Chapter

Unraveling Primary Membranous Nephropathy Using Proteogenomic Studies

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Abstract

Membranous nephropathy is one of the leading causes of nephrotic syndrome in adults. The disease manifests in different forms with varying severity and outcomes range from spontaneous remission to rapid disease progression. The effects of the disease are so far best understood using conventional histopathological morphology and clinical phenotype. Being an autoimmune condition subject to a multi-hit hypothesis, the notion of underlying genetic risks is being examined in recent times. Current evidence points to significant heterogeneity in the gene expression profiles in both the immune system and at the glomerular level, with potential implications for disease management. Further proteomic and transcriptomic analysis can instruct classification, prognostication, and treatment pathways. This chapter focuses on the links identified between primary membranous nephropathy and underlying gene polymorphism, and pathways using both proteomics and transcriptomic analysis. We discuss the potential impact this could have on future management to try to minimize the patient's immunosuppression exposure and find the most effective targeted immunosuppressive therapy.

Keywords: membranous nephropathy, gene polymorphism, HLA, Transcriptomics, Proteomics

1. Introduction

Management of membranous nephropathy remains a continuing challenge in the field of nephrology. Over the last 50 years, we have been classifying and managing membranous nephropathy based on both histological and clinical phenotyping, which is the key feature guiding treatment decisions. However, in recent years other mechanisms have come to shed light on the heterogeneity of membranous nephropathy and clinical outcomes. With the emergence of proteogenomic analysis, the classification of other nephrotic syndromes has advanced immensely [1]. Therefore, the potential value for membranous nephropathy is to be considered.

Varied clinical manifestation may be due to the polymorphic gene expression in the immune system as well as at the glomerular filtration barrier site, which comprises podocytes, endothelial cells, and intervening glomerular basement membrane [2]. In addition to the multiple immune cascade pathways, underlying complex molecular and cellular processes are identified by proteomics and transcriptomics in glomerular disorders like Minimal Change Disease and focal

segmental glomerulosclerosis (FSGS) [1]. Early data suggest that gene expression and molecular pathways are both potential emerging therapeutic targets in the era of precision medicine in these disorders.

Primary membranous nephropathy is considered an autoimmune disease, associated with autoantibodies recognizing a target antigen on the podocytes. The connection between the immune system and underlying cellular pathways in the pathophysiology of membranous nephropathy has been an area of extensive research. Even though membranous nephropathy does not follow the mendelian trait, the role of underlying genetic factors was examined in previous studies.

Although clinical and histological apparency of membranous nephropathy are seemingly similar, response to immunosuppressive therapy can be variable with poor response to treatment in some patients whilst about a third of the patients have minimal to no long-term consequence following spontaneous remission. This heterogenicity may be due to differences in underlying biological processes influenced by genetic polymorphism and protein interaction networks and this warrants more in-depth understanding to unravel precise prognostication and therapeutic options.

The genome-wide association study (GWAS) provided evidence on the susceptibility of specific HLA regions and the susceptibility to primary membranous nephropathy [3]. With the emergence of PLA_2R antigen as an immune system target in primary membranous nephropathy, more evidence has shown a link between PLA_2R antigen and specific HLA alleles [4, 5].

This chapter intends to review the current understanding of gene expression analysis, proteomics, and metabolomics in membranous nephropathy with a focus on the utility of these methods in clinical practice.

2. Integrating genomics, transcriptomics, and proteomics

The major histocompatibility complex found on chromosome 6 p21 consists of polymorphic genes that code cell surface proteins and plays an important role in adaptive immunity. MHC I is found on all nucleated cells whereas MHC II is found mainly on antigen-presenting cells. The human genome project has been a turning point in our understanding of human genomes and our understanding of disease pathophysiology [6], revealing the link between genetic downstream pathways for both target antigens and immune cells.

It has been found that immune-mediated glomerular injury involves activation of both innate and adaptive immunity, which leads to the clinical and pathological manifestation of the disease. Over the past 10 years, more than 80 genes causing glomerular barrier dysfunction have been identified [2]. With the emergence of human genetic studies, understanding which gene sequence is the main trigger for the immune system malfunction became a very powerful tool to uncover the molecular drivers of these diseases and help find potential targeted therapy.

The understanding of genome sequence will reveal the gene expression and how the cell behaves in a static state by looking at the amino acid sequence of the DNA. To identify the effect of variation in genetic architecture on proteins and correlate with diseases, one needs to move to undertake transcriptomics and proteomics, to study mRNA from DNA code and tRNA decoding the mRNA sequence into protein. This function can further vary by an alternative splicing process where the single genome can be translated into different proteins [7]. Hence, this is a process that captures the expressed portion of the genome and the expressed protein set in the genome, which will reveal how the cells act in a dynamic state as well as a static state [8].

Proteomic analysis is performed directly from tissue microdissection and mass spectrometry. The NEPTUNE group identified clusters of patients based on their mRNA expression in the tubulointerstitial portion of renal tissue [1]. Also, they performed non-invasive techniques to evaluate the proteomic biomarkers and found common downstream gene expression pathways from the one found in the renal tissues. The utility of proteomics in autoimmune kidney diseases has been previously tested and has shown common cellular pathways that might help in targeting therapy for different autoimmune renal conditions [9, 10].

Membranous nephropathy is one of the leading causes of nephrotic syndromes. At the histological level, both glomerular immunoglobulin and complement deposition are hallmarks in making the diagnosis. PLA_2R1 antigen was the first membrane-bound glycoprotein identified as a target antigen in adults with primary membranous nephropathy. This was identified by Beck *et al* in a pivotal study in 2009. With the use of laser microdissection and mass spectrometry LM/MS, new target antigens are being discovered, some of which may present opportunities to identify as protein biomarkers for secondary autoimmune or malignancy-associated membranous nephropathy [11, 12]. The absence of PLA_2R antigen on histological staining and the presence of Exostosin1/2 identified on mass spectrometry following laser microdissection can pave the way for identifying Lupus class 5 at an early stage [13]. Also identifying NELL1 by laser microdissection and mass spectrometry can allow early evaluation for underlying malignancy and may warrant further follow-up [14]. Validation of these target antigens in independent studies will add further value to the clinical management of these patients.

Another attractive area for the use of laser microdissection and mass spectrometry is Amyloidosis. Due to the abundance of amyloid protein in the tissue, LM/MS has gained investigators' attention and showed very encouraging outcomes when performed on amyloid renal tissue samples [15].

Even though both transcriptomics and proteomics yield very rich information, they have some limitations. This may be due to the alterations that might happen after mRNA translation or failure to identify proteins resulting from the alternative splicing process. Yet, these are complementary information and one can still conclude within these limitations [16].

3. Role of immunoglobulins in the disease pathophysiology

During the early development of T and B cells as the immune system line of defense, a VDJ (variable diversity joining) gene segment is created. Due to the heterogeneous gene sequence resulting from mutation triggers, diverse antibodies and T cell receptors are formed given the variable amino acid sequence identified on these cell's genes [17]. IgG3 is the least VDJ mutated followed by IgG1, IgG2 then IgG4. Based on this finding, a temporal model for immunoglobulin subclass transformation during an inflammatory response has been hypothesized [18]. This suggests that cells first switch from IgM to IgG3, to IgG1, to IgG2 finally to IgG4 following a genomic ordering. That was partially similar to the Markov chain except for IgG2 emerging before IgG1 [19]. IgG1 is a complement-fixing antibody with IgG4 lacking the effector response of antibody-dependent cell-mediated cytotoxicity and complement-dependent cellular toxicity but is known for its anti-inflammatory blocking response, which can dampen down the overactivity of other IgG subclasses [20].

The role of immunoglobulin subtypes and their effect on complement when identified by immunofluorescent stain on renal tissue of membranous nephropathy is an area that requires further research. In one study, there has been a correlation

between serum IgG4 and PLA2R associated membranous nephropathy disease activity [21]. Subclassing the immunoglobulin types can also be performed using laser microdissection and mass spectrometry at the tissue level. It has been found that IgG1 is the most abundant subclass followed by IgG4 in Exostosin1/2 associated membranous nephropathy compared to IgG4 in PLA_2R associated membranous nephropathy [11]. This is in line with other studies [19, 22]. This raises the question if the response to immunosuppression varies based on these antibodies' amino acid sequence or not. Same for the downstream cellular and molecular pathways resulting from the inflammatory cascade rather than relying on the immunoglobulin subtype staining.

4. Role of complements in the disease pathophysiology

Identifying complements on routine renal biopsy evaluation gives us a limited understanding of the role of these complements in the disease process. Complement proteins and their cleavage products have a vital role in attacking the glomerular basement membrane and podocytes [23]. Yet, most of the randomized controlled trials were designed to use immunosuppression medication directed towards blocking the immune system at the immunoglobulin level rather than targeting complements and the underlying cellular and molecular pathways identified by proteogenomic analysis [24–27] (RI-CYCLO NCT03018535).

Although the 3 complement pathways (classical, lectin, and alternative) converge at the same downstream target, they differ from each other at the point of origin [23]. The classical pathway is mainly triggered by IgM and IgG3/1 complexing with the antigen. This immune complex activates C1, which is the main classical complement pathway protein. The C1 complex then breaks down C2 and C4 into C2b and C4a, which then merge to form the C3 convertase. The lectin pathway is activated when the mannose-binding lectin attaches to the bacterial surface. This complex further aids the classical pathway by the breakdown of C2 and C4 and leads to the formation of C3 convertase. The alternative pathway is activated by the spontaneous hydrolysis of C3 into C3b, which binds to factor B and forms the other C3 convertase. This pathway abrogation is being investigated in the ongoing clinical trial utilizing LNP023 (NCT04154787). Finally, c3 convertase from classical, lectin, and alternative pathway complexes with c3b to form two different types of c5 convertase that cascades to form the membrane attack complex as the final complement effector.

Eculizumab is a humanized monoclonal antibody that inhibits the cleavage of C5 into C5a and C5b and hence inhibits the deployment of membrane attach complex. In 2002, an abstract of a randomized control trial in 200 patients with idiopathic membranous nephropathy received 2 dose regimens of eculizumab with no significant effect on neither proteinuria nor renal function [28]. This was probably due to an inadequate complement inhibition response and further trials with higher doses may be required.

The role of complement activation in membranous nephropathy glomerular injury is supported by the presence of C3, C5b-9, and IgG in the subepithelial space. With the discovery of IgG4 as the main subclass antibody identified in primary membranous nephropathy, it should be noted that these antibodies are incapable of binding to C1q and therefore unable to activate the classical complement pathway and are unlikely to be present in primary membranous nephropathy. The absence of C1q in the presence of C4d which is a classical pathway protein break down from C4b has been identified in cases of primary membranous nephropathy. The most likely explanation is that either there is an element of classical pathway activation

that has not been discovered yet, or the lectin pathway has been activated. Lectin pathway activation has shown to carry a worse prognosis in PLA_2R associated membranous nephropathy [29].

With the emergence of proteogenomic analysis, recent evidence has shed the light on the significance of complement activation in the pathophysiology of membranous nephropathy [22]. To further analyze, common target antigens for both primary and secondary autoimmune membranous nephropathy were identified using mass spectrometry following laser microdissection. Using these methods, they found PLA_2R antigen and Exostosin1/2 were the two most common target antigens for primary and secondary autoimmune membranous nephropathy respectively and were analyzed accordingly [11].

It has been found that the three complement pathways play a role in disease pathogenesis. Regardless of the antigen identified, C3 and its regulatory protein CFH as the main alternative pathway downstream proteins showed the highest spectral counts, whereas C1q as the main classical pathway downstream protein showed the lowest spectral count [22]. Moreover, they found high spectral counts from the terminal complement pathway cascade (C5, C6, C7, C8, and C9). Thus, targeting the terminal complement pathway might be a future therapeutic option. On a further note, investigators identified C4 as the second most abundant protein in the absence of Manon-binding lectin serine protease 1 and 2, which points to the role of the classical pathway in the underlying disease process.

The mannose-binding lectin molecule is a major recognition molecule of the lectin pathway. Genetic polymorphism has been shown to play a role in the disease downstream pathways. By performing genotyping, one study has found that patients with MBL deficiency can develop membranous nephropathy with the downstream complements being activated primarily from the alternative pathway. Whereas patients with wild type of MBL2 have their complements mainly derived from the lectin pathway [30]. These studies show that classical, lectin, and alternative complement pathways play a significant role in disease pathogenesis.

As previously discussed, the lectin pathway plays an important role in disease pathogenesis [30]. In a case–control Brazilian study, an association was noted between membranous nephropathy and mannose-binding lectin 2(MBL2) polymorphism in patients carrying O allele, in particular, A/O genotype [31]. Investigators also found a defective MBL production in patients with YA/O, XA/O, and O/O genotypes. As shown previously, activating the lectin pathway in patients with PLA_2R associated membranous nephropathy carries a worse prognosis [29].

The use of LM/MS is not limited to identifying proteins but also can aid in classifying disease severity and treatment response by analyzing the complement protein spectral counts [22]. The persistence of complements on glomerular capillaries may explain the lag in clinical remission behind immunological remission and provide a reason for persistent proteinuria after the disappearance of PLA_2R-Ab in serum [32]. More studies using proteogenomic analysis can further analyze these complement proteins to identify a more targeted therapy and inform about disease severity and likely response.

5. Single nucleotide polymorphism and genotyping in HLA class II allele and PLA_2R1 antigen

The immune system recognizes peptide sequences processed from a target antigen when presented by the antigen-presenting cells. In PLA_2R associated membranous nephropathy, the immune system will interpret the PLA_2R1 presented by the HLA Class II molecule as the target antigen. The GWAS (genome-wide

association studies) identified a strong association of single nucleotide polymorphisms (SNPs) on both HLA-DQA1, PLA_2R1 antigen, and Primary Membranous nephropathy [4]. Due to the proximity of HLA-DQ and HLA-DR on chromosome 6 and the probability of linkage disequilibrium that can result in the coinheritance of common haplotype, both alleles were found to be associated with membranous nephropathy. When imputation was performed it has confirmed the same signals on the same loci identified in other studies [33]. That can also be interpreted from one study where they have identified a significant association between PLA_2R1 antigen and both HLA DQA1/DRB1 [34]. In the former study, it was noted that using a combined genetic risk score and serum anti PLA_2R antibody using the ELISA method can potentially mitigate the need for a diagnostic renal biopsy in high-risk patients.

Not all cases of primary membranous nephropathy are associated with PLA_2R1 antigen. After the discovery of the PLA_2R antigen, THSD7A was the 2nd antigen implicated in primary membranous nephropathy and is found in 2–5% of patients [35]. Investigators have identified similarities and differences between THSD7A and PLA_2R antigens [36]. This can be extrapolated to suggest that there may be an HLA link with THSD7A either similar or different to PLA_2R antigen. In a large case–control study, the link between the HLA-DQA1 and PLA_2R1 antigen was found to be more prevalent in patients with confirmed PLA_2R1 associated membranous nephropathy compared to PLA_2R1 negative patients [37]. For now, the specific association is yet to be noted in anti-PLA_2R1 negative cases. Also, association with these SNPs is seen in patients with Caucasian backgrounds compared to patients from Afro-American origins.

Another Asian study found a strong association between HLADQA1 and PLA_2R1 antigen [38]. In this study investigators not only identified SNP variation on HLA-DQA1 but also found that AA and AG genotype carriers are at increased risk of developing primary membranous nephropathy compared to those carrying GG genotype. On the other hand, GG genotype on PLA_2R1 antigen SNP and AA genotype on another were encountered more frequently in subjects with primary membranous nephropathy. Two other Asian studies have identified other HLA risk alleles other than HLA-DQA1 in their cohorts of patients with primary membranous nephropathy [39, 40]. Again this raises the possibility of linkage disequilibrium.

Correlating the treatment response to the underlying genetic polymorphism is another area that is being investigated. A Chinese-Taiwanese study observed that haplotype H1 might carry a higher risk for disease progression when compared to H3 haplotype. The group found no relation between disease progression and underlying genetic polymorphism in PLA2rR1 antigen without the incidence of ESRD or death after therapy [41].

In another Spanish study, they found no association between survival and single nucleotide polymorphism in PLA_2R1 antigen. In the same study, AA and AG genotypes in HLA-DQA1 and AA genotype on PLA_2R1 antigen were shown to be associated with a trend towards immunosuppression treatment response compared to other genotypes [42]. Moreover, AA and AG genotypes on HLA-DQA1 SNP have shown significant protection for doubling of serum creatinine and progression to end-stage renal disease, without identifying any protective genotypes on PLA2R1 antigen.

A Chinese paper has highlighted the ethnic distribution difference in membranous nephropathy based on their HLA types [34]. They have found that DRB1*1501 is the major risk allele in the East Asian population, DQA1*0501 in Europeans, and DRB1*0301 in both ethnicities. This new finding can allow us to categorize high-risk patients from different background ethnicities based on their HLA type.

6. Genetic polymorphism other location than HLA alleles and PLA_2R1 antigens

In addition to the previously mentioned HLA alleles and PLA_2R1 polymorphism, many other genes were discovered to be linked with primary membranous nephropathy. It has been shown that TH2 cells are predominant in primary membranous nephropathy due to the presence of IgG4, which belongs to type 2 immune response [43]. TH2 cells are responsible for the secretion of IL4, IL 10, and TNF **α** as major cytokines. These cytokines can enhance the expression of the HLA molecule and lead to disease pathogenesis.

In a case–control study, TNF**α** and TNFd genotypes belonging to MHC class III were associated with primary membranous nephropathy [44]. It was noted in this study that for that association to occur, the underlying HLA was B8/DR3/DQ2. The influence of TNF **α** gene polymorphism on disease progression was not present for either of the TNF genotypes separately or in combination. The importance of TNF in clinical practice was examined in a small study of 10 patients who did not respond to maximum RAS blockade. Pentoxifylline is a phosphodiesterase inhibitor that is capable of lowering TNF **α** levels were used, and patients were followed up for 6 months. 9 out of 10 patients achieved remission with TNF **α** levels trending down in both plasma and urine [45].

Another study showed an association between specific IL4 and IL10 genotypes and membranous nephropathy [46]. This raises a particular question for future studies using interleukin inhibitors in membranous nephropathy.

7. Genetic polymorphism and the risk of cancer and thrombosis

Identifying PLA_2R antibodies has not yet changed our approach for excluding secondary causes for membranous nephropathy. KDIGO recommendations include performing a secondary work up to rule out autoimmune conditions, infections, and malignancies [47]. Recently with the use of laser microdissection and mass spectrometry, NELL1 antigen has shown an association with cancers [14]. Genotyping by Polymerase chain reaction has shown an association between specific gene polymorphism and cancers, and progression to end-stage renal disease. In one study investigators sub-classified patients with urokinase plasminogen activator polymorphism (Gene 3'-UTR) into groups based on their allele distribution either C/C or C/T. Although the number of patients was significantly higher in the CC group, patients with T/C genotype had a better trend towards renal survival and lower cancer incidence [48]. It should be noted that this gene polymorphism was not different from the control group.

A plasminogen activator inhibitor 1 gene polymorphism was examined to assess for correlation with disease activity, treatment response, and long-term prognosis [49]. Patients carrying 5G/5G genotype were more likely to attain complete remission, whereas 4G/4G and 4G/5G were more likely to develop renal disease progression, and 4G/4G showing no signs of remission. Patients carrying the 4G allele (4G/4G or 4G/5G) were more likely to develop coronary artery disease and peripheral vascular disease in comparison to carriers of the 5G allele, which was in line with another meta-analysis that showed a high risk of myocardial infarction in plasminogen activator inhibitor 1 4G/5G carriers [50]. It should be noted that in the former study, gene polymorphism was not different between membranous nephropathy patients and controls. Moreover, the number of patients carrying 4G allele was almost double that for 5G allele. These studies are single-center studies

and need validation through well-designed multi-center trials to establish the relationship between polymorphism and disease outcomes. The framework of bio registries can help to validate the significance of these studies.

8. Genetic polymorphism on TLR, MYH9, NF-κB, and IRF4

The toll-like receptor expressed on the surface of the macrophage plays an important role in the link between innate immunity and adaptive immunity. TLR recognizes microbes leading to activation of downstream signals that result in the production of INF gamma. It is speculated that the association of TLR9 in membranous nephropathy might explain why and how infections can trigger the occurrence of membranous nephropathy. In a previous study, overexpression of TLR in the renal tissues was confirmed in lupus nephritis [51]. The same idea was tested in an Asian study to identify the link between TLR9 and the incidence of membranous nephropathy. Genotyping for TLR9 found a statistically significant difference between AA and GG genotypes on two specific SNPs loci on TLR9 when compared to controls [52]. Although the incidence of tubulointerstitial fibrosis was higher in the A-G haplotype when compared to the non-A-G haplotype, survival did not differ between the two groups. Another study investigated the association between membranous nephropathy and TLR4 specific gene polymorphism and found a statistically significant difference between A/G TLR4 genotype in membranous nephropathy and control, and no difference in haplotype frequency [53]. These observations suggest a possible association between membranous nephropathy and TLR4/9.

Myosin heavy chain 9 (MYH9) is expressed on most of the tissues that participate in the process of cell division and migration. Mutation in MYH9 was found to be associated with many renal diseases [54]. A difference in specific gene pleomorphism on MYH9 between membranous nephropathy and control was found [55]. On haplotype frequency, C-A was the common haplotype in membranous nephropathy and that was significantly different from controls.

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a complex protein that controls cytokine production and plays a key role in regulating the immune response to infection.

The most abundant SNP (rs230540) found on the NKFB1 locus was predicted to have a functional impact on specific immune cells. Investigators have found that the previously mentioned SNP locus is associated with higher mRNA expression of NFKB1 in whole blood [34, 56]. Coinciding with the NFKB1 pro-inflammatory effect, another UK study has found that membranous nephropathy risk haplotype at this same locus has shown a higher leucocyte signal [57]. Another SNP locus (rs9405192) on IRF4 has shown a major role in innate immunity activation. IRF4 IS A lymphocytic gene that regulates toll-like receptor activation signaling, which is under the control of NFKB1 complex [58]. This highlights that certain loci on NFKB1 and IRF4 play a crucial role in the underlying membranous nephropathy pathophysiology at the cellular pathway level.

9. Conclusion

The heterogenicity of clinical phenotypes and outcomes in membranous nephropathy could be related to the underlying complex molecular, cellular, and biological pathways. Both genomic and proteomic analysis are becoming widely available tools to interrogate these possibilities. Membranous nephropathy being

an autoimmune condition, requires identification of potential antigenic peptides at the podocyte, glomerular capillaries, and basement membrane levels. The proteogenomic analysis will help reveal downstream pathways that arise from the interaction between these genes, which can occur either at the glomerular level or in the immune system pathway. Hence, understanding the pathophysiology and the close interaction between various arms of the immune system and ancillary pathways is crucial for future targeted therapy in membranous nephropathy.

Laser microdissection and mass spectrometry will likely be crucial in revealing the link between the antibody subtypes and newly discovered glomerular antigens, and the three different complement pathways. Even though the classical pathway does not play a major role, some evidence has shed the light on its involvement. Thus, further investigations into types of complement activation with targets at the molecular level should be an area for future research.

The advent of proteogenomic analysis has shown a link between HLA and PLA_2R antigen, but evidence for the link with THSD7A and other antigens is yet to be discovered. Also, the correlation between HLA and disease outcome is another area of interest that might further aid our future choice for immunosuppression treatment. A well-designed trial is required to correlate that link with disease outcome and treatment response.

Identifying certain high-risk alleles in patients with urokinase plasminogen activator gene polymorphism and plasminogen inhibitor activator 1 pleomorphism, has shown a probable association with cancers, thrombosis, disease progression, and remission response. This might pave the way for future discovery of certain genes that can identify cancer as a trigger for membranous nephropathy even if it would occur years after diagnosis. Also, can help with identifying patients who are more prone to venous thromboembolism and would benefit from anticoagulation. Moreover, patients who are more liable for disease progression can benefit from early immunosuppression treatment without the need for a period of watchful wait on RASS inhibition compared to other patients who might attain complete remission without the need for aggressive immunosuppression therapy.

Hence, the proteogenomic analysis is the way forward for identifying targeted immunosuppression therapy for membranous nephropathy in the era of precision medicine.

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