Identification of cell-type-specific mutations in nodal T-cell lymphomas

TB Nguyen1,2,3, M Sakata-Yanagimoto1,4,5, Y Asabe1, D Matsubara6, J Kano7, K Yoshida8, Y Shiraishi9, K Chiba9, H Tanaka9, S Miyano9, K Izutsu10,11, N Nakamura12, K Takeuchi13, H Miyoshi14, K Ohshima14, T Minowa15, S Ogawa16, M Noguchi17 and S Chiba1,4,5

Recent genetic analysis has identified frequent mutations in ten-eleven translocation 2 (TET2), DNA methyltransferase 3A (DNMT3A), isocitrate dehydrogenase 2 (IDH2) and ras homolog family member A (RHOA) in nodal T-cell lymphomas, including angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma, not otherwise specified. We examined the distribution of mutations in these subtypes of mature T-/natural killer cell neoplasms to determine their clonal architecture. Targeted sequencing was performed for 71 genes in tumor-derived DNA of 87 cases. The mutations were then analyzed in a programmed death-1 (PD1)-positive population enriched with tumor cells and CD20-positive B cells purified by laser microdissection from 19 cases. TET2 and DNMT3A mutations were identified in both the PD1+ cells and the CD20+ cells in 15/16 and 4/7 cases, respectively. All the RHOA and IDH2 mutations were confined to the PD1+ cells, indicating that some, including RHOA and IDH2 mutations, being specific events in tumor cells. Notably, we found that all NOTCH1 mutations were detected only in the CD20+ cells. In conclusion, we identified both B- as well as T-cell-specific mutations, and mutations common to both T and B cells. These findings indicate the expansion of a clone after multistep and multilinear acquisition of gene mutations.

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INTRODUCTION

Nodal T-cell lymphomas are subtypes of mature T-/natural killer-cell neoplasms, including angioimmunoblastic T-cell lymphoma (AITL); nodal peripheral T-cell lymphoma (PTCL) with T follicular helper (TFH) phenotype; peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), and follicular T-cell lymphoma. Among them, AITL is a distinct subtype, accounting for 16.0–28.7% of all mature T-/natural killer-cell neoplasms.1–3 AITL is characterized by specific clinical features, including generalized lymphadenopathy, high fever, skin rash and autoimmune-like manifestations. AITL tumor cells share characteristics with TFH cells, expressing B-cell lymphoma protein 6, a transcription factor; C-C motif chemokine receptor 5, a chemokine receptor; C-X-C motif ligand 13, a chemokine; and programmed death-1 (PD1), a member of the CD28 costimulatory receptor family.4,5 AITL tissues display prominent infiltration of inflammatory cells, follicular dendritic cell meshwork formation and branching vascular structures. Some nodal T-cell lymphomas exhibit several features reminiscent of AITL, although they do not show the typical morphology of AITL (nodal PTCL with TFH phenotype).6,7 The massive infiltration of inflammatory cells in AITL has been explained by cytokines and chemokines being released from TFH-like tumor cells.4

Recurrent gene mutations have been identified in nodal T-cell lymphomas, including those in ten-eleven translocation 2 (TET2) in 20–83%, isocitrate dehydrogenase 2 (IDH2) in 0–45%, and ras homolog family member A (RHOA) in 17–71%, depending on the subtypes and DNA methyltransferase 3A (DNMT3A) in approximately 30%, independent of the subtypes.8–13 Mutations in TET2 encoding a methylcytosine dioxygenase and those in DNMT3A encoding a DNA methyltransferase presumably result in epigenetic abnormalities in nodal T-cell lymphomas. IDH2 mutations also affect epigenetic modifications by inhibiting TET and histone demethylation enzymes through production of 2-hydroxyglutarate.14 Mutations in RHOA encoding a small GTPase are almost always located at the hotspot site, resulting in conversion from glycine to valine at the seventeenth position of the RHOA protein (G17V RHOA mutation). The G17V RHOA mutants could not be converted to an active GTP-bound form, although the downstream signaling of the G17V RHOA mutants in nodal T-cell lymphomas development has yet to be clarified.8,9,13

TET2 and DNMT3A mutations are proposed to arise in hematopoietic stem/progenitors upstream of T-lineage commitment. This hypothesis is based on the fact that identical TET2 and DNMT3A mutations were found in both tumor tissues and apparently normal blood cells in some AITL and PTCL-NOS patients.8,10,15–17 In contrast, the origins of the G17V RHOA mutation remain to be elucidated: it may be a tumor-specific event, considering that the allele frequencies of G17V RHOA mutations were lower than those of TET2 mutations and that G17V
**MATERIALS AND METHODS**

**Patients and samples**

Samples, obtained from 87 patients (Supplementary Table S1) with AITL (n = 48), nodal PTCL with TFH phenotype (n = 5) and either of PTCL-NOS or of nodal PTCL with TFH phenotype (PTCL-NOS/nodal PTCL with TFH

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*RHOA* mutations were found in only CD4+ T lymphocytes in 1 AITL and 1 PTCL-NOS case. Here we describe the clonal architecture of nodal T-cell lymphomas by determining the distribution of mutations in enriched tumor cells and infiltrated B cells.

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**Figure 1.** Targeted sequencing result of 87 nodal T-cell lymphoma samples. *TET2-, RHOA-, IDH2- and DNMT3A-mutated cases are indicated by blue, red, orange and green boxes, respectively. Other recurrently mutated genes are in purple. The laser-microdissected samples are indicated in red letters.
phenotype, n = 34), were used after approval was obtained from the local ethics committees of all the participating institutes.

Genomic DNA was extracted from 56 fresh frozen samples using the Puregene DNA Blood Kit (Qiagen, Hilden, Germany) and 31 periodate-lysine-paraformaldehyde (PLP)-fixed frozen samples using the QIAamp DNA FFPE Tissue Kit (Qiagen).

Targeted sequencing

Targeted sequencing was performed for 71 genes, which are listed in Supplementary Table S2. Sixty-one of the genes were previously screened by whole-genome sequencing, while 6 were the family genes of those whose mutations were identified by the whole-exome sequencing. The other four genes were deemed susceptible to mutations in PTCLs on the basis of the mutational profiles of other lymphoid malignancies. All the exons of the selected genes were captured by use of a SureSelect Target Enrichment Kit (Agilent, Santa Clara, CA, USA) and then massively sequenced using HiSeq2000 (Illumina, Santa Clara, CA, USA). For each sample, all the sequencing reads were aligned to hg19 using BWA version 0.5.8 with default parameters. After all the duplicated reads and the low-quality reads and bases were removed, the allele frequencies of single-nucleotide variants and indels at each genomic position were calculated by enumerating the relevant reads using SAMTools (http://www.htslib.org).

Initially, all the variants showing allele frequencies > 0.02 were extracted and annotated using ANNOVAR for further consideration, if they were found in > 6 reads of > 10 total reads and appeared in both the positive- and the negative-strand reads. All synonymous variants and known single-nucleotide polymorphisms in public and private databases, including dbSNP131, the 1000 genomes project as of 2012/05/21 and our in-house database, were removed. To exclude germline variants, nonsynonymous variants were excluded when the allele frequencies were from 0.45 to 0.55. Candidate mutations were validated by amplicon-based deep sequencing using Ion PGM (Life Technologies, Carlsbad, CA, USA) and/or Sanger sequencing (see below).

In the cohort of 87 cases, 79 were analyzed for RHOA, TET2, DNMT3A and IDH2 mutations, and the results of this analysis were described in the previous paper. Now, eight were new cases. We re-analyzed all the 87 samples for targeted sequencing of 71 genes.

Amplicon-based sequencing

The libraries were prepared using the Ion Plus Fragment Library Kit according to the protocol for preparing short amplicon libraries (Life Technologies). Briefly, PCR amplicons were ligated to the barcode adapters and P1 adapters and then amplified. The amplified libraries were quantitated by quantitative PCR with the Ion Library Quantitation Kit according to the manufacturer’s instructions (Life Technologies). The libraries were then subjected to deep sequencing on the Ion Torrent PGM platform according to the standard protocol for 300 base-pair single-end reads (Life Technologies). The data were analyzed using Variant Caller 3.4 (Life Technologies).

Immunohistochemistry

PLP-fixed frozen samples were cut in a cryostat at ~ 22 °C into 5-μm sections and mounted on PEN-Membrane slides (Leica, Wetzlar, Germany). The tissue sections were stained with mouse anti-human PD1 (NAT105 ab52587, Abcam, Cambridge, UK) and anti-human CD20 (clone L26, Dako, Michigan, MI, USA) antibodies, diluted 1:2000 and 1:1000, respectively, and detected by use of the Envision Dual Link System-HRP (Dako). The tissue sections were then counterstained with hematoxylin (Mayer’s hematoxylin, Muto Pure Chemical, Tokyo, Japan) for 20 s at room temperature. After staining, tissue sections were dehydrated with ethanol and dried at room temperature before laser microdissection (LMD).

LMD, DNA extraction and PCR

Nineteen of the 87 cases (13 AITL, 1 nodal PTCL with TFH phenotype and PTCL-NOS/nodal PTCL with TFH phenotype) were analyzed by LMD, which was performed using LMD7000 (Leica). The cells being positive for either PD1 or CD20 were dissected and collected into 0.2-ml PCR tubes (Takara, Shiga, Japan) containing 20 μl of distilled water. Stained cells at approximately 100,000 cells/l μl of distilled water. Stained cells at approximately 100,000 cells/l were used for PCR under the following conditions: 95 °C for 15 min, 60 °C for 4 min, 72 °C for 4 min, 35 to 40 cycles at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min using the AmpliTaq Gold 360 K (Applied Biosystems, Foster City, CA, USA) with each primer set (Supplementary Table S6). PCR amplicons were used for amplicon-based sequencing and Sanger sequencing.

IgH gene rearrangement analysis and subcloning of the PCR product

Multiplex PCR assays were used to detect the clonality of B cells according to the European BIOMED-2 collaborative study. PCR products migrating at the expected size were extracted and sequenced using the Sanger method. Subcloning was performed if the Sanger sequencing indicated a polyclonal

| Table 1. Targeted sequencing result of 87 nodal T-cell lymphoma samples |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Gene                        | AITL (n = 48)               | Nodal PTCL with TFH phenotype (n = 5) | PTCL-NOS/nodal PTCL with TFH phenotype (n = 34) |
| TET2                        | 36 75 5 100                 | 19 55.9 60 69                |
| RHOA                        | 33 68.8 5 100               | 3 8.8 41 47.1                |
| DNMT3A                      | 11 22.9 1 20                | 11 32.4 23 26.4              |
| IDH2                        | 13 27.1 0 100               | 0 0 13 14.9                  |
| NAV2                        | 0 0 0 0                    | 4 11.8 4 4.6                 |
| ODZ1                        | 2 4.2 1 20                 | 1 2.9 4 4.6                  |
| COL19A1                     | 1 2.1 0 0                   | 2 5.9 3 3.4                 |
| FAT2                        | 1 2.1 0 0                   | 2 5.9 3 3.4                 |
| MTERFD3                     | 2 4.2 0 1                   | 1 2.9 3 3.4                 |
| NOTCH1                      | 3 6.3 0 0                   | 0 0 3 3.4                   |
| B2M                         | 0 0 0 0                    | 2 5.9 2 2.3                 |
| HMCN1                       | 1 2.1 0 1                   | 1 2.9 2 2.3                 |
| LAMA2                       | 0 0 0 0                    | 2 5.9 2 2.3                 |
| MLL2                        | 0 0 0 0                    | 2 5.9 2 2.3                 |
| TET3                        | 2 4.2 0 0                   | 0 0 2 2.3                   |
| LYN                         | 2 4.2 0 0                   | 0 0 2 2.3                   |
| EBF2                        | 1 2.1 0 0                   | 1 2.9 2 2.3                 |

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; nodal PTCL with TFH phenotype, nodal peripheral T-cell lymphoma with T follicular helper phenotype; PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified.

| Table 2. Mutation profiles of 19 laser microdissected samples |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Gene                        | AITL (n = 13)               | Nodal PTCL with TFH phenotype (n = 1) | PTCL-NOS/nodal PTCL with TFH phenotype (n = 5) |
| TET2                        | 12 92.3 1 100               | 3 60 16 84.2                |
| RHOA                        | 9 69.2 1 100               | 0 0 10 52.6                 |
| DNMT3A                      | 6 46.2 1 100               | 0 0 7 36.8                  |
| IDH2                        | 4 30.8 0 100               | 0 0 4 21.1                  |
| NAV2                        | 0 0 0 0                    | 1 20 1 5.3                 |
| ODZ1                        | 1 7.7 0 0                   | 0 0 1 5.3                  |
| COL19A1                     | 1 7.7 0 0                   | 1 20 2 10.5                |
| FAT2                        | 1 7.7 0 0                   | 0 0 1 5.3                  |
| MTERFD3                     | 1 7.7 0 0                   | 0 0 1 5.3                  |
| NOTCH1                      | 3 23.1 0 0                  | 0 0 3 15.8                 |
| B2M                         | 0 0 0 0                    | 1 20 1 5.3                 |
| HMCN1                       | 1 7.7 0 0                   | 0 0 1 5.3                  |
| MLL2                        | 0 0 0 0                    | 1 20 1 5.3                 |
| TET3                        | 1 7.7 0 0                   | 0 0 1 5.3                  |
| LYN                         | 1 7.7 0 0                   | 0 0 1 5.3                  |

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; nodal PTCL with TFH phenotype, nodal peripheral T-cell lymphoma with T follicular helper phenotype; PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified.

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background by use of the pGEM-T Easy Vector System I (Promega, Madison, WI, USA). At least 12 colonies were picked up and sequenced to confirm the clonal expansion. The sequence results were analyzed using the IMGT tools\textsuperscript{27} and aligned to the closest match with the germline IGHV segment. Sequencing results with a germline identity of < 98% were regarded as mutated and vice versa according to previous study.\textsuperscript{28}

RESULTS

Novel recurrent mutations in nodal T-cell lymphomas

Targeted sequencing for 71 genes was performed in 87 samples (Supplementary Table S1), including AITL (n = 48), nodal PTCL with TFH phenotype (n = 5) and PTCL-NOS/nodal PTCL with TFH phenotype (n = 34). TET2, DNMT3A, RHOA and IDH2 mutations were identified in 60 (68.7%), 23 (26.4%), 41 (47.1%) and 13 (14.9%) of 87 cases, respectively (Figure 1, Table 1, Supplementary Table S3). The mutational profiles of these 4 genes in 79 of the 87 samples are described elsewhere.\textsuperscript{7}

Thirty-four novel recurrent mutations were identified in 13 of the 71 (18.3%) genes and in 24 of the 87 (26.4%) cases (Figure 1, Table 1 and Supplementary Table S4). Mutations in genes associated with lymphoid malignancies, for example, Notch homolog 1, translocation-associated (NOTCH1), β2 microglobulin (B2M) and mixed-lineage leukemia 2 (MLL2) were identified in 3, 2 and 7 samples, respectively.

Figure 2. RHOA mutations are specific to PD1+ cells. (a) An example of the immunostaining pattern for PD1 and CD20 in AITL. Left, PD1+ cells; right, CD20+ cells. (b) Sequences of G17V RHOA mutations in whole tumor, PD1+ cells and CD20+ cells. The numeric values indicate allele frequencies of mutations defined by amplicon-based deep sequencing. The AITL samples are indicated in black letters. The nodal PTCL with TFH phenotype sample is indicated in red letters *: RHOA c.A51T:p.G17V, silent mutation. The filled and dashed red arrows indicate mutations and no mutations, respectively.
2 cases, respectively. Mutations in FAT atypical cadherin 2 (FAT2), a gene associating with several cancers, and those in TET3, a member of the TET gene family, were identified in 3 and 2 cases, respectively.

Nineteen of the 87 samples were analyzed by the use of LMD (Figure 1). The frequencies of TET2, DNMT3A, RHOA and IDH2 mutations in the laser-microdissected samples were similar to those found in the entire cohort (TET2 mutations, 16/19 (84.1%); DNMT3A mutations, 7/19 (36.8%); RHOA mutations, 10/19 (52.6%) and IDH2 mutations, 4/19 (21.1%)) (Table 2). A total of 26 TET2 mutations were found in 16 cases, while 2 TET2 mutations were found in 10 samples each (Supplementary Table S5). NOTCH1 and COL19A1 mutations were identified in 3 and 2 cases, respectively. Other
gene mutations, including NAV2, ODZ1, FAT2, MTERFD3, B2M, HMCN1, MLL2, TET3 and LYN, were identified in a single case each.

Specific existence of G17V RHOA mutations in tumor cell-enriched cells of nodal T-cell lymphomas

Previously, we reported that G17V RHOA mutations were detected by flow cytometry only in CD4-positive (CD4+) cells but not in other cell lineages purified from the skin tumor of a PTCL-NOS patient and the pleural effusion cells of an AITL patient. These preliminary results suggested that the G17V RHOA mutation may specifically exist in mature CD4+ T cells in PTCL-NOS and AITL. To gain further insight into the origin of the G17V RHOA mutation, we examined the mutation in laser-microdissected PD1+ and CD20+ B cells, which were assumed to be enriched and depleted in tumor cells, respectively, in 10 nodal T-cell lymphomas (1 nodal PTCL with TFH phenotype and 9 AITL cases). The G17V RHOA mutation was detected only in the PD1+ cells but not in the CD20+ cells in all 10 cases (Figure 2). The allele frequencies of the G17V RHOA mutations in the dissected PD1+ cells were substantially higher than those in the matched whole tumor samples in 7 of the 10 cases. The efficiency of mutation allele enrichment was not substantial in three cases (PTCL63, PTCL78 and PTCL127). In these cases, PD1+ cell selection was not successful enough to purify the tumor cells because of the presumed abundance of PD1- non-tumor cells or the very high tumor cell content before the selection. Additionally, using flow cytometry, we found an AITL case showing that the G17V RHOA mutation existed in PD1+CD4+ cells sorted from bone marrow mononuclear cells (Supplementary Figure S2). This finding strongly supports our hypothesis that the acquisition of the G17V RHOA mutation is a specific event in TFH cells.

| Gene | Mutation | Whole tumor | PD1+ | CD20+ |
|------|----------|-------------|------|-------|
| PTCL 60 | MTERFD3 | c.A673T | p.T225S | 9.3 | 16.9 | 0 |
| PTCL 61 | HMCN1 | c.A2484T | p.R828S | 15.2 | 42.0 | 0 |
| PTCL 77 | TET3 | c.2089+1G>T | splicing | 29.3 | 78.9 | 0 |
| PTCL 78 | LYN | c.C1145A | p.A382D | 28.9 | 76.6 | 18.4 |
| PTCL 142 | COL19A1 | c.3377delA | p.E1126fs | 18.7 | NA | NA |
| PTCL 121 | B2M | c.68-1G>C | splicing | 35.5 | 39.9 | 0 |
| PTCL 126 | NAV2 | c.G2432A | p.R811Q | 66.8 | 66.9 | 83.5 |
| PTCL 132 | COL19A1 | c.G764A | p.G255D | 18.4 | NA | NA |

Figure 4. Distribution of newly identified gene mutations in nodal T-cell lymphomas. The results of Sanger sequencing and/or amplicon-based deep sequencing for some newly identified gene mutations in whole tumor, PD1+ cells and CD20+ cells are shown. The numeric values indicate allele frequencies of mutations defined by deep sequencing. The AITL samples are indicated in black letters. The PTCL-NOS/nodal PTCL with TFH phenotype sample is indicated in blue letters. NA, not analyzed by deep sequencing. The filled and dashed red arrows indicate mutations and no mutations, respectively.
Distribution of TET2, IDH2 and DNMT3A mutations

We and others have previously reported that TET2 and DNMT3A mutations were found in apparently normal blood cells, including bone marrow mononuclear cells, and in immature progenitors and blood cells of various lineages isolated from peripheral blood of a few PTCL patients.7,9,15–17 We examined the distribution of TET2, IDH2 and DNMT3A mutations in PD1+ and CD20+ cells. Twenty of the 26 TET2 mutations were identified in both the PD1+ and the CD20+ cells (Supplementary Table S7), and 15 of the 16 TET2-mutated samples had at least one mutation in both the PD1+ and the CD20+ cells (Figure 3). Concomitantly, DNMT3A mutations were identified in both the PD1+ and CD20+ cells in four of the seven DNMT3A-mutated samples (Figure 3, Supplementary Table S8). In myeloid malignancies, TET2 and IDH2 mutations are known to be mutually exclusive.14,29 However, we and others reported that IDH2 mutations often coexist with TET2 mutations in PTCL.8,10,30 IDH2 mutations were identified in PD1+ cells but not in CD20+ cells in all 4 TET2- and IDH2-comutated samples (PTCL8, PTCL61, PTCL63 and PTCL70) (Figure 3). Each of these samples had at least one TET2 mutation in both the PD1+ and CD20+ cells and the G17V RHOA mutation only in the PD1+ cells. That is, TET2, IDH2 and G17V RHOA mutations coexisted in the PD1+ cells in these cases. In addition, we also found the coexistence of IDH2, TET2 and G17V RHOA mutations in PD1+CD4+ cells sorted from the bone marrow mononuclear cells of an AITL patient (Supplementary Figure S2).

B-cell-specific mutations in nodal T-cell lymphomas

To clarify the cellular origin of newly identified gene mutations, we also checked the distribution of these mutations in PD1+ and CD20+ cells (Table 2). We identified B2M, COL19A1, HMCN1, MTERFD3 and TET3 mutations only in PD1+ cells but not in CD20+ cells. COL19A1, LYN, NAV2 and NOTCH2NL mutations were identified in both the PD1+ and CD20+ cells (Figure 4). Interestingly, three NOTCH1 and one FAT2, MLL2 and ODZ1 mutations each were found only in the CD20+ but not in the PD1+ cells in four samples (PTCL 63, PTCL70, PTCL78 and PTCL128) (Figure 5). Especially, all three NOTCH1 mutations identified by

![Table and Figure]

Figure 5. B-cell-specific mutations in nodal T-cell lymphomas. The results of Sanger sequencing and/or amplicon-based deep sequencing for some newly identified gene mutations in whole tumor, PD1+ cells and CD20+ cells are shown. The numeric values indicate allele frequencies of mutations defined by deep sequencing. The AITL samples are indicated in black letters. The PTCL-NOS/nodal PTCL with TFH phenotype sample is indicated in blue letters. NA, not analyzed by deep sequencing. The filled and dashed red arrows indicate mutations and no mutations, respectively. NOTCH1 is marked by red letters because this is repetitive.
targeted sequencing were identified only in the CD20+ cells with high allele frequencies. The NOTCH1 gene encodes a transmembrane protein. One of the NOTCH1 mutations was a frameshift mutation residing in the PEST domain of the Notch1 protein. This would be an activating mutation, because deletion of the PEST domain enhances Notch signaling after ligand binding. The other two mutations were located in one of the epidermal growth factor-like and in the ankyrin repeat domains (Supplementary Figure S1). One of the NOTCH1-mutated samples simultaneously had two TET2 mutations and G17V RH OA mutation (PTCL 63, Supplementary Table S9). In this case, both TET2 mutations were detected in both the PD1+ and CD20+ cells, while the G17V RH OA mutation was confined to the PD1+ cells. We used the multiplex PCR method to also check the clonality of immunoglobulin genes in the samples with B-cell-specific mutations. Interestingly, only one sample showed monoclonal rearrangement while the others showed oligoclonal rearrangement (Table 3).

DISCUSSION

By determining the distribution of the mutations, we elucidated the clonal architecture of nodal T-cell lymphomas. RH OA mutations were identified only in PD1+ cells in 100% cases, while TET2 and DNMT3A mutations were identified in both the PD1+ cells and CD20+, tumor-cell-depleted cells in the majority of cases. In addition, IDH2 mutations were actually found only in the PD1+ cells and coexisted with TET2 mutations. These data suggest that, in nodal T-cell lymphoma development, multistep tumorigenesis may progress in association with the differentiation of blood cells/lymphocytes. Surprisingly, some of the mutations resided in a B-cell-specific manner.

Recent genetic studies have revealed that, in several hematological cancers, several gene mutations existed in preleukemic hematopoietic cells as well as in tumor cells; examples are TET2 and/or DNMT3A mutations in acute myeloid leukemia and NOTCH1 and SF3B1 mutations in chronic lymphocytic leukemia. Moreover, somatic mutations have been demonstrated in elderly individuals without hematological malignancies: DNMT3A, ASXL1, and TET2 mutations frequently observed in hematological malignancies were the most frequent in these cohorts. Similarly, our data indicated that in nodal T-cell lymphomas, premalignant cells having TET2 and/or DNMT3A mutations may differentiate not only into T-lineage tumor cells but also into B cells. In contrast, the G17V RH OA mutations specifically existed in the T cells of nodal T-cell lymphomas in all 13 cases (11 cases have been described in this paper, while 2 were previously described elsewhere), indicating that the G17V RH OA mutation is the event after the B- and T-cell specification. This could happen right after the T/B specification, after differentiation into TFH cells or even after malignant transformation establishing a subclone. One possibility is that the G17V RH OA mutation occurs in TET2-mutated premalignant cells and facilitates the selective differentiation of TET2-mutated premalignant cells into tumor cells with the TFH phenotype. This needs to be proven in the future.

IDH2 mutations were also specifically identified in the tumor-cell-enriched cells, suggesting that IDH2 mutations are also tumor-cell-specific events in AITL, although the number of samples was not large enough to allow a definite conclusion. We have previously showed that the IDH2-mutated cases were almost a subcohort of G17V RH OA-mutated cases. This result could be interpreted that the acquisition of IDH2 mutations may be the event occurring after the acquisition of RH OA mutation and thus the IDH2-mutated cells may, at least in some cases such as PTCL70, constitute a subclone in the RH OA-mutated clone. TET2- and IDH2-mutated AITL samples were reported to have more extensive histone modification profiles than those with TET2 mutations without an IDH2 mutation, while the difference in genome-wide cytosine methylation profiles between these samples was only moderate.

Our data showed that B cells that have infiltrated AITL tissues also have gene mutations: the multilineal mutations represented by those in TET2 and DNMT3A, and B-cell-specific mutations represented by those in NOTCH1 and other genes. Monoclonal or oligoclonal expansion of B cells has been found in up to 30% of AITL cases. Furthermore, approximately 10% of AITL cases develop B-cell malignancies during their clinical course. Some lymphoma cells are infected by Epstein–Barr virus. In such cases, Epstein–Barr virus is proposed to contribute to the transformation of B cells. This hypothesis, however, needs to be re-evaluated because Epstein–Barr virus was not detected in a substantial proportion of B-cell malignancies accompanying AITL. TET2 mutations are found in diffuse large B-cell lymphomas. Tet2-deficient mice show the expansion of both B- and T-cell lineages in addition to prominent myeloproliferation. Combinational loss of Tet1 and Tet2 provokes B-cell malignancies in mice. Activating NOTCH1 mutations were reported in diffuse large B-cell lymphomas, chronic lymphocytic leukemia, mantle cell lymphoma, and follicular lymphoma. In our cohort, all three NOTCH1 mutations were defined only in B cells with very high allele frequencies (Figure 5, Supplementary Table S9) and two of the three samples showed oligoclonality of B cells (Table 3). This implies that the origin of NOTCH1 mutation is earlier than the acquisition of hypermutation of the CDR3 region in the immunoglobulin gene. Anyway, acquisition of these mutations in B-cell lineage may account for the frequent occurrence of B-cell lymphomas in AITL. Moreover, our data alert us to the need for careful interpretation of the mutational profiles of PTCLs because some of the mutations may not exist in tumor cells.

In conclusion, our findings illustrate the concept of multistep and multilineal tumorigenesis in nodal T-cell lymphomas (Supplementary Figure S3). Understanding the pathogenesis will lead us to better management of nodal T-cell lymphomas in future.

| Table 3. VDJ rearrangement status of B-cell clones in B-cell-specific mutated samples |
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| **Sample** | **Diagnosis** | **Number of colonies having the identical VDJ gene usage/total number of colonies analyzed** | **Common VDJ gene usage** | **Identity of V gene (%)** | **Amino-acid sequences of junctions** |
| PTCL63 | AITL | 2/12 | V3-21/D2-2/J5 | 72.2 | CARSTQTYQOLLWNG#NWFDPSW* |
| PTCL74 | AITL | 2/12 | V1-2/J1 or J2 or J3 | 84.4 | Not identified at http://www.imgt.org |
| PTCL78 | AITL | 2/12 | V3-23/J6/D4-17 | 72.2 | CAGKNDGYDSSSYGGMDW |
| PTCL126 | PTCL-NOS/nodal PTCL with TFH phenotype | 2/12 | V6-1/J6/D3-3 | 71.0 | CATTPTSFQVTAGYYGMDW |

*Out of frame junction. **NA**, not applicable because direct sequencing demonstrated monoclonality.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS
TBN, MS-Y and YA performed experiments and data analysis; KY, YS, KC, HT, SM and SO participated in discussions and interpretation of the data and results. NN, KT, HM and MN and KO were responsible for pathological diagnosis; DK, JK, TM, NN, KT, HM and MN established the LMD procedure; KI and KO collected specimens and were involved in planning the project; TBN, MS-Y and SC generated figures and tables and wrote the manuscript; SC led the entire project; and all authors were involved in planning the project; TBN, MS-Y and SC generated figures and tables and wrote the manuscript; SC led the entire project; and all authors participated in discussions and interpretation of the data and results.

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