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Multiple productive IGH rearrangements denote oligoclonality even in immunophenotypically monoclonal CLL

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Typically, all cells of a chronic lymphocytic leukemia (CLL) clone share a unique structure of B-cell receptor, as they are the progeny of a single B-lymphocyte. This is due to the mechanism of allelic exclusion that ensures the expression of only one allele in immunoglobulin (IG) heavy chain locus (IGH) and one either in IG light chain Kappa or Lambda loci (IGK, IGL) that were productively rearranged. However, current evidence suggests that up to a quarter of CLL patients express not only one but multiple clonal productive IGH rearrangements.[1,2]

In our systematic study of CLL cases with multiple productive IGH rearrangements (MP-IGH CLL),[3] we previously showed that one-third of them exhibit two CLL populations with distinct immunophenotypes. We also pointed to the presence of multiple clones as a likely cause of MP-IGH detection even in cases with an immunophenotypically monoclonal disease, on the basis of IGK, IGL and incomplete IGH gene rearrangement analyses. Still, the underlying biological cause of MP-IGHs possibly involves lack of allelic exclusion[4] or IGHV gene replacement.[5] The extent to which these mechanisms are involved in MP-IGH CLL cases with homogeneous immunophenotype is still not known, and the lack of conclusive evidence hinders further research on CLL oligoclonality. To clarify this phenomenon, we performed extensive analysis of clonality in MP-IGH CLL combining conventional methods (Sanger sequencing, IGH fragment analysis), immunophenotyping, single-cell analysis (SCA), and next-generation sequencing (NGS).

First, we assessed IGH rearrangements from complementary DNA (cDNA) in a consecutive cohort of 1534 CLL patients using Sanger sequencing and fragment analysis (Supplementary Methods). Single clonal productive IGH rearrangement was detected in the vast majority of cases, 75 (4.9%) cases exhibited two clonal productive IGH rearrangements (67/75 cases; 89%) or three (8/75 cases; 11%) concurrently. In all MP-IGH CLL cases, we also analyzed IG light chain rearrangements; a list of all IG rearrangements detected in individual MP-IGH CLL cases including their changes over time is provided in Supplementary Table S1.

The immunophenotype of separated B-lymphocytes was analyzed in detail in 40/75 MP-IGH CLL patients (Supplementary Methods). In 13/40 (32.5%) cases, we identified two leukemic populations being either sIgK$^+$ or sIgL$^+$; using FACS sorting and subsequent IG rearrangement analysis, we matched productive IGH rearrangements to the corresponding IGK and IGL rearrangements (Supplementary Table S1). Nevertheless, the number of detected clonal productive IGH rearrangements exceeded the number of distinct CLL populations in the majority of cases (27/40, 67.5%; 24/27 were immunophenotypically monoclonal). In total 22 of them were submitted to SCA: 20 cases with one leukemic population of homogeneous immunophenotype and two cases with two leukemic populations, but three productive IGH rearrangements.

For SCA, CLL samples containing $>95\%$ of CLL cells were used. Single CD19$^+$ cells were sorted into 96-well plates and transcribed IGH, IGK and IGL alleles were analyzed using multiplex nested RT-PCR and Sanger sequencing (Supplementary Methods). Two confirmed biclonal CLL cases and B-lymphocytes obtained from a healthy donor served as the technique accuracy controls (Supplementary Figure S1).

More than one clone (each in $\geqslant 3$ wells) was distinguished in 18/22 analyzed MP-IGH CLL cases unexplained by immunophenotyping. Each of the clones was characterized by a specific productive IGH rearrangement and all but one also by a productive IGK/IGL rearrangement. The estimated clonal ratios are presented in Figure 1 for all 18 cases and 2 CLL controls. The detailed SCA results are presented in Supplementary Results and Supplementary Table S2. In 4 out of the 22 analyzed MP-IGH CLL cases (nos. 523, 885, 1087 and 1440) only one clone characterized by single productive IGH rearrangement and single productive IGK/IGL rearrangement was detected, presumably due to the insufficient representation of a minor clone in a sample. This assumption was supported by the ASO-qPCR and IG-NGS results (see Supplementary Methods and Supplementary Tables S3 and S4).

Twenty from a total of 111 clonal IG rearrangements (18%) detected in SCA, including both heavy and light chains, were newly identified. This resulted in the identification of an additional clone in patients no. 319 and 1502 (to a total of four clones in both cases). Surprisingly, in patients no. 1049 and 1352, we found a different clone than one of the expected, likely due to unequal rearrangement amplification and/or their low representation in bulk samples. The IG-NGS results confirmed the presence of all minor clonal IGH rearrangements detected by either method, each in a low proportion (Supplementary Tables S3 and S4). Only in few clonal IG rearrangements detected by SCA did we observe intraclonal heterogeneity (see Supplementary Results, Supplementary Table S5 and Supplementary Figure S2).

In all 75 MP-IGH CLL cases, we tested the possibility that any of the IGH rearrangements evolved from a co-detected rearrangement by replacing an IGHV gene.[5] No signs of IGHV gene replacement were observed in any of them. We also did not observe a lack of allelic exclusion in IGH,[4] though in case of IG light chains, co-expression of two productive rearrangements was identified by SCA in two cases. Nevertheless, taking our present data and recently published observations together,[2,6] we assume that IGHV replacement and lack of allelic exclusion are infrequent in CLL.

Our results are in agreement with two recent works,[6,7] in which the clonality of MP-IGH CLL was also studied at the single-cell level. Both studies identified the coexistence of multiple leukemic clones in all cases tested. However, the number of cases involved was limited (seven[6] and three[7] cases) and the extent of SCA was considerably less than in the present data. In summary, we brought the conclusive evidence for the coexistence of multiple B-lymphocyte clones in the vast majority of the tested cases. Using SCA in the cohort of 22 MP-IGH CLL patients unexplained by immunophenotype, we show that each detected productive IGH rearrangement belonged to a separate clone.

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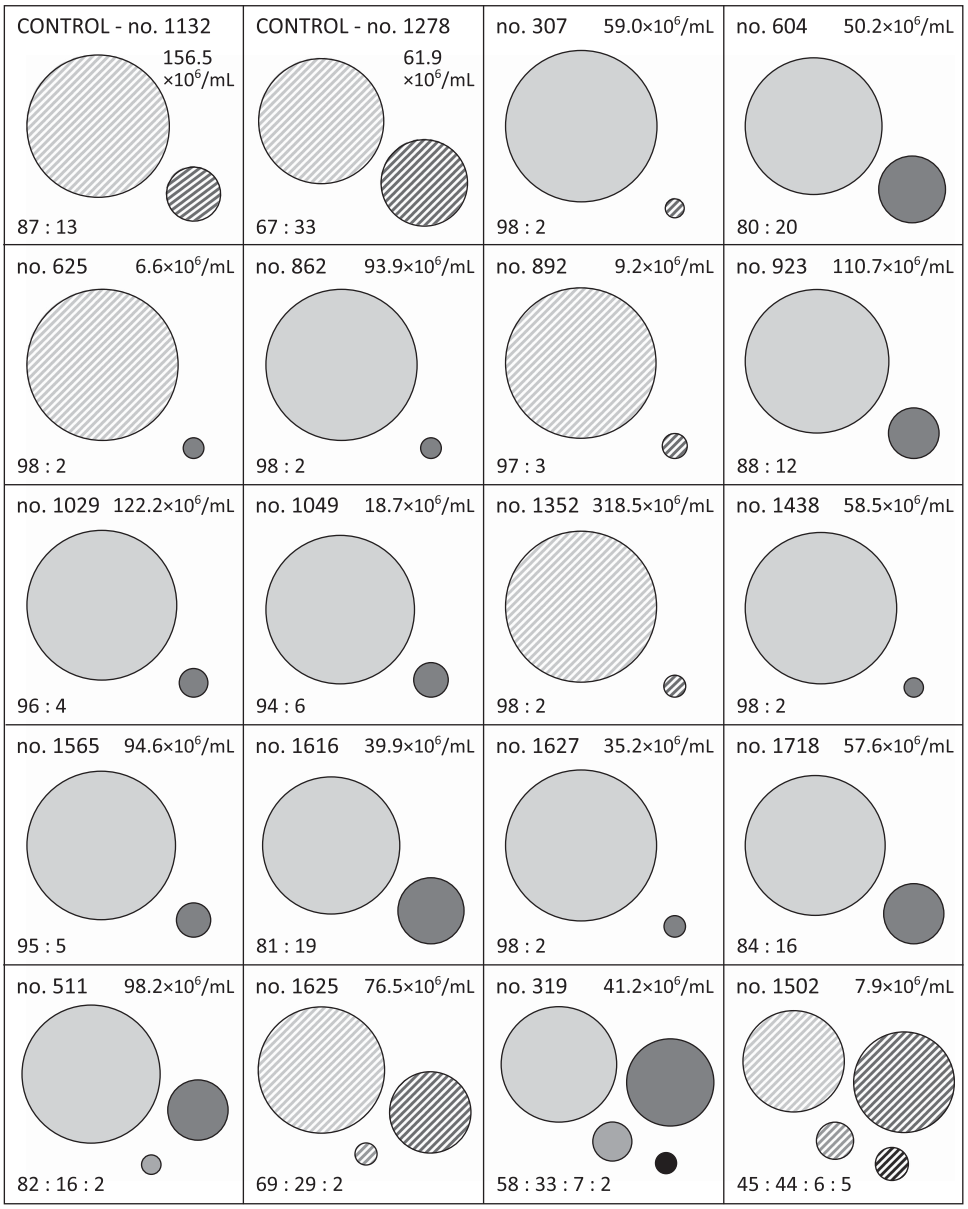

Figure 1. SCA of MP-IGH CLL cases. Individual CLL clones are depicted as rounds with areas that correspond to the estimated ratio of coexisting clones (based on numbers of IGH positive wells). Clones with mutated productive IGH rearrangement ($<$ 98% IGHV germline identity) are hatched, clones with unmutated productive IGH rearrangement ($\geqslant$ 98% IGHV germline identity) are in full color. Counts of $CD19^+$ $CD5^+$ cells in peripheral blood are shown upper right. In cases no. 523, 885, 1087 and 1440, only one clone characterized by single productive IGH rearrangement was detected at the single cell level (not shown).

To clarify whether any specific disease characteristics associate with MP-IGH CLL, or its subgroups according to the IGHV somatic hypermutational status following the 98% germline identity cut-off value,[1] we analyzed and compared clinico-biological parameters including gender, age and Rai stage at diagnosis, time to first treatment, overall survival, *TP53* mutational status, and hierarchical cytogenetics between 52 MP-IGH and 1039 single productive IGH rearrangement (SP-IGH) cases and their subgroups (see Supplementary Methods, Supplementary Figures S3 and S4, and Supplementary Tables S6 and S7). We did not identify any statistically significant difference between MP-IGH and SP-IGH CLL in our cohort, with the exception of higher age at diagnosis in MP-IGH CLL ($P = 0.008$; Supplementary Figure S3A). The difference in age was, however, not observed in other published cohorts[2,8,9] possibly due to the low numbers of cases involved in the studies, different criteria for group assigning, and different methods used. Furthermore, we noted an inclination of the MP-IGH CLL subgroup with discordant IGHV somatic hypermutation status to behave as high-risk cases, being skewed towards higher Rai stages and more frequent *TP53* mutations and 11q deletions (Supplementary Table S7). Similar findings were reported in other studies.[2,9] This is likely related to our previously reported observation[3] that in discordant cases it is mostly the mutated productive IGH rearrangement that eventually diminishes, which is followed by a reassignment of a patient to the less favorable prognostic category. Such an observation is important, as the discordant MP-IGH cases represent an interpretation challenge with regards to CLL prognostic stratification.[1] Of note, it has been shown that an initially minor, or even undetectable clone defined by a productive IGH rearrangement can overgrow the major one.[3,10] Nevertheless, in the majority of MP-IGH CLL cases studied longitudinally, major clones eventually displaced the minor ones or clonal ratios did not change dramatically over time and/or treatment.[2,3,6]

The presence of multiple clones in CLL raises questions regarding their relation and origin. As oligoclonality has frequently been observed at a stage of pre-malignant monoclonal B-cell lymphocytosis,[11,12] it is possible that clones at various stages of malignant transformation could coexist in oligoclonal CLL. The uneven clonal representation in cases tested in our cohort is supportive of that. Of note, the occurrence of concordant status in the co-detected productive IGH rearrangements was significantly higher ($P = 0.0004$; calculated only in 65 cases with 2 productive IGH rearrangements) than expected on the basis of incidence of mutated and unmutated productive IGH rearrangements in the cohort (see Supplementary Methods, Supplementary Table S8, and Supplementary Figure S5). This was strikingly prominent in both cases with four productive IGH rearrangements, where the rearrangements were either all mutated (no. 1502) or all unmutated (no. 319). This phenomenon is also noticeable in other published MP-IGH CLL cohorts.[1,9] The frequent concordance in IGHV mutational status of coexisting clones could indicate common factors affecting them during maturation and selection; recent evidence suggests that shared epitope reactivity[7] or origin from a common progenitor[13,14] might be such factors.

Employing the SCA, this is the first study demonstrating on the representative cohort that the number of productive IGH rearrangements detected in CLL patients corresponds to the number of B-lymphocyte clones, even in cases with immunophenotypically monoclonal disease. This represents a stepping stone for further research as many questions concerning oligoclonality in CLL still remain unresolved.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

KP and BT designed the study. KB, KP, HSF, HK, MB, VB, JMal and AO performed experiments. JK, YB, JMay and MD provided samples and/or clinical data. KB, KP and HSF evaluated results. KP, MD and SP supervised the study. KB, KP and SP wrote and edited the manuscript. All authors provided final approval of the manuscript.

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