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Exploring tumor clonal evolution in bone marrow of patients with diffuse large B-cell lymphoma by deep IGH sequencing and its potential relevance in relapse

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Dear Editor,

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoma and accounts for 30–40% of all B-cell non-Hodgkin lymphoma (NHLs)1,2. Many patients respond to the first-line regimens such as R-CHOP3. However, up to one-third of patients have refractory and relapse disease associated with poor outcome. DLBCL is heterogeneous with respect to morphologic, immunophenotypic, and molecular features4, and encompasses numerous variants, subgroups, and subtypes. Approximately 10–15% of DLBCL patients have bone marrow (BM) involvement5–7. Morphologically, lymphoma involvement in the BM of DLBCL patients can either be classified as large B-cell lymphoma (histologically concordant) or indolent, low-grade B-cell lymphoma (histologically discordant).

Tumor clonal dynamics and evolution in the BM of patients with DLBCL have not yet been studied in detail. Questions remain whether the lymphomatous involvement in BM in DLBCL patients represents a direct dissemination of tumor cells from the dominant tumor clones in the nodal or extranodal location to the BM. It is also unknown whether tumor-related cells can be detected in the BM when there is no morphologic evidence of lymphoma, and whether detection of these cells can have prognostic utility. Deep immunoglobulin heavy-chain gene enables the study of tumor evolution in B-cell malignancies, and has been previously employed in elucidating the patterns of clonal evolution in DLBCL relapse8 based on a combination of VDJ determination, somatic hypermutation profiling, and phylogenetic analysis.

In our study, we selected 28 DLBCL cases with paired staging bone marrows (BM) (Supplementary Table 1). IGH VDJ deep sequencing and phylogenetic analysis were performed (Supplementary Figs. 1, 2) on the diagnostic DLBCL samples and their matched BM samples (n = 29). In addition, two patients (#22 and #45) had posttreatment BM only, and are included for separate case analysis (see below). The dominant rearrangements identified in the DLBCL are listed in Supplementary Table 2. The phylogenetic relationships between the dominant tumor clones of the diagnostic DLBCLs and the tumor-related clones identified in the corresponding staging BM samples are summarized in the heatmap (Fig. 1a, b), and representative phylogenetic trees are shown (Fig. 1c).

Minor tumor-related clones were detected in 19 (82.6%) of the 23 BM samples which did not exhibit morphologic evidence of lymphomatous involvement. These analyzes demonstrate that these minor clones are present in the BM in minute quantity, ranging from ~0.01% to 1.98%. (Supplementary Table 2A). Among the minor tumor clones identified in these 23 BM samples, 6 (26.1%) were divergent/ancestral (DA)-type only, 7 (30.4%) were identical (I)-type only and 5 (21.7%) were both DA and I. No tumor clones were detected in five BM samples. Our results indicate frequent minimal
disease involvement by tumor-related clones detectable by deep IGH sequencing in morphologically normal BM of patients with DLBCL.

We also studied the clonal patterns in BM with morphologic evidence of involvement. Major clones were detected in all six BM specimens with morphologic evidence of lymphoma (DLBCL or low-grade B-cell lymphoma) (Fig. 1b; Supplementary Table 2C). One of them (16.7%) is of DA-type only, and two are of I-type only (33.3%). Minor I-type clones were also found in two of the three BM cases with major DA-type clones. The BM sample with low-grade B-cell lymphoma harbored I-type and DA-type tumor clones of approximately equal abundance. No significant difference in distribution of the DA and I clone types was observed between the BM with or without morphologic evidence of lymphoma.

As DLBCL relapses occur primarily via divergent evolution6, we hypothesize that there may be an association between the presence of DA tumor clones in the BM and subsequent relapse. Among the 22 DLBCL patients with no morphologic evidence of involvement, 12 had clinical follow-up. Four of these 12 patients relapsed with DLBCL. Interestingly, DA-type minor tumor clones were detected, either solely or with I-type clones, in the staging marrow of all four patients. For the other eight patients who did not relapse, DA-type clones were detected in only two of them. None of the six patients without detectable tumor clones or I-type clone only in the BM developed relapse.
generating the pre-R clone. (Fig. 2b). Similar DA clones ancestral precursors which are potentially capable of VDJ sequencing identiﬁed the dominant lymphoma clone in the diagnostic DLBCL. They are sub-classiﬁed as identical (designated as “†”) if they showed a SHM pattern that was identical or immediately descendant to the dominant lymphoma clone in the diagnostic DLBCL. In addition, a major clone is deﬁned as a clone for which the VDJ rearrangement is the most abundant among other VDJ rearrangements; whereas a minor clone is deﬁned as a clone not representing the most abundant VDJ rearrangement. The presence and subtype(s) of tumor-related clones (DA = divergent or ancestral, I = identical) in the BM for each patient is indicated in a heatmap-like chart. Orange denotes that the clones in the BM are major, and green denotes that the tumor clones in the marrow are minor. Cases without any detectable tumor clones are shown as blank. In five of these 23 samples without morphologic evidence of lymphoma (#28, 32, 41, 42, and 43), ﬂow cytometric abnormalities were detected. VDJ analysis identiﬁed dominant B-cell clones unrelated to the DLBCL tumor clones in four of the ﬁve cases (see Supplementary Table 2B), implying that the abnormalities seen in the ﬂow cytometry do not represent tumor involvement in the BM but instead are incidental ﬁndings. In the remaining case (#41), no dominant B-cell clone was detected in the BM; however, CLL-like B-cells were detected by ﬂow cytometry, which are most consistent with monoclonal B-cell lymphocytosis of undetermined signiﬁcance (CLL-type). Patients in which comparisons were made between relapsed DLBCL samples and matched staging BM samples because of the unavailability of the original diagnostic DLBCL were marked with asterisks. For patients with no morphologic evidence of lymphoma involvement in BM, the presence or absence (indicated by R or 0 in the clinical status) of subsequent relapse was indicated. Patients who were lost to follow-up or did not have staging BM samples at diagnosis are excluded (colored gray). Representative phylogenetic trees comparing tumor-related clones detected in the bone marrow and in the diffuse large B-cell lymphomas present in the lymph nodes. The dominant tumor clones identiﬁed in the DLBCL are marked with asterisks. BM tumor clones identical to the dominant DLBCL clones in the LN (I-type clones, also see Fig. 1) are marked with dashed lines. BM tumor clones divergent or ancestral to the dominant DLBCL clones in the LN (DA-type clones) are marked with solid lines. Sequence reads derived from the LN and BM are indicated in blue and red, respectively, and the lengths of the horizontal bars reﬂect the numbers of reads. The somatic hypermutation proﬁle in the IGH VJ region (right) corresponding to each horizontal bar (left) is shown, with each nucleotide mismatch from the germline sequences indicated by a vertical colored bar. Representative ﬁgures in four different cases are shown (i–iv). (i) No histologic evidence of lymphoma in the BM (Pt. #42). Minor DA tumor clones are detected. (ii) No histologic evidence of lymphoma in the BM (Pt. #19). Both minor DA and I clones are detected. (iii) BM involvement by DLBCL (pt. #35). Tumor clones of the DA-type are identiﬁed. (iv) BM involvement by DLBCL (Pt. #15). The DA tumor clones predominate in the BM, and I-type clones are present in relatively low abundance (Supplementary Table 3). These ﬁndings suggest a possible association of detection of minor DA-type tumor clones in the staging marrow with subsequent relapse (p = 0.06, Fisher’s exact test).

In our cohort, we identiﬁed three patients (#29, #45, and #22) who had posttransplant DLBCL relapse (Supplementary Table 1). Patient #29 serves as an excellent example to illustrate a direct link between these minor DA clones in the BM and subsequent DLBCL relapse (Fig. 2a). Both the pre-transplant and posttransplant BMs showed no morphologic evidence of lymphoma or ﬂow cytometry abnormalities. However, we identiﬁed in the staging BM a minor DA-type clone which persisted in the posttransplant BM, implying chemoresistance. Importantly, this DA clone has an identical SHM pattern to the dominant tumor clone in the relapsed DLBCL, and therefore represents the actual relapse precursor clone (pre-R).

For #45, a pre-transplant marrow taken during remission post R-CHOP was not diagnostic for lymphoma based on morphologic grounds, but ﬂow cytometry identiﬁed a small monotypic B-cell population with similar immunophenotypic proﬁle as the diagnostic DLBCL which represented ~2.5% of analyzed cells. Deep VDJ sequencing identiﬁed minor DA clones representing ancestral precursors which are potentially capable of generating the pre-R clone. (Fig. 2b). Similar DA clones were also seen in the posttransplant BM involved by a low-grade B-cell lymphoma with ~10–20% of involvement, suggesting that these ancestral minor DA clones are most likely chemoresistant and have expanded to form frank lymphoma in the BM. Interestingly, in the peripheral blood stem cell harvest (45-SC) sample, minor DA clones closely related and slightly divergent to the relapsed DLBCL were detected. However, the actual pre-R clones might be exceedingly rare in the BM or PB and escape detection. Similar clones representing ancestral precursors were also observed in the posttransplant marrow in patient #22 (Supplementary Fig. 3). Patient #22 eventually relapsed; however, the relapse sample was not available for molecular analysis.

Our ﬁndings demonstrate the feasibility of this NGS method to detect minimal lymphoma involvement in the marrow. Our analysis based on DLBCL and the corresponding staging marrows revealed that tumor-related clones can be detected frequently in BM without morphologic evidence of lymphoma using this highly sensitive technique. A major ﬁnding from our study is the frequent detection of DA-type tumor clones. These ﬁndings underscore tumor heterogeneity and also suggest distinct biological properties of these DA tumor clones, which may have higher intrinsic propensity to disseminate to the BM compared with the dominant tumor clones despite their low abundance in the
Fig. 2 (See legend on next page.)
diagnostic tumor. In addition, our study shows that morphologically concordant involvement of the BM does not necessarily imply genetic concordance. Three of the 5 BM involved by DLBCL that were analyzed harbored dominant/major DA-type clones, suggesting that overt BM involvement in patients with DLBCL is not simply a direct extension of the nodal/extranodal DLBCL to the BM but can represent a tumor divergently evolving in parallel to the main tumor. These findings may have important therapeutic implications, as it is conceivable that the tumor in the bone marrow may demonstrate different drug responsiveness from the extramedullary tumors because of the diverse genetic composition.

Significant difference was not detected in the distribution of clonal types (i.e., DA- or I-type) between the involved or uninvolved BM samples based on analysis of a small cohort. Similar patterns of clones can be seen in both involved and uninvolved BM. These observations suggest a model of tumor progression in the BM, initiating from minute tumor-related clones not detected by morphology and expanding to overt lymphoma (Supplementary Fig. 4).

There seems to be a possible association between the detection of minor DA-type clones in the staging BM of patients without evidence of lymphoma involvement, and subsequent relapse. This association is in line with the previous observation that DLBCL relapse occurs predominantly via divergent evolution. A statistical trend (p ~ 0.06) could be detected based on analysis of a limited number of samples. However, a larger sample size is needed to confirm this association.

Our study of two illustrative cases suggest that identification of minor DA-type clones in the BM/circulation in a posttreatment setting may signify the presence of pre-R (relapse precursor) clones and herald increased risk of relapse. Recent studies have demonstrated the utility of monitoring clonal VDJ rearrangements and gene mutations in cell-free DNA in predicting DLBCL relapse. Our assay provides a simple, feasible, and highly sensitive methodology to detect relapse precursor clones directly. Our study serves as a pilot proof-of-principle investigation that suggests serial monitoring of divergent circulating tumor clones from post-therapy bone marrow, peripheral blood or plasma at different time points to decipher clonal dynamics of these clones may be of clinical value to predict relapse. It is conceivable that emergence or rise in the abundance of one of these divergent subclones may serve as a predictive marker of impending relapse. Additional investigation on a larger cohort is necessary to examine in detail the association between the minor post-therapy tumor-related clones, chemoresistance, and subsequent DLBCL relapse.
Authors’ contributions
J.M. designed and performed the experiments, analyzed the data, and wrote the paper; D.R. performed analysis of the data; A.M. performed the experiments and analyzed the data; A.S.N. assisted in analyzing the data; K.N. performed the experiments; S.M. provided critical clinical material; W.T. and O.E. conceptualized and directed the project, designed the experiments, interpreted the data, and wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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