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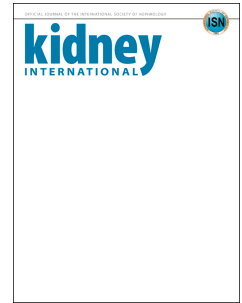
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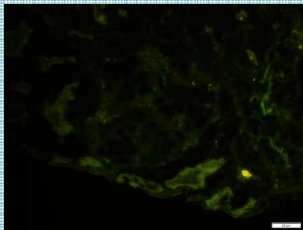
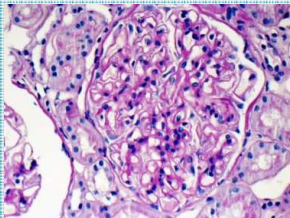
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Patient Selection

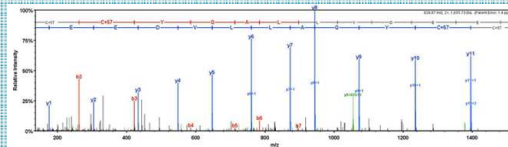
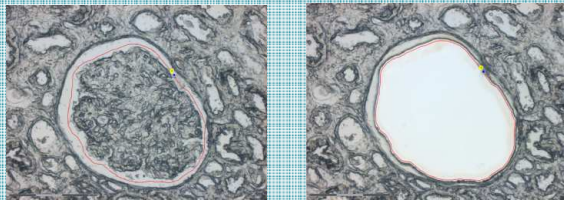


PLA2R-negative MN



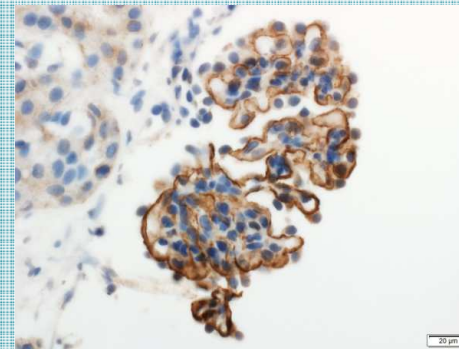
Laser Microdissection and Mass Spectrometry

Identification of novel protein
Sema 3B



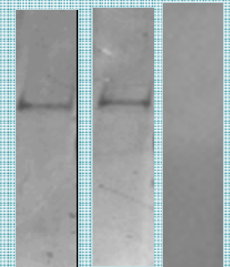
Immunohistochemistry /Immunofluorescence

Granular anti-Sema3B
GBM staining



Western Blot Analysis

Serum antibodies to
Sema3B



CONCLUSION:

Semaphorin 3B defines a distinct type of membranous nephropathy

[QUERY TO AUTHOR: title and abstract rewritten by Editorial Office – not subject to change]

Semaphorin 3B-associated membranous nephropathy is a distinct type of disease predominantly present in pediatric patients.

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Conflict of interest: None

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ABSTRACT

Membranous nephropathy results from subepithelial antigen-antibody complex deposition along the glomerular basement membrane. Although PLA2R, THSD7A, and NELL-1 account for a majority (about 80%) of the target antigens, the target antigen in the remaining cases is not known. Using laser microdissection of PLA2R-negative glomeruli of patients with membranous nephropathy followed by mass spectrometry we identified a unique protein, Semaphorin 3B, in three cases. Mass spectrometry failed to detect Semaphorin-3B in 23 PLA2R-associated cases of membranous nephropathy and 88 controls. Semaphorin 3B in all three cases was localized to granular deposits along the glomerular basement membrane by immunohistochemistry. Next, an additional eight cases of Semaphorin 3B-associated membranous nephropathy were identified in three validation cohorts by immunofluorescence microscopy. In four of 11 cases, kidney biopsy also showed tubular basement membrane deposits of IgG on frozen sections. Confocal microscopy showed that both IgG and Semaphorin 3B co-localized to the glomerular basement membrane. Western blot analysis of five available sera showed reactivity to reduced Semaphorin 3B in four of four patients with active disease and no reactivity in one patient in clinical remission; there was also no reactivity in control sera. Eight of the 11 cases of Semaphorin 3B-associated membranous nephropathy were pediatric cases. Furthermore, in five cases, the disease started at or below the age of two. Thus, Semaphorin 3B-associated membranous nephropathy appears to be a distinct type of disease; more likely to be present in pediatric patients.

Key words: Membranous nephropathy, Semaphorin 3B, mass spectrometry, kidney biopsy

INTRODUCTION

Membranous nephropathy (MN) results from antibodies targeting an antigen in the glomerular basement membrane (GBM). MN is typically classified as primary MN that has no identifiable underlying disease association and secondary membranous MN where the MN may be associated with an autoimmune disease, infection, malignancy, etc.¹⁻³ The target antigen in primary MN has been identified as M-type phospholipase A2 receptor (PLA2R), Thrombospondin Type-1 Domain-Containing 7A (THSD7A) and the recently described Neural epidermal growth factor like-1 protein (NELL-1), respectively.⁴⁻⁶ Exostosin 1/exostosin 2 (EXT1/EXT2) have also recently been identified as the putative antigen(s) in secondary (autoimmune) MN.⁷ NELL-1 and EXT1/EXT2 were identified using laser microdissection of PLA2R-negative MN glomeruli followed by mass spectrometry. Using a similar approach, we sought to identify other novel antigen(s) in MN.

RESULTS

Discovery cohort:

Laser dissection and mass spectrometry (MS/MS) detection of Semaphorin 3B in PLA2R-negative MN biopsies: We detected a unique protein Semaphorin 3B (Sema3B) by MS/MS in the glomeruli of 3 cases (patients 1-3) of MN (Figure 1). The counts ranged from 10 to 42 with an average total spectral count of 23.7 (SD $\pm$ 16.5). The average spectral counts of Sema3B were lower than PLA2R (86.1, S.D $\pm$ 27.5), NELL-1 (63.1, S.D $\pm$ 21.6), and EXT1/EXT2 (EXT1 65.3, S.D $\pm$ 34.6, EXT2 83.4, S.D $\pm$ 38.4) in PLA2R-, NELL-1- and EXT1/EXT2-associated MN, respectively.^{6, 7} However, the presence of Sema3B was unique in this subset of MN and importantly, all control cases including 15 time 0 transplant biopsies, 73 other glomerulopathies, and 23 PLA2R positive MN cases were negative for Sema3B. The spectral counts of Sema3B in the 3 cases, along with representative sequence coverage map of Sema3B is shown in Figure 2. The MS/MS spectra match from one case is shown in Figure S1. None of the cases showed any spectral counts for THSD7A. Subsequently, we screened over 2500 kidney biopsies, including pediatric cases, from our database (over 12500 data files) from our amyloid laboratory (both amyloid and non amyloid cases) and we did not find any kidney biopsy that showed any spectral counts for Sema3B. We included both adult and pediatric cases of C3 glomerulopathy. On analysis of other organs, we found trace amounts of Sema3B (1-3 spectral counts) in the heart and liver of 23 cases (over 2000 non-kidney cases were screened for Sema3B).

All four classes of Ig were detected in Sema3B associated MN, with average spectral counts of IgG1 25.0 (S.D $\pm$ 4.0), IgG2 22.7 (S.D $\pm$ 4.5), IgG3 25.0 (S.D $\pm$ 9.5), and IgG4 17.0 (S.D $\pm$ 5.3).

Immunohistochemical (IHC) staining for Sema3B in PLA2R-negative MN biopsies:

We performed IHC staining for Sema3B in all 3 cases positive on MS/MS studies. All cases showed positive (2-3+/3) granular staining for Sema3B along the GBM. Importantly, there was no significant mesangial staining (Figure 3A). There was no staining along the Bowman's capsule, tubular basement membranes or in vessel walls. The positive Sema3B granular staining mirrored the granular IgG along the GBM seen in each case. All 45 control cases were negative for Sema3B, this included 3 pediatric cases of minimal change disease/FSGS. Representative negative staining for Sema3B in diabetes, FSGS, IgA nephropathy, minimal change disease, and PLA2R-associated MN is shown in Figure 3B.

Validation cohorts:

Given the results obtained in the Mayo cohort showing 2 adult #1 and #2 and one pediatric patient #3 of Sema3B-associated MN, we analyzed 3 validation cohorts, one adult (*French cohort 1*) and 2 pediatric (*Italian cohort, French cohort 2*) cohorts (Figure 1).

Sema3B was detected using immunofluorescence microscopy (IF) studies on paraffin sections after antigen retrieval. Patient 4 of the validation cohort had both IHC and IF studies performed.

French cohort 1 (Tenon Hospital)

Two adult cases (patients 4 and 5) out of 59 PLA2R-, THSD7A-, EXT1/EXT2-, and NELL-1- negative primary MN were positive for Sema3B. One case (patient 4) was detected by IHC at the Mayo Clinic and was then confirmed by IF studies on paraffin sections at Tenon Hospital (Figure 4A, 4B). Patient 5 had 3 biopsies performed at the age of one, 6 and 19 years, showing granular staining for Sema3B along the GBM (Biopsy at age one and 19 shown in Figure 4C, 4D, and supplementary Figure 3e, f).

Once it became clear that Sema3B-associated MN was enriched in pediatric patients. Following this unique finding, we then screened for Sema3B in a larger cohort of MN in the pediatric age group (Italian cohort and French cohort#2).

Italian cohort (Bambino Gesù Pediatric Hospital, Rome)

Four cases (patients 6, 7, 8, 9) out of 43 pediatric cases negative for PLA2R-, THSD7A-, EXT1/EXT2-, and NELL-1 showed bright granular staining for Sema3B along the GBM (Figure 4E, 4F, 4G, 4H). It should be pointed out that patient 7 and 8 are siblings. Of the 43 cases, 6 had lupus class V MN including 3 with III + V, and were all negative for Sema3B.

French cohort 2 (Trousseau Pediatric Hospital, Paris)

An additional 2 cases (patients 10, 11) out of 16 pediatric cases negative for PLA2R-, THSD7A-, EXT1/EXT2-, and NELL-1 showed the bright granular deposits of Sema3B along the GBM (Figure 4I, 4J). Of the 16 cases, 9 had lupus class V MN and were all negative for Sema3B. Note the granular GBM staining and absence of mesangial or Bowman capsule staining in all cases (Figure 4). An adult and pediatric case of Sema3B negative-MN is also shown (Figure 4K and 4L).

Confocal microscopy

We performed confocal immunofluorescence microscopy to determine whether Sema3B and IgG co-localize along the GBM (Figure 5, 19-years old). Bright granular staining for Sema3B (green) and IgG (red) was seen along the GBM (Figure 5A and 5B). Furthermore, superimposition of the two signals showed co-localization resulting in a yellow signal (Figure 5C). Laser quantitative analysis (Figure 5D and 5E) also confirmed the co-localization of Sema3B and IgG further corroborating that the subepithelial deposits contain both Sema3B and IgG. A second case (patient 6, 2-years old) is shown in the supplementary Figure 2.

Western blot analysis

Western blot analyses in non-reducing and reducing conditions were performed using human Sema3B full length recombinant protein to determine the presence of circulating anti-Sema3B antibodies in the available serum of 5 patients (patient 3, 4, 5, 8 and 11), (Figure 6). Four of the 5 patients showed reactivity against Sema3B under reducing but not under non-reducing conditions. A band was seen at around 81kDa representing the full-length protein. Serial serum samples of patient 4 who was positive for anti-Sema3B antibody were also available for time-course analysis of antibody levels. Results show progressive decrease with disappearance of antibodies after rituximab treatment. Patient 5 who was sampled after a long-term immunosuppressive treatment was negative by Western blot analysis. In contrast, sera from patients with proteinuric conditions including PLA2R, THSD7A, and NELL-1-associated MN, IgA nephropathy, focal segmental glomerulosclerosis, and minimal change disease, and control healthy people did not show reactivity with Sema3B when analyzed under identical conditions.

Clinical and Kidney Biopsy Findings of Sema3B associated MN

We identified 11 cases of Sema3B-associated MN, 3 from the Mayo Clinic cohort, 2 from the French validation cohort #1, 4 from the Italian validation cohort, and 2 from the French validation cohort #2 (Table 1). There were 7 males and 4 females. Of the 11 cases, 8 (73%) were pediatric patients (<18

years of age) and 3 (27%) were adult patients. Of the Mayo Clinic cohort (patients 1-3), 1 was a pediatric patient (1 out of 4 pediatric PLA2R-negative MN cases tested for Sema3B, 25%) and 2 patients were adults (2 out of 160 primary and secondary PLA2R-negative MN including class V membranous lupus nephritis tested, 1.25%). Of the validation cohorts (Paris and Rome combined), there were 8 pediatric patients (of 60 PLA2R negative pediatric MN tested including case 5, 13.3%) and 1 adult patient (1 out of 58 PLA2R negative adult MN tested excluding case 5, 1.7%). In patient 5, MN was diagnosed at the age of 1 year (biopsy performed because of steroid-resistant NS) and repeat biopsy performed 6 and 18 years later showed grade II MN. Each of the pediatric cohorts included 9 patients with lupus MN class V. In total, 6/41 (14.6 %) non-lupus pediatric cases tested were Sema3B positive.

The average age of the pediatric patients at disease onset was 6.9 years ($S.D \pm 6.8$), and the average age of the 3 adult patients was 36.3 ($S.D \pm 7.2$). Two children had extra-renal features of auto-immunity (type 1 Diabetes in patient 10 and idiopathic thrombocytopenic purpura with positive ANA in patient 11). The average serum creatinine was 0.5 mg/dl ($S.D \pm 0.2$) and 24-hour urinary protein was 7.4 grams ($n=7$, $S.D \pm 5.7$).

Kidney biopsy in all cases showed features of MN with thickened glomerular basement membranes. Immunofluorescence microscopy showed granular IgG and Sema3B deposits along the glomerular capillary walls. Interestingly, 4 cases (patients 3, 5, 6, 9, all pediatric, <2 years of age at onset) also showed tubular basement membrane staining for IgG in a granular pattern (Figure 7, supplementary Figure 3a, b). In patient 5, granular IgG deposits along tubular basement membranes were noted in the first biopsy (one year of age) but disappeared thereafter. However, the tubular basement membrane deposits were negative for Sema3B staining in all biopsies (Figure 7, Supplementary Figure 3d, e). Complete IgG subtyping done in 4 cases (3 in Mayo Clinic cohort and 1 in French cohort #1) showed IgG1 in 3 cases and IgG1 and IgG4 in 1 case. IgG4 staining only was done in the 4 cases of the Italian cohort and all 4 cases were negative for IgG4 (Supplemental Table 1). Electron microscopy was performed in all patients except patients 4 and 11. It showed glomerular subepithelial deposits in all cases that were graded as stage II in most cases. The deposits seen along tubular basement membranes were confirmed or revealed by EM in 3 cases (patients 3, 6, 9). Tubuloreticular inclusions were noted in these cases as well.

Treatment and follow up

Mayo Clinic cohort: Patient 1 and 2 received only angiotensin converting enzyme inhibitors and went to remission with minimal proteinuria (<500 mg/24 hours) at last follow up of 18 and 24 months respectively.

Patient 3 (Figure 6 and 7) received steroids, tacrolimus and angiotensin converting enzyme (ACE) inhibitors and also went into remission. At last follow-up (24-months) the proteinuria was 150 mg/24 hours.

French adult cohort:

Patient 4 was treated for 4 months with angiotensin converting enzyme inhibitors. Because of persisting severe nephrotic syndrome, she was given rituximab, 2 infusions of 375 mg/m² at 2-week interval, followed by a third infusion at 3 months. Thereafter, she reached complete remission, which is maintained after 10 years (daily proteinuria <500mg, no more detectable anti-Sema3B antibody in serum, Figure 6).

Patient 5 was diagnosed at the age of one year (Figure S3b, e). He failed to respond to cyclosporine and from the age of 5 to the age of 7 years, he was treated with mycophenolate mofetil that induced complete remission. Six months after mycophenolate mofetil withdrawal, a relapsed occurred. A second biopsy showed MN stage 2 with disappearance of granular tubular deposits. Mycophenolate mofetil (500 mg twice daily) was resumed and increased to 1 gram twice daily 10 years later because of reappearance of isolated proteinuria without nephrotic syndrome. The patient was referred to us (PR) at the age of 19 years to discuss immunosuppressant withdrawal. Because of persisting low level of proteinuria (400 mg/day) with normal renal function (serum creatinine, 0.9 mg/dL), a third kidney biopsy (Figure 5 and S3 c, f) was performed. The kidney biopsy showed MN stage 2 with abundant deposits by IF (IgG1, C3, Sema3B) and EM (subepithelial electron dense deposits). Mycophenolate mofetil was then tapered. One year later, the patient is still in complete remission. Anti-Sema3B antibodies are not detected (Figure 6).

Italian pediatric cohort:

Patient 6 (onset < 2 years of age with nephrotic-range proteinuria), was biopsied at disease onset (Figure S2 and 3a, d) because his father had MN. He received oral prednisone tapered in 6 months and cyclosporine. This led to a complete remission, with a follow-up of 13 months.

Patient 7 and 8 are siblings. Patient 7 presented at age 17 years with proteinuria of 16 grams/day and was treated with oral prednisone monotherapy. Due to partial response after 2 months, a renal biopsy was done which showed MN. The patient was started on an angiotensin converting enzyme inhibitor

and cyclosporine. Prednisone was tapered and discontinued at the end of 4 months. The patient went into remission and has no proteinuria at 19 month follow-up.

Patient 8 presented at age of 2 years with nephrotic-range proteinuria, normal renal function and negative ANA. Kidney biopsy showed a MN. Because the biopsy showed a full house (IgG3+, IgM 2+, IgA 3+, C3 2+, C1q 3+) pattern of staining on immunofluorescence microscopy, the patient was treated with intravenous cyclophosphamide and methylprednisolone bolus followed by maintenance low-dose prednisone and azathioprine. Treatment was tapered and discontinued after 2 years. The child experienced a first relapse (UP/CR ratio ≥ 1) 2 years later that showed a MN on kidney biopsy that responded to cyclosporine. The patient had a relapse again at 9 years of age another repeat kidney biopsy (the one examined in this study) confirmed the MN. He was treated with intravenous rituximab with relapse upon tapering of cyclosporine despite repeated doses, and therefore was changed to tacrolimus with full response. At last follow-up (14 years from onset) he is in remission with normal renal function on low-dose tacrolimus and ACE-inhibitor.

Patient 9 presented with nephrotic-range proteinuria (UP/CR ratio 16.75) at 2 years of age and was treated with oral prednisone with partial response after 4 weeks of treatment. A renal biopsy was performed that showed MN. The patient went into complete remission following treatment with cyclosporine and oral prednisone. However, each time cyclosporine was tapered, proteinuria reappeared, and at the age of 7 years he was treated with rituximab. At last follow-up, he is in complete remission.

French pediatric cohort:

Patient 10 was lost to follow-up.

Patient 11 (Figure 6) progressed to end stage kidney disease within 3 years, being unresponsive to rituximab, mycophenolate mofetil and cyclosporine.

DISCUSSION

MN is the most common cause of nephrotic syndrome in Caucasian adults. On the other hand, it is a rare cause of nephrotic syndrome in children. In the last decade, groundbreaking research has led to the identification of some of the target MN antigens including PLA2R, THSD7A, NELL-1 and putative antigens EXT1/EXT2.⁴⁻⁷ Yet, these antigens do not account for all cases of MN and in a significant number of MN the target antigen is still elusive. Using a combination of laser microdissection, MS/MS and IHC techniques we studied a subgroup of PLA2R-negative MN to determine whether we could identify novel target antigens in this remaining group of MN.

Our previous MS/MS studies confirmed high spectral counts NELL-1 and EXT1/EXT2 in a cohort PLA2R-negative MN.^{6,7} Using the same cohort, we then searched for other proteins that were unique to this group of PLA2R-negative MN. The criteria used to identify a putative antigen/protein from over 1500-2000 proteins detected on MS/MS was the identification of a unique protein and the absence (or low baseline spectral counts) of the protein in control cases and other cases of MN including both PLA2R-positive and -negative MN. Using this approach, we found moderate spectral counts of a unique protein Sema3B in 3 cases of MN. We confirmed the MS/MS finding by IHC and further validated our findings in 2 French cohorts and an Italian cohort.

Eight (72.7%) of the 11 Sema3B-associated MN were pediatric patients of which 5 patients developed MN on or before the age of 2 years. The remaining 3 patients developed MN at the age of 14, 16 and 17 years. Of the 3 adult cases identified, the average age was 36.3 years which is still significantly lower than primary MN age group. Thus, Sema3B-associated MN appears primarily to involve pediatric patients and young adults.

In the Mayo Clinic cohort, there were only 4 pediatric patients tested of which 1 case was positive for Sema3B. However, the combined French and Italian pediatric cohorts included a large number of patients and detected Sema3B in 6 (10.1 %) patients out of 59 pediatric MN. Because 9 patients in each pediatric cohort had lupus class 5, the real prevalence of Sema3B associated disease among non-lupus pediatric patients in France and Italy is 6/41 (14.6 %). It is remarkable that five of the 8 patients with Sema3B-positive MN were 2 years old or less; this makes Sema3B a new target antigen in young children with the disease. Interestingly, 2 patients had an autoimmune disease that included type 1 diabetes and thrombocytopenic purpura. One child at disease onset had a full-house immunofluorescence (patient 8 with no systemic or serologic features of lupus) which was not present in kidney biopsies performed at subsequent relapses.

The kidney biopsy findings of the glomeruli in Sema3B-associated MN were typical for MN. Granular Sema3B was present uniformly along the subepithelial region of the GBM suggesting that Sema3B is shed from the podocytes rather than from the mesangial cells or endothelial cells since there is no mesangial or subendothelial staining for Sema3B. It is also likely that Sema3B does not represent trapped circulating antigen-antibody complexes as these are more likely to be present in the subendothelial region of the GBM and have a patchy irregular localization along the capillary walls unlike the subepithelial uniform Sema3B staining in our cases. IgG subtypes were done in 4 cases and revealed IgG1 in 3 cases and IgG4 predominance along with IgG1 and IgG2 in 1 case. In addition, IgG4 (only) staining was done in all 4 cases of the Italian cohort and all 4 cases were negative for IgG4, pointing to a

non-IgG4 dominant IgG in Sema3B-associated MN, most likely IgG1. This is different from PLA2R-associated MN where IgG4 is the dominant IgG subclass.

Interestingly, tubular basement membrane deposits were present in 4 pediatric cases. All four cases had onset < 2 years and showed tubular basement membrane deposits that stained for IgG.

Interestingly, the tubular basement membrane deposits were negative for Sema3B staining although glomerular deposits were brightly stained for Sema3B in the same sections (Figure 7, supplementary Figure 3). Lupus serologies were evaluated and were negative in all cases. Thus, tubular basement membrane deposits in setting of MN in a child <2 years may indicate a Sema3B-associated MN. The finding of Sema3B-associated MN in the pediatric age group, in particular in very young patients, and the fact that in the Italian cohort patients 7 and 8 are siblings and patient 6 has a father with MN raise the possibility of a genetic basis of the disease. Further studies are needed to determine whether alterations in the sequence of the protein enhancing its antigenicity or specific immune response genes (HLA-D allotypes) are involved.

Semaphorins are a group of secreted and transmembrane/membrane bound proteins containing a conserved extracellular Semaphorin (sema) domain of about 500 amino acids that is characterized by highly conserved cysteine residues.⁸⁻¹⁰ The sema domain is the critical component through which semaphorins mediate their effects. The first semaphorins were identified as proteins that guided neuronal axons to their targets. Since then more than 20 semaphorins have been identified and are classified into 8 subclasses: class I and IV-VII are membrane associated (I, IV, V, VI are transmembrane and VII is membrane bound) whereas class II, III and VIII are secreted. In addition to the nervous system, semaphorins are also widely expressed in other organ systems including cardiovascular, endocrine, gastrointestinal, hepatic, immune musculoskeletal, renal, reproductive and respiratory systems. The primary semaphorin receptors are the receptors plexins and neuropilins; plexins consist of 4 subclasses A through D and neuropilins consist of neuropilin 1 and 2. The expression of various semaphorins is diverse in different organ systems suggesting that they serve varying functions during development and in adults. Semaphorins also have a role in immune system by modulating cytoskeletal organization.¹¹ Furthermore, the semaphorins also play a role in disease pathogenesis including tumorigenesis and metastasis, retinal degeneration, rheumatoid arthritis, and decreased bone mineral density.¹² However, the exact mechanisms, function and disease association (if any) of most semaphorins remains unknown.

Semaphorin 3 is a secreted protein with a sema domain, PSI (plexin-semaphorin-integrin) domain, Ig domain and a basic domain (Figure 6B). Semaphorin 3 and their receptors have been

detected in endothelial cells, podocytes and tubular epithelial cells.^{8, 13} In particular semaphorin 3A has been shown to regulate slit diaphragm proteins such as podocin, and decreased its interaction with CD2-associated protein and nephrin. Semaphorin 3A also induced podocyte apoptosis.⁸ The role and function of Sema3B in the kidney is not known and to the best of our knowledge overexpression of Sema3B has not been reported in any kidney disease.

Unexpectedly our results show reactivity of Sema3B antibodies only under reducing conditions. This could suggest that the epitope is unmasked by disruption of the disulfide bonds. Sema3B contains conserved cysteine residues forming intra-subunit disulfide bonds. In some conditions early in life, disruption of these disulfide bonds would expose new protein domains that could serve as neoepitopes. It would be interesting to determine the three dimensional structure of Sema3B to localize the site of conformational change and to determine whether reduced conditions are required for the antibodies to recognize Sema3B throughout the course of the disease. These *de novo* created structures could lead to autoimmunization. It is tempting to speculate that the anti-Sema3B antibodies are formed to an epitope on the Sema domain or even the basic domain since mass spectrometry detected extensive sequence coverage of these segments of the Sema3B protein (Figure 2B).

Since this is a retrospective study, limitations include sera availability in only 5 patients, and , complete IgG subtypes were performed in only 4 patients. There are also important questions that future studies need to address including: Why TBM staining for IgG is present in the very young patients? Why is the TBM staining negative for Sema3B? Is the antigen in the TBM deposits different from Sema3B present in the MN deposits? Is the Sema3B-associated MN different in pediatric patients compared to adult patients?

Circulating antibodies against the recombinant protein were detected by Western blot in the patients with active disease, and they disappear upon clinical remission. These antibodies were present in Sema3-associated MN patients that were negative for anti-PLA2R, anti-THSD7A and anti-NELL-1 antibodies, and thus not reflective of a secondary immunological phenomenon.¹⁴ Further studies are required to confirm whether Sema3B is a true antigen or merely a biomarker in Sema3B-associated MN. These include studies that localize the Sema3B on podocyte surface and studies that determine the specificity of circulating antibodies to glomerular Sema3B.

In conclusion, Sema3B-associated MN represent a unique type of primary MN that is more likely to be present in pediatric patients, particularly in very young patients with onset <2 years. Sema3B-associated MN should be added to the list of serologically defined MN, including PLA2R, THSD7A and NELL-1 associated MN.

METHODS AND PATIENT SELECTION

Mayo Clinic Cohort

We performed MS/MS in selected kidney biopsies of 70 cases of PLA2R-negative MN received at the Mayo Clinic (2015-2018) for analysis by tandem mass spectrometry (MS/MS), and detected the unique protein, Sema3B in 2 cases. The 70 cases included both primary and secondary membranous nephritis. These cases were also used to detect recently reported novel antigens EXT1/EXT2 and NELL1.^{6,7} Immunohistochemistry (IHC) was then performed in to confirm the results the MS/MS results. In addition, we screened 160 PLA2R negative MN (including the 68 negative and 2 positive on MS/MS) by IHC for Sema3B. We detected only 1 more positive case. Other than 4 pediatric cases (<18 years of age), the remaining cases were all adult cases.

French and Italian Validation Cohort

A total of 118 biopsies were stained for Sema3B, of which 59 were from the French adult cohort, 43 from the Italian pediatric cohort, and 16 from the French pediatric cohort. All cases were PLA2R, THSD7A, EXT1/EXT2, and NELL1-negative MN. Each pediatric cohort included 9 lupus class V cases.

Control cases

For control cases, we performed MS/MS on 111 cases that included 15 cases of time 0 transplant biopsies, 17 cases of minimal change disease, 44 cases of focal segmental glomerulosclerosis, 7 cases of diabetic glomerulosclerosis, 5 cases of IgA nephropathy, and 23 cases of PLA2R-positive membranous nephropathy. For control IHC, we used 45 cases that included: 9 cases of minimal change disease, 9 cases of focal segmental glomerulosclerosis, 4 cases of IgA nephropathy, 7 cases of diabetes and 15 cases of PLA2R-positive MN and 1 case of time 0 transplant protocol biopsy.

These biopsies were received in the Renal Pathology Laboratory, Department of Laboratory Medicine and Pathology, Mayo Clinic, for diagnosis and interpretation between January 2015 and May 2018. Light microscopy, immunofluorescence microscopy including PLA2R studies, and electron microscopy was performed in each case of MN. The clinical information was obtained from the accompanying charts. The study was approved by the Mayo Clinic Institutional Review Board.

Laser microdissection and mass spectrometry: Protein identification by laser capture microdissection, trypsin digestion, nano-LC orbitrap tandem mass spectrometry (MS/MS)

For each case 10 micron thick formalin-fixed paraffin sections (FFPE) were obtained and mounted on a special PEN membrane laser microdissection slide and using a Zeiss Palm Microbeam microscope, the glomeruli were microdissected to reach approximately 250-500,000µM² per case. Resulting FFPE

fragments were digested with trypsin and collected for MS/MS analysis. The trypsin digested peptides were identified by nano-flow liquid chromatography electrospray tandem MS/MS (nanoLC-ESI-MS/MS) using a Thermo Scientific Q-Exactive Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to a Thermo Ultimate 3000 RSLCnano HPLC system. All MS/MS samples were analyzed using Mascot and X! Tandem set up to search a Swissprot human database. Scaffold (version 4.8.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted at greater than 95.0% probability by the Scaffold Local FDR algorithm with protein identifications requiring a 2 peptide minimum and a 95% probability using Protein Prophet.¹⁵

Immunohistochemical Staining for Semaphorin 3B

Tissue sectioning and IHC staining was performed at the Pathology Research Core (Mayo Clinic, Rochester, MN) using the Leica Bond RX stainer (Leica). FFPE tissues were sectioned at 5 microns and IHC staining was performed on-line. Slides for Semaphorin 3B stain were retrieved for 20 minutes using Epitope Retrieval 1 (Citrate; Leica) and incubated in Protein Block (Dako) for 5 minutes. The Semaphorin 3B primary antibody (Rb. Polyclonal; Abcam #ab48197) was diluted to 1:200 in Background Reducing Diluent (Dako) and incubated for 15 minutes. The detection system used was Polymer Refine Detection System (Leica). This system includes the hydrogen peroxidase block, post primary and polymer reagent, DAB, and Hematoxylin. Immunostaining visualization was achieved by incubating slides 10 minutes in DAB and DAB buffer (1:19 mixture) from the Bond Polymer Refine Detection System. To this point, slides were rinsed between steps with 1X Bond Wash Buffer (Leica). Slides were counterstained for five minutes using Schmidt hematoxylin and molecular biology grade water (1:1 mixture), followed by several rinses in 1X Bond wash buffer and distilled water, this is not the hematoxylin provided with the Refine kit. Once the immunochemistry process was completed, slides were removed from the stainer and rinsed in tap water for five minutes. Slides were dehydrated in increasing concentrations of ethyl alcohol and cleared in 3 changes of xylene prior to permanent cover slipping in xylene-based medium.

Immunofluorescence staining for Sema 3B and co-localization analysis

Immunofluorescence staining was performed on FFPE sections retrieved for 30 min using target retrieval solution high pH (Dako) in pressure cooker equipment (BioSB). The Sema 3B primary antibody (rabbit polyclonal, Abcam antibodies) was diluted to 1:100 in blocking solution (2% calf fetal serum and 2% normal goat serum) and incubated overnight at 4°C with retrieved biopsy sections. Next, the slides were

incubated with secondary antibody the goat Alexa488-conjugated anti-rabbit Fab IgG antibodies (dilution 1:400, Life technologies). Next anti-human IgG Alexa Fluor 647 rabbit monoclonal antibody (dilution 1:400, Sigma) was reacted with the retrieved tissue following the staining for Sema 3B as described above. Finally slides were mounted in mounted medium (Thermo Scientific) and covered with LDS2460EP cover slips. Co-localization of Sema3B and IgG along the glomerular basement membranes was examined by confocal microscopy using a Leica TCS-SP2 and analyzed with Leica Confocal Software version 2.61, Wetzlar, Germany.

Western blot analysis

Sema3B recombinant human protein (AVIVA System Biology) was diluted with non-reducing or reducing Laemmli sample buffer (Bio-Rad) and boiled for 10 min. Samples were loaded into Criterion 4-15% TGX gels (Bio-Rad) and electrophoresed in Tris-glycine-SDS running buffer. Proteins were transferred to polyvinylidene difluoride membranes according to standard protocols, and then membranes were blocked with Pierce Protein-Free Blocking buffer (Thermo Scientific). Membranes were incubated overnight at 4°C with sera from patients, controls (dilution 1:50) and rabbit polyclonal antibodies (dilution 1:2000) against Sema3B (Abcam). Subsequently, blots were washed and incubated for 2 h at room temperature with goat-anti human or goat anti-rabbit IgG, AP conjugate (both dilution 1:10000, Sigma). Immunoreactive proteins were visualized with BCIP/NBT liquid substrate system (Sigma).

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Author contribution: SS and FCF designed the study. SS wrote the manuscript, interpreted the kidney biopsy, clinical, IHC and MS data. BM and CC performed the laser microdissection and mass spectrometry. AR helped in gathering clinical data. LG performed the IHC. HD, DB and FDC provided tissue for the validation cohorts. HD performed the immunofluorescence, confocal studies and Western blot analysis. PR, MV, FE, and TU provided clinical information. The manuscript was drafted and written by the first author, with input as appropriate from the investigators.

Table 1. Clinical findings in Sema3B-associated MN

Case	Age/Sex	Urinary protein gms/24 hour	Serum Cr (mg/dL)	Spontaneous remission versus immunosuppressive treatment	S. Cr/ Urinary protein/24 hour (FU months)
1	41/F	7.9	0.74	Spontaneous	0.6/no proteinuria (16 months)
2	28/F	6.2	0.4	Spontaneous	0.43/400 mg (18 months)
3	2/M	5	0.5	Immunosuppressive	0.35/150 mg (24 months)
4	40/F	17.3	0.9	Immunosuppressive	0.6/no proteinuria (10 years)
5	19/M** (onset 1 year)	0.4	0.7	Immunosuppressive	0.9/400 mg (18 years)
6	1 year and 9 month/F	UP/CR ratio 6.81, Micro- hematuria ++	0.21	Immunosuppressive	UP/CR ratio 0.23 (13 months)
7	17/M	UP/CR ratio 0.78	0.6	Immunosuppressive	UP/CR ratio 0.1 (absent) (19 months)
8	9/M (onset 2 years)	UP/CR ratio 0.94	0.45	Immunosuppressive	UP/CR ratio 0.09 (absent) (14 years)
9	2/M	UP/CR ratio 1.95	0.13	Immunosuppressive	UP/CR ratio 0.12 (absent) (5 years)
10	14/M	3	0.64	Lost to follow up	NA
11	16/M	12	0.83	Immunosuppressive	Dialysis

* MN was diagnosed at the age of 1 year (biopsy performed because of steroid-resistant NS), repeat biopsy in 2006 and in 2019 at the age of 19 years, FU-follow up, UP/CR- urinary protein/urinary creatinine ratio , NA- not available.

Table 2. Kidney biopsy findings in Sema3B-associated MN

Case	Sclerosed/ total glomeruli	IFTA	Immunofluorescence microscopy	Electron microscopy	Tubular basement membrane deposits
1	1/22	0	IgG 3+ C3 2+	I-II	No
2	1/36	0	IgG 3+ C3 2+	I-II	No
3	0/60	10	IgG 3+ C3 3+	I-II	Yes
4	0/19	0	IgG 1+ C3 1+ C1q 1+	Not done	Not done
5	0/6	0	IgG 3+ C3 2+	II	Yes
6	0/20	0	IgG 3+ C3 2+ C1q 1+	III	Yes
7	1/30	0	IgG 3+ C3 3+ C1q 2+	II	No
8	3/16	30	IgG 3+ C3 2+	II	No
9	1/18	0	IgG 3+ IgM 1+ C3 +	II	Yes
10	0/9	0	IgG 3+ IgA 2+ C3 3+ C1q 1+	II	No
11	0/9	5	IgG 3+ IgM 3+ C1q2+	Not done	Not done

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Figure Legends

Figure 1: Flowchart of the discovery and validation cohorts. Initial studies were done by mass spectrometry (MS) studies using 70 PLA2R negative MN. We detected Sema3B in 2 cases which was then confirmed by immunohistochemistry (IHC). All 70 cases were negative for NELL-1 and EXT1/EXT2 by MS. We then studied an additional number (n=90) of PLA2R negative MN for Sema3B that yielded one positive Sema3B MN case. Only 4 cases were pediatric cases of the 160 cases of PLA2R-negative cases. Three validation cohorts were studied. The first (French) validation cohort included 59 adult cases of which one had a childhood onset (patient 5), the second (French) cohort included 16 pediatric cases, and the Italian cohort included 43 pediatric cases. * All cases were negative for PLA2R, THS7DA, NELL-1, and EXT1/EXT2 staining by IF. Yellow boxes indicate detection and confirmation of Sema3B by MS, grey boxes indicate detection by IHC/IF.

Figure 2: Proteomic Identification of Sema3B in PLA2R-negative MN. Glomeruli were microdissected and analyzed using mass spectrometry as described in methods.

Figure 2A shows detection of Sema3B in 3 cases of PLA2R-negative MN. Numbers in green boxes represent spectral counts of MS/MS matches to a respective protein. Top panel shows spectral counts of Sema3B in the 3 cases. Panels 2-4 show the immunoglobulins IgG1, IgG2, IgG3, and IgG4, and bottom panel shows baseline spectral counts of PLA2R.

Figure 2B: Representative sequence coverage map of Sema3B from 1 case. Amino acids highlighted in bold letters over yellow background are the amino acids detected. Note the extensive coverage. Green highlighted boxes indicated amino acids with artefactual chemical modification induced by mass spectrometry such as oxidation of methionine.

Figure 3. Immunohistochemical stain for Sema3B in Sema3B-associated MN (Mayo Clinic cohort), PLA2R-associated MN and control cases

Figure 3A shows bright granular capillary wall staining for Sema3B along the glomerular basement membranes in 3 cases of Sema3B-associated MN.

Figure 3B shows negative Sema3B staining in a representative case of (A) diabetic glomerulosclerosis, (B) focal segmental glomerulosclerosis, (C) IgA nephropathy, (D) minimal change disease, (E) time zero transplant biopsy, and (F) PLA2R-positive MN.

Figure 4. Immunofluorescence microscopy for Sema3B in the validation cohorts

Figure 4 shows bright granular staining for Sema3B. Figure 4A and 4B are the same case (patient 4) stained by IHC and IF. Figure 4C and 4D are the same patient (patient 5, with biopsies at the ages of 1 and 19). Figure 4E-J represents patient 6, 7, 8, 9, 10 and 11, respectively. Patients 6 to 11 are pediatric patients. Figure 4K represents an adult PLA2R-positive MN control case and Figure 4L represents a pediatric PLA2R-negative MN control case.

Figure 5. Detection of Sema3B and IgG in glomerular immune deposits in Sema3B associated MN by confocal immunofluorescence microscopy analysis.

Glomeruli were double-labeled with anti-Sema3B (A- green) and anti-human IgG (B- red), with the right panel (C) showing the merged image. Figure 5D shows the enlarged image of the merged image. The white lines shows the places where fluorescence was recorded across sections of a representative capillary loop. Figure 5E shows quantitative analysis of the fluorescence recorded across sections of a representative capillary loop (arrows). Note the superimposition of the two signals, which indicates that subepithelial immune deposits contain Sema3B (green) and IgG (red). The confocal microscopy studies shown are from the biopsy of patient 5 (biopsy done at the age of 19 years).

Figure 6. Detection of anti Sema3B antibodies in the serum by Western blot analysis.

Figure 6A shows reactivity of serum samples with recombinant Sema3B protein. Under non-reducing conditions Sema3B is detected by control anti-Sema3B rabbit antibody as a band at around 80 kDa. This band is not detected by patient's sera and control sera. Under reducing conditions Sema3B is detected as a sharp band at around 80 kDa by the control anti-Sema3B antibody. In this condition, the same band is also detected by patient's sera but not by sera from patients with other kidney diseases or healthy controls. In patient 4, samples were available at the indicated time points. Treatment with Rituximab was started on 18.03.2009. Note disappearance of the band afterward. Patient 5 was sampled after long-term immunosuppressive treatment. Patients 3, 4, 5, 8, 11 are labeled as MN3, MN4, MN5, MN8, MN11, respectively.

Figure 6B shows the molecular structure of Sema3B, with large Sema domain region, PSI domain (plexin-semaphorin-integrin), Ig domain and short C terminal basic domain.

Figure 7. Representative kidney biopsy findings (patient 3). A: Light microscopy showing slightly thickened glomerular capillary walls (40x), B: Immunofluorescence microscopy showing glomerular and

tubular basement membrane staining for IgG, C-D: Electron microscopy showing (C) subepithelial and (D) tubular basement membrane deposits. E- Sema3B staining along the glomerular basement membrane. (F) Higher power showing that the tubular basement membranes are negative for Sema3B (60x). Arrow points to electron dense deposits (C-9300x, D-4800x).

Supplementary Material

Methods: Details of laser microdissection and mass spectrometry

Supplemental figure 1: An example of MS/MS spectra match to a sequence from Sema3B.

Supplemental figure 2: Confocal microscopy of additional Semaphorin 3B positive MN case.

Supplemental figure 3: Immunofluorescence staining for IgG and Semaphorin 3B in additional cases.

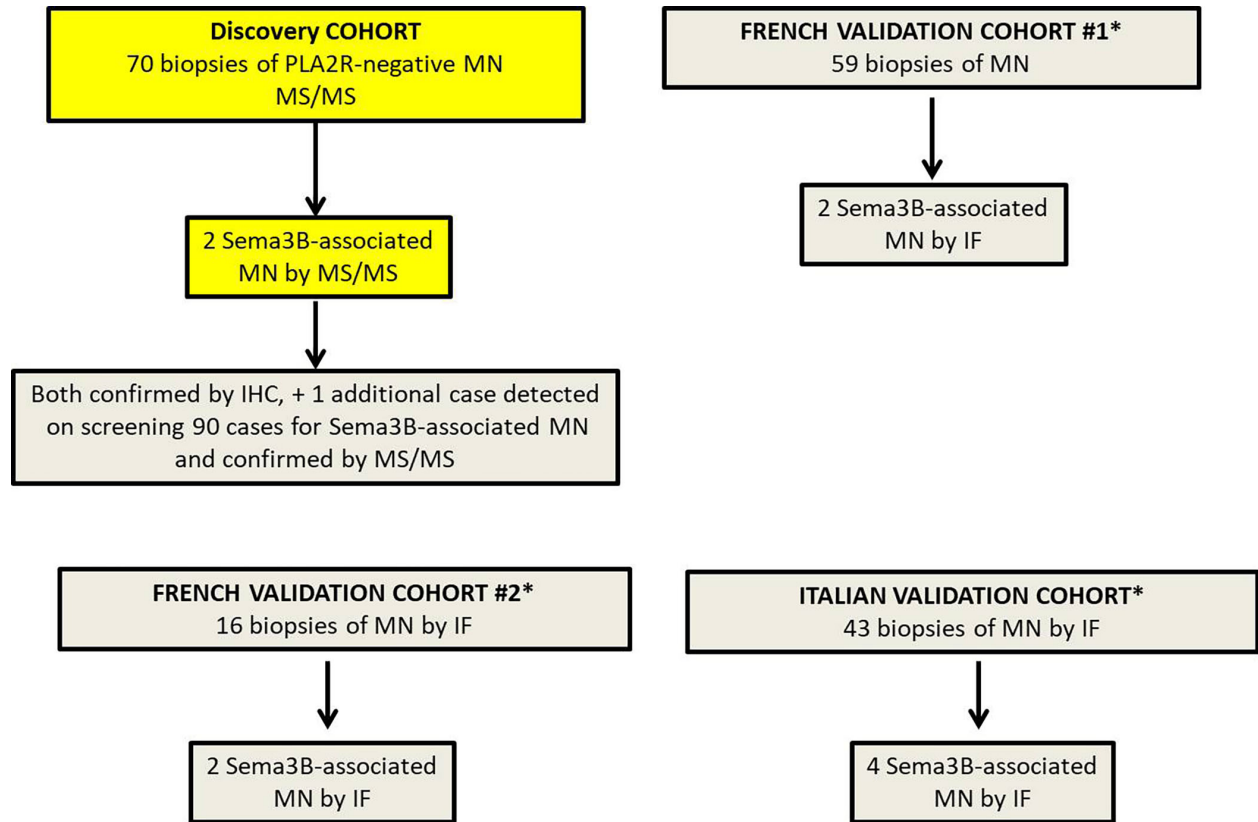
Supplemental table 1: Immunofluorescence staining in Semaphorin 3B positive cases.

Supplementary items are available on Kidney International's website

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Probability Legend: over 95% 80% to 94% 50% to 79% 20% to 49% 0% to 19%	Accession Number	Molecular Weight	Case 01	Case 02	Case 03	
Bio View:						
1850 Proteins in 1674 Clusters						
With 1849 Filtered Out						
Semaphorin-3B OS=Homo sapiens OX=9606 GN=SEMA3B PE=2 SV=1		sp Q13214 SEM3B_HUMAN	83 kDa	19	42	11
Immunoglobulin gamma-1 heavy chain OS=Homo sapiens OX=9606 PE=1 SV=...		sp P0D0X5 IGG1_HUMAN	49 kDa	25	29	21
Immunoglobulin heavy constant gamma 2 OS=Homo sapiens OX=9606 GN=I...		sp P01859 IGHG2_HUMAN	36 kDa	24	18	28
Immunoglobulin heavy constant gamma 3 OS=Homo sapiens OX=9606 GN=I...		sp P01860 IGHG3_HUMAN	41 kDa	20	19	36
Immunoglobulin heavy constant gamma 4 OS=Homo sapiens OX=9606 GN=I...		sp P01861 IGHG4_HUMAN	36 kDa	20	11	21
Secretory phospholipase A2 receptor OS=Homo sapiens OX=9606 GN=PLA2R1 ...		sp Q13018 PLA2R_HUMAN	169 kDa	2	5	(0)

sp|Q13214|SEMA3B_HUMAN (100%), 83,121.5 Da

Semaphorin-3B O5=Homo sapiens OX=9606 GN=SEMA3B PE=2 SV=1

31 exclusive unique peptides, 37 exclusive unique spectra, 45 total spectra, 415/749 amino acids (55% coverage)

MGRAGAAAVI	PGLALLWAVG	LGSAAAPSPPR	LRLSFQELQA	WHGLQTFSL	RTCCYQALLV	DEERGRLFVG	AENHVASLNL
DNISKRAKKL	AWPAPVEWRE	ECNWAGKDIG	TECMNFVKLL	HAYNRTHLLA	CGTGAFHPTC	AFVEVGHRAE	EPVLRRLDPGR
IEDGKGKSPY	DPRHRAASVL	VGEELYSGVA	ADLMGRDFTI	FRSLGQRPSL	RTEPHDSRWL	NEPKFVKVFW	IPESENPDDD
KIYFFFRETA	VEAAPALGRL	SVSRVQGICR	NDVGGQRSLV	NKWTTFLLKAR	LVCSPVGVGG	DTHFDQLQDV	FLLSSRDHRT
PLLYAVFSTS	SSIFQGSAVC	VYSMNDVRRR	FLGPFHKEG	PMHQWVS YQG	RVPYPRPGMC	PSKTFGTFS	TKDFPDDVIQ
FARNHPLMYN	SVLPTGGRPL	FLQVGANYTF	TOIAADR VAA	ADGHYDVLFI	GTDVGTVLKV	ISVPKGSRPS	AEGLLLEELH
VFEDSAVTS	MQISSKRHQL	YVASRSAVAQ	IALHRCAAHG	RVCTECCLAR	DPYCAWDGVA	CTRFPQSAKR	RFRQDVRNG
DPSTLCSGDS	SRPALLEHKV	FGVEGSSAFL	ECEPRSLQAR	VEWTFQRAGV	TAHTQVLAEE	RTERTARGLL	LRRLRRRDSG
VYLCAAVEGG	FTQPLRRLSL	HVLSATQAER	LARAEAAAPA	APPGPKLWYR	DFLQLVEPGG	GGSANSLRMC	RPQPALQSLP
LESRRKGRNR	RTHAPEPRAE	RGPRSATHW					

