Repertoire analysis of memory T-cell receptors in Japanese patients with inflammatory bowel disease

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Abstract

Background and Aim: The T-cell receptor (TCR) repertoire was assessed in response to various antigens and was considered to be associated with the pathogenesis of inflammatory bowel disease (IBD). Thus, we performed TCR repertoire analysis to examine the pathology of IBD from changes in the TCR repertoire of memory T cells in the intestinal lamina propria mononuclear cells (LPMCs) and peripheral blood mononuclear cells (PBMCs) of patients with IBD.

Methods: LPMCs in the surgical specimens and PBMCs were isolated from 12 patients with IBD (5 patients with ulcerative colitis [UC] and 7 patients with Crohn’s disease [CD]). PBMCs were collected from 10 healthy individuals as controls. Comprehensive TCR sequence analyses of adaptor-ligation polymerase chain reaction (PCR) products were performed using MiSeq.

Results: The diversity of TCR-α and TCR-β in PBMCs was significantly lower in patients with IBD than that in controls (P = 0.00084 and 0.0013, respectively). Comparisons of TCR diversity in LPMCs and PBMCs between CD and UC showed that the diversity in LPMC was not affected by diseases, whereas that in PBMCs was significantly lower in CD than in UC (P = 0.045 and 0.049, respectively). Some TCR clones may have shown a specific increase or decrease in CD and UC, and many clones were common to both LPMCs and PBMCs in the same patients.

Conclusion: The diversity of TCR clones in LPMCs and PBMCs in patients with IBD was significantly lower than that of PBMCs in controls. TCR diversity in PBMCs was particularly low in patients with CD.

Introduction

Inflammatory bowel disease (IBD) includes Crohn’s disease (CD) and ulcerative colitis (UC). Its various symptoms and complications are a result of chronic inflammation in the gastrointestinal tract. Genetic and environmental factors have been reported to be involved in the onset of this disease. To date, over 200 genomic regions contributing to disease susceptibility have been identified as genetic factors.1–3 In addition, environmental factors, such as intestinal bacterial flora and dietary antigens, have attracted attention. Regardless of the strength of the genetic factors, enteritis does not develop in genetically modified enteritis model mice in a sterilized environment; environmental factors, especially intestinal microbiota, are considered important in the pathogenesis of IBD.4,5

On the other hand, there have been studies on remission induction treatment of CD that resets the immune system using bone marrow transplantation.6 Clinical trials on the effects of these studies are being conducted, and the treatment is effective not only with stem cells with different genetic background, but with autologous stem cells.7 This suggests that the acquired changes of host immune system for intestinal flora play an important role in disease onset of IBD. Memory T cells in intestine play an important role in the process by which such an immune system is selected by the intestinal environment. Lymphocytes, such as memory T cells, express the T-cell receptor (TCR) (i.e. a receptor molecule that recognizes antigens), recognize and bind to antigens (digested peptide antigens, such as cancers and bacteria presented in major histocompatibility complex molecules of antigen-presenting cells), and initiate an immune response. Very diverse TCRs have been created through mechanisms such as gene rearrangement and somatic hypermutation. The TCR repertoire refers to a collection of lymphocytes with TCRs of different specificities.8 Various methods have been
utilized to conduct such an analysis since early times.\textsuperscript{9,10} Analysis of the TCR repertoire may clarify whether antigen-specific T cells are induced by immunization and identify the characteristics of common T cells in specimens.

Many studies about TCR repertoires in IBD had been reported from the 1990s, which reported that T-cell clonal expansion in intestinal tissue\textsuperscript{11–13} or peripheral blood\textsuperscript{13,14} was observed in IBD patients, suggesting contribution of acquired immunity to IBD pathogenesis. In IBD, memory T cells in lamina propria mononuclear cells (LPMCs) play an important role in the intestinal tract immune response. The TCR repertoire in LPMCs may have a remnant of immune response to antigens, which could be associated with disease onset.\textsuperscript{15} IBD has various extraintestinal complications that could be caused by an immune response for systemic antigens.\textsuperscript{16} It would be interesting to determine whether the remnants of systemic immune response are present in the TCR repertoire in peripheral blood mononuclear cells (PBMCs). However, in these previous studies, polymerase chain reaction (PCR)-based methods (i.e. PCR with multiple primers or PCR-based enzyme-linked immunosorbent assay) had been used to compare TCR repertoires. In recent years, advances in next generation sequencing (NGS) system enabled large-scale analysis of sequence data, several TCR repertoire analyses using NGS have been developed.\textsuperscript{17,18} By using these high-throughput TCR repertoire analysis system, there have been several studies on the TCR repertoire in LPMCs of patients with CD in Western countries.\textsuperscript{19–21} Some TCR clones which were characteristic of CD have also been confirmed. There are only a few studies that compare the LPMCs and PBMCs from the same patients or examine the TCR repertoire in UC. There are no studies that compare CD and UC. Although the TCR repertoire of LPMCs has been reported to correlate with the recurrence of CD,\textsuperscript{19} it is not easy to collect LPMCs in clinical practice. If PBMCs had specific patterns reflecting disease phenotypes (i.e. prognosis and response to drugs), they may be used as a disease biomarker in TCR repertoire analysis in patients with IBD; PBMCs can be collected relatively easily. Therefore, it is necessary to confirm the relationship of the TCR repertoire between PBMCs and LPMCs to clarify the characteristics of the TCR repertoire in patients with IBD.

**Methods**

**Subjects.** Twelve patients (5 patients with UC and 7 patients with CD), who underwent bowel resection in the Department of Surgery, Tohoku University Hospital, Sendai, Japan, between February 2018 and February 2019, and 10 healthy volunteers, as controls, were included in this study. The diagnoses of CD and UC were based on clinical symptoms, endoscopic findings, X-ray findings, and tissue findings. All participants were Japanese. This study was approved by the Ethics Committee of the Tohoku University School of Medicine (approval number: 2018-1-378). Patients provided informed consent prior to the study. Table I and Table S1, Supporting information, show the characteristics of patients and controls after quality control.

**Isolation of LPMCs.** Regardless of the reasons for surgery, all patients had active inflammation in the resection samples. We isolated LPMCs from inflamed small or large intestines by the method reported by Fiocchi \textit{et al.}\textsuperscript{22} LPMCs were subsequently isolated from the extracted intestine. Specifically, each intestine sample was divided into pieces (2–3 cm) using Hank’s balanced salt solution (HBSS, Wako, Osaka, Japan) after removing feces. Ultrasonic washing with HBSS containing 0.15% diithiothreitol (DTT, Wako Pure Chemical Industries Ltd., Osaka, Japan) was performed for 30 min. Subsequently, ultrasonic washing was performed again for 90 min with HBSS containing 1 mM ethylenediaminetetraacetic acid (EDTA, Wako Pure Chemical Industries Ltd., Osaka, Japan). The procedures were performed until there was no epithelial cell layer. After removing the epithelial cell layer, ultrasonic washing was again performed with HBSS. The remaining intestinal tract was then finely cut into 5-mm pieces. The cells were then digested with 1 mg/mL collagenase type III (Worthington Biochemical Corporation, Lakewood, NJ, USA) and DNase I (Roche, Basel, Switzerland) at 37°C for 8–10 h. The cell suspension was collected after passing it through a 100-μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). After centrifugation at 700g, cell pellets were suspended in HBSS and isolated using Ficoll–Hypaque gradient (GE Healthcare, Little Chalfont, United Kingdom). After centrifugation at 1000g for 20 min, cells in the interface between HBSS and Ficoll–Hypaque gradient were collected and used as LPMCs.

**Isolation of PBMCs.** Peripheral blood (10 mL) was collected from participants and isolated using a Ficoll–Hypaque gradient (GE Healthcare). After centrifugation at 1000g for 20 min, cells in the interface between HBSS and Ficoll–Hypaque gradient were collected and used as PBMCs.

**RNA extraction from PBMCs and LPMCs and storage.** RNAProtect Cell Reagent (Qiagen, Hilden, Germany), diluted fivefold, was added after cell suspension was adjusted so that the number of isolated PBMCs/LPMCs was within the range of 1 × 10\textsuperscript{6} to 5 × 10\textsuperscript{6}. The total RNA was then extracted using the RNeasy Plus Universal Mini Kit (Qiagen). RNA content and purity were measured using an Agilent 2200 Tapestation (Agilent Technologies, Palo Alto, CA, USA).

**TCR repertoire analysis.** Unbiased repertoire analysis technology developed by Repertoire Genesis Inc. (Osaka, Japan) was used to perform TCR sequence analysis according to previous studies.\textsuperscript{18,23} Sequence performance utilized the Illumina MiSeq paired-end platform. Samples with ≥ 280 000 reads were used for the analysis as a quality control measure.

**Statistical analysis.** The reciprocal of Simpson’s index was used to evaluate the diversity of the TCR repertoire. A paired t-test was used to compare PBMCs and LPMCs of patients with IBD. Student’s t-test was used for comparisons between patients with IBD and controls and between UC and CD. TCR clones with the number of reads accounting for >0.01% of the total number of reads tested positive. Regarding LPMCs, clones with a positive rate of >15% in both CD and UC and clones with a positive rate of >15% in either CD or UC alone were extracted as candidate clones common to IBD and CD/UC-specific clones, respectively. Regarding PBMCs, clones with a positive rate of >15% in both CD and UC and clones with a positive rate of ≤15% in the control group were extracted as candidates of TCR clones characteristic of CD or UC. In contrast, clones with a
positive rate of ≥80% in controls and ≤20% in patients with CD or UC were also extracted as those that are specifically missing in CD or UC.

The above analysis was performed using JMP, version 14 (SAS Institute Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant.

**Results**

**TCR repertoire analysis of PBMCs and LPMCs.**

Analysis of all 12 specimens was performed after prior quality control (QC) of the specimens. The average number of reads of the obtained data was 234,444 ± 74,719 (range: 79,080–356,019). The subsequent analysis included 11 specimens that met the criteria. Overall, 260,411 types of TCR-α clones and 200,494 types of TCR-β clones were extracted.

**Comparison of the diversity of TCR repertoire between LPMCs and PBMCs in patients with IBD.**

The diversity of TCR-α and TCR-β in patients with IBD was compared between LPMCs and PBMCs (Fig. 1). Regarding TCR-α, the reciprocal of Simpson’s index (mean ± SD) tended to be lower in PBMCs than in LPMCs but not to a significant degree (153.4 ± 140.0 vs 406.0 ± 333.9, respectively; $P = 0.053$). Regarding TCR-β, the reciprocal of Simpson’s index also tended to be lower in PBMCs than in LPMCs but not to a significant degree (228.8 ± 203.3 vs 535.6 ± 468.2, respectively; $P = 0.051$).

![Figure 1](image-url)
Comparison of the diversity of TCR repertoire in PBMCs between controls and patients with IBD. The diversity of TCR repertoire in PBMCs was compared between controls and patients with IBD (Fig. 2). Regarding TCR-\( \alpha \), the diversity was significantly lower in patients with IBD than in controls (154.0 \( \pm \) 140.0 vs 1350.1 \( \pm \) 993.6, respectively; \( P = 0.00084 \)). In addition, the diversity of TCR-\( \beta \) was significantly lower in patients with IBD than in controls (228.8 \( \pm \) 203.3 vs 1094.4 \( \pm \) 730.6, respectively; \( P = 0.0013 \)).

**Figure 2** Comparison of T-cell receptor (TCR) diversity in peripheral blood mononuclear cells between patients with inflammatory bowel disease (IBD) and controls. (a) Comparison of TCR-\( \alpha \). TCR diversity was significantly lower in patients with IBD than in controls (154.0 \( \pm \) 140.0 vs 1350.1 \( \pm \) 993.6, respectively, \( P = 0.00084 \)). (b) Comparison of TCR-\( \beta \). The diversity of TCR-\( \beta \) was significantly lower in patients with IBD than in controls (228.8 \( \pm \) 203.3 vs 1094.4 \( \pm \) 730.6, respectively, \( P = 0.0013 \)).

Comparison of the diversity of TCR repertoire in PBMCs between controls and patients with IBD. The diversity of TCR repertoire in PBMCs was compared between controls and patients with IBD (Fig. 2). Regarding TCR-\( \alpha \), the diversity was significantly lower in patients with IBD than in controls (154.0 \( \pm \) 140.0 vs 1350.1 \( \pm \) 993.6, respectively; \( P = 0.00084 \)). In addition, the diversity of TCR-\( \beta \) was significantly lower in patients with IBD than in controls (228.8 \( \pm \) 203.3 vs 1094.4 \( \pm \) 730.6, respectively; \( P = 0.0013 \)).

**Figure 3** Comparison of T-cell receptor (TCR) diversity in Crohn’s disease (CD) and ulcerative colitis (UC). (a) Comparison of TCR-\( \alpha \). There was no significant difference in the diversity of lamina propria mononuclear cells (LPMCs) between CD and UC (\( P = 0.85 \)). Conversely, TCR diversity in peripheral blood mononuclear cells (PBMCs) was significantly lower in CD than in UC (79.3 \( \pm \) 54.7 vs 243.6 \( \pm \) 164.0, respectively, \( P = 0.045 \)). (b) Comparison of TCR-\( \beta \). There was no significant difference in the diversity of TCR-\( \beta \) in LPMCs between CD and UC (\( P = 0.67 \)). TCR diversity in PBMCs was significantly lower in CD than in UC (122.0 \( \pm \) 121.5 vs 357.0 \( \pm \) 217.4, respectively, \( P = 0.049 \)).
Comparison of TCR repertoire diversity by disease. We examined whether the diversity of the TCR repertoire differed between PBMCs and LPMCs depending on the disease (CD and UC) (Fig. 3). In UC, TCR-α and TCR-β in LPMCs were 428.9 ± 369.8 and 607.4 ± 482.7, respectively. In CD, TCR-α and TCR-β in LPMCs were 386.9 ± 335.6 and 475.7 ± 492.5, respectively. Neither of the groups showed significant differences (P = 0.85 and P = 0.67, respectively). However, the diversity of TCR-α and -β in PBMCs was significantly lower in CD than in UC (79.3 ± 54.7 vs 243.6 ± 164.0 for TCR-α and 122.0 ± 121.5 vs 357.0 ± 217.4 for TCR-β; P = 0.045 and P = 0.049, respectively).

Evaluation of IBD-specific TCR clones. This study did not detect previously reported TCR clones in LPMCs or PBMCs specimens of UC or CD and PBMCs in controls, which were observed with a high incidence in patients with CD in Western countries (Table S2).19 Conversely, multiple common TCR clones were found among Japanese patients with UC or CD (Table S2). Regarding TCR-α, the positive rate of CASSLAGGTDQYF (TRBV7-6, TRBJ2-3) was 40% in LPMCs of UC and 20% in PBMCs of UC. The clone was not present in patients with CD or controls and was identified as a clone that is found only in UC (Table 2A). Additionally, the positive rate of CAVRAGGKNFQ (TRAV20, TRAJ31) was 40% for PBMCs of UC and 33% for PBMCs of CD. The clone was not positive in PBMCs of controls; however, the positive rate of the clones in UC was low.

In patients with IBD, the number of common TCR-β clones was lower than that of TCR-α clones (Table S3). Although the positive rate of CASSLEGLQPQHF (TRBV5-1, TRBJ1-5) was 33% in LPMCs of CD and 17% in PBMCs of CD, the remaining TCR clones were negative and were only found in patients with CD (Table 2B). Regarding TCR-β, the positive rate of CASSLAGGTDQYF (TRBV7-6, TRBJ2-3) was 20% in LPMCs of UC and 40% in PBMCs of UC; it was only observed in samples from UC patients.

Evaluation of the homology of TCR repertoire between PBMCs and LPMCs in the same patients. Comparisons of the frequencies of the top 300 TCR clones (i.e. the sum of the frequency of LPMCs and PBMCs for each patient) showed that most were clones specific to LPMCs and PBMCs. However, some common clones were found (Table 3A, B, Figs. S1A and S1B); the number of common clones between LPMCs and PBMCs of the same patient was significantly higher than that of the different patients.

Discussion

Major findings of this study are as follows. (i) in Japanese patients with IBD, TCR diversity in PBMCs was significantly lower than that in LPMCs in the same patients, (ii) TCR diversity was significantly lower in patients with IBD than that in controls, (iii) although there was no difference in the TCR diversity of LPMCs between CD and UC, TCR diversity in PBMCs was significantly lower in CD than that in UC, (iv) TCR clones

Table 2

| Clones with a high positive rate in LPMCs |
| Clones with a low positive rate in UC |
| V | J | CDR3 |
|---|---|---|
| TRAV1-2 | TRAJ33 | CAVRAGGKNFQ |
| TRAV2-2 | TRAJ33 | CAAMDSNYQLIW |

CDL, Crohn’s disease LPMCs; CDP, Crohn’s disease PBMCs; CtrlP, control PBMCs; UCL, ulcerative colitis LPMCs; UCP, ulcerative colitis PBMCs.

Table 2 Positive rate of T-cell receptor (TCR) clones specific to Japanese patients with inflammatory bowel disease (IBD)
frequently found in CD in patients in Western countries were not detected in IBD patients in Japan, (v) there are candidates of TCR clones that may be specific to Japanese patients with IBD, and (vi) many TCR clones are common to LPMCs and PBMCs of the same patients.

Various factors are involved in the onset and pathology of IBD. Among these factors, memory T cells are considered to play an important role. In this study, we performed a TCR repertoire analysis of LPMCs and PBMCs in the intestinal tract of Japanese patients with IBD as well as PBMCs of controls. Inflammation occurs mainly in the intestinal tract in patients with IBD. Although the TCR repertoire in the intestinal tract was expected to be less diverse than that in the peripheral blood, the diversity of the memory T cells in PBMCs was lower than that in the local intestine. The results were the opposite of those previously reported in the TCR repertoire analysis of the Western population. Although the reason for this is unclear, the pathogenesis of IBD may differ between patients in Western countries and patients with IBD in Japan because of the differences in the genetic background. A possible reason for the difference in pathogenesis is that the participants in this study were surgical patients with more severe systemic inflammation. Future studies are necessary to confirm these findings.

A comparison showed that the TCR diversity in PBMCs was significantly lower in patients with IBD than that in controls. This suggests that an increase in T cells with specific TCR that reacts with certain antigens leads to systemic inflammation and inflammation in the intestinal tract in patients with IBD. The above results were similar to those of patients with CD in Western countries, which stated that there was also a significantly lower diversity of the TCR repertoire in the peripheral blood of Japanese patients with IBD.

Comparisons between UC and CD showed significantly lower TCR diversity in PBMCs of CD but no significant difference in the TCR diversity in intestinal LPMCs. The results suggested a higher incidence of a systemic immune response in CD than in UC. The finding of this study that TCR diversity in PBMCs is lower in patients with CD may be attributable to the phenotype differences in systemic complications of CD, and further research is warranted. The results of repertoire analysis in this study supported the previous assumption that a systemic immune response is more likely induced by CD than by UC.

The TCR clones, which have been reported to show a high positive rate in patients with IBD in Western countries, were examined in this study. However, none was found in either LPMCs or PBMCs in Japanese patients with IBD or PBMCs in CD, Crohn's disease; UC, ulcerative colitis.

### Table 3
The number of T-cell receptor (TCR) clones that are common to peripheral blood mononuclear cells (PBMCs) and lamina propria mononuclear cells (LPMCs) of the same patients

| LPMCs | PBMCs | UC1 | UC2 | UC3 | UC4 | UC5 | CD1 | CD2 | CD3 | CD4 | CD5 | CD6 |
|-------|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| UC1  | 14    | 4   | 1   | 2   | 3   | 3   | 3   | 5   | 1   | 0   | 2   |     |
| UC2  | 5     | 13  | 2   | 0   | 1   | 2   | 1   | 2   | 1   | 0   | 0   |     |
| UC3  | 1     | 17  | 0   | 3   | 1   | 1   | 0   | 0   | 1   | 2   |     |     |
| UC4  | 3     | 2   | 4   | 22  | 3   | 1   | 1   | 1   | 1   | 1   | 1   |     |
| UC5  | 4     | 2   | 2   | 4   | 45  | 4   | 3   | 2   | 10  | 1   | 6   |     |
| CD1  | 6     | 2   | 7   | 12  | 4   | 71  | 5   | 0   | 2   | 1   | 3   |     |
| CD2  | 3     | 2   | 2   | 2   | 6   | 6   | 29  | 3   | 7   | 2   | 4   |     |
| CD3  | 0     | 2   | 1   | 2   | 4   | 1   | 20  | 4   | 0   | 0   |     |     |
| CD4  | 0     | 2   | 0   | 0   | 0   | 0   | 0   | 2   | 0   | 0   |     |     |
| CD5  | 8     | 4   | 5   | 1   | 8   | 3   | 4   | 0   | 4   | 14  | 8   |     |
| CD6  | 2     | 0   | 1   | 1   | 4   | 6   | 5   | 3   | 1   | 3   | 50  |     |

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controls. Because environmental factors, such as related antigens (e.g., Enterobacteria), differ between patients in Western countries and those in Japan even when they have the same disease, the results of the repertoire analysis may have greatly varied depending on race or regions. This study examined TCR specific to IBD in the Japanese population. Some TCRs were common to patients with IBD but were not found in controls. In contrast, there were clones that were common to controls and patients with CD but had a low positive rate in patients with UC. Regarding TCR-β, there were only a few TCRs common to controls and patients with IBD, whereas some TCRs were found only in patients with IBD. Continued evaluation of TCR may lead to the identification of disease-specific TCR. It would be interesting to examine whether the presence (or absence) of these clones is related to pathogenesis, response to treatment, or prognosis.

This study has the following limitations: (i) the number of specimens was small, (ii) all participants were patients with IBD who required surgery (i.e., patients with mild IBD, who did not require surgery, were excluded from this study), (iii) LPMC samples from colon and ileum were analyzed all together, and (iv) the age range of the controls was narrower than that of patients with IBD. We focused on TCR repertoires of LPMCs in inflammation site regardless of whether the samples were from colon or ileum. However, the difference of T-cell population could affect the results. Including other limitations, future studies with a larger sample size are necessary to further examine the results of this study and to obtain new findings.

Although this study failed to identify TCR as a promising biomarker, the results suggest that the sensitivity and specificity of TCR as a biomarker of autoimmune diseases (e.g., rheumatoid arthritis and systemic lupus erythematosus) are higher than those of conventional immunological serum markers, such as anti-antinuclear antibody (ANA) antibodies and anti-double-stranded DNA (anti-dsDNA) antibodies. In pediatric UC patients, Werner et al. reported correlation between TCR repertoires and disease severity. In addition, the identification of disease-specific TCR may lead to the discovery of dietary antigens and enteric bacteria that correspond to the TCR and serve as a target for drug discovery. Especially in CD, dietary antigens are considered to be involved in disease progression, an elemental diet is effective in IBD. Therefore, examination of TCR in patients with IBD may lead to the identification of dietary antigens to be avoided.

The signatures of TCR in PBMCs and LPMCs were similar in the same patient; therefore, if the pathogenesis may be characterized by the TCR repertoire in PBMCs, we may use repertoire analysis of PBMCs in clinical practice, which is easier to perform than that of LPMCs. Based on these findings, further research analyzing a large number of patients to find correlation of TCR repertoires in PBMCs with disease diagnosis, phenotypes, prognosis, and response to drugs in Japanese patients with IBD is warranted.

In conclusion, a TCR repertoire characteristic of IBD in the Japanese population has been confirmed. The diversity of the TCR repertoire was significantly lower in PBMCs of patients with IBD than that in PBMC of controls or LPMC of patients with IBD. The signatures of TCR in PBMCs and LPMCs were similar in the same patient; therefore, this suggests that a TCR repertoire analysis of PBMCs, which can be collected more easily, is useful for patient stratification.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s website:

Appendix S1 Supporting Information.