The clinicopathological implications of endothelial tubuloreticular inclusions found in glomeruli having histopathology of idiopathic membranous nephropathy

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Abstract

Background. The pathological recognition of secondary membranous nephropathy (MN) is sometimes difficult, especially in those showing primary idiopathic MN-like histomorphology. The ultrastructural finding of tubuloreticular inclusions (TRIs) in MN always evokes suspicion of their association with underlying diseases such as viral infections and autoimmune diseases. However, it is not clear whether some other underlying diseases are associated with TRI expression in MN. Since treatment of the underlying diseases is the primary consideration for the management of secondary MN, it is important to make out the clinical significance of TRI expression in MN.

Methods. Excluding the patients fully qualified for systemic lupus erythematosus (SLE) diagnostic criteria, we recruited 36 cases having a renal biopsy featured with idiopathic membranous nephropathy. We investigated their clinical and pathological profiles and focused on the potential connections with the underlying diseases and treatment outcomes.

Results. One-third of our cases showed no identifiable underlying aetiology. Other underlying disease groups included autoimmune disease (25%), hepatitis (14.7%), potential Helicobacter pylori infection (13%), diabetes (5.6%) and lymphoma (5.6%). Pathologically, patients in the autoimmune group tended to have more heterogeneous membranous deposits with frequent mesangial and subendothelial deposits. While all patients of the autoimmune group presented complement C1q in glomeruli, more than two-thirds of the patients in others groups were negative for C1q. Clinically, the patients in autoimmune and hepatitis groups were younger in age and had less remission of proteinuria following treatment, while the other groups of patients achieved partial or complete remission more frequently.

Conclusion. The underlying diseases of our patients were consistent with the major disease categories that have been frequently linked to secondary MN. The HP group was more akin to undefined groups regarding their pathological and clinical profiles. Since the MN in the undefined group might be the only renal manifestation antedating other clinical presentations of the corresponding underlying disease, a
long-term follow-up and meticulous search for aetiological factors are required to validate this assumption.

**Keywords:** interferon; membranous nephropathy; tubuloreticular inclusion

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### Introduction

Tubuloreticular inclusions (TRIs) are subcellular organelles characterized by small clusters of anastomosing tubule-like structures within cisternae of endoplasmic reticulum [1,2]. As suggested by previous investigations, the morphogenesis of TRIs might relate to the biological activities of alpha or beta interferons (IFN-α and IFN-β), and correlate clinically with systemic IFN treatment or endogenous overproduction of IFNs [3–6]. In the kidney, TRIs are frequently seen in glomerular endothelial cells (GECs) under certain pathological conditions such as lupus nephritis, virus-associated nephropathies and renal transplant [7,8]. There was a report of the appearance of TRIs in glomerular endothelial cells before the development of full blown disease [9]. However, the role of TRIs as a reliable indicator of secondary membranous nephropathy (MN) with a wide spectrum of aetiologies has not been fully studied. In this report, we analysed 36 cases of MN showing unequivocal TRIs in glomerular endothelial cells but otherwise showing conventional idiopathic-type histopathology of MN. We investigated their clinical and pathological profiles and addressed specifically on the potential connections with the underlying diseases, clinicopathological correlation and the outcome of treatment.

### Materials and methods

Thirty-six cases were selected from a total of 287 pathologically diagnosed MN in a period of 6 years in Taipei Veterans General Hospital. The selection criteria were based on the ultrastructural finding of TRIs in GECs of MN. The glomerular pathology of 21 cases was non-proliferative by light microscopy while the remaining 15 cases showed mild focal segmental mesangial hypercellularity (involving <20% of all glomeruli and <50% of involved glomeruli). Our cases did not include patients who were qualified for systemic lupus erythematosus (SLE) based on current clinical diagnostic criteria [10]. All cases had adequate specimen for routine pathological examination, which included histochemical stains (haematoxylin and eosin, periodic acid-Schiff, methenamine silver-periodic acid, Masson trichrome), immunofluorescent stains (IgG, IgM, C1q, C3 and C4) and electron microscopy. All cases were also surveyed for the *Helicobacter pylori* antigen in glomeruli using an immunohistochemical method (mouse anti-*H. pylori*, Dako, Denmark, 1:100 dilution). A previous stomach biopsy of one case was also included for anti-*H. pylori* staining. Four gastric mucosal tissues infected with *H. pylori*, obtained by an endoscopic biopsy, were used as positive control. The renal tissues from patients who had positive hepatitis B serum markers were surveyed for the presence of hepatitis B surface antigen (mouse anti-HBsAg, Novocastra, Newcastle, UK), and core antigen (mouse anti-HBcAg, Novocastra, Newcastle, UK) in glomeruli. Thromboglobulin (TG) (mouse anti-human TG, Dako, Denmark, 1:250 dilution) immunohistochemical stains were performed on sections from two patients who had a history of autoimmune thyroiditis and hypothyroidism. Renal biopsy specimens were processed according to standard protocols. All histochemical and immunohistochemical stains were prepared using 3 μ paraffin sections. Routine immunofluorescence staining was performed using FITC-conjugated rabbit anti-human antibodies (Dako) on 6 μ frozen sections. The renal biopsy tissues for electron microscopy were fixed in 2.5% glutaraldehyde and processed according to routine protocols. Thin sections were viewed and digitally recorded using a transmission electron microscope (JEM-1230, JEOL, Japan) equipped with a multiscan CCD camera (791 MSC, Gatan, USA). The cases positive for TRIs were retrieved by retrospective review of pathological records of renal biopsies. An average of 1.7 glomeruli/case was examined by electron microscopy.

To search for the potential aetiologic factors, we carefully reviewed the patient records with respect to their clinical and laboratory information related to autoimmune diseases, infection episodes, oncology profiles, tumour markers, diabetes mellitus, drug uses and toxic agent exposures. All patients had been routinely screened for serum HBsAg, anti-HCV, anti-HIV, antinuclear antibodies (ANA), anti-double-stranded DNA (anti-dsDNA) and complements. Other optional serum markers included nuclear ribonucleoprotein (RNP), Smith (Sm), SS-A, SS-B, Scl-70, Jo-1, cardiolipin, TG, thyroid peroxidase (TPO) and cryoglobulins. Based on the clinically identified underlying diseases, profiles of autoimmune markers and immunostaining results, we categorized all patients into six groups (Table 1, Figures 4 and 5). We categorized the patients (A1–A9) into the autoimmune group based on their autoantibody profiles, which included positive ANA (A1, A3, A4, A6 and A8; serum titres: 320–2560) or positive dsDNA (A7; serum titre: 126), or the presence of other autoantibodies (A1, A2, A3, A4, A5, A6 and A9; RNP, Sm, SS-A, Jo-1, RF, cardiolipin, TPO). The patients who only showed marginal ANA titre (≥ 80) were not included in the autoimmune group.

The disease process of each patient was categorized into either complete remission (proteinuria <0.3 g/day) or partial remission (reduction in proteinuria <3.5 g/day and a 50% reduction from peak proteinuria at the end of the follow-up period) [11,12]. The patients who had either complete or partial remission were designated as the remission group, and all others categorized into the non-remission group. Statistical analyses were carried out using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). Continuous variables were tested using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for multiple comparisons. Categorical variables were compared using Fisher’s exact test. Differences were considered significant if two-sided \( P < 0.05 \).

### Results

We identified a cohort of patients (\( n = 36 \)) who had renal biopsy specimens with histopathological features similar to idiopathic MN but ultrastructural appearance of TRIs in GECs (Figures 1 and 2). One-third of them (\( n = 12 \), UD1–UD12) showed no identifiable underlying aetiology (Figure 3). Others had the underlying diseases such as autoimmune or autoimmune-like diseases (\( n = 9 \)), hepatitis (\( n = 5 \), HB1–HB4 and HC1, 14.7%), *H. pylori* infection-associated (\( n = 6 \), HP1–HP6, 13%), diabetes (\( n = 2 \), D1 and D2, 5.6%) and non-Hodgkin lymphoma (\( n = 2 \), T1 and T2, 5.6%). There was no significant overlap between any two groups except that one patient in the HP group also showed clinical signs of diabetes.

Immunohistochemical analyses found one patient (HB3) of hepatitis B to be positive for HBsAg (Figure 3B). Six patients (HP1–HP6) were unexpectedly positive for *H. pylori* antigen on glomerular capillary walls (Figure 3B and C). One of the HP patients (HP6) was found to have systemic *H. pylori* infection by the endoscopic biopsy (Figure 3D). We failed to detect TG antigen in two patients (A2 and A4) with hypothyroidism in the autoimmune group (Figure 3A). Ultrastructurally, subepithelial deposits were either heterogeneous (multistage) or homogeneous (synchrony) in all groups (Figure 2). Although relatively more patients in the autoimmune group (78%) showed multistage deposits, there was no statistical difference between groups. Neither homogeneous nor heterogeneous deposition was...
significantly associated with the clinical remission of proteinuria ($P = 0.5$). In addition, extramembranous (subendothelial and/or mesangial) deposits were more frequently found in patients of the autoimmune group as compared with those in the non-autoimmune group ($P = 0.04$, Figure 5). Immunofluorescent analyses showed the presence of complement C1q in all cases of the autoimmune group (Figure 5). Conversely, significantly lower frequencies of C1q deposition were observed in hepatitis ($P = 0.027$), HP ($P = 0.011$) and undefined groups ($P = 0.001$) than in the autoimmune group.

Clinically, the serum titres of anti-dsDNA or ANA were generally negative or marginal in non-autoimmune groups (Figure 4). Although the autoimmune patients were more likely to have positive or marginal ANA than other groups of patients ($89\%$ versus $22\%$, $P = 0.047$), only one patient (A7) had anti-dsDNA in positive range (Figure 4). The clinical screening for other serum markers had disclosed that the majority of patients in the autoimmune group tended to have one or more of the aforementioned antibodies (Figure 4). Patients in autoimmune and hepatitis groups were younger than in HP and undefined groups ($37 \pm 14$ and $39 \pm 11$ versus $67 \pm 11$ and $57 \pm 15$, respectively; ANOVA, $P < 0.05$). The patients in combined autoimmune ($56\%$) and hepatitis groups ($60\%$) had higher incidence of hypocomplementemia than those in combined HP ($0\%$) and undefined groups ($33\%$) ($P = 0.027$). As for the clinical outcome of proteinuria, the remission rate (PR + CR) among treated patients was $53\%$ overall, but differed significantly between the HP group and the hepatitis or autoimmune group (Table 1). However, Fisher’s tests failed to show a meaningful difference in remission rate between the undefined group and the hepatitis or autoimmune group despite that the undefined group had higher remission rate ($67\%$ versus $25\%$ and $21\%$, respectively, Table 1).

**Discussion**

Pathologically, TRI is very much like a footprint of INF action on target cells. In our study, the common finding
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Fig. 3. Representative immunohistochemical stains of glomerular (A, B, and C) and gastric mucosal tissues (D). (A) Anti-thyroglobulin of patient A2, (B) Anti-HBsAg of patient HB3, (C and D) Anti-\textit{H. pylori} of patient HP6. Bar: 50 \(\mu\)m.

Table 1. The outcome of proteinuria of treated patients

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>NR % (n)</th>
<th>PR % (n)</th>
<th>CR % (n)</th>
<th>(F_{(HP)}) (RP + CR versus NR)</th>
<th>(F_{(UD)}) (RP + CR versus NR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune</td>
<td>71 (5)</td>
<td>0 (0)</td>
<td>21 (2)</td>
<td>0.028/24.2</td>
<td>0.169/–</td>
</tr>
<tr>
<td>Diabetes</td>
<td>100 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>75 (3)</td>
<td>25 (1)</td>
<td>0 (0)</td>
<td>0.048/25.7</td>
<td>0.262/–</td>
</tr>
<tr>
<td>HP</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>100 (5)</td>
<td>–</td>
<td>0.261/–</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>50 (1)</td>
<td>50 (1)</td>
<td>0 (0)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Undefined</td>
<td>33 (4)</td>
<td>25 (3)</td>
<td>42 (5)</td>
<td>0.261/–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>47 (15)</td>
<td>16 (5)</td>
<td>37 (12)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NR: non-remission of proteinuria; PR: partial remission; CR: complete remission.

\(F_{(HP)}\): Fisher’s test, HP versus autoimmune/hepatitis/undefined respectively.

\(F_{(UD)}\): Fisher’s test, undefined versus autoimmune/hepatitis/HP respectively.

\(n\): the number of patients in each group.

of TRIs in the fraction of MN suggests direct or indirect INF interactions with GECs. Under normal physiological conditions, IFN genes are generally silent in cells [13]. Production of IFNs requires stimulation by viruses, microbial products or specific chemicals via triggering the signalling systems linked to toll-like receptors (TLRs) [13,14]. In early innate immune response to pathogens, tissue-associated and circulating dendritic cells, specifically the plasmacytoid dendritic cell (pDC), produce type I IFN [13]. On the other hand, IFN-\(\gamma\) is produced by NK cells, or by activated T cells during the development of an adaptive immune response [13]. In SLE and other autoimmune diseases, uncontrolled production of type I INF by pDC plays a pivot role in the pathogenesis of these diseases [15]. Other organ specific autoimmune diseases such as thyroiditis, Sjogren’s syndrome, diabetes, psoriasis and autoimmune dermatitis have also been described to express IFN-\(\alpha/\beta\) and to have pDC infiltration in the lesion sites [15]. In addition to the circulating INF, local activation of INF may also play a role in the pathogenesis of viral nephropathy [16]. The viral RNA alone or as a part of immunocomplex may activate TLR3 in glomerular cells, boost local innate immunity and produce proinflammatory cytokines, chemokines and INF [17]. The deposition of IgG1 and IgG2 in addition to IgG4 in paraneoplastic MN might activate Th1-related immunity and enhance local production of INF-\(\gamma\) in glomeruli [18,19]. Furthermore, INF-\(\gamma\) is able to modulate the expression of HLA-DR and cell-adhesion molecules in GECs [20,21]. However, we still cannot determine whether the INF-\(\gamma\) is able to induce TRI formation since the past experimental
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Fig. 4. Clinical profiles of individual patients in different groups. Additional positive serum markers of individual patients are labelled beside the dsDNA bars. ‘T’ column represents regimens for specific treatment: (S) steroid, (C) cyclosporine, (A) cyclophosphamide, (M) mycophenolate mofetil, (Z) azathioprine, (Q) hydroxychloroquine, (V) anti-viral drugs lamivudine, entecavir, or IFN-α. ‘F’ column represents a follow-up period in month. Patient groups: UD1–UD12 (no identifiable underlying aetiology); T1–T2 (non-Hodgkin lymphoma); HB1–HB4 and HC1 (viral hepatitis); HP1–HP6 (positive for H. pylori antigen on glomerular capillary walls); D1–D2 (diabetes); A1–A9 (autoimmune or autoimmune-like diseases).

An interesting finding in our study was the detection of H. pylori antigen in glomeruli of six patients (HP1–HP6). However, it remained obscure whether the anti-H. pylori antibody tested in our study indeed detected mimic antigens or, alternatively, in situ implanted antigens or deposited circulating immunocomplex. Recently, many extragastric manifestations of H. pylori infection have been reported, which included idiopathic thrombocytopenic purpura, sideropenic anaemia, Sjögren syndrome, hypothyroidism, Schönlein-Henoch purpura, chronic urticaria, rosacea, asthma, bronchiectasis, acute immune polyneuropathy and atherosclerosis [22,23]. There is more evidence to suggest that molecular mimicry, resulting in autoantibodies following H. pylori infection, may play an important role in extragastric diseases [24,25]. Several Japanese studies also proposed the pathogenic association of H. pylori infection with MN [26–28]. They not only found a higher prevalence of H. pylori infection in Japanese patients with MN but also detected H. pylori antigen in the glomeruli of patients with MN. Remission of proteinuria following eradication of H. pylori infection in some patients further addressed the pathogenic role of H. pylori infection in MN [27,28]. Although the question remains whether the H. pylori antigen immunohistochemically detected in MN is the true culprit or just an epiphenomenon, our finding of glomerular TRIs in the patients of the HP group implies the potential connection of INF-associated tissue response with H. pylori infection.

As for the undefined group, the patients showed an apparent idiopathic MN at presentation, apart from ultrastructural demonstration of TRIs in glomerular endothelial cells. Since previous reports have shown that lupus nephritis could manifest as a sole disease that antedated other clinical and immunological features of the diseases by years [29–31], a long-term follow-up and more meticulous search for its underlying aetiological factors are required to validate the presumptive nature of secondary MN in our patients. On the other hand, the underlying diseases of the patients in the autoimmune group and perhaps some in undefined or other groups are very similar to the so-called...
undifferentiated connective tissue disease (UCTD) [32]. However, many reports have shown that a few patients with UCTD have major organ involvement, and the majority will remain in undifferentiated stable status over time [32]. In contrast, the dominant manifestation of MN and the presence of additional autoantibodies in some of our patients imply a different biological nature for their diseases, and maybe predict a tendency to evolve in them.

Most of our patients have been treated, in addition to symptomatic remedies, as idiopathic MN because their underlying diseases were not easy to be recognized at presentation. The different remission rate of proteinuria among groups might come from the different aetiological nature of MN, or from the individualized therapeutic arrangement for each patient. However, if secondary nature of MN could be verified before planning a therapeutic modality, more optimal results and fewer complications might have been achieved.

In summary, the cure of secondary MN can be dramatic if its underlying cause can be eradicated. When reviewing renal biopsies, any unusual pathological findings that might point to a diagnosis of secondary MN are thus essential in terms of therapeutic consideration. In this report, we address the clinicopathological profiles of 36 patients with implication of secondary MN by ultrastructural finding of TRI in glomerular endothelial cells. However, further investigation is required to understand pathophysiology of INF–GEC interactions, which might reveal clues of glomerular injury in secondary MN.

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Conflict of interest statement. None declared.

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