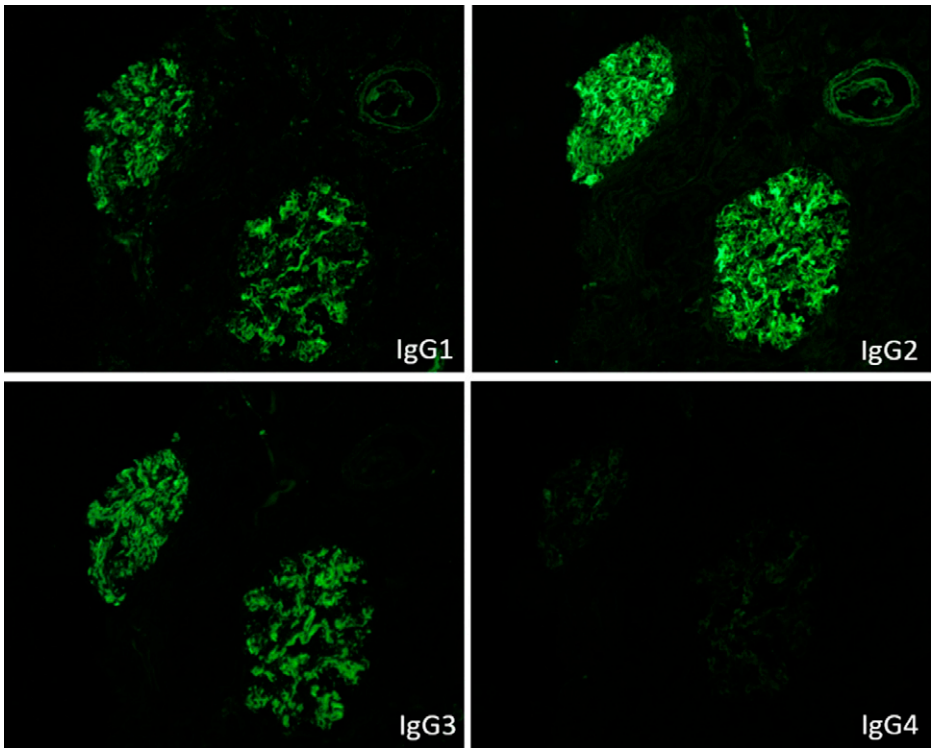

Figure 4. | IgG subclass staining shows that TGFBR3-associated membranous nephropathy typically does not express IgG4. IgG subclass staining using FITC-conjugated monoclonal antibodies of a representative TGFBR3-associated MN case is shown.

autoimmune disease, particularly SLE. In this study, 16 of 17 patients with TGFBR3-associated MN had SLE, with 14 of 17 diagnosed before kidney biopsy, and two of whom met criteria for SLE after biopsy. TGFBR3-associated MN shows similar clinical and histopathologic features to the two other known antigenic targets implicated in MLN: NCAM1 and EXT1/2. Patients with all three of these forms of MN are of a younger mean age, are more likely to express multiple immune reactants within subepithelial deposits (IgA, IgM, C3, C1q, or full-house staining), and to have mesangial and/or subendothelial immune deposits, when compared with patients with idiopathic MN.

TGFBR3, also known as betaglycan, is an accessory receptor for TGF-β signaling that promotes binding to TGFBR1 and TGFBR2. It is an 851-amino acid TGF-β–binding transmembrane proteoglycan that migrates at 280–330 kD on gel electrophoresis, due to its heavy glycosylation pattern, and can alternatively exist as a soluble form within serum and extracellular matrix. TGFBR3 is expressed within glomerular podocytes, mesangial cells, and endothelial cells (18). TGFBR3 has been shown to be protective against autoimmunity, reducing T cell–dependent antibody responses with reduced differentiation of $CD4^+$ T cells to proinflammatory T-helper 1 cells, the latter of which secrete IFN-γ (19), a cytokine implicated in multiple autoimmune diseases, including SLE. Absence of TGFBR3 exacerbates experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis, demonstrating protection of TGFBR3 in autoimmunity (20). We have identified TGFBR3 as a biomarker of a novel subtype of MN. TGFBR3 deposits in glomeruli may arise *via* deposition of circulating immune complexes or through *in situ* immune complex formation in the subepithelial space.

The lack of detection of antibodies against TGFBR3 *in vitro* raises several questions. First and foremost, is this protein an antigenic target of IgG antibodies in patients with glomerular TGFBR3 deposition? The presence of coimmunoprecipitation with IgG from the tissue supports an antigen-antibody reaction, although antibodies were not detected in serum and this antigenic specificity cannot be demonstrated *in vitro* to conclusively prove the presence of circulating autoantibodies directed against TGFBR3. An almost identical issue is present with the reported MLN antigen EXT (21). This commonality with two different lupus antigens could be coincidental but also raises the possibility of a unique pathogenic mechanism driving these antigen-antibody interactions. It also raises the possibility that antibodies are present against these proteins and, for unknown reasons, the autoantibody epitopes are not available for binding *in vitro*.

The number of proteins associated with MN continues to increase at a rapid rate and now stands at eight, including PLA2R, THSD7A, EXT1/2, NELL1, NCAM1, HTRA1, protocadherin 7, and anti-neutral endopeptidase. However, many cases of MN do not have a known associated protein, particularly in patients with SLE. Therefore, we anticipate that there will be more MN-associated proteins discovered in the coming years. Although discovery of new MN-associated proteins might not have immediate implications in the clinic, we expect it will eventually enable precision medicine–based testing and interventions for this rare disease. As such, we recommend attempting to determine the associated membranous protein for all cases of MN so that when specific treatments or laboratory tests become available for different types of MN, patients and their physicians would then be able to benefit.

In summary, mass spectrometric analysis identified TGFBR3 as a distinct type of MN, which was enriched in glomeruli in a subset of patients with MN and could be eluted as IgG immune complexes from kidney biopsy tissue. We showed that TGFBR3 colocalizes with IgG within glomerular immune deposits in this subset of patients. TGFBR3-associated MN is increased in patients with autoimmune disease, comprising 6% of patients with MLN. Whether TGFBR3 is an antigenic target in MN remains to be determined because circulating autoantibodies were not detected. However, further studies will be required to elucidate whether TGFBR3-associated MN associates with other clinical manifestations of SLE or has prognostic significance.

Disclosures

J. M. Arthur reports serving as a scientific advisor for, or member of, *Kidney360*; and receiving honoraria from Travere Pharmaceuticals. L. H. Beck reports serving on the advisory boards of Alexion, Genentech, Ionis, and Visterra; serving on the editorial boards of *Kidney International Reports* and *Kidney Medicine*; receiving research funding from Pfizer and Sanofi-Genzyme; receiving honoraria from UpToDate, Inc.; and being a coinventor on, and receive royalties related to, the US patent "Diagnostics for Membranous Nephropathy." D. J. Kenan reports having patents and inventions with EMD Millipore. All remaining authors have nothing to disclose.

Funding

This work was funded by the National Institute on Minority Health and Health Disparities grant MD014110, awarded to C. P. Larsen, D. J. Kenan, J. M. Arthur, and R.D. Edmondson.

Acknowledgments

We thank Mr. Sudhir Joshi for technical assistance in the staining of various cohorts in this manuscript.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Author Contributions

J. M. Arthur, L. H. Beck, T. D. Bourne, T. N. Caza, S. I. Hassen, C. Herzog, D. J. Kenan, C. P. Larsen, and A. Storey reviewed and edited the manuscript; J. M. Arthur, T. N. Caza, and C. P. Larsen conceptualized the study; J. M. Arthur, D. J. Kenan, and C. P. Larsen were responsible for project administration; L. H. Beck, T. D. Bourne, T. N. Caza, S. I. Hassen, C. Herzog, C. P. Larsen, and A. Storey were responsible for investigation; L. H. Beck, T. N. Caza, R. D. Edmondson, S. I. Hassen, C. Herzog, and A. Storey were responsible for data curation; L. H. Beck, R. D. Edmondson, C. P. Larsen, and A. Storey were responsible for resources; T. N. Caza, R. D. Edmondson, S. I. Hassen, C. Herzog, C. P. Larsen, and A. Storey were responsible for formal analysis; T. N. Caza, S. I. Hassen, and C. P. Larsen were responsible for visualization; T. N. Caza, S. I. Hassen, C. P. Larsen, and A. Storey were responsible for methodology; T. N. Caza and C. P. Larsen wrote the original draft; D. J. Kenan, R. D. Edmondson, J. M. Arthur, and C. P. Larsen were responsible for funding acquisition; C. P. Larsen provided supervision; and A. Storey was responsible for software.

Data Sharing Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium, *via* the PRIDE partner repository, with the dataset identifier PXD026506 and 10.6019/PXD026506.

Supplemental Material

This article contains the following supplemental material online at http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0001492021/-/DCSupplemental.

Supplemental Methods.

Supplemental Table 1. IgG subclass staining in 13 cases of TGFBR3-associated membranous lupus nephritis.

Supplemental Figure 1. Immunofluorescence staining on TGFBR3 compared to negative control cases.

Supplemental Figure 2. TGFBR3 immunofluorescence staining on non-membranous proteinuric kidney diseases.

Supplemental Figure 3. Diagram of study cohorts.

Supplemental Figure 4. TGFBR3 immunofluorescence staining of a representative glomerulus from each of the 17 cases included in the series.

Supplemental Figure 5. Subendothelial tubuloreticular inclusions were present within 3 of 17 TGFBR3-associated MN cases.

Supplemental Figure 6. Serum from TGFBR3-associated MN patients does not react with TGFBR3 recombinant protein.

Supplemental Figure 7. A cell-based indirect immunofluorescence assay through transient transfection of HEK-293 cells failed to detect anti-TGFBR3 antibodies within serum from patients with positive TGFBR3 staining within glomeruli.

Supplemental Figure 8. Sera from TGFBR3-associated MN patients does not immunoprecipitate with human glomerular extract.

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Received: March 15, 2021 **Accepted:** June 10, 2021