Polytypic B cells, monotypic/monoclonal B-cell proliferations, and neoplastic T cells diverge from *TET2-/DNMT3A*-mutant clonal hematopoiesis in follicular helper T-cell lymphomas

Follicular helper T-cell lymphomas (TFHL) frequently develop upon a background of *TET2-/DNMT3A*-mutant clonal hematopoiesis (CH).[1] Monotypic/monoclonal B-cell proliferations (MBP), which are often, but not invariably, Epstein-Barr virus-positive (EBV$^+$), commonly complicate TFHL.[2-5] While rare reports found B cells can share *TET2/DNMT3A* mutations with TFHL,[6,7] MBP pathogenesis is incompletely understood. Here, we assessed mutational profiles of paired TFHL and polytypic B cells (PolyBC) or MBP in the context of EBV status in 25 TFHL patients.

Cases with sufficient tissue for sequencing from both a TFHL and either PolyBC or an MBP were genotyped utilizing targeted next-generation sequencing (NGS) or, in select cases (N=4), droplet digital polymerase chain reaction as previously described[1] on bulk samples or flow cytometry (FC)-sorted cells, including T-, B-, and myeloid, as available (*Online Supplementary Table S1*), with an allele frequency (VAF) cut-off of >0.02. In bulk samples, mutant VAF and percentages of cell types were compared to determine the cellular compartment in which a variant was present. (See *Online Supplementary Table S1* for details.) The study was approved by the Memorial Sloan Kettering Institutional Review Board.

First, we analyzed FC-sorted PolyBC from 11 TFHL patients (median age 67 years [range, 38-81 years]; 7 females and 4 males). PolyBC were immunophenotypically normal with polytypic light chain expression by high-resolution FC (sensitivity at least 0.01%) and, if available, lacked morphologic evidence of a B-cell proliferation. PolyBC were collected pre- (N=6) or post- (N=5) TFHL-directed therapy (Figure 1) and included 7 EBV$^+$ and 3 EBV-negative (EBV$^-$) patients (one unknown) (Figure 2). Two patients (patient 1 and patient 8) whose PolyBC were obtained post therapy had histories of MBP; however, 0/11 PolyBC patients subsequently developed an MBP.

PolyBC harbored identical mutations as corresponding TFHL in 7/11 patients (64%), consisting of *TET2* (6/7) and *DNMT3A* (3/7) (median VAF, 0.11 [range, 0.02-0.27] and 0.04 [range, 0.03-0.49], respectively) (Figure 2, *Online Supplementary Table S1*). Only one PolyBC sample (9% of total, 14% [1/7] of samples genotyped by NGS) harbored a private mutation absent in its TFHL (patient 2, *TET2*). The incidence of shared mutations in PolyBC and TFHL was not significantly different among EBV$^+$ and EBV$^-$ patients or among those with PolyBC sampled pre- and post-treatment (*Online Supplementary Figure S1A, B*). *TET2/DNMT3A* mutant VAF in PolyBC did not differ among EBV$^+$ and EBV$^-$ patients while a trend towards lower VAF was present among pre-treatment samples (*Online Supplementary Figure S1C, D*). This suggests PolyBC commonly arise from the same CH-harboring precursor as TFHL, irrespective of EBV or treatment status, but uncommonly harbor private mutations.

Next, paired TFHL and MBP from 14 patients were evaluated (median age, 74 years [range, 67-82 years]; 3 females and 11 males). MBP were collected prior to (N=8) or following TFHL- or MBP-directed treatment (N=6) (Figure 1) and included large B-cell lymphoma (LBCL)-like (N=5), polymorphic (N=6), and follicular lymphoma-like (N=2) proliferations, and plasma cell myeloma (N=1) (Figures 2, 3A-S). Four of 5 LBCL-like, 6/6 polymorphic, and 1/2 follicular lymphoma-like proliferations were EBV$^+$ (Figure 2). Identical mutations within MBP and TFHL were identified in 9/14 patients (64%), consisting of *TET2* (9/9), *DNMT3A* (5/9), and *TET3* (1/9) (Figure 2, *Online Supplementary Table S1*). Additionally, 2 MBP without shared mutations (CH$^-$) harbored private *TET2* mutations, resulting in 11/14 (79%) MBP exhibiting *TET2* and/or *DNMT3A* mutations in total (median VAF, 0.42 [range, 0.10-0.56] and 0.27 [range, 0.05-0.50], respectively). The shared *TET2/DNMT3A* mutant VAF in MBP were significantly higher than those in PolyBC (Figure 3T). Twelve of 14 (86%) MBP harbored private mutations absent in corresponding TFHL (VAF, 0.10-0.99 [median, 0.34]) (Figure 2, *Online Supplementary Table S1*), an incidence significantly higher than in PolyBC (Figure 3U). Although few private mutations were recurrent, overall, they most affected signaling (N=22; e.g., *DTX1, KRAS, EPHA5*), epigenetic / transcriptional regulation (N=14; e.g., *TET2, KMT2D, SETD5*), and DNA damage response (N=3; e.g., *ATM, CHEK2, BRCA2*) genes. This suggests MBP commonly arise from CH-harboring B cells but likely require additional genomic aberrations for transformation.

Neither the incidences of shared or private mutations nor the VAF of shared mutations differed among EBV$^+$ and EBV$^-$ or between pre- and post-treatment MBP (*Online Supplementary Figure S1E-J*). The private mutant VAF did not differ among EBV$^+$ and EBV$^-$ or between CH$^+$ and CH$^-$ MBP (*Online Supplementary Figure S1K, L*). We saw no significant difference in the incidence of EBV positivity or of shared or private mutations or obvious difference in the types of private mutations among the MBP types (*data not*

shown). CH mutations and EBV may thus not function in a mutually exclusive manner but may act separately or in concert to promote B-cell survival and proliferation.

We also sequenced separate samples containing PolyBC from 4 MBP patients (patients 12, 15, 17, and 19) (*Online Supplementary Figure S2*). Three samples with PolyBC were genotyped in bulk, precluding assessment of shared mutations; however, none of them harbored the private mutations detected in the corresponding MBP despite a high PolyBC content (20-35%) and high private mutant VAF in the MBP. The FC-sorted PolyBC from patient 17 demonstrated the same *TET2* and *DNMT3A* mutations as those in their TFHL, MBP, and myeloid compartment, although at lower VAF (all around 0.2 *vs*. 0.47-0.50 in the MBP). They also harbored the MBP's private *TET2* and *PIK3C3* mutations, also at significantly lower VAF (0.11 and 0.04 *vs*. 0.48 and 0.52 in the MBP). This MBP shortly followed the PolyBC-containing sample (4.6 months). This supports the concept that gain of private mutations occurs with MBP development and acquisition of certain private mutations may portend imminent transformation.

Myeloid compartments in 4/8 (50%) PolyBC and in 9/12 (75%) MBP patients shared *TET2* and/or DNMT3A mutations with PolyBC and TFHL (median VAF, 0.22 [range, 0.06-0.47] and 0.26 [range, 0.07-0.46]) and with MBP and/or TFHL (median VAF, 0.26 [range, 0.05-0.97] and 0.14 [range, 0.07-0.48]), respectively (Figure 2, *Online Supplementary Table S1*), supporting the concept that the same CH-harboring precursor often gives rise to all 3 hematopoietic compartments in TFHL patients. The shared *TET2/DNMT3A* mutant VAF did not differ between myeloid compartments of PolyBC and MBP patients (Figure 3V), suggesting the level of background CH may not predict MBP risk.

While proposed contributors to clonal B-cell outgrowth in TFHL patients include latently EBV-infected B-cell expansion due to defective immune surveillance and pro-proliferative properties inherent in neoplastic TFH cells,[5] contributions of genomic alterations were unknown. We show both PolyBC and MBP in TFHL patients often harbor EBV and share *TET2/DNMT3A* mutations with TFHL, supporting origin from a common CH-harboring precursor. In contrast, private mutations are nearly exclusive to MBP, which in conjunction with higher shared *TET2/DNMT3A* mutant VAF than PolyBC, suggest MBP may arise from preferential outgrowth of pre-existing CH^+ clones, which is driven by additional private alterations. Indeed, among

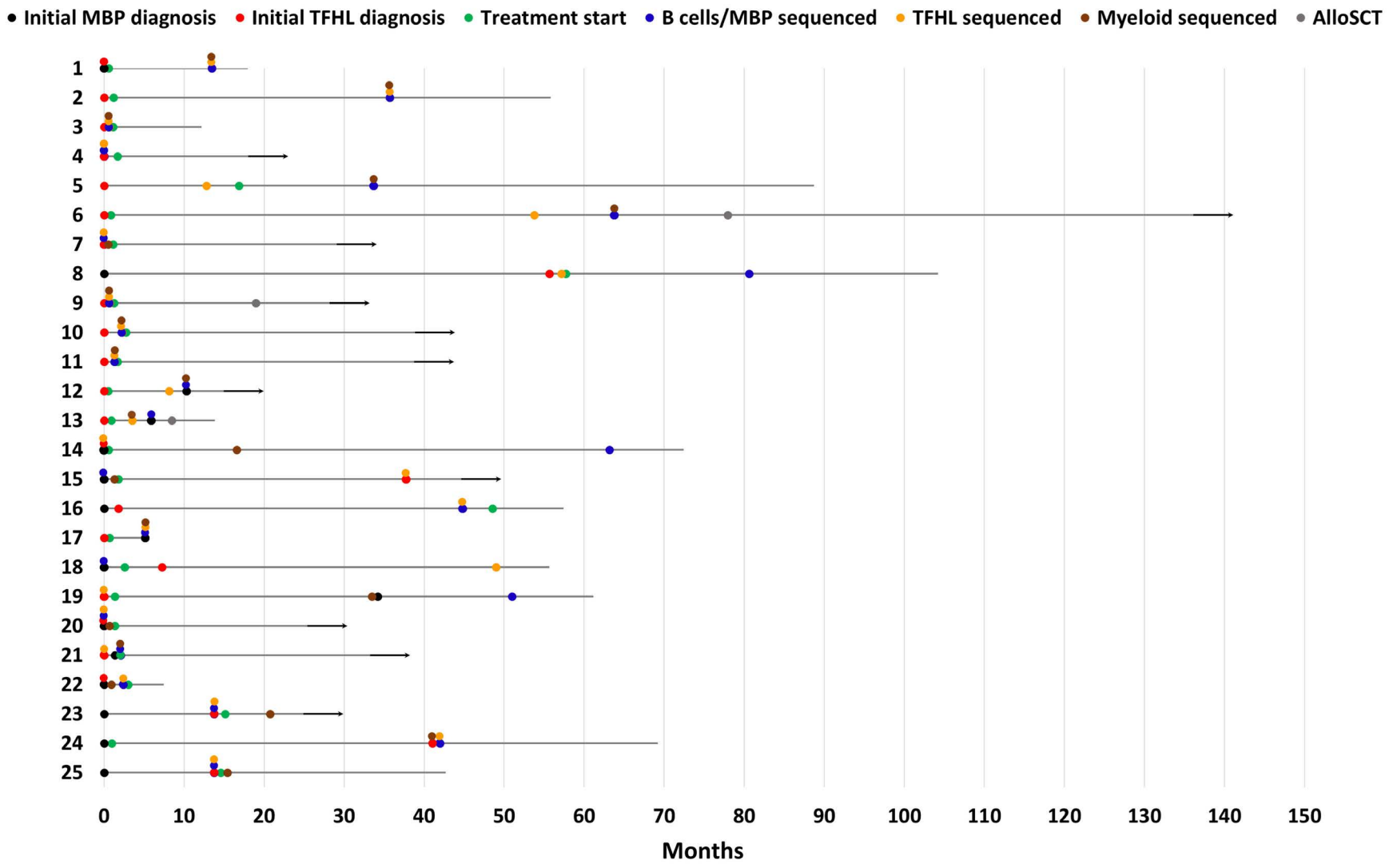

Figure 1. Clinical timepoints and outcomes of 25 follicular helper T-cell lymphoma patients. Swimmer plot depicting timepoints at which samples from each patient were collected, systemic therapy was initiated, allogeneic stem cell transplant (alloSCT) occurred, and clinical outcomes. Each row depicts one patient and is indicated by the patient identification number. Month 0 indicates time at which a follicular helper T-cell lymphoma (TFHL) or monotypic/monoclonal B-cell proliferation (MBP) was initially diagnosed. Horizontal arrows indicate patients alive at last follow-up while no arrow indicates a deceased patient.

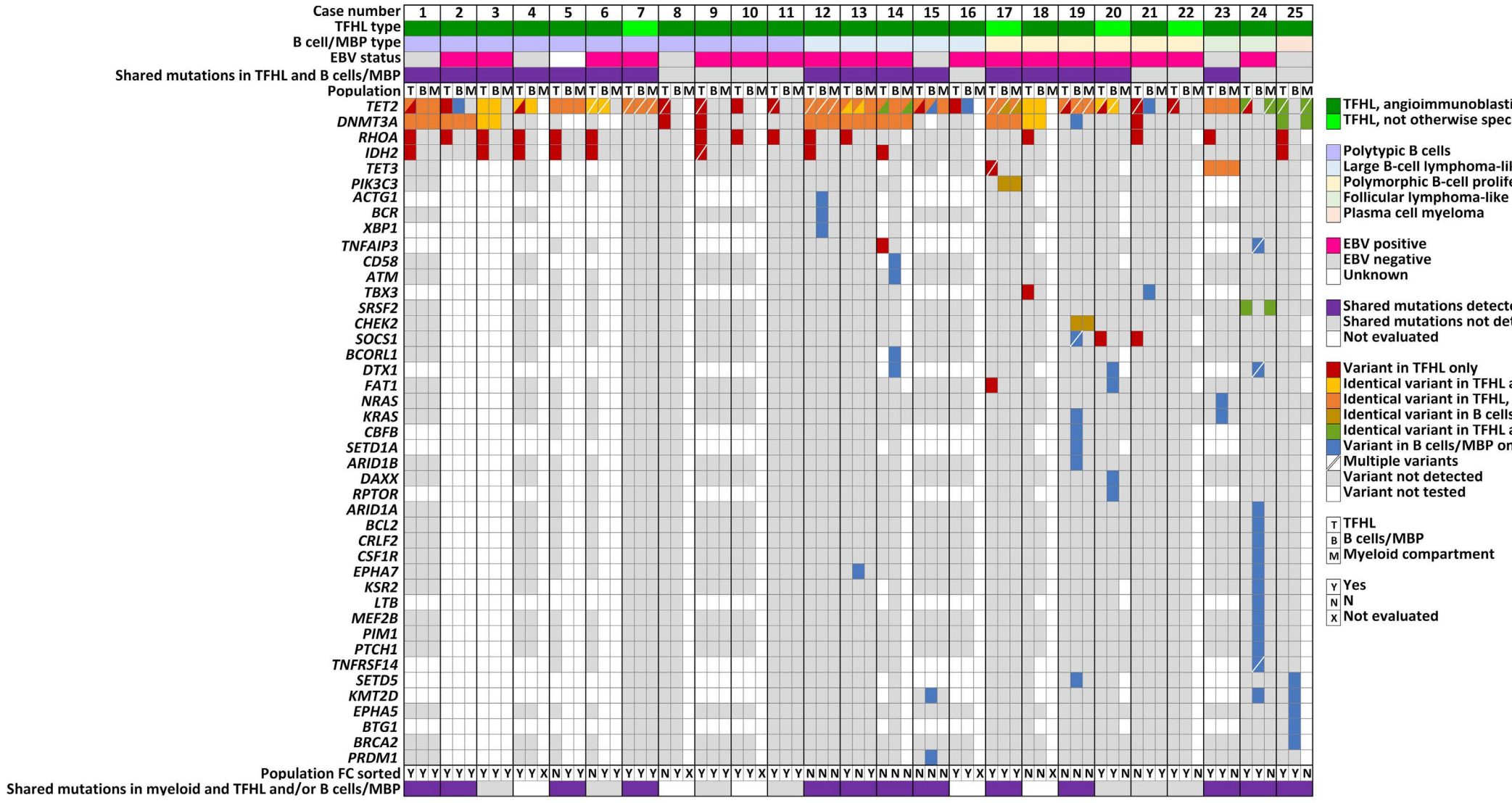

Figure 2. Mutational profiles of various cellular compartments and Epstein-Barr virus status of 25 follicular helper T-cell lymphoma patients. Oncoplot detailing mutations detected in follicular helper T-cell lymphomas (TFHL), polytypic B cells (PolyBC), monotypic/monoclonal B-cell proliferations (MBP), and myeloid compartments. Each column represents one patient, labeled with patient identification numbers, and the subcolumns indicate the cellular compartment in which a mutation was evaluated (T: TFHL, B: PolyBC/MBP, M: myeloid). Only select private mutations identified in TFHL are included (e.g., *RHOA*, *IDH2*). (See *Online Supplementary Table S1* for all identified mutations.) Whether a given cell population was sorted by flow cytometry (FC) is detailed at the bottom and the Epstein-Barr virus (EBV) status of each patient at the time of PolyBC or MBP collection is indicated at the top. EBV positivity was called based on either: i) *in situ* hybridization for EBV-encoded RNA (EBER) reactivity anywhere within tissue sections for PolyBC patients or within the majority of cells for MBP; ii) detection of plasma EBV DNA by quantitative polymerase chain reaction analysis (PolyBC patients only); or iii) off-target EBV reads of ≥10 by next-generation sequencing, as previously described.[15]

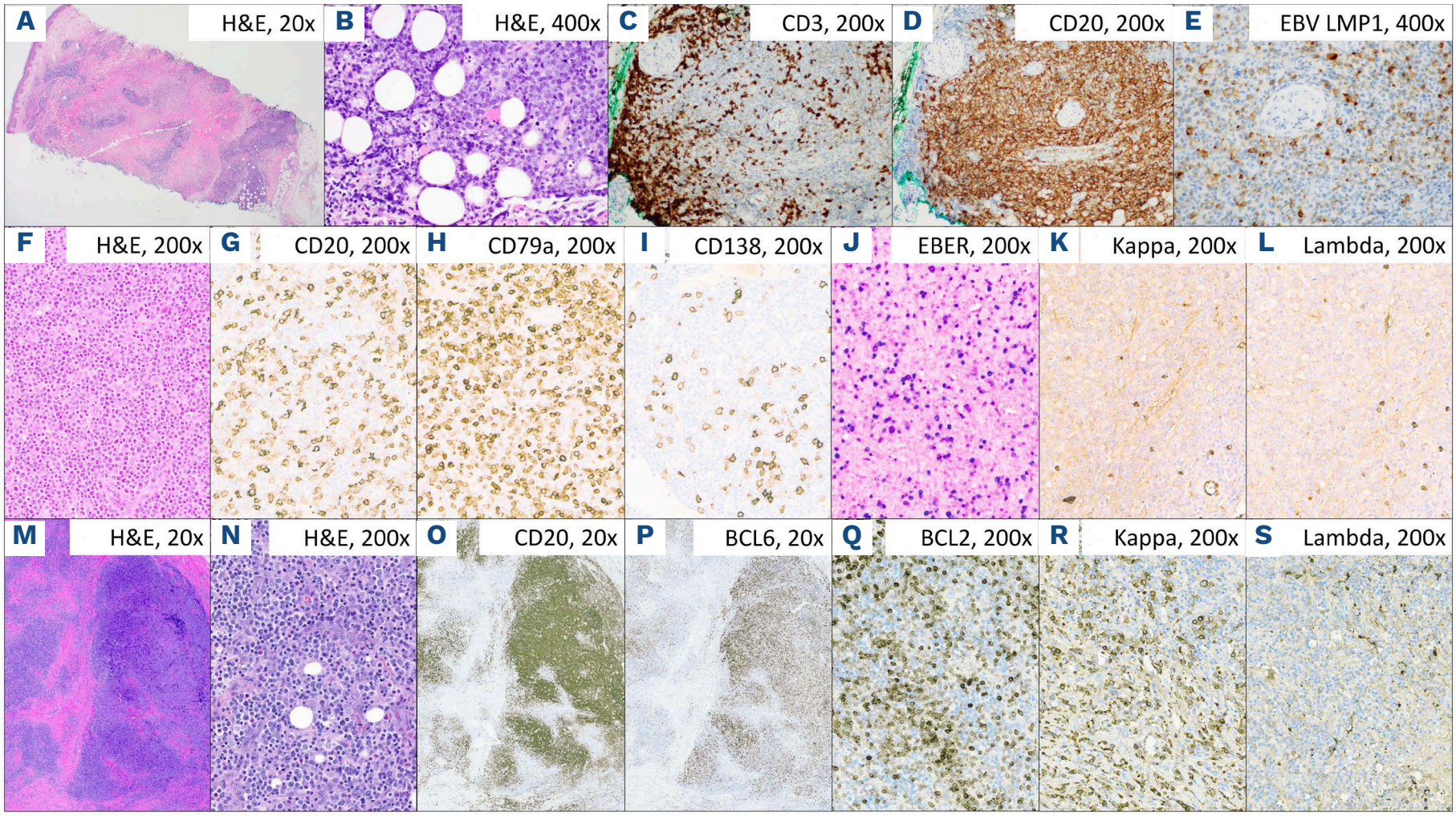

Figure 3. Morphologic and immunohistochemical features of monotypic / monoclonal B-cell proliferations and comparisons between polytypic B cells and monotypic / monoclonal B-cell proliferations. The large B-cell lymphoma-like and follicular lymphoma-like B-cell proliferations, as well as the plasma cell myeloma, all fulfilled 2022 International Consensus Classification / 5th edition World Health Organization criteria for diagnosis of diffuse large B-cell lymphoma, follicular lymphoma, and plasma cell myeloma, respectively. The polymorphic B-cell proliferations showed atypical polymorphic morphology along with an abnormal B-cell immunophenotype by flow cytometry and/or a clonal immunoglobulin heavy chain gene rearrangement. (A-E) Epstein-Barr virus (EBV)-positive large B-cell lymphoma-like proliferation from patient 13. (A and B) This pan-dermal cutaneous proliferation demonstrated vague nodules and sheets of large lymphoid cells with distinct nucleoli and extensive necrosis. The neoplastic cells lacked (C) CD3 expression and expressed (D) CD20 and (E) EBV LMP1. (F-L) EBV-positive polymorphic B-cell proliferation from patient 20. (F) The lymph node was effaced by scattered small B cells, frequent plasmacytoid cells, plasma cells, and few large B cells in a background of scattered intermediate sized neoplastic T cells. (G) CD20 strongly labeled the B cells and weakly labeled the plasmacytoid cells while (H) CD79a highlighted B, plasmacytoid, and plasma cells. (I) CD138 highlighted plasma cells. The B lineage cells expressed (J) EBER and aberrantly lacked both (K) kappa and (L) lambda light chain expression. (M-S) Lymph node from patient 23 involved by an EBV-negative, grade 3B follicular lymphoma-like B-cell proliferation with plasmacytoid differentiation. (M and N) It demonstrated nodules of intermediate to large sized B cells with rounded nuclei with admixed apoptoses and mitoses in a background of increased vascularity, sclerosis, eosinophils, and small to intermediate sized neoplastic T cells. The B cells expressed (O) CD20, (P) BCL6, and (Q) partial, weak BCL2, and showed kappa light chain restriction with (R) kappa and (S) lambda immunostains. (T) The allele frequencies (VAF) of shared *TET2/DNMT3A* mutations (present in both follicular helper T-cell lymphoma and B cells) were significantly higher in the monotypic / monoclonal B-cell proliferations (MBP) than in the polytypic B cells, (U) as was the incidence of private mutations. (V) The VAF of shared *TET2/DNMT3A* mutations within the myeloid compartments (mutations present in myeloid and follicular helper T-cell lymphoma and/or B cells) did not significantly differ between polytypic B cell and MBP patients. In the analyses in (T) and (V), only the variant with the highest VAF was used from patients harboring multiple mutations in the same gene. Horizontal lines in (T) and (V) indicate medians. H&E: hematoxylin&eosin; NS: not significant.

4 patients from whom both MBP and PolyBC were genotyped, only one harbored MBP-associated private mutations in PolyBC with significantly lower VAF. This clone's rapid growth and acquisition of an abnormal monotypic immunophenotype support the concept that gain of such alterations may promote neoplastic progression. Thus, the multi-step model in which epigenetic dysregulation via *TET2/DNMT3A* mutations in pre-malignant hematopoietic precursors precedes lineage commitment and secondary alterations like that described in TFHL and myeloid neoplasms[1,8] also likely applies to MBP. While inclusion of post-treatment samples may have overestimated the incidence of shared mutations, as treatment may select for CH-harboring clones, the lack of significant difference in CH incidence among pre- and post-treatment samples in both PolyBC and MBP patients is reassuring. Nonetheless, this is a limitation of our study.

As CH and EBV positivity in B cells are detected more commonly than MBP occur in TFHL patients (approx. 10%[2]), it is likely that neither is sufficient for MBP development without secondary private genomic alterations. Supporting this, our data showed no correlation between the incidence or level of CH-related or private mutations and EBV status in either PolyBC or MBP. However, given MBPs' relative frequency among TFHL, this unique biology of CH mutations in B cells, EBV reactivation, and unchecked neoplastic TFH cell-mediated stimulation may all increase MBP risk. One TFHL mouse model found *TET2* loss in all blood cells (including B cells) led to expansion of genomically aberrant B cells while *TET2* loss restricted to T cells did not, suggesting CH mutations in B cells may promote their proliferation and clonal expansion.[9] Additionally, our trend towards higher *TET2/DNMT3A* mutant VAF in post-treatment PolyBC suggests treatment could promote CH^+ B-cell outgrowth in a manner akin to therapy-related myeloid neoplasms. Larger studies would be needed to assess whether CH-derived B cells increase MBP risk.

Prior work demonstrated *NOTCH1* variants in microdissected B cells in 3/87 TFHL.[6] While we interrogated *NOTCH1, NOTCH2, NOTCH3*, and *NOTCH4* in all 14 MBP and in 7/11 PolyBC, no mutations were found, possibly due to sample size. However, we detected missense variants in exons 1 and 2 of *DTX1* in 3/14 MPB, which have been described in various B-cell lymphomas.[10-12] DTX1 is a ubiquitin ligase that regulates NOTCH signaling and plays a role in B-cell development.[13] Mutations in *DTX1* appear to hinder its ability to negatively regulate NOTCH signaling.[11] NOTCH pathway activation may thus be important in MBP development in TFHL patients, which can occur via alterations of multiple genes in the pathway.

Lastly, our data support the concept that MBP in TFHL patients are biologically unique. Compared to *de novo* EBV^- LBCL, EBV^+ LBCL more frequently harbor *TET2/DNMT3A* mutations and lack mutations common in activated B-cell-type LBCL (e.g., MYD88, CD79B),[14] similar to our results, suggesting a distinct pathogenesis and potential co-operation between EBV and CH mutations. Additionally, while LBCL are typically aggressive and require intensive treatment, we have observed MBP in TFHL patients often favorably respond to anti-CD20 therapy alone, suggesting an individualized approach is warranted.

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Contributions

NEL, KP-D, RS and AD conceived the study, collected and analyzed the data, and wrote the manuscript. KP-D also annotated the sequencing data. CV, SH, SS, JB and HB collected data. QG and MR helped with flow cytometric analyses and flow cytometry cell sorting. AJM and SMH provided critical clinical information. WX and MR analyzed data and provided critical guidance.

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Data-sharing statement

Data are available upon request from the corresponding authors.

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