Clinicopathological and genetic features of limited-stage diffuse large B-cell lymphoma with late relapse: targeted sequencing analysis of gene alterations in the initial and late relapsed tumors

According to the long-term analysis of several clinical studies, late relapse (LR) is more common in patients with limited-stage diffuse large B-cell lymphoma (DLBCL) than in those with advanced-stage DLBCL,\(^3\) therefore a unique but poorly understood tumor biology has been proposed for limited-stage DLBCL.\(^3\) Cell of origin (COO), defined by gene expression profiling\(^4\) or immunohistology,\(^5\) and patterns of gene alterations, identified by next-generation sequencing, have recently allowed the classification of DLBCL into biologically and clinically distinct subgroups.\(^6,7\) Several studies have evaluated the genetic alterations in DLBCL that occur between initial diagnosis and relapse;\(^8,9\) however, there is limited information concerning such alterations in LR. This study focused on patients with limited-stage DLBCL who developed LR and evaluated the clinicopathological and genetic features of paired specimens obtained at initial diagnosis and LR. We aimed to reveal the biological background and mechanisms of LR.

The retrospective study protocol was approved by the Institutional Review Board of the National Cancer Center Hospital (NCCH). Detailed methods are available in the Online Supplementary Appendix. We defined LR as the first relapse of any B-cell lymphoma (including indolent B-cell lymphoma) that occurred at >5 years after the initial diagnosis. Between 1997 and 2012, 334 consecutive patients with limited-stage \textit{de novo} DLBCL were treated using CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) with or without rituximab therapy at the NCCH. Regular recurrences were observed (Figure 1), and of the 334 patients, 16 developed LR during a median follow-up period of 8.9 years (interquartile range [IQR]: 6.2–12.3 years). Additionally, three patients developed LR after initial treatment at other institutions and were subsequently referred to the NCCH. Then, 19 patients (patients #1-19) were subjected to the clinicopathological and genetic evaluations (Tables 1, Online Supplementary Table S1–2).

Immunohistochemical analysis of formalin-fixed, paraffin-embedded (FFPE) tissues was performed using antibodies against MYC, BCL2, programmed death-ligand 1 (PD-L1), and COO markers (CD10, BCL6, MUM1) (Online Supplementary Table S3). Fluorescence in situ hybridization (FISH) was performed using probe mapping 9p24.1 for specimens with PD-L1 tumor expression and commercial MYC break-apart probe.

Genomic DNA (gDNA) was extracted from (i) the primary tumor, (ii) LR tumor, and (iii) control bone marrow without lymphoma infiltration in 12 patients (patient #1-12) who were eligible for further genetic evaluations. Immunoglobulin H (IgH) clonality assays and target sequencing of 64 lymphoma-related genes (Online Supplementary Table S4) were performed using the paired gDNA samples from the initial diagnosis and relapsed tumor. Mutations in the paired tumor specimens, which were defined as described in the Online Supplementary Appendix and Online Supplementary Table S5, were compared between samples, and shared/non-shared mutations among the samples in the pair were identified.

The median age at the initial diagnosis of the 19 patients was 60 years (range: 32–77 years), 13 (68%) patients had stage I disease, and all had low or low-intermediate International Prognostic Index (IPI) risk. The median duration from the initial diagnosis to LR was 8 years (range: 5–18 years). At LR, 13 (68%) patients had an advanced-stage disease, and 10 (53%) had high-intermediate or high IPI risk (Table 1).

At initial diagnosis, 14 of the 19 patients (78%) had a non-germinal center B-cell like (non-GCB) DLBCL, although 4 patients experienced relapse that involved a different COO subtype (from the initial diagnosis). At LR, three patients relapsed with indolent lymphoma as...
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Table 1. Characteristics of 19 patients who developed late relapsed diffuse large B-cell lymphoma

|                      | At initial diagnosis | At late relapse |
|----------------------|----------------------|-----------------|
| Median age, years (range) | 60 (32–77)           | 71 (45–84)      |
| Sex, male : female, n | 11 : 8               |                 |
| Ann Arbor clinical stage, I / II / III or IV, n | 13 / 6 / 0 | 5 / 1 / 13 |
| Number of extranodal diseases, 0/1/2, n | 8 / 11 / 0 | 2 / 10 / 7 |
| IPI risk, n        |                      |                 |
| Low/low-int        | 19                   | 9               |
| High-int/high      | 0                    | 10              |
| Site of extranodal disease, n |          |                 |
| Gingiva            | 3                    | 0               |
| Nasal or paranasal cavity | 3            | 2               |
| Testis             | 3                    | 3               |
| Skin or subcutaneous | 0                  | 6               |
| Bone or bone marrow | 0                  | 4               |
| Intestine or colon | 0                    | 3               |
| Liver              | 0                    | 2               |
| Other sites        | 2                    | 6               |
| Immunohistochemistry |                     |                 |
| Cell of origin     | 4 / 14 / 1 / 0       | 5 / 12 / 0 / 2  |
| GCB / non–GCB / NA / non–DLBCL, n |           |                 |
| PD-L1 on tumor cells, +/-       | 0 / 19               | 2 / 17         |
| BCL2, +/-/NA, n        | 9 / 7 / 3            | 11 / 5 / 3     |
| MYC, +/-/NA, n         | 6 / 7 / 6            | 5 / 8 / 6      |

DLBCL: diffuse large B-cell lymphoma; IPI: International Prognostic Index; GCB: germinal-center B-cell-like; NA: not assessed; PD-L1: programmed death-ligand 1; int: intermediate.

defined by histology. Two of the 19 patients (#8 and #16) exhibited tumor expression of PD-L1 at LR, despite having negative expression at initial diagnosis. The FISH analysis revealed a 9p24.1 gain in patient #8 (Online Supplementary Figure S1). The expression of MYC was evaluated in 13 pairs; it turned positive at LR in four patients, one of whom gained an MYC translocation detected by FISH analysis. BCL2 expression was evaluated in 15 pairs; seven pairs were positive, and three became positive at LR (Online Supplementary Table S6).

The summary of the results and representative examples of the IgH clonality assays are shown in the Online Supplementary Table S7 and Online Supplementary Figure S2. Of the 12 pairs, 7 (58%) were judged to be clonally related, 2 (17%) clonally unrelated, while the clonal relationship could not be determined in 3 (25%).

The summary of the target sequencing results is described in the Online Supplementary Table S8-9. Patient #9 was excluded from further analysis due to the poor specimen sequence quality at initial diagnosis.

The key results of the targeted sequencing, plus the clinicopathological information and IgH clonality findings are shown in Figure 2. Shared mutations were detected in 9 of the 11 patients. The most frequent shared mutation (n=7) was a CD79B missense mutation that involved a single substitution in the immunoreceptor tyrosine-based activation motif domain (Y196 [n=5] or L199 [n=2]). Next to this was an MYD88 missense mutation (L265P) (n=5). Four patients had shared mutations in both of the CD79B and MYD88 genes. Of the eight patients with shared mutations in the CD79B and/or MYD88 genes, all had non-GCB DLBCL. From the IgH clonality analysis, 6 of the 8 patients had a clonally related relapse. All except 1 (patient #11) with tumor mutations in the CD79B and/or MYD88 genes had initially presented with an extranodal disease in the testis (n=3), nasal/paranasal cavity (n=2), or gingiva (n=2).

In our study, non-GCB types were more frequent in patients who developed LR, which is contrary to several previous studies. The difference in clinical characteristics of patients might explain this discrepancy. Our study focused only on the patients with limited-stage DLBCL and excluded those with transformed B-cell lymphoma at initial diagnosis, which might have lowered the number of patients with the GCB type.

Two recent large-scale studies, which examined the genetic characteristics of untreated DLBCL reported a similar genetic subtype of DLBCL, which typically involved somatic mutations in the CD79B and MYD88 genes. These subtypes were described as the MCD type and cluster-5 type, which was associated with the activated B-cell type of COO and extranodal involvement. Our results suggest that tumors with CD79B and/or MYD88 mutations in eight patients may belong to this genetic subgroup; at least, tumors with both MYD88 and MYD88 mutations would belong to this subgroup. In the current study, patients with MYD88 and MYD88 tumor mutations all had the non-GCB type and extranodal sites involvement (except for patient #11).

The survival curves of patients with the MCD type and cluster-5 type showed an acute decline within 12 months; after that, a gradual but continuous deterioration. On the one hand, it can be speculated that most patients with these subtypes show aggressive clinical courses. However, limited-stage DLBCL, especially with low IPI risk of these subtypes, might respond to
chemotherapy once, but be prone to continuous relapse, as observed in our study. The paired specimens of 2 patients (#8 and #10) were judged to be clonally unrelated based on the IgH clonality analysis. However, patient #8 had shared mutations in the paired specimens that involved CREBBP, KMT2D, MEF2B, and PIM1. Furthermore, FISH analysis using FFPE specimens revealed BCL2/IGH translocations in the paired tumors (data not shown). Abnormalities in the CREBBP, KMT2D, MEF2B, and PIM1 genes are reportedly associated with lymphomagenesis, and lymphoma-progenitor cells acquiring these mutations may develop DLBCL with different IgH rearrangements in the same patient. The background of the clonally unrelated relapse could not be determined in patient #10, and a rare second primary DLBCL might occur in this patient.

Our findings suggested that the mechanisms of LR were heterogeneous. The acquired overexpression of MYC or BCL2 might be associated with LR in some patients, although it is not considered specific for LR. We cannot definitively comment on the possibility that recurrent mutations were associated with LR; the two most common non-shared mutations involved PIM1 (five samples) and KMT2D (two samples), although they were not consistently detected at initial diagnosis or LR. At LR, two patients expressed PD-L1, and three had acquired loss of function mutations involving B2M, CD58, or CIITA, which might be associated with an immune evasion mechanism related to avoidance of antigen presentation.

The present study has several limitations. First, only a small number of patients developed LR. Second, many of the initial tumor specimens were old, which might have affected the results of the genetic analyses. However, the reliability of shared mutations is likely robust, as it is extremely rare to identify the same mutation in paired samples by chance. Furthermore, we only considered 64 lymphoma-related genes, and other important genetic alterations might be missed. Again, we did not perform the genetic analyses or COO evaluations for all patients with limited-stage DLBCL; thus, it remains unclear whether our findings are truly specific for LR development.

In conclusion, the present study revealed that patients with limited-stage DLBCL and LR tended to have CD79B and/or MYD88 tumor mutations, the non-GCB type of DLBCL, and extranodal disease at the initial presentation. However, the mechanisms of LR appear to be heterogeneous, and further studies are needed to confirm our results.

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doi:10.3324/haematol.2019.235598

Disclosures: TS reports personal fees from Chugai Pharmaceutical, outside the submitted work; DM reports grants and personal fees from Chugai, and personal fees from Takeda, during the conduct of the study; grants from Sanofi, grants and personal fees from Janssen, and personal and professional fees from Eisai, grants and personal fees from Celgene, and personal and professional fees from Kyowa Hakko Kirin.
grants and personal fees from Ono, grants and personal fees from Mundipharma, grants and personal fees from MSD, grants and personal fees from Zenyaku, personal fees from Sumitomo, personal fees from AstraZeneca, personal fees from Bristol-Myers Squibb, personal fees from Ipsen, personal fees from Astellas, personal fees from Amgen, personal fees from Novartis, personal fees from Pfizer, grants from Astellas, grants from Bayer, grants from Symbio, grants from CMIC, grants from Quintiles, grants from IQVIA, outside the submitted work; KJ reports grants and personal fees from Eisai, grants and personal fees from MSD, grants and personal fees from Takeda, grants and personal fees from Janssen, personal fees from Bristol-Myers Squibb, personal fees from Daiichii Institute, grants and personal fees from Mundipharma, personal fees from Nikon Mediphysics, grants and personal fees from Chugai, grants and personal fees from AstaZeneca, grants and personal fees from Abbvie, personal fees from Bayer, grants and personal fees from Ono, grants from Celgene, grants from Zenyaku, grants and personal fees from Celgene, grants from Solasia, grants from Symbio, grants from Astellas, grants from Astellas Amgen, grants from Bayer and Daiichii Institute, personal fees from Kyowa Hakko Kirin, outside the submitted work; KT reports personal fees from Zenyaku Kogyo, grants and personal fees from Eisai, grants and personal fees from Takeda, grants and personal fees from Mundipharma, personal fees from THAYA Bioscience International, grants and personal fees from Kyowa Hakko Kirin, grants and personal fees from Celgene, grants and personal fees from Pfizer, grants and personal fees from Ono Pharmacuetical, personal fees from Yakult, personal fees from Daiichii Institute, personal fees from Bristol-Myers Squibb, personal fees from Meiji Seika Kaisa, personal fees from Solasia Pharma, personal fees from Vasenetch, grants from Janssen, grants from Abbvie, outside the submitted work; YK reports grants, personal fees and other from Pfizer, personal fees from Astellas, personal fees from Symbio, outside the submitted work.

Contributions: ToS collected the data. ToS, SF, YI, SH, SY, SM, WM, TaS, DM, KJ, KT, and YK provided the cases. AM and HT performed the immunohistological evaluations. ToS, JN, SY, and TU performed the genetic analyses and interpreted the data. ToS and SF wrote the preliminary draft of the manuscript. All authors revised the subsequent drafts and approved the final version for submission.

Acknowledgments: the authors would like to thank laboratory technicians MS, ST, MM and IM for performing FISH analysis and extra immunostaining for this study.

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