TCR sequencing analysis of cancer tissues and tumor draining lymph nodes in colorectal cancer patients

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ABSTRACT

Tumor draining lymph nodes (TDLNs) are located in the routes of lymphatic drainage from a primary tumor and have the highest risk of metastasis in various types of solid tumors. TDLNs are also considered as a tissue to activate the antitumor immunity, where antigen-specific effector T cells are generated. However, T cell receptor (TCR) repertoires in TDLNs have not been well characterized. We collected 23 colorectal cancer patients with 203 lymph nodes with/without metastatic cancer cells (67 were metastasis-positive and the remaining 136 were metastasis-negative) and performed TCR sequencing analysis using mRNAs and applied our technology for immunogenomics. TDLNs showed a significantly lower TCR diversity and shared TCR clonotypes more frequently with primary tumor tissues compared to metastasis-negative TDLNs. Principal component analysis indicated that TDLNs with metastasis showed similar TCR repertoires. These findings suggest that cancer-reactive T cell clones could be enriched in the metastasis-positive TDLNs.

Introduction

Lymphocytes are one of the major factors to define tumor immune microenvironment, and many studies suggested that the presence of higher numbers of tumor infiltrating lymphocytes (TILs) at primary tumor sites is related to the better prognosis of cancer patients. To overcome immunosuppressive tumor microenvironment and enhance the antitumor activity of TILs, adoptive TIL transfer therapy has been attempted for many years. However, the response rate of adoptive TIL infusion therapy is still very limited.

The tumor draining lymph nodes (TDLNs), which are located in the routes of lymphatic drainage from a primary tumor, are the most likely sites to have metastatic cancer cells in various types of solid tumor. TDLNs are usually removed at the time of surgical treatment and are considered to be tissues to initiate the antitumor immune responses. The tumor cells or antigen-presenting cells (APCs) with a tumor antigen(s) migrate from the primary tumor site to TDLNs and present the antigen(s) to naive T cells. Effector T cells such as cytotoxic T lymphocytes (CTLs) and helper T cells are generated in the TDLNs, are released to the bloodstream, and then infiltrate into the primary tumor. The TDLNs have been examined for their roles in cancer immunity for decades. Some investigators have used TDLNs as a source of T cells for adoptive T cell therapy and demonstrated their anti-tumor activity in clinical trials. However, T cell repertoires in TDLNs have not been well characterized.

T cell receptors (TCR), most of which consists of TCR alpha (TCRA) and beta (TCRB) chains, are expressed on T cells and recognize antigens presented on the major histocompatibility complex (MHC) molecules. To recognize a large variety of antigens, TCR genes undergo the somatic recombination process of variable (V), diversity (D) (for beta chain), and joining (J) exons, termed the V(D)J recombination, and generate an extremely high degree of the diversity. We established next-generation sequencing-based TCR repertoire analysis using mRNAs and applied our technology for the TCR analysis of various types of tumor. A detailed TCR transcript analysis has provided a new insight into T cell responses in various disease conditions including characterization of the tumor microenvironment.

We hypothesized that TDLNs contain tumor-reactive T cells, including T cells enriched in primary tumors and can be a good source of T cells for cancer immunotherapy.
Especially metastasis-positive TDLNs might enrich tumor-reactive T cells because of the direct stimulation by tumor antigens carried by cancer cells or APCs in these lymph nodes. To test this hypothesis, we enrolled 23 colorectal cancer patients with lymph node metastasis and performed TCR sequencing of colorectal cancer tissues, normal tissues, and a total of 203 regional lymph nodes.

Results

Whole-exome sequencing

We obtained surgically resected TDLNs along with primary tumors and normal tissues from 23 colorectal cancer cases. Characteristics of the patients are summarized in Table 1. We first performed whole-exome sequencing and identified a median of 97 (range: 55–1,103) somatic non-synonymous single nucleotide variants (SNVs) and 9 (range: 1–568) insertion/deletion mutations (indels) in individual tumors (Figure 1). Among them, two patients (CCLN2 and 31) showed a very high number of SNVs and indels, probably due to mutation in the \( MSH6 \) gene (T1085fs) in both cases; \( MSH6 \) is one of the well-known DNA mismatch-repair genes associated with Lynch syndrome. Mutation profiles in each patient for previously reported driver genes are summarized in Figure 1. \( TP53 \), \( APC \), and \( KRAS \) mutations were found in 73.9%, 69.6%, and 34.8% of 23 colorectal cancer tumors examined, respectively.

Comparison of TCR repertoire between primary tumor and TDLNs

To assess the TCR repertoires in primary tumors and TDLNs, we isolated RNAs from tumors, normal tissues and dissected TDLNs from 23 colorectal cancer patients. We performed next-generation sequencing-based TCR\( \beta \) sequencing for 23 pairs of tumor:normal tissues and 203 regional lymph nodes, including 67 metastasis-positive and 136 metastasis-negative lymph nodes (the positivity was assessed by pathological analysis). We obtained an average of 648,691 ± 260,920 total sequence reads (average ± one standard deviation (SD)), 367,313 ± 138,822 sequence reads (average ± one SD) mapped to V, J, and constant (C) segments for TCR\( \beta \). From these TCR\( \beta \) reads, we identified an average of 57,862 ± 40,519 (average ± one SD) unique TCR\( \beta \) complementarity determining region 3 clonotypes (Supplementary Table 1).

To investigate T-cell clonality, we evaluated TCR diversity using inverse Simpson’s diversity index by which higher values indicate higher diversity of TCR repertoire. The TCR diversity index of primary tumors is significantly lower than dissected TDLNs (\( P = 0.0009 \); Figure 2a). When we compared lymph nodes by metastatic status, the TCR diversity index of metastasis-positive TDLNs was significantly lower than that of metastasis-negative TDLNs (\( P < 0.0001 \)), indicating that certain T cell clones expanded in tumors and metastasis-positive TDLNs but did not in metastasis-negative TDLNs.

To further examine the expanded TCR clonotypes in the metastasis-positive TDLNs, we calculated the percentage of abundant TCR clones (TCR\( \beta \) sequences observed at the frequencies of 0.05% or higher in the primary tumors) in metastasis-positive and metastasis-negative TDLNs. Metastasis-positive TDLNs shared TCR clonotypes with primary tumors at a significantly higher percentage than metastasis-negative TDLNs (\( P < 0.0001 \), Figure 2b). These results indicate that tumor-reactive lymphocytes expanded in metastasis-positive TDLNs, and then circulated and infiltrated into primary tumors although we cannot exclude a possibility that tumor-reactive lymphocytes expanded in the primary tumor microenvironment and then further expanded when cancer cells were present in TDLNs.

To exclude a possibility that the difference of the diversity and clonotypes might reflect the physical locations of the lymph nodes (how close to the primary tumors), but not the presence or absence of cancer cells in TDLNs, we compared the percentage of abundant TCR clones shared with primary tumors among different lymph node groups, which are classified into three groups, paracolic, intermediate and main lymph nodes;\(^ {22,23} \) paracolic lymph nodes are the closest lymph nodes from the primary tumor and main lymph nodes are far from the primary tumors. As shown in Figure 2c, the presence or absence of cancer cells, but not the physical locations, was associated with the TCR repertoires/diversities of these TDLNs.

Amount of lymph node metastasis affects the TCR repertoire of TDLNs

Since we used a half of each dissected lymph node for TCR analysis and defined the metastatic status by the pathological
Figure 1. Genomic profiles of 23 colorectal cancers.
The number of SNVs and indels detected in each sample and summary of driver gene mutations in 23 colorectal cancers. *Significantly mutated genes; q < 0.2.

Figure 2. TCR repertoire analysis of primary tumors and TDLNs.
(a) TCR diversity index in primary tumors, metastasis-positive lymph nodes, and metastasis-negative lymph nodes. TCR diversity index of metastasis-positive TDLNs is significantly higher than that of primary tumor and lower than metastasis-negative TDLNs. TCR diversity index was calculated as inverse Simpson’s diversity index, which shows a higher value when the diversity of TCR repertoire is higher. (b) Percentage of shared abundant TCR clonotypes between TDLNs and primary tumors. The TCRβ sequences which were observed above 0.05% in the primary tumor were defined as abundant TCR clonotypes. Metastasis-positive TDLNs shared TCR clonotypes with primary tumors at a significantly higher percentage than metastasis-negative TDLNs. (c) Percentage of shared abundant TCR clones between metastasis-negative TDLNs and primary tumors. TDLNs were classified into three groups based on the physical locations (paracolic, intermediate, or main). The percentage of shared TCR clonotypes between primary tumors and TDLNs were not different by physical locations of TDLNs.
diagnosis of the remaining half of the dissected lymph nodes, the half of the lymph node which we analyzed might be different in the metastatic status from the remaining half. Thus, we estimated the proportions of cancer cells using somatic KRAS mutations as a marker of cancer cells. We selected eight patients who had the KRAS mutations in their primary tumors and quantified proportion of cancer cells in the lymph node tissues based on the allelic frequencies of KRAS mutant using TaqMan KRAS mutation detection assay (CCLN1 (G12V), CCLN3 (G13D), CCLN12 (G12V), CCLN22 (G13C), CCLN27 (Q61H), CCLN29 (G12D), CCLN30 (G12D), and CCLN51 (G12D)). The pathological diagnosis of the remaining half part of the lymph nodes is compatible with our KRAS mutational assay (Figure 3a). The 23 of 25 (92%) pathologically metastasis-positive TDLNs showed a KRAS mutation with the frequencies of 1% or higher. KRAS mutation was not detected in 40 of 43 (93%) pathologically metastasis-negative TDLNs, but we detected a KRAS mutation with the frequency of <1% in the three remaining TDLNs (CCLN22-2, CCLN29-7, CCLN51-7).

To examine the effect of tumor cell proportions to define the TCR patterns in TDLNs, we calculated the proportions of shared abundant TCR clones in primary tumors in four TDLNs groups that were classified according to the quantitative KRAS mutation results. Interestingly, the higher KRAS mutation rate (higher proportion of cancer cells in lymph nodes) is associated with the higher percentage of abundant TCR clonotype sharing between the primary sites and TDLNs (Figure 3b).

**The similarity of TCR repertoire based on the metastatic status of TDLNs**

We then compared the similarity of TCR repertoires among primary tumors, normal tissues, and TDLNs using principle component analysis (PCA) for 8 patients with KRAS mutations (Figure 4) and the remaining 15 patients (Supplementary Figure 1). In most of the samples, metastasis-positive TDLNs made a cluster(s), which was separated from the cluster(s) of metastasis-negative TDLNs. We also found the tendency that the TDLNs with the KRAS mutation frequencies of >10% were tightly clustered, suggesting these TDLNs may have similar TCRs, some of which are probably reactive to tumor-specific antigens (Figure 4). Interestingly, two pathologically metastasis-positive TDLNs (CCLN29-4 and CCLN51-1), which showed very low KRAS mutation frequency (<1%) by a quantitative analysis, revealed more similar TCR repertoire patterns with metastasis-negative TDLNs than other metastasis-positive TDLNs.

**Discussion**

In this study, we have examined the TCR repertoires in primary tumors, corresponding normal tissues and TDLNs in colorectal cancer patients. This is the first report which characterized TCR repertoires in detail in TDLNs of human cancer. Through the TCR analysis, we found that infiltration of tumor cells into TDLNs induced certain levels of expansion of clonal T cells (possibly cancer-antigen-specific T cells) which were also present abundantly in the primary tumors and that the proportion of cancer cells in TDLNs significantly influenced immune microenvironment characterized by TCR repertoires.

The recent success of immune checkpoint inhibitors such as anti-programmed cell death 1 and anti-programmed cell death ligand 1 antibodies demonstrated the importance of host immune response against cancer cells. Cytotoxic T cells targeting neoantigens have been shown to play critical roles to eradicate cancer cells in patients treated with immune

![Figure 3. Quantification of cancer cells in the TDLNs by qPCR of KRAS mutation.](image)
checkpoint inhibitors or by the adoptive TIL infusion therapy. However, the response rate of either immune checkpoint inhibitors or adoptive TIL infusion therapy is still limited. Thus, it is critically important to develop new strategies for additionally enhancing immune response against cancer cells. Our findings suggest that TDLNs with metastatic tumor cells might serve as good sources for further improvement of adoptive immunotherapy.

Marits et al. investigated the immunological role of sentinel lymph nodes, which are considered to be the first TDLNs that receive the lymphatic drainage from primary tumors. Tumor-reactive T cells were likely to be enriched in freshly isolated lymphocytes from sentinel lymph nodes of colorectal cancer, compared to TILs in primary tumors, lymphocytes from non-sentinel lymph nodes or PBMCs. Based on this finding, they conducted a pilot study of an adoptive T cell therapy using autologous in vitro expanded lymphocytes isolated from sentinel lymph nodes and reported the promising potentiality of sentinel node-based adoptive immunotherapy for stage IV colon cancer; four of nine patients with stage IV colorectal cancer who received sentinel node-based adoptive immunotherapy achieved complete tumor regression. Median survival of these patients was 2.6 years as compared with 0.8 years in conventionally treated controls. Another group also reported the possibility of sentinel node-based adoptive immunotherapy for stage IV colorectal cancer patients who underwent palliative surgery; the 2-year survival rate of the sentinel node-based adoptive immunotherapy group was significantly higher than that of the control group (55.6% vs 17.5%). These two reports prompted us to characterize the TCR repertoire of TDLNs of colorectal cancer patients. Based on the findings in this study, we assume metastasis-positive TDLNs might be a better source of T cells for adoptive T cell therapy compared to the metastasis-negative TDLNs due to the higher percentage of shared T cell clones with primary tumor, which are likely to be tumor-reactive lymphocytes.

As another promising immunotherapy to enhance the host immune system, researchers including us have been working on adoptive T cell therapy with TCR-engineered T cells. Rapoport et al. reported the clinical benefit of NY-ESO-1-specific TCR-engineered T cell transfer therapy for multiple myeloma patients. Clinical responses observed in this trial was impressively high as 80% (16 of 20 patients) with a median progression-free survival of 19.1 months. The biggest challenging part of this type of therapy is an identification of antigen (neoantigen and/or shared antigen)-specific T cells. TILs or lymphocytes from PBMCs have been used to induce and identify antigen-specific T cells, and we reported a method to identify antigen-specific TCRs using PBMCs in as short as two weeks. Since our results suggest that TDLNs with metastatic cancer cells seem to be enriched with tumor-reactive T cells, TDLNs with cancer cells may also be a good source of T lymphocytes to induce antigen-specific T cells and we reported a method to identify antigen-specific TCRs using PBMCs in as short as two weeks. Since our results suggest that TDLNs with metastatic cancer cells seem to be enriched with tumor-reactive T cells, TDLNs with cancer cells may also be a good source of T lymphocytes to induce antigen-specific T cells. In this study, we found that metastasis-positive TDLNs tended to reveal similar TCR repertoire patterns each other (Figure 4, Supplementary Figure 1). However, some metastasis-negative TDLNs (CCLN2-1, CCLN22-3 and CCLN22-6) showed similar TCR repertoire patterns with metastasis-positive TDLNs and primary tumors. In these cases, cancer cells could be eradicated by cancer-reactive T cells at the time of surgical operation.

Figure 4. PCA of TCRβ repertoire in 8 colorectal cancers having KRAS mutation. Metastasis-positive and metastasis-negative TDLNs formed separate clusters. In particular, TDLNs with the high KRAS mutation rates were tightly clustered each other. Metastasis-positive TDLNs reveal highly similar TCR repertoire patterns with the primary tumors than metastasis-negative TDLNs. Metastatic status of TDLNs was classified by the KRAS mutational assay.
In conclusion, through the genome and TCR sequencing of tumor/normal tissues and TDLNs from 23 colorectal cancer patients, we found that metastasis-positive TDLNs showed a significantly lower TCR diversity and contained TCR clonotypes that shared with primary tumor tissues. PCA analyses supported that metastasis-positive TDLNs showed similar TCR repertoires, which are different from TCR repertoires in metastasis-negative TDLNs. These findings suggest that certain cancer-reactive T cell clones may be enriched in the metastasis-positive TDLNs, but not in metastasis-negative TDLNs.

Materials and methods

Study design

Patients with colorectal cancer who received tumor resection and lymphadenectomy were enrolled. Informed consent was obtained before surgery. Tumor staging was classified according to the Japanese Classification of Colorectal Carcinoma, 8th Edition. A primary tumor, a normal tissue (normal mucosa of large intestine) and multiple lymph nodes were collected from a surgical specimen of individual patients. Blood samples were also obtained from several patients as a normal control. The study protocol was approved by the Institutional Review Board of the University of Chicago (approval number 13–0797 and 15–1738) and Cancer Institute Hospital of Japanese Foundation for Cancer Research (approval number 2015–1142). Our study’s involvement with human subjects complies with the Declaration of Helsinki.

Whole-exome sequencing

Genomic DNAs from tumor and normal tissues (PBMC or mucosa of large intestine) were extracted using AllPrep DNA/RNA mini kit (Qiagen). Whole-exome libraries were prepared from genomic DNAs using SureSelectXT Human All Exon V6 kit (Agilent Technologies) and sequenced by 100-bp paired-end reads on a HiSeq2500 Sequencer (Illumina).

Sequence alignment and mutation calling were performed as described previously. Briefly, sequence reads were mapped to the human reference genome GRCh37/hg19 using Burrows-Wheeler Aligner (BWA) (v0.7.10). Possible PCR duplicated reads were excluded using Picard v1.91 (http://broadinstitute.github.io/picard/). Read pairs with mismatches more than 5% of read length and with a mapping quality of < 30 were also excluded. Finally, somatic variants (single nucleotide variations and indels) were called using Fisher’s exact test-based method with the following parameters, (i) base quality of ≥15, (ii) sequence depth of ≥ 10, (iii) variant depth of ≥ 4, (iv) variant frequency in tumor of ≥ 10%, (v) variant frequency in normal of < 2%, and (vi) Fisher p-value of < 0.05. The significance of mutated genes was calculated using MutSigCV v1.4.

TCR sequencing analysis

TCR sequencing was performed using the methods described previously. In brief, we extracted total RNAs from surgical resected tumors, normal tissues, and TDLNs. cDNAs with common 5’-RACE adapter were synthesized from total RNA using SMART library construction kit (Clontech). The TCRβ cDNAs were amplified by PCR using a forward primer for the SMART adapter and a reverse primer corresponding to the constant region of TCRβ. After adding the Illumina index sequences with barcode using the Nextera XT Index kit (Illumina), the prepared libraries were sequenced by 300-bp paired-end reads on Illumina MiSeq platform, using MiSeq Reagent v3 600-cycles kit (Illumina). Obtained sequence reads were analyzed using Tcrip software. The TCRβ clonality was evaluated using the inverse Simpson’s diversity index calculated as follows:

\[
1/D_s = \left( \frac{\sum_{i=1}^{k} n_i(n_i - 1)}{N(N - 1)} \right)^{-1}
\]

where \(K\) is the total number of CDR3 clonotypes, \(n_i\) is the number of sequences belonging to the \(i\)-th clonotype, and \(N\) is the total number of identified CDR3 sequences.

PCA of pattern of TCRβ repertoire

We applied PCA to compare TCRβ clonotype patterns among the primary tumor, normal tissue, and TDLNs in each patient. We used only the information of abundant TCRβ clonotypes (TCRβ sequences observed at the frequencies of 0.05% or higher) in at least one samples. PCA was performed using R command prcomp on R environment 3.4.3.

KRAS mutation detection assay

KRAS somatic mutations (G12V, G12D, G13C, G13D, and Q61H) were identified in eight colorectal cancer tissues through whole-exome sequencing. TaqMan Mutation Detection Assay (Thermo Fisher Scientific) was applied for analyzing KRAS mutation frequencies (to measure the proportions of cancer cells in individual tissues) in normal tissues, tumor tissues, and lymph nodes. KRAS reference probe was also applied as a qPCR experiment was performed on ViiA 7 Real-time PCR system with 384-well plate (Thermo Fisher Scientific). Mutation rates were acquired by using Mutation Detector Software v.2.0 (Thermo Fisher Scientific).

Statistical analysis

Statistical significance for the difference between the two groups were all determined using the Mann–Whitney U test. Statistical significance for the difference between three or four groups were determined using the Kruskal–Wallis test. Statistical analyses were done using GraphPad Prism version 6.0 (GraphPad software). P value of <0.05 was considered to be statistically significant.

Abbreviations

| Term          | Definition                                |
|---------------|-------------------------------------------|
| TDLNs         | Tumor draining lymph nodes                |
| TCR           | T cell receptor                            |
| TILs          | Tumor infiltrating lymphocytes             |
| APCs          | Antigen-presenting cells                   |
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Author contributions

Y.N. planned and supervised the entire project and completed the article. Y.K. provided clinical samples, designed the study and provided scientific advice. S.N., S.M., M.U., W.Y., H.T., H.N., S.H., N.S., K.F., provided clinical samples, provided scientific advice. K.K. and J.P. provided the study design and the working hypothesis and revised the article; T. M. designed the study, conducted experiments, performed data analysis, and drafted the article. E.M., Y.H., M.Z., T.K., M.H. conducted experiments, performed data analysis.

Competing interests

YN is a stockholder and a scientific advisor of OncoTherapy Science Ltd. J-H P was an advisor of and is now an employee of OncoTherapy Science Ltd. MH is an employee of OncoTherapy Science Ltd.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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