



The Landscape and Prognosis Potential of the T-Cell Repertoire in Membranous Nephropathy

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Membranous nephropathy (MN), a common pathological type of adult nephrotic syndrome, is an antibody-mediated kidney disease. It is widely accepted now that MN is an immune-related disease that involves the whole immune system. In this study, we analyzed the T-cell receptor beta chain (TCR β) repertoire of the circulating T lymphocytes of MN patients and healthy controls using high-throughput sequencing. We compared multiple aspects of the TCR β repertoire, including diversity and the V β and J β genes between MN patients and healthy controls, and we found that the diversities within the VJ cassette combination in the peripheral blood of MN patients were lower than in the healthy controls. We also found the TCR β repertoire similarity between pre- and post-therapy could reflect the clinical outcome, and two V β genes in pre-therapy had the potential to predict the therapeutic effect. These findings indicated the potential of the TCR β repertoire as non-invasive biomarkers for the prognosis prediction of MN. The characteristics of circulating T-lymphocyte repertoires shed light on MN detection, treatment, and surveillance.

Keywords: T-cell receptor repertoire, membranous nephropathy, clinical response, high-throughput sequencing, non-invasive biomarkers

INTRODUCTION

Membranous nephropathy (MN), a common pathological type of adult nephrotic syndrome, is an antibody-mediated kidney disease clinically characterized by proteinuria (1). The typical pathological characteristics are the deposition of immune complexes on the subepithelial side of the glomerular basement membrane (GBM), resulting in diffuse thickening of GBM (2, 3). Recently, a large multicenter retrospective study, which investigated 71,151 cases of renal puncture in China from 2004 to 2014, found that the proportion of MN was 23.4%, only lower than IgA nephropathy, and was growing rapidly, and this was associated with long-term exposure to air pollution (4).

MN was regarded as an organ-specific autoimmune disease in which IgG autoantibodies form subepithelial immune complexes with autoantigens expressed on podocyte cell surfaces (2, 3). The autoantibodies targeting phospholipase A2 receptor (PLA2R) and thrombospondin type-1 domain-containing 7A (THSD7A) were found in some MN patients (5, 6). It is thus widely accepted that MN is an immune-related disease with the involvement of the whole immune system.

The diagnosis of MN relied on the histopathology of renal biopsy, which was examined by light microscopy, immunofluorescence, and electron microscopy (7). It was invasive and could lead to

infection and potential damage to the kidney. Performing it repeatedly during and after treatment to monitor the disease progress was consequently not practicable. In recent years, many novel biomarkers have been developed in plasma or urine. Of all them, the most commonly used was APLA2R (8). Although APLA2R showed some potential, there are still considerable false positives and false negatives.

At present, studies of MN have focused on the B-cell immunity, and rituximab is currently the most widely used anti-CD20 monoclonal antibody. Some studies also found that there was a correlation between MN and T cells. Previous studies have shown that there has been an increase in the CD4/CD8 subset ratio of some patients with MN with or without nephrotic syndrome (9–11), and a recent study reported that the evaluation of Tregs in patients with severe idiopathic MN could predict an early response to rituximab (12). These studies indicated that the pathogenesis of MN was also closely related to T cells.

Recently, using T-cell receptor repertoire high-throughput sequencing (TCR-HTS), a series of studies, including our own, have characterized the signatures of T-cell repertoires and revealed their diagnostic and prognostic implications in various types of immune-mediated diseases, including infectious diseases (13, 14), autoimmune diseases (15–17), and tumors (18–20). The TCR repertoire could reflect the status of T-cell immunity in MN, and investigating the diversity of T lymphocytes by TCR-HTS could help understand the pathogenesis of MN.

In this study, we analyzed the T-cell receptor beta chain (TCR β) repertoire of the circulating T lymphocytes of MN patients (pre-therapy and post-therapy) and healthy controls using TCR-HTS. We compared multiple aspects of the TCR β repertoire, including diversity and V β and J β genes between MN patients and healthy controls, and we found that the diversities of VJ cassette combination in peripheral blood of MN patients were lower than healthy controls. We also found the TCR β repertoire similarity between pre- and post-therapy could reflect the clinical outcome and two V β genes in pre-therapy also had the potential to predict the therapeutic effect.

MATERIALS AND METHODS

Sample Collection

This study was approved by the ethics committee of the Affiliated Foshan Hospital of Sun Yat-Sen University. Twenty patients with membranous nephropathy were recruited in this study from 2016 to 2018. Clinical characteristics of the patients are showed in **Table 1** and **Table S1**. The inclusion criteria were MN diagnosis established by renal biopsy. The renal biopsy specimens of all the patients were examined by light microscopy, immunofluorescence, and electron microscopy. Most of the patients accepted the therapy with prednisone (1 mg/kg body weight) or tacrolimus (0.05 mg/kg body weight, two patients with contraindication to prednisone) once per day for about 6 months. But one of the patients rejected the treatment because of insurance issues, and they were thus excluded. Peripheral blood samples were collected before therapy initiation and after about 6 months of treatment for all the patients. The laboratory assessment included APLA2R status,

TABLE 1 | Summary of clinical and laboratory information of the recruited people.

Variable		MN patients	Healthy control	P-value
Gender (Female/male)		7/12	7/12	1.0
Age (years)	Median (range)	48 (16-67)	47 (23-68)	0.93
24 h UP (g)		5.1 (1.7–15.9)	/	/
Serum creatinine (μ mol/l)		75 (49–123)	/	/
Serum urea nitrogen (mmol/l)		5.5 (3.4–9.7)	/	/
eGFR (ml/min/1.73 m ²)		73.8 (52.2–108.5)	/	/
APLA2R (Ru/ml)		109.7 (1.19–1500.00)	/	/
Serum albumin(g/l)		24.7 (15.7–35.3)	/	/

serum albumin, proteinuria, 24-h urine protein (24 hUP), urea nitrogen, creatinine, and eGFR levels, and these were collected at the time of renal biopsy and at 6-months follow-up. We evaluated the therapeutic effects of the patients according to KIDNEY DISEASE IMPROVING GLOBAL OUTCOMES (KDIGO) (21, 22). We also recruited 19 healthy volunteers without a history of cancer, autoimmune disorder, or surgery as a control, and blood samples were drawn from each healthy volunteer. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh anticoagulant peripheral blood by density gradient centrifugation, lysed with TRIzol[®] reagent (Life, US), and frozen at -80°C until further processing.

High-Throughput Sequencing of TCR β Chains

Total RNA was extracted from PBMC lysates using a total RNA Kit (OMEGA Bio-tek, US) according to the manufacturer's instructions. For every sample, 1 μ g of total RNA was reverse transcribed into 5' RACE-ready cDNA using a SMARTer PCR cDNA synthesis kit (Clontech, US). In order to amplify the completely arranged TCR β fragments, the first-strand cDNA was used as templates for a 5'RACE-PCR with forward universal primer and reverse primer specific to the TCR β constant (C) region (5'-AACACSTTKTTCAGGTCCT-3'). The PCR reaction conditions were the same as in our previous paper: 94 $^{\circ}\text{C}$ for 3 min, carried out with 35 cycles of denaturing at 94 $^{\circ}\text{C}$ for 15 s, annealing at 58 $^{\circ}\text{C}$ for 30 s, extension at 72 $^{\circ}\text{C}$ for 45 s, and a final extension at 72 $^{\circ}\text{C}$ for 10 min. The PCR products were purified by 2% agarose gel electrophoresis and the gel extraction kit (QIAGEN, German). The Illumina Hiseq sequence adaptors were ligated to construct sequencing libraries, which were then sequenced on an Illumina Hiseq2500 platform. The raw data of the sequencing will be available on request.

Bioinformatics Analysis of TCR β Repertoire Data

The sequencing data were stored in a FASTQ format. Firstly, the low-quality sequences were filtered according to four strict criteria: (1) contaminated by the adapter sequence; (2) had

more than 5% uncalled bases (N); (3) had an average Phred-type Q-score <15; and (4) had PE reads with low-quality base readings (Q-score <10) at the ends of reads. Secondly, the high-quality sequences were aligned with the TCR β reference genes by BLAT (-stepSize=5 -minIdentity = 0 -minScore = 0) (23). The TCR reference gene sequences were downloaded from the IMGT/GENE database (24). If V, J, and C genes in a given sequence were all identified, we further translated them into an amino acid (aa) sequence. The aa sequences without a terminator were selected as the productive TCR sequences. Lastly, at the V-D-J junctions, the sequences that started with cysteine and ended with the FGXG motif [C...FGXG] were defined as CDR3 (23).

Diversity Indices

We selected the Shannon entropy as our fundamental measure of the diversity; it has been widely used in previous TCR repertoire studies for its inclusion of both richness (number of members) and diversity (evenness of distribution) and its applicability to more complex models of repertoire size (25–27). The formula of the Shannon entropy index is as follows

$$\text{Shannon entropy} = - \sum_i \left(\frac{n_i}{N} \right) \log_2 \left(\frac{n_i}{N} \right)$$

where i is an index that is chosen between 1 and the number of species s , n_i is the number of sequencing reads in species i , and N is the total number of reads.

Similarity Between Two TCR β Repertoires

To evaluate changes in the TCR repertoire after therapy, we calculated the Morisita-Horn similarity index (MH) of two different TCR repertoires. The index between two TCR repertoires was calculated based on the number of shared sequences between the two samples and the contribution of the shared sequences to each repertoire, and the index ranged from 0 to 1 (28, 29). The formula is as follows

$$MH = \frac{2 \sum_{i=1}^s x_i y_i}{\left(\frac{\sum_{i=1}^s x_i^2}{X^2} + \frac{\sum_{i=1}^s y_i^2}{Y^2} \right) XY}$$

where x_i is the number of times clonotype i is represented in the total X sequences from one sample, y_i is the number of times clonotype i is represented in the total Y sequences from another sample, and S is the number of unique clonotypes.

Statistical Analysis

GraphPad Prism version 5.1 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) were used for statistical analysis of the data. Comparisons between groups were conducted using the Mann-Whitney U test or the Wilcoxon signed ranks test if appropriate, and $P < 0.05$ were considered statistically significant. The receiver operating characteristic (ROC) curve was used to illustrate the diagnostic ability of a binary classifier system. The area under the curve (AUC) was calculated by the Hanley and McNeil method.

RESULTS

Clinical and Pathological Characteristics of the Patients

The demographic and clinical characteristics data of the MN patients is summarized in **Table 1**. For MN patients, 12 of them were male and seven were female. The average age of the patients was 48 years old. There were no significant differences in age ($P = 0.93$) or gender ($P = 1.0$) between the MN patients and healthy controls (**Figure S1**). The median 24-h proteinuria was 5.1 g for patients with MN and varied largely among patients (1.7 g/24 h–15.9 g/24 h). The serum creatinine, serum urea nitrogen, serum albumin, eGFR, and APLA2R also varied extensively among MN patients, with a median of 75 $\mu\text{mol/l}$, 5.5 mmol/l, 24.7 g/l, 73.8 ml/min/1.73m², and 109.7 Ru/ml, respectively.

Profiling of the TCR β Sequencing Data

A total of 83,414,134 productive amino acid sequences were obtained from 57 blood samples of the patients and healthy control with an average of 1,463,406 productive sequences generated per sample. The average number of productive unique sequences per sample was 96,934. All the 65 distinct V β and 13 distinct J β segments were identified, and the usage frequencies of these segments were analyzed in each sample, which are listed in **Table S2**. The five most frequent genes detected in almost all the samples were V β 5.1, V β 20.1, V β 12.4, V β 7.9, and V β 29.1 as well as J β 1.1, J β 2.1, J β 2.7, J β 1.2, and J β 2.3.

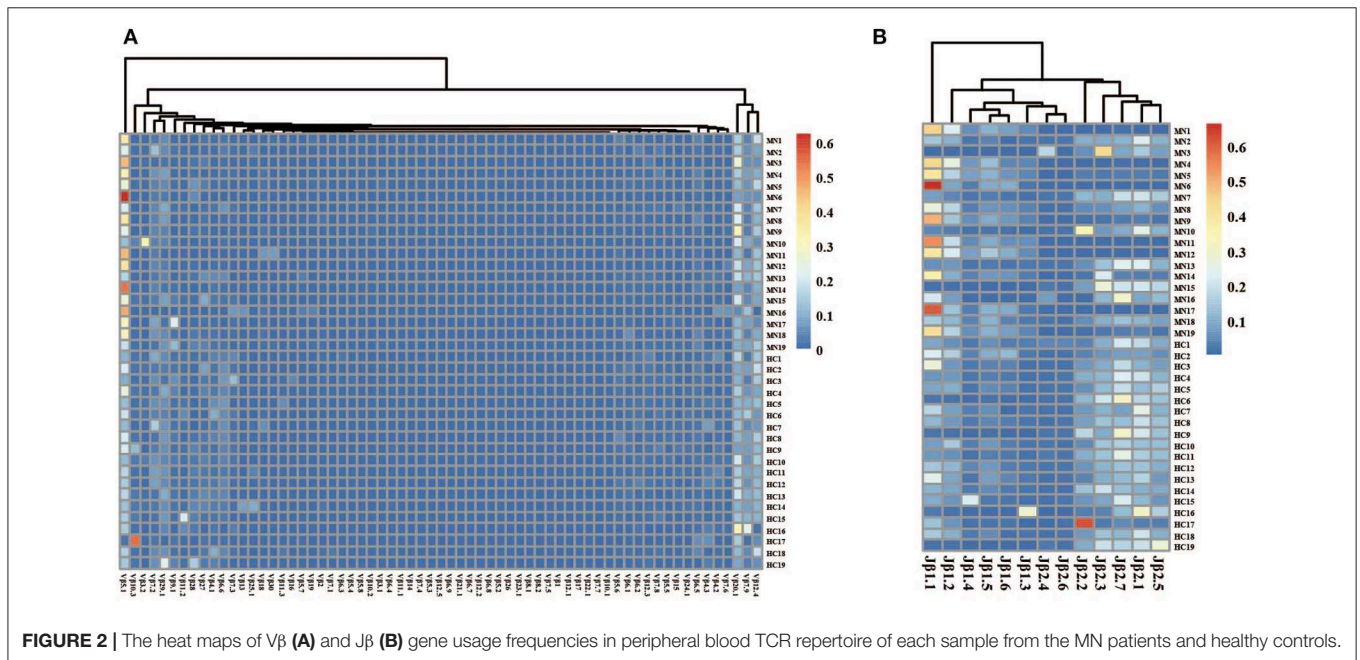
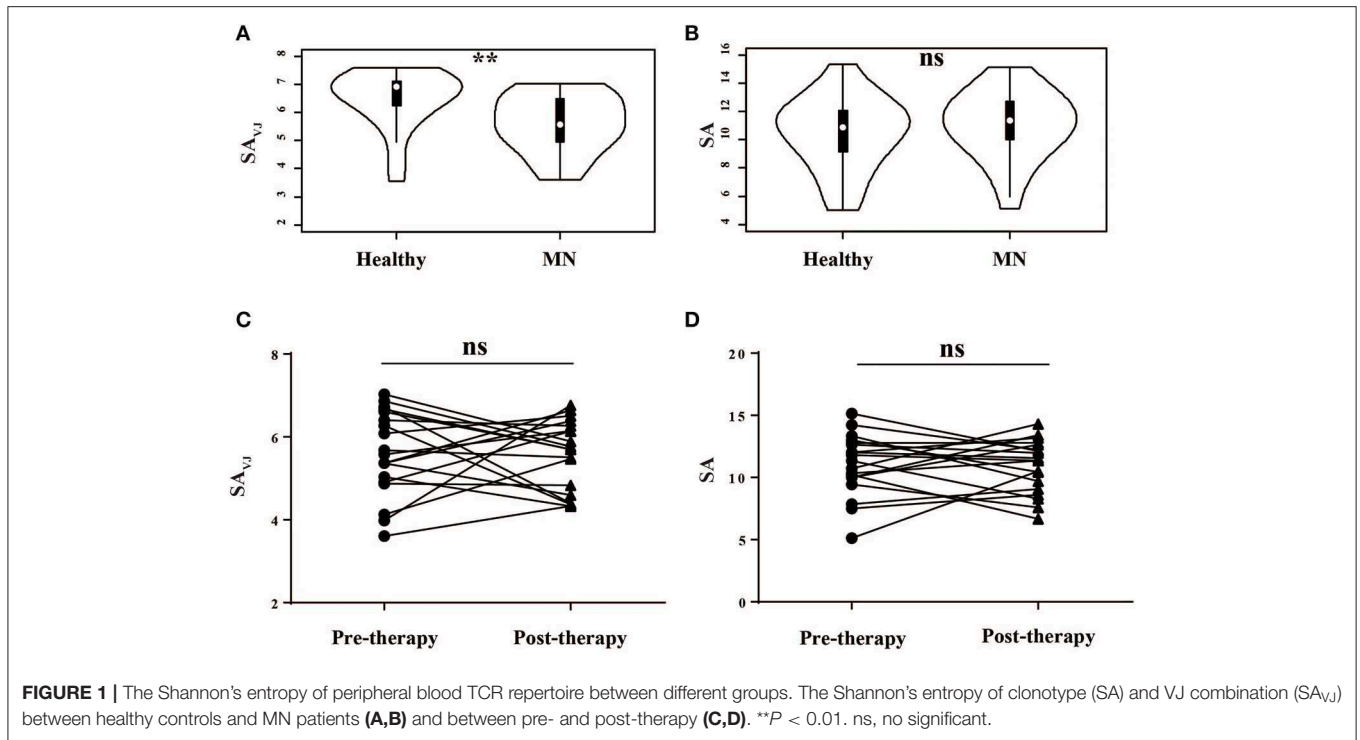
Lower Diversity of VJ Cassette Combination in Peripheral Blood of MN Patients

As a measure of diversity, we calculated the Shannon entropy—a function of the number of unique elements in the population and their frequencies—for VJ cassette combinations (SA_{VJ}) and clonotypes (SA) (27). We firstly compared the diversity between MN patients and healthy controls, and the VJ combination diversity in MN patients was significantly lower than that in healthy controls ($P = 0.003$, **Figure 1A**), which indicated antigenic stimulation and expansion of some specific VJ combinations. No significant difference was, however, observed by clonotype diversity ($P = 0.448$, **Figure 1B**), which might be due to the increased random insertions and deletions independent of VJ combinations in MN patients.

We then analyzed the correlation between the diversity and clinical characteristics (**Figure S2**), and we compared the diversity between pre-therapy and post-therapy (**Figures 1C,D**)—no significant difference was observed. Moreover, we did not observe the relationship between diversity change and clinical response.

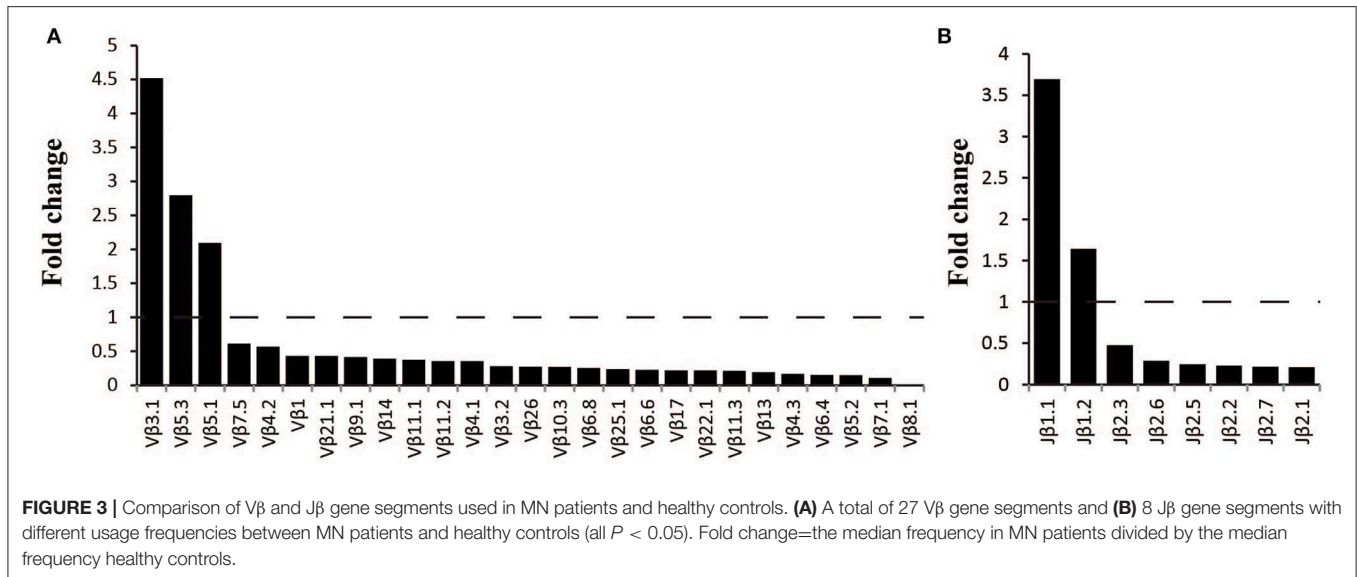
The Different Usage Patterns of V β , J β Genes and CDR3 Motifs in MN Patients

The frequency of the TCR V β and J β genes in MN patients and healthy controls were delineated in heat maps (**Figures 2A,B**). Consistent with previous reports of other researchers (30–32), some V β genes exhibited high-frequency usage in almost all of the samples, such as V β 5.1 and V β 20.1. Comparing the V β



and $J\beta$ gene usage between two groups, significant differences were observed in almost half of the $V\beta$ and $J\beta$ genes (27/65 $V\beta$ gene and 8/13 $J\beta$ genes, **Figures 3A,B**), which indicated that the usage patterns of $V\beta$ and $J\beta$ genes were skewed in MN patients. These changes may reflect an altered immune status associated with MN. We also compared the $V\beta$ and $J\beta$ genes between the patients with positive anti-PLA2R antibodies and those

with negative antibodies and found that six $V\beta$ genes ($V\beta 4.2$, $V\beta 6.4$, $V\beta 7.3$, $V\beta 7.5$, $V\beta 13$, and $V\beta 24.1$) and three $J\beta$ genes ($J\beta 1.6$, $J\beta 2.3$, and $J\beta 2.7$) had significant differences ($P < 0.05$). Moreover, we found that $V\beta 10.2$ decreased significantly after therapy in the patients with anti-PLA2R antibodies turning negative. Given that TCRs with similar CDR3 sequences might recognize the same antigenic peptides, we identified CDR3



motifs based on similarities in their CDR3 aa sequences using the CD-HIT program (90% quantile). Two CDR3 motifs were associated with MN with the most significance (Chi-square test, $P < 0.0001$, **Figure 4**), which might be valuable in immunotherapy.

The Usage Patterns of V β Genes Are Associated With Clinical Response

In our cohort, the patients were divided into two groups—complete remission (CR) and non-CR—according to KDIGO after 6 months of treatment. We compared the V β and J β gene usage in pre-therapy blood between the two groups, and we found that the frequency of V β 4.1 and V β 13 were significant higher in the CR group who had good clinical response, and these two V β genes had the potential to predict the therapeutic effect (**Figures 5A,B**). The areas under the curve (AUC) of V β 4.1 and V β 13 were 0.82 and 0.80, respectively (**Figures 5C,D**). Moreover, we also found that the frequency of V β 4.1 and V β 13 were significant higher in healthy control than MN patients (**Figure 3A**), which indicated their potential value in diagnosis and prognosis.

Dramatic Changes in TCR Repertoire After Treatment Are Associated With Good Outcomes

For patients, the TCR repertoire would change after treatment. We calculated the Morisita-Horn similarity index between pre-therapy and post-therapy TCR β repertoires for every patient. Interestingly, the indexes in the CR group were significant lower (**Figure 6**, $P < 0.01$, AUC = 0.88). This result indicated that dramatic changes in the TCR repertoire after treatment were associated with good outcomes, which might be due to remission and a change of autoantigens.

DISCUSSION

In this study, we performed TCR β repertoire HTS of the PBMC of MN patients in pre- and post- therapy stages, as well as of PBMC of healthy controls, to investigate the relevance of T-cell immunity and the clinical characteristics of MN. Many studies indicated aberration in the usage of the TCR β V gene and J gene in autoimmune diseases and cancers (15–20). In this study, we found a similar situation in MN patients. The usage pattern of V β and J β genes was skewed significantly in MN patients, and the diversity of the VJ cassette combination was narrowed, which reflected an altered immune status associated with MN.

As we know, the usage of prednisone and tacrolimus in MN patients has many bad side effects, such as infection, centripetal obesity, osteoporosis, and so on. The prediction of treatment effects before and during therapy could thus prevent the overuse of drugs and minimize the harm to patients. In fact, some studies have reported several predictors of responses to drugs in MN, such as baseline proteinuria, the reduction of proteinuria at month 3, a lower percentage of Tregs at baseline, and an increased percentage of Tregs at day 8 (12, 33). In this study, we found that the TCR β repertoire could predict the response to treatments, which had not been reported in MN. Two V genes were found to be the candidate biomarkers—V β 4.1 and V β 13. High-usage frequencies of them were associated with good outcomes, and their usage frequencies were significant higher in the healthy controls. These results suggested that the T cells in these two TCR subfamilies might be the antigen-specific Tregs that help to counter T-cell-mediated immune responses. Alternatively, they also might mark the effector T cells with altered recognition of an undefined autoantigen, which might skew or attenuate the immune response. There is no evidence for these hypotheses, and further studies are thus needed. We also compared the other clinical characteristics (24 hUP, Serum albumin, APLA2R, eGFR, Serum Creatinine, and Serum Urea Nitrogen) between CR and non-CR patients, and there were

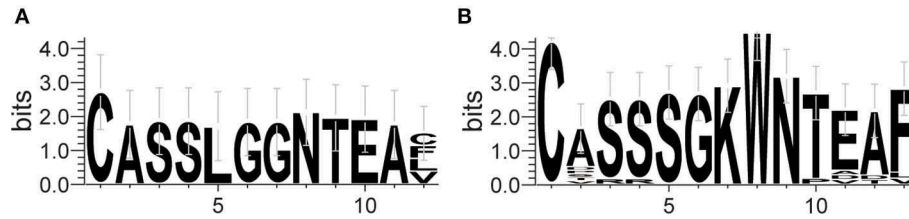


FIGURE 4 | Sequence logo figures of the CDR3 motifs associated with MN. Each logo consists of stacks of symbols, with one stack for each amino acid in the sequence. The overall height of the stack indicates the degree of sequence conservation at that position, while the height of the symbols within the stack indicates the relative frequency of each amino acid at that position. The width of the stack is proportional to the fraction of valid symbols at that position.

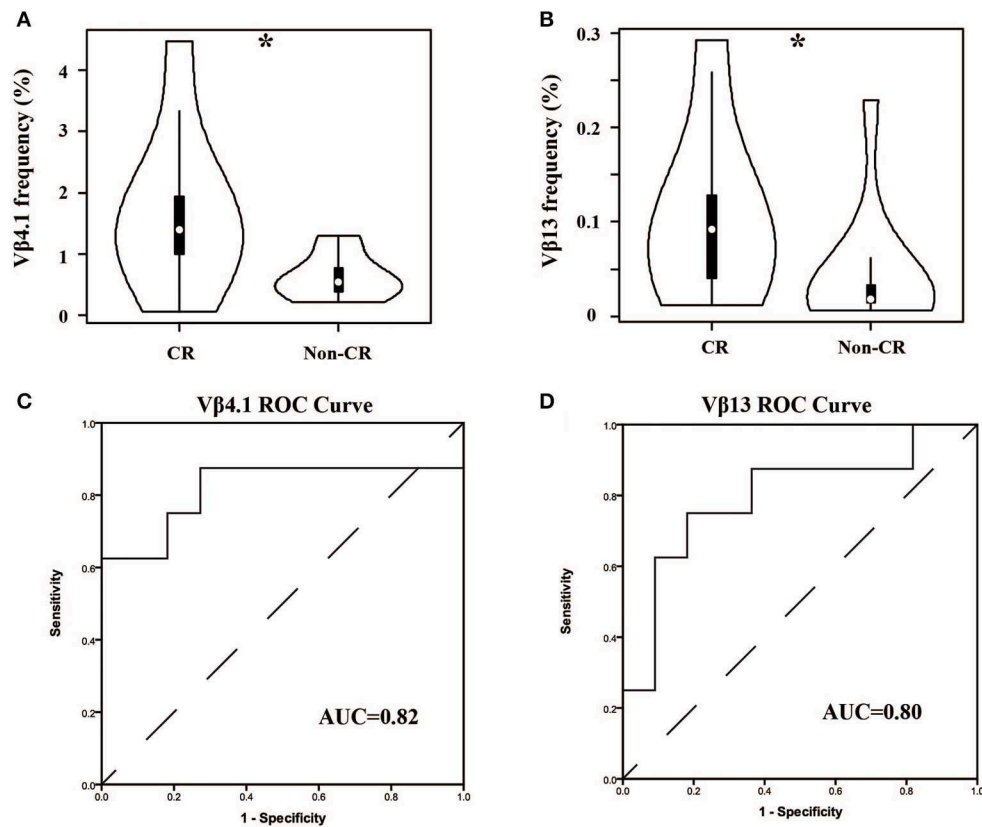
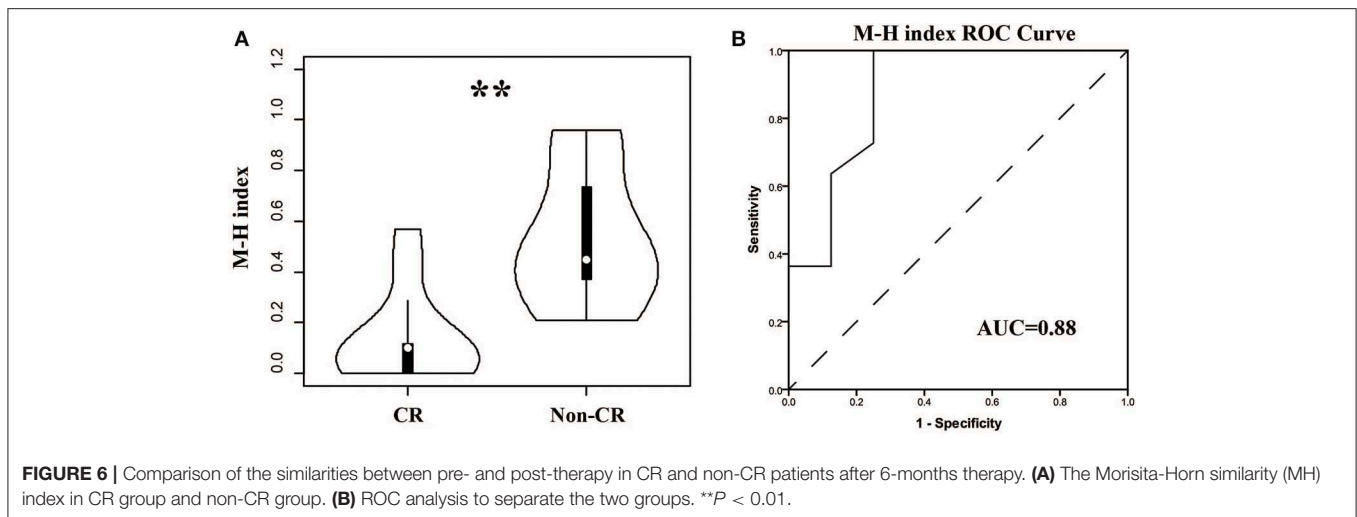


FIGURE 5 | Comparison of V β 4.1 and V β 13 gene segments used in patients with or without urinary protein after 6-months therapy. **(A,B)** The usage frequencies of V β 4.1 and V β 13 gene segments in CR group and non-CR group. **(C,D)** ROC analysis for the frequencies of V β 4.1 and V β 13 gene segments in MN patients to separate the two groups. * $P < 0.05$.

no significant differences. It indicated that, in our study, these characteristics could reflect the status of MN, but their prediction potential might be weaker than the TCR β repertoire. Moreover, limited by the number of samples in our study, validation of the performance of those biomarkers and demonstrating the accuracy and reproducibility of this method by more studies from other laboratories is needed.

In this study, two patients were treated with tacrolimus because they had the contraindication to prednisone, and they

did not go into complete remission. If we excluded these two patients, the V β 4.1 and V β 13 also had the potential to predict the therapeutic effect (AUC = 0.81 and 0.76). It is very interesting to determine whether patients receiving tacrolimus have similar TCRV β usage compared to CR and non-CR in patients receiving prednisone. But the number of the patients receiving tacrolimus is much lower than prednisone in the first 6 months. We will extend the follow-up period in our further study to further explore the answer of this question.



In theory, MN is an immune-related disease. If the treatments were effective, the immune status of the body will be different from the pre-treatment, and the TCR β repertoire will also change significantly; this has been reported in various types of immune-mediated diseases (19, 34, 35). We therefore calculated the similarity of the TCR β repertoire between pre- and post-therapy in each patient. As expected, the similarity was significantly lower in patients with good outcomes, which indicated that the autoimmune antigen had been inhibited or cleared. However, peripheral blood samples were only collected at 6 months after treatment, and it was impossible to judge whether the change of TCR β repertoire could reflect the treatment effects earlier than the current biomarkers in use. We plan to focus on it in our future studies.

We separated responders from non-responders in the analyses shown by **Figures 1C,D** and found no significant correlation between the Shannon entropy index and therapeutic outcome. The Shannon entropy index and the Morisita-Horn similarity index reflect different dimensionalities of the TCR repertoire. The Morisita-Horn similarity index reflects the overlap between two TCR repertoire, and the Shannon entropy index reflects the TCR diversity. In our results, the TCR diversity did not change significantly after therapy, but the overlap between pre- and post-therapy was low, which indicates that, in the responders group, the old T cell clonotypes in the pre-therapy samples disappeared or were exhausted, and many new clonotypes emerged in the post-therapy samples.

A combination of steroids and cytotoxic agents are used as first-line therapy according to KDIGO. But some studies in patients from East Asia showed that the steroid alone was also useful. One study from Japan reported that the steroid alone seems to be useful in Japanese patients, and a remission from heavy proteinuria likely resulted in a favorable outcome ($N = 949$) (36). Another study found that there was a similar treatment response to the steroid alone or in a combination of steroids and cytotoxic agents in Chinese patients, which was contrary to reports in

Caucasians (37). Moreover, a study in Korean patients showed that 5-years CR rates of C (corticosteroids alone) and C+C (corticosteroids plus cyclosporine) were 88.5 and 86.2%, respectively. Ten-year event-free rates in these groups were 91.7 and 79.9%, respectively, and they concluded that stepwise treatment using corticosteroids alone and in combination with cyclosporine is warranted in these patients (38).

In summary, we have presented here a comprehensive landscape of the T-cell repertoire in MN, including the restricted VJ repertoire size and skewed usage patterns of V β and J β genes. The most important discovery was that the TCR β repertoire similarity between pre- and post-therapy could reflect the clinical outcome, and two V β genes in pre-therapy were associated with a therapeutic effect. These findings demonstrated the potential of the TCR β repertoire as alternative non-invasive biomarkers for the prognosis prediction of MN. However, the independent validation cohort study was still needed.

DATA AVAILABILITY STATEMENT

The data of this article will be available upon request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the Affiliated Foshan Hospital of Sun Yat-Sen University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YZ, HL, CX, XL, PY, and YK provided patients samples and clinical information. YJ, ZG, ZS, XC, YP, and LZ performed the

experiments. YZ and YJ performed the statistical analysis. YZ, YJ, and WL analyzed and interpreted the data. YK and WL designed and supervised the study. YJ and YZ wrote the manuscript. WL carried out an extensive revision. All authors participated in the writing and approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00387/full#supplementary-material>

Figure S1 | Comparison of the age (A) and gender (B) between healthy controls and MN patients.

Figure S2 | No significant correlation between the diversity and clinical characteristics. ns, no significant.

Table S1 | Clinical and sequencing information of the patients.

Table S2 | The V and J genes usage in the blood samples.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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