Autonomous B-cell receptor signaling and genetic aberrations in chronic lymphocytic leukemia-phenotype monoclonal B lymphocytosis in siblings of patients with chronic lymphocytic leukemia

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Received: March 1, 2023.
Accepted: June 30, 2023.
Early view: July 13, 2023.

https://doi.org/10.3324/haematol.2022.282542

Abstract

Clonal expansion of CD5-expressing B cells, commonly designated as monoclonal B lymphocytosis (MBL), is a precursor condition for chronic lymphocytic leukemia (CLL). The mechanisms driving subclinical MBL B-cell expansion and progression to CLL, occurring in approximately 1% of affected individuals, are unknown. An autonomously signaling B-cell receptor (BCR) is essential for the pathogenesis of CLL. The objectives of this study were functional characterization of the BCR of MBL in siblings of CLL patients and a comparison of genetic variants in MBL-CLL sibling pairs. Screening of peripheral blood by flow cytometry detected 0.2-480 clonal CLL-phenotype cells per microliter (median: 37/μL) in 34 of 191 (17.8%) siblings of CLL patients. Clonal BCR isolated from highly purified CLL-phenotype cells induced robust calcium mobilization in BCR-deficient murine pre-B cells in the absence of external antigen and without experimental cross-linking. This autonomous BCR signal was less intense than the signal originating from the CLL BCR of their CLL siblings. According to genotyping by single nucleotide polymorphism array, whole exome, and targeted panel sequencing, CLL risk alleles were found with high and similar prevalence in CLL patients and MBL siblings, respectively. Likewise, the prevalence of recurrent CLL-associated genetic variants was similar between CLL and matched MBL samples. However, copy number variations and small variants were frequently subclonal in MBL cells, suggesting their acquisition during subclinical clonal expansion. These findings support a stepwise model of CLL pathogenesis, in which autonomous BCR signaling leads to a non-malignant (oligo)clonal expansion of $CD5^+$ B cells, followed by malignant progression to CLL after acquisition of pathogenic genetic variants.

Introduction

Chronic lymphocytic leukemia (CLL) is a monoclonal expansion of more than 5,000 mature CD5-expressing B cells per microliter of peripheral blood.[1,2] The clinical behavior of CLL is highly variable and depends strongly on structural and functional properties of the B-cell receptor (BCR) as well as the presence of various recurrent genetic

aberrations.[1-6] Autonomous signaling of the clonotypic BCR expressed by CLL cells, i.e. signaling without engagement of an external antigen, is an indispensable oncogenic signal in CLL.[7] This peculiar property of CLL BCR is absent in other types of indolent B-cell lymphoma and in antigen-specific normal B cells.[7] Autonomous BCR signaling is caused by physical interaction between neighboring BCR complexes on the cell surface, as demonstrated by seminal crystallographic studies in paradigmatic CLL BCR.[8] In the Eμ-TCL1 transgenic mouse model, autonomous BCR signaling is indispensable for CLL development even when all B cells primarily express an antigen-specific transgenic BCR and are stimulated by their cognate antigen.[9]

CLL is characterized by remarkable skewing in the BCR immunoglobulin (BCR IG) gene repertoire, culminating in the existence of subsets of cases with restricted BCR IG features.[5] The largest immunogenetic CLL subset, stereotyped subset #2L, is defined by the expression of a BCR with a light chain utilizing the IGLV3-21 gene that carries a G110R mutation at the junction between the variable and constant domains.[10] This seminal IGLV3-21 G110R mutation facilitates autonomous BCR signaling.[8]

Numerous gene polymorphisms predispose to the development of CLL.[11-13] Formally, CLL develops through a precursor stage, a subclinical expansion of mature B cells with the characteristic $CD5^{+}CD20^{low}CD79^{low}$ CLL phenotype, commonly referred to as CLL-phenotype monoclonal B lymphocytosis (MBL).[14] The prevalence of MBL is approximately 100 times higher than that of CLL and is relatively increased in siblings of CLL patients.[15,16] Similarly to cases of indolent CLL, MBL cells carry relatively few genetic aberrations.[17,18] However, the mechanisms leading to benign, longitudinally stable clonal expansion of CLL-phenotype B cells, and the hierarchy and sequence of events causing eventual progression to overt CLL are not fully elucidated yet.

In order to investigate the early stages in the ontogeny of CLL, we screened siblings of CLL patients for the presence of MBL with CLL phenotype. Specifically, we sought to clarify whether these MBL cells express BCR with autonomous signaling capacity. In addition, we compared the prevalence of inherited risk loci and acquired genetic aberrations between CLL patients and their first-degree MBL relatives.

Methods

Patients, probands, and samples

CLL patients under management of the outpatient clinics at the participating centers were asked to inform their respective living siblings about this study and to provide their contact information. These siblings were asked to provide a single blood sample. Both CLL patients and their siblings gave written informed consent to participation in the study, which was conducted according to the Declaration of Helsinki and approved by the Ethical Committees of both participating institutions (Leiden: P12.059; Freiburg: 44/08). These approvals explicitly excluded longitudinal analyses and mandated providing siblings with a categorical screening result.

Detection, isolation, and processing of chronic lymphocytic leukemia-phenotype cells

MBL was detected as discrete $CD19^{+}CD5^{+}CD20^{low}CD79^{low}$ populations (Figure 1) by six-color flow cytometry. DNA and RNA were isolated from MBL cells sorted to >95% purity (Allprep DNA/RNA mini kit; Qiagen, Hilden, Germany). Frequencies of MBL cells were calculated from gated cells and absolute lymphocyte counts.

Identification of clonal B-cell receptor transcripts

VDJ and VJ sequences were determined by ARTISAN PCR.[19,20] Ig sequences were analyzed by Geneious 10.2.6 (Geneious, Auckland, New Zealand) and IMGT/V-QUEST.[21,22] Assignment to CLL stereotypes was performed by an established purpose-built algorithm.[5,23]

Analysis of B-cell receptor signaling activity

Murine TKO pre-B cells were transduced with retroviral pMIZCC and pMIZYN vectors encoding paired Ig heavy and light chain sequences from individual cases.[7,10] TKO cells are genetically deficient of Rag2, Lambda5, and Slp65. Slp65 function is reconstituted by a 4-hydroxytamoxifen-inducible Slp65-ERT2 fusion gene.[7,24] Calcium mobilization was measured in indo-1 AM-loaded (ThermoFisher, Waltham, MA, USA), live-gated TKO cells by flow cytometry prior to and after addition of 2 μM 4-hydroxytamoxifen (Sigma Aldrich, St Louis, MO, USA). Maximum BCR signaling was measured after addition of crosslinking anti-IgM or anti-IgG antibodies (clones 2022-01 and 2042-01; Southern Biotech, Birmingham, AL, USA). Autonomous BCR signaling was quantitated with correction for totally unresponsive cells during BCR crosslinking (*Online Supplementary Material*) at least twice at approximately 3 and 4 weeks after transduction.

Genetic characterization

Variants in 52 B-cell lymphoma-related genes were analyzed by the LYMFv1 custom targeted sequencing panel on an Ion S5 system (ThermoFisher).[25] Sequencing reads were aligned to the GRCh37/hg19 human reference genome with TMAP 5.0.7 software under default parameters. CLL-associated copy number variations (CNV) were detected by single nucleotide polymorphism (SNP) arrays.[26] Whole exome sequencing (WES) was performed with SureSelect Human All Exon V7 kit (Agilent, Santa Clara, CA, USA) capture on the HiSeq2000 (Illumina, San Diego, CA,

USA) platform to an average coverage of 50x. If necessary, DNA was amplified by isothermal alkaline genome amplification (REPLI-g kit; Qiagen). Detected variants were annotated for predicted pathogenicity and occurrence in both CLL and MBL siblings (*Online Supplementary Material*). The global distribution of variant allele frequencies (VAF) in CLL and MBL was estimated using the Wilcoxon rank sum test with a continuity correction (Mann-Whitney U test) R function 'wilcox.test()' (*www.R-project.org*) and the probability density function of VAF calculated by kernel density estimation using the R function 'density()'. Monoallelic and subclonal variants present in CLL and MBL samples from siblings were tested using the Wilcoxon signed-rank test R function 'wilcox.test(paired=TRUE)'. All tests were only performed after checking the corresponding test assumptions.

Inherited CLL risk loci were genotyped by targeted Sanger sequencing of 24 CLL susceptibility alleles, genes and loci covered by WES and the SNP array.[12,27-31] Observed frequencies of risk alleles were compared to the most appropriate reference population from the gnomAD database by the Fisher exact test.[32] CLL risk SNP were compared with WES-based data of Northwestern Europeans in gnomAD v2.1.1 and with whole genome sequencing-based data of non-Finnish Europeans in gnomAD v3.1.2. Polygenic risk scores were calculated for individual MBL probands and CLL patients for their genotyped risk loci based on the most recent reported odds ratios.[12,13]

The extent of genetic analyses was restricted in individual cases by insufficient amounts of DNA due to the paucity of clonal CLL-phenotype B cells (*Online Supplementary Figure S1; Online Supplementary Table S1*).

Results

Prevalence and frequency of chronic lymphocytic leukemia-phenotype cells in siblings of patients with chronic lymphocytic leukemia

Peripheral blood samples from 191 siblings of CLL patients were received and analyzed at two academic medical centers. Flow cytometry revealed a discrete population of CLL-phenotype $CD19^{+}CD5^{+}CD20^{low}CD19^{low}$ cells, in the majority with evident light chain restriction, in 34 siblings (17.8%) of 26 CLL patients (Figure 1; *Online Supplementary Table S1*). The median age of siblings with CLL-phenotype cells was 68 years (range, 43-80 years). No sibling with detectable MBL was a member of a family with a CLL back-

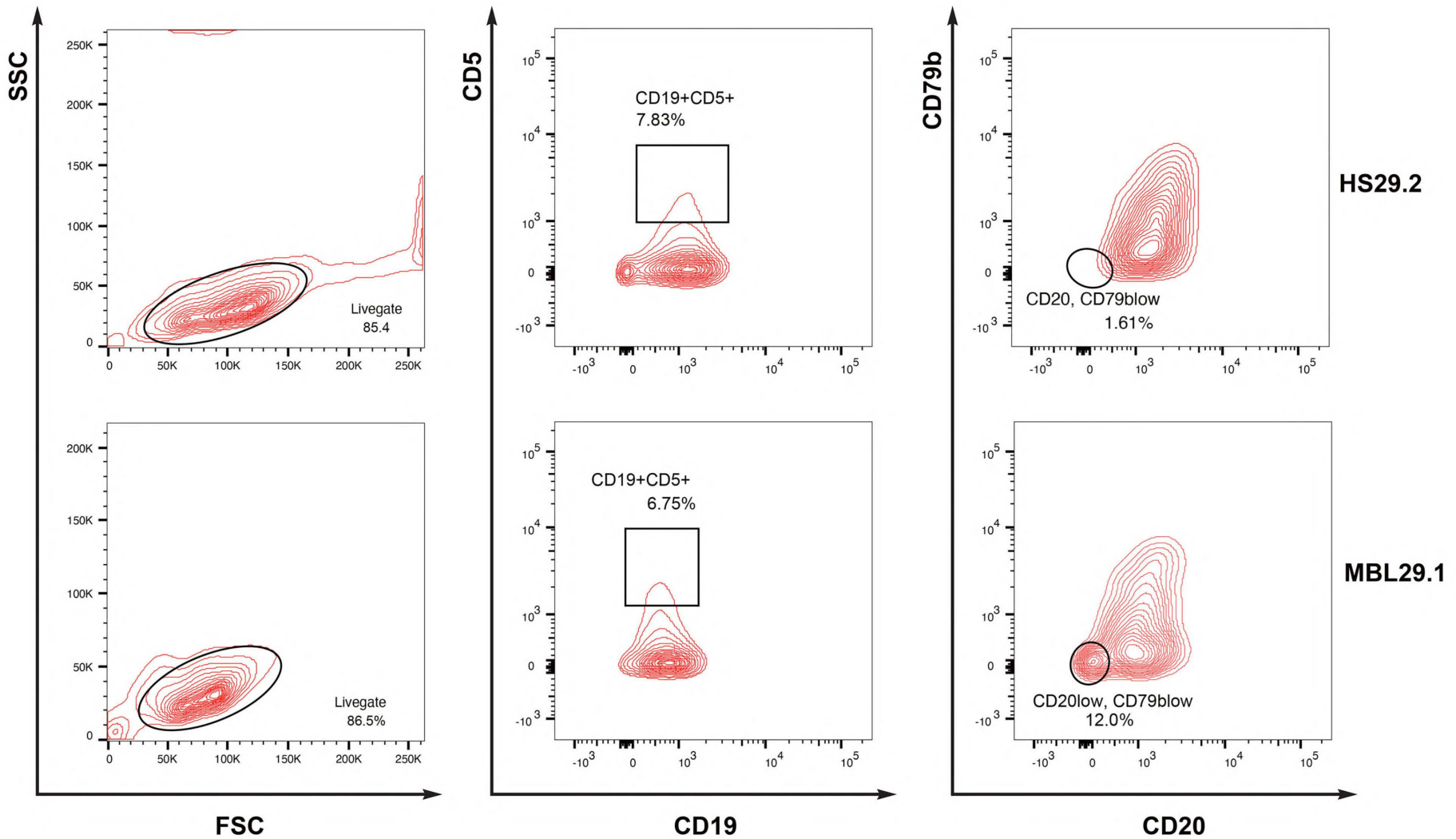

Figure 1. Detection of chronic lymphocytic leukemia-phenotype monoclonal B lymphocytosis cells. Flow cytometry screening of two healthy siblings of chronic lymphocytic leukemia (CLL) patient 29.1. *Upper row*: Healthy sibling HS29.2 without detectable CLL-phenotype cells. *Lower row*: Healthy sibling MBL29.1 with the lowest detected expansion of CLL-phenotype cells ($CD19^{+}CD5^{+}CD20^{low}CD79^{low}$) in this study. SSC: side scatter; FSC: forward scatter.

ground. The absolute number of CLL-phenotype MBL cells ranged from 0.2/μL to 1,863/μL of blood, and 32 siblings (94%) were classified as having low-count MBL with fewer than 500 clonal CLL-phenotype cells per microliter (Figure 2A).[33]

Sequence characteristics of clonally dominant B-cell receptor transcripts of chronic lymphocytic leukemia-phenotype cells in chronic lymphocytic leukemia patients and their healthy siblings

BCR IG transcripts from highly purified CLL-phenotype cells were sequenced in 17 siblings of CLL patients. In two siblings, two different productive VDJ gene rearrangements were identified within the CLL-phenotype MBL cell population (MBL01.1, MBL04.1); all other siblings carried an apparently monoclonal MBL population. Five MBL clones in healthy siblings could be assigned to CLL stereotyped subsets according to expanded immunogenetic criteria (*Online Supplementary Table S1*).[5,23] In particular, the BCR of MBL07.1 and MBL27.2 belonged to CLL subset #2 and #2L, respectively, based on expression of a BCR light chain utilizing the IGLV3-21 gene with the G110R mutation.[10] An IgG-expressing MBL fulfilled the criteria of CLL subset #4.[34] These MBL BCR were predicted to exert autonomous signaling based on structural analyses.[8] Furthermore, all sequenced MBL BCR contained one or both of the FR2 VRQ and FR3 YYC motifs proposed as structural requirements for autonomous BCR signaling in the majority of CLL cases.[7,35] The somatic mutation status of the clonotypic rearranged IGHV gene did not correlate to the degree of expansion of CLL-phenotype cells (Figure 2B). In addition, IGHV gene mutational status could differ between CLL patients and their siblings; in particular, three non-subset #2L MBL (17.6%) expressed unmutated BCR IG, whereas the corresponding CLL BCR was mutated (Figure 2C).

Functional characteristics of monoclonal B lymphocytosis B-cell receptors from siblings of patients with chronic lymphocytic leukemia

BCR from clonally dominant MBL cells of 11 siblings of CLL patients, including all five MBL assigned to a CLL stereotyped subset, were expressed in TKO cells and tested for calcium mobilization prior to and after tamoxifen-induced reconstitution of the BCR signaling cascade and after BCR crosslinking. As predicted,[10] both CLL subset #2L MBL BCR showed robust calcium flux upon addition of 4-hydroxytamoxifen without antigen exposure or BCR crosslinking (Figure 3). In addition, all other tested MBL BCR from siblings of CLL patients had autonomous BCR signaling activity. On average, autonomous BCR signaling was slightly stronger in CLL than in MBL in each individual sibling pair (Figure 4A). There was no quantitative correlation between BCR signaling activity of CLL and MBL sibling pairs (Figure 4B). BCR signaling strength was positively correlated with MBL cell counts at the time of sampling (Figure 4C).

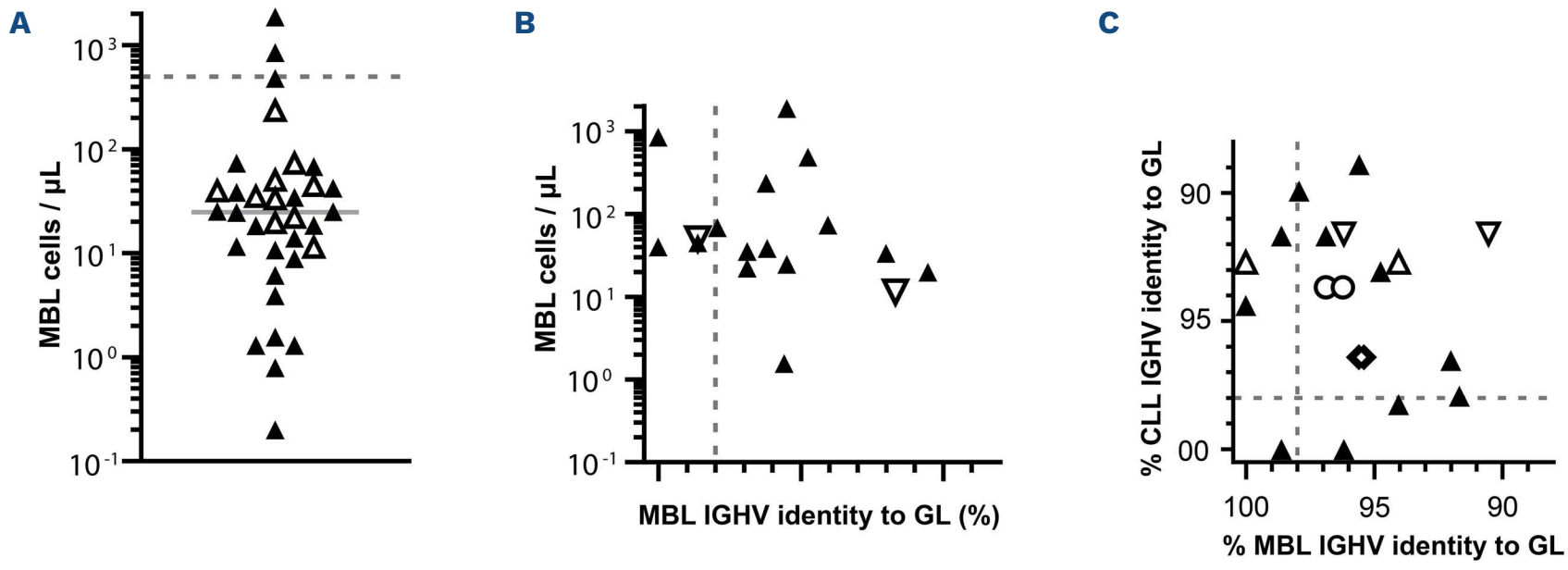

Figure 2. Characteristics of monoclonal B lymphocytosis cells in chronic lymphocytic leukemia siblings. (A) Monoclonal B lymphocytosis (MBL) counts of all 34 siblings with a detectable chronic lymphocytic leukemia (CLL)-phenotype B-cell population ($CD19^{+}CD5^{+}CD20^{low}CD79^{low}$). Solid line: median. Dotted line: commonly accepted threshold between low- and high-count MBL. Open triangles: MBL selected for functional B-cell receptor (BCR) testing. (B) IGHV gene somatic hypermutation status (x axis) and counts of MBL cells (y axis). Dotted line: commonly accepted threshold between mutated and unmutated BCR status in CLL. Inverted open triangles: MBL cells from two CLL siblings expressing a CLL subset #2 BCR predicted to exert autonomous signaling.[10] (C) IGHV gene identity to germline in MBL cells (x axis) and CLL cells (y axis) in siblings. Dotted lines: commonly accepted boundary between mutated and unmutated BCR status in CLL. Open triangles: two cases of MBL with expression of two immunoglobulin heavy chains (MBL01.1: upright triangles, MBL04.1: inverted triangles). Open diamonds: patient CLL24.1 with two MBL siblings (MBL24.1, MBL24.2). Open circles: patient CLL30.1 with two MBL siblings (MBL30.1; MBL30.2). Closed triangles: all other CLL-MBL pairs. GL: germline.

Comparative genetics of chronic lymphocytic leukemia patients and monoclonal B lymphocytosis siblings

Analysis of known germline variants from up to 26 different gene loci associated with increased incidence of CLL revealed a significantly higher prevalence of 12 risk alleles within our cohort (*Online Supplementary Table S2*). A polygenic CLL risk score composed of 24 risk loci was higher in both CLL and MBL siblings than the average reference score calculated from reference population data (Figure 5A).[32] An extended analysis incorporating five to eight CLL risk loci, as assessable by WES and SNP array data, in additional cases (*Online Supplementary Table S2*) yielded no evidence of higher polygenic risk scores in CLL cases in a matched-pair comparison to their MBL siblings. Taken together, these findings suggest that CLL risk loci predispose to clonal expansion of CLL phenotype cells in both low-count MBL and CLL. *Vice versa*, we did not find evidence of a particular association of CLL risk alleles with malignant progression from MBL to clinical CLL.

Fifteen MBL samples with sufficient DNA were genotyped by SNP arrays to detect recurrent CLL-associated CNV. Ten MBL carried recurrent CLL-associated CNV, including a del(17)(p13-q21) in one case and del(13)(q14) in nine cases. One additional MBL case carried a del(14)(q22.2) in combination with a trisomy 18q12, resulting in a total of 11 MBL samples with informative CNV (Table 1). Eleven of 15 genotyped CLL cases within this study carried informative CNV, suggesting a similar overall prevalence of CNV between CLL cases and MBL in their siblings. Quantitative analysis of the SNP array data indicated that all malignant cells harbored the respective CNV in nine of 11 CLL samples. In contrast, individual CNV were present in only a minor fraction of the highly purified MBL cells in ten of 11 samples (P=0.0019; Fisher exact test).

We corroborated these quantitative CNV data with analysis of small variants, i.e., single nucleotide variants and indels from WES in ten CLL-MBL sample pairs. Identical variants detected in a CLL case and the patient's MBL sibling were presumed to be inherited and were considered noninformative with respect to a differential pathogenesis between MBL and CLL. In contrast, variants that were not shared between siblings, i.e., variants observed exclusively

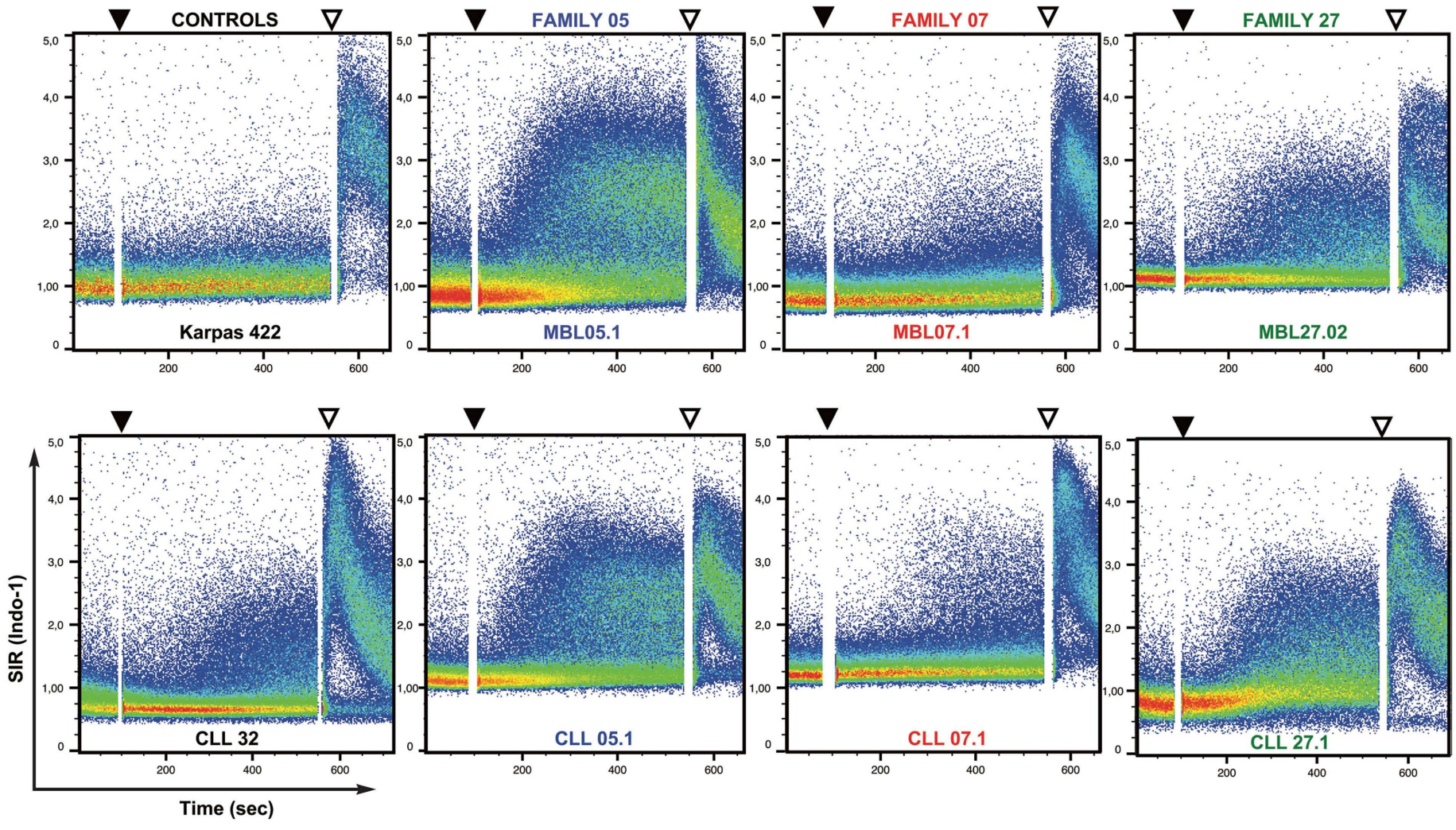

Figure 3. Autonomous B-cell receptor signaling of chronic lymphocytic leukemia and monoclonal B lymphocytosis cells in three representative sibling pairs. Calcium mobilization was measured by flow cytometry as indo-1 AM signal intensity ratios of B-cell receptor (BCR)-transduced TKO cells. Black triangle: timepoint of addition of 4-hydroxytamoxifen to reconstitute a functional BCR signaling cascade. White triangle: timepoint of addition of anti-IgM/IgG crosslinking antibody. K422: TKO cells transduced with BCR of the lymphoma cell line Karpas 422 as a negative control. CLL 32: TKO cells transduced with BCR of patient 32 with chronic lymphocytic leukemia (CLL) as a positive control.[7] MBL05.1: TKO cells transduced with BCR of monoclonal B lymphocytosis (MBL) cells in a sibling expressing a CLL subset #60 BCR.[5,23] MBL07.1, MBL27.2: TKO cells transduced with BCR of MBL cells in siblings expressing a CLL subset #2 BCR. TKO cells transduced with CLL BCR are depicted below the MBL of their respective siblings. SIR: signal intensity ratio.

in either a CLL sample or that from the sibling with MBL, could be either inherited or acquired somatically and were considered as potentially informative for differential MBL-CLL pathogenesis. Initially, we focused on 120 genes recurrently mutated in CLL according to the COSMIC database (*Online Supplementary Table S3*). As determined by targeted panel sequencing and/or WES analysis in ten CLL-MBL sample pairs, CLL and MBL cases carried similar numbers of unique gene variants with predicted pathogenic relevance (*Online Supplementary Table S4*). Variants in these genes were infrequent, and their VAF did not differ significantly between CLL and MBL (Figure 5B). Genome-wide, VAF of non-shared variants were significantly lower in MBL samples than in CLL cases (Figure 6A). Kernel density estimation indicated that this difference was mainly attributable to variants with an allele frequency of 0.15 to 0.3 (Figure 6B). In order to formally test whether this difference could be attributed to a higher fraction of subclonal

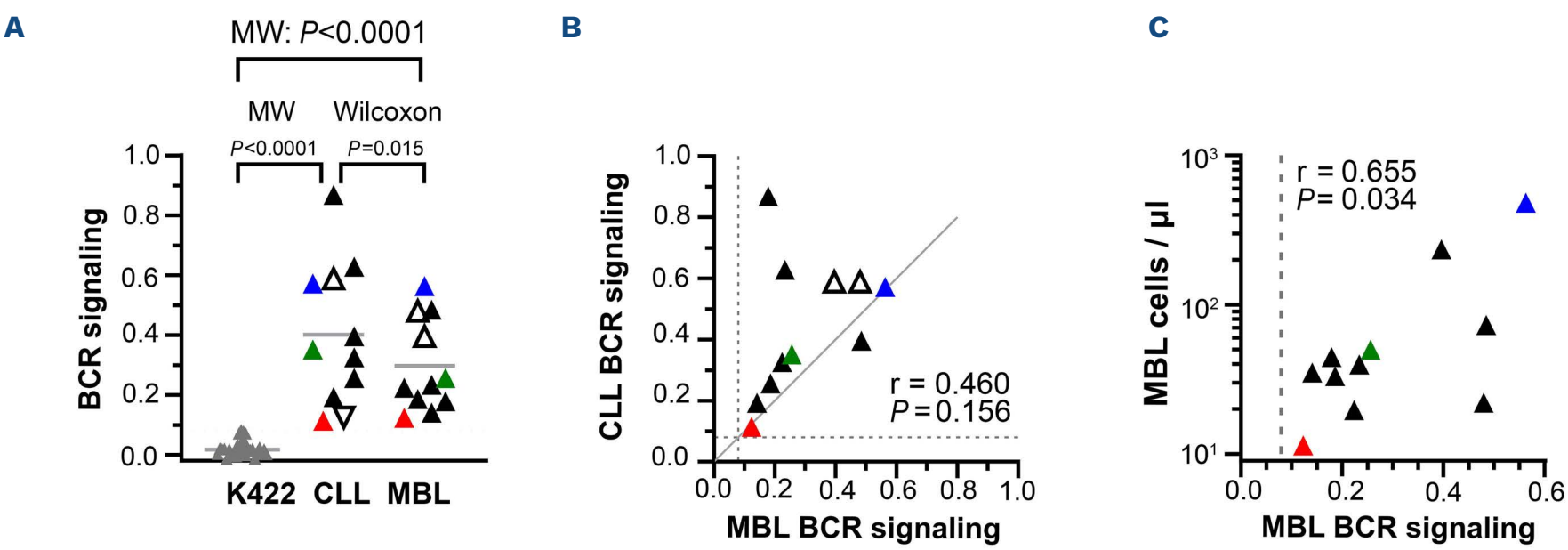

Figure 4. Quantitative analysis of autonomous B-cell receptor signaling of cells from subjects with chronic lymphocytic leukemia cells or monoclonal B lymphocytosis. (A) Autonomous B-cell receptor (BCR) signaling strength in TKO cells transduced with clonal BCR. K422: all measurements of Karpas 422 BCR (negative control) performed during this study. Inverted open triangles: CLL 32 (positive control). Open triangles: a case of chronic lymphocytic leukemia (CLL) with two siblings with monoclonal B lymphocytosis (MBL). Other triangle colors correspond to those in Figure 3. Black triangles: all remaining tested CLL and MBL cases. MW: unpaired comparison by the Mann-Whitney test. Wilcoxon: paired comparison between CLL and MBL within siblings by the Wilcoxon matched-pair signed rank test. (B) Correlation of autonomous BCR signaling strength between CLL and MBL siblings. Colors correspond to those in Figure 3 and 4A. Dashed lines indicate maximum BCR signaling strength observed in the Karpas 422 negative controls. (C) MBL counts (y axis) according to autonomous BCR signaling strength (x axis). Colors correspond to those in Figures 3 and 4A, B. The dashed line indicates maximum BCR signaling strength observed in the Karpas 422 negative controls.

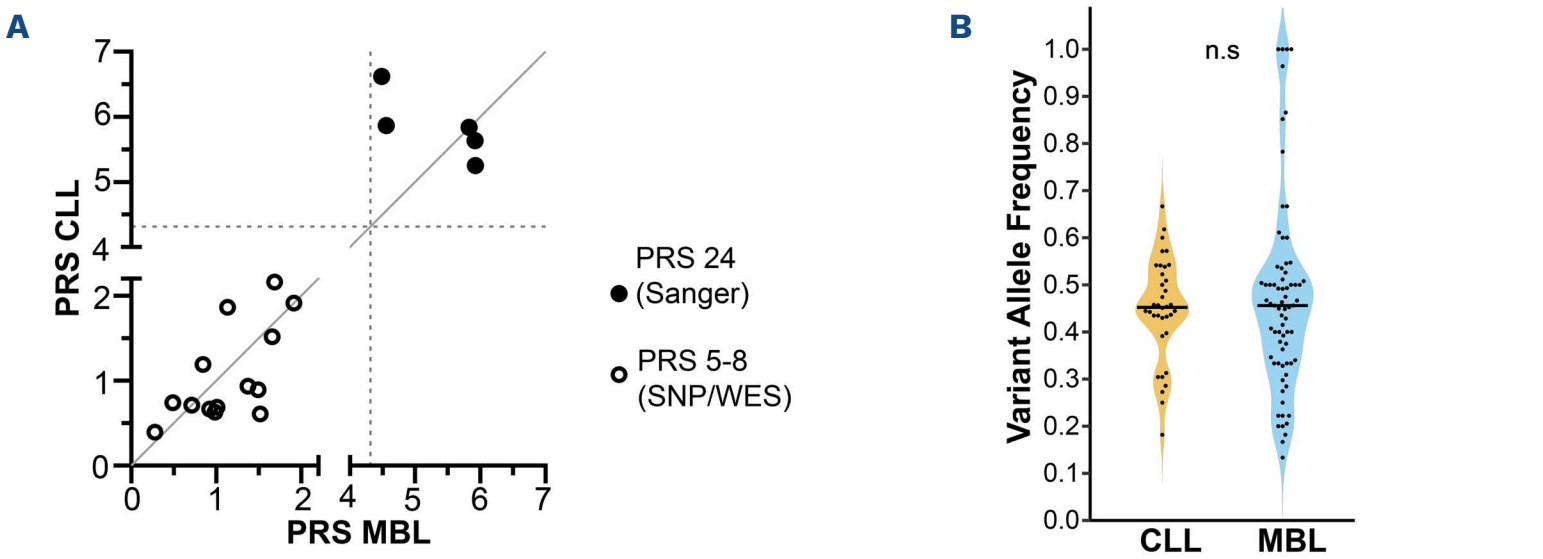

Figure 5. Comparison of chronic lymphocytic leukemia-associated variants in monoclonal B lymphocytosis-chronic lymphocytic leukemia siblings. (A) Polygenic risk score (PRS) for chronic lymphocytic leukemia (CLL) in probands with monoclonal B lymphocytosis (MBL) (x axis) and their CLL sibling (y axis). Depending on available data, the PRS was calculated for 24 CLL risk loci (black dots) or five to eight loci as assessed by single nucleotide polymorphism array and/or targeted Sanger sequencing (open circles). Dashed lines indicate the mean PRS of the reference European population for the 24 loci analyzed. (B) Distribution of variant allele frequencies of non-shared variants in 120 CLL driver genes as detected by whole exome sequencing in seven CLL cases and ten MBL siblings from seven families. CLL and MBL were compared by the Wilcoxon signed rank test. SNP: single nucleotide polymorphism; WES: whole exome sequencing; n.s.: not significant.

variants in MBL compared to CLL, we separately compared in sibling pairs the numbers of non-shared monoallelic variants (VAF=0.43-0.55), presumably carried by all cells in a given sample, and numbers of monoallelic subclonal variants (VAF=0.1-0.33). In accordance with this hypothesis, monoallelic variants were detected at similar frequencies in CLL samples and their respective sibling MBL samples (Figure 6C). In contrast, the prevalence of subclonal variants was higher in MBL cells than in the sibling CLL counterparts (Figure 6D).

Discussion

This systematic study was undertaken to clarify whether the presence of an autonomous BCR signal could explain

Table 1. Copy number variations in monoclonal B lymphocytosis and chronic lymphocytic leukemia samples as detected by single nucleotide polymorphism array.

Family	MBL		CLL	
	Case	Aberration	Case	Aberration
1	MBL01.1	13q14.2q14.3x0[0.7]	CLL01.1	13q14.2q14.3x1
2	MBL02.1	13q14.2x1[0.6] 13q14.2q14.3x0[0.6] 13q14.3x1[0.6]	CLL02.1	13q14x1 8q21.3qterx3 15q26.1q26.3x1
3	MBL03.1	no CNV	CLL03.1	2p22.1p16.1x1 8q24.22x1
4	-	-	CLL04.1	no CNV
5	MBL05.1	no CNV	CLL05.1	13q14.13q14.2x1[0.5] 13q14.2q14.3x0[0.5] 13q14.3x1[0.5]
6	MBL06.1 MBL06.2	13q14.2q14.3x1 14q22.2q32.12x1[0.6] 18q12.3q23x3[0.6]	CLL06.1	14q23.2q32.13x1
7	MBL07.1 MBL07.2	13q14.2q14.3x1[0.15] 13q14.13q31.1x1[0.15]	CLL07.1	13q14.11q14.2x1
8	MBL08.1	13q14.2q14.3x1[0.85]	CLL08.1	5q33.1q35.3x3[0.3] 6q14.1q27x1 8q23.1q24.3x3 17p13.3p12x0[0,5]
9	MBL09.1	no CNV	CLL09.1	no CNV
10	MBL10.1	13q14.2q14.3x1[0.5]	CLL10.1	13q14x1
11	MBL11.1	13q14.2q14.3x1[0.7]	CLL11.1	no CNV
12	MBL12.1	13q14.2q14.3x0[0.5]	CLL12.1	13q14.2q14.3x1
13	-	-	CLL13.1	11q22.3q25x1 3q26.1q29x3 4p16.3p14x1 17q21.32q25.3x3
15			CLL15.1	no CNV
16	MBL16.1	2q36.3q37.3x1[0.7] 17p13.3q21.2x1[0.7]	CLL16.1	4p16.3p15.2x1[0.3] 4p14x1[0.3] 4p13x1[0.3] 4p13x1[0.3] 5p15.33p15.2x1[0.3] (11)cx[0.3~0.8] 13q14.11q14.2x1[0.5] 13q14.2q21.33x0[0.8] 13q21.33q22.3)x1[0.5]
Total analyzed	14		15	
With CNV	11		11	
With clonal CNV	1		9	
With subclonal CNV only	10		2	

MBL: monoclonal B lymphocytosis; CLL: chronic lymphocytic leukemia; CNV: copy number variation.

the different clinical behavior between non-malignant clonal expansions of cells with a CLL phenotype (MBL) and clinically malignant CLL. In order to minimize potential confounding effects of inherited germline variants, we investigated this hypothesis in a comparative manner in siblings of CLL patients.

The detection limit and range of clonal B cells with a CLL phenotype were very similar to those in a recently published study of members of CLL families.[16] However, the observed MBL prevalence of 17.8% was relatively high compared to that in previous reports on CLL siblings and in the range of individuals belonging to CLL families defined by more than one first-degree relative diagnosed with CLL.[15,16]

Unbiased amplification of expressed heavy and light IG chain gene rearrangements revealed single dominant BCR in 15 and potential biclonality in two MBL subjects. Potential MBL oligoclonality has been recognized in MBL, especially in the low-count subtype.[36,37] The identification of a fraction of MBL with unmutated BCR IG in our series is also in line with a published study.[36] Whereas low-count MBL was reported to belong only rarely to any immunogenetically defined CLL stereotype subset,[36,38] we encountered five instances of BCR IG from low-count MBL (33.3%) that could be assigned to such CLL subsets.[5,23] It remains hypothetical whether this apparent discrepancy is due to focusing on MBL carriers with CLL siblings in our study. In healthy people who eventually developed CLL, approximately 20% of dominant clonotypes identified during the pre-diagnostic period could be assigned to CLL stereotypes.[39]

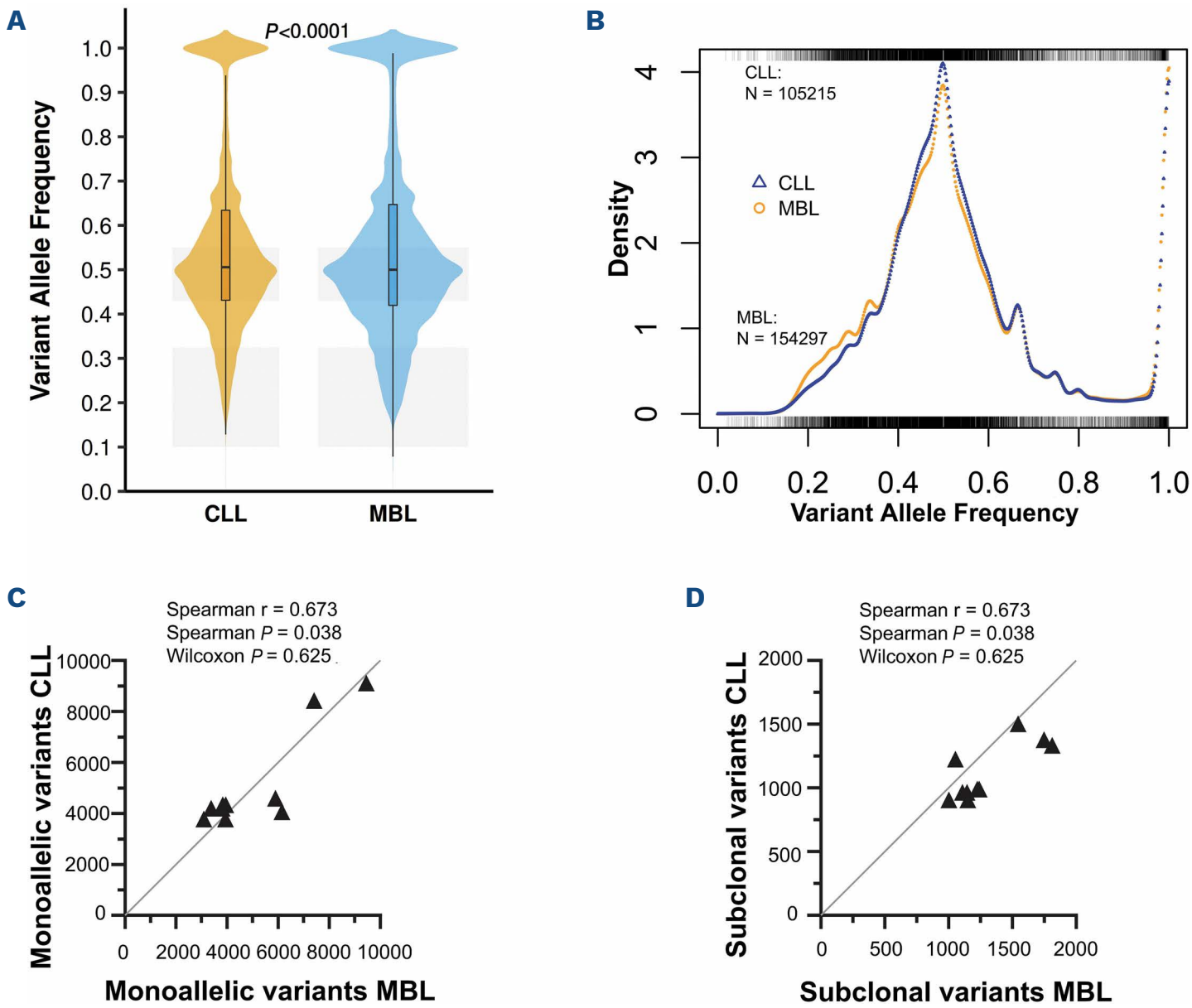

Figure 6. Comparison of genome-wide variants in siblings with monoclonal B lymphocytosis or chronic lymphocytic leukemia. (A) Global distribution of variant allele frequencies (VAF) in cases of chronic lymphocytic leukemia (CLL) and monoclonal B lymphocytosis (MBL). VAF were compared by the Wilcoxon rank sum test with continuity correction (Mann–Whitney U test). Shaded boxes indicate the ranges of VAF analyzed in (C) and (D). (B) Kernel density estimation distribution and rug plots of VAF in CLL and MBL. The rug plot marks along the x-axis (top and bottom) indicate positions of the VAF for CLL and MBL, respectively. A total of 105,215 CLL data points and 154,297 MBL data points were analyzed. (C) Numbers of monoallelic variants (VAF=0.43-0.55; present in all cells) of CLL and MBL samples from siblings. Correlations between CLL and MBL samples were calculated by the nonparametric Spearman correlation. CLL and MBL samples were compared by the Wilcoxon matched-pair signed rank test. (D) Numbers of subclonal variants (VAF=0.1-0.33) in CLL and MBL samples from siblings. Correlations between CLL and MBL samples were calculated by the nonparametric Spearman correlation. CLL and MBL samples were compared by the Wilcoxon matched-pair signed rank test. CLL: chronic lymphocytic leukemia; MBL: monoclonal B lymphocytosis.

With respect to the primary aim of our study, MBL BCR IG resembling certain CLL subsets were predicted to have autonomous BCR signaling activity akin to that in CLL. This prediction applied especially to the herein first reported low-count MBL expressing an isotype-switched BCR of CLL subset #4, and two subset #2L MBL cases expressing an IGLV3-21 light chain with the seminal G110R mutation. Both of these structural BCR IG characteristics mechanistically cause autonomous BCR signaling by facilitating steric interactions between neighboring BCR complexes on the cell surface.[8,10] Limited by the sample size, we found no evidence for correlations in structural features of the BCR, e.g., mutational status or IG gene usage, between CLL-MBL sibling pairs.

As already suggested by the MBL BCR IG sequences, our findings unequivocally establish, by quantifiable functional testing, that the BCR of MBL, i.e., a premalignant clonal expansion of CD5-positive B cells with CLL phenotype, exerts antigen-independent, autonomous signaling. All known origins of autonomous BCR signaling in CLL were also encountered in our MBL cases, i.e., acquisition during primary V(D)J recombination in cases with unmutated BCR, acquisition by somatic hypermutation as exemplified best by the single G110R mutation of the IGLV3-21 gene,[10] and acquisition by class switch recombination.[8]

Since our study specifically addressed CLL-MBL sibling pairs, we were able to compare the autonomous BCR signaling strength quantitatively in a matched-pair fashion. This analysis revealed significantly lower intensity of BCR signaling in MBL compared to CLL. While autonomous BCR signaling is generally dependent on individual HCDR3 in conjunction with recurrent motifs in FR2 and interactions of defined amino acid residues in some instances,[7,8,10,35] quantitative differences in autonomous BCR signaling cannot be assigned yet to defined structural BCR characteristics. However, BCR signaling strength was directly correlated to the expansion of the MBL clone at the time of sampling. This novel observation requires validation in independent cohorts. Nevertheless, the quantitative differences of BCR signaling between CLL and MBL and in MBL expansion suggest a possible causal and differential role of BCR signaling in growth kinetics and subsequent clinical behavior of a neoplastic CLL-phenotype B-cell clone.

It would have been highly desirable to validate the correlation between BCR signaling strength and clonal B-cell expansion by longitudinal sampling. However, the approvals by the institutional ethics committees explicitly excluded longitudinal analyses and mandated that the healthy siblings were provided with a categorical result of the screening. Since low-count MBL does not represent a definite pathological condition and rarely progresses to CLL,[16,33] it was reported as absence of pathological findings. As a consequence, the ethical committees concluded that repeat sampling could potentially give rise to anxiety given the prior absence of pathology in the healthy siblings, and explicitly denied approval for longitudinal analysis.

In order to interpret BCR function in the context of genetic mechanisms in CLL pathogenesis, we addressed genetic susceptibility, genetic instability, and recurrent CLL-associated genetic changes in CLL cases and their MBL siblings. These studies were restricted by limited quantities of DNA extracted from the MBL samples. Nevertheless, analysis of CLL susceptibility loci indicated that both CLL and MBL probands have similar polygenic risk scores for CLL, which were higher than in the general (Northern) European reference population from the gnomAD database. While these findings were predictable from the study design in CLL-MBL sibling pairs, on the other hand they do not provide any evidence that these CLL risk loci drive malignant progression rather than initial premalignant expansion of the CLL-phenotype clone. With respect to the possible inherited shared predisposition to MBL and CLL, it would have been highly desirable to strengthen this conclusion by showing lower polygenic risk scores in the CLL siblings without detectable MBL cells. The study design, however, did not include informed consent for genetic analyses in non-MBL probands. Notwithstanding this limitation of our study, there appears to be no plausible causal relationship between the presence of any risk allele and the stochastic acquisition of a BCR with autonomous signaling based on the known function of the genes located at the CLL risk loci.

The overall prevalence of CLL-associated CNV and gene variants was not evidently different between CLL and MBL in matched comparisons. In accordance with the literature, del(13)(q14) was the most abundant CNV in MBL, but the detected del(17)(p) has also been described occasionally.[40] Our observed low prevalence of potentially pathogenic variants in CLL driver genes is also in accordance with published data.[18] However, we noted that CLL-associated CNV were frequently subclonal in MBL, suggesting that these aberrations might be gradually acquired at this stage. This observation is in accordance with findings from a longitudinal study in low-count MBL, in which the categorical presence of CLL-associated CNV approximately doubled, compared to the primary sample, after a median interval of 7 years.[41] The impression of gradual acquisition of mutations in MBL was independently supported by the observation that MBL cells carry more subclonal variants detected by next-generation sequencing than CLL, whereas the number of variants carried by all clonal cells did not differ between CLL and MBL. In CLL, the presence of subclonal drivers is associated with clinical outcome.[42]

In summary, our data demonstrate that autonomous BCR signaling operates in MBL in similarity to CLL. A hypothetical absence of this mechanism therefore does not explain the difference between benign MBL and malignant CLL. However, relatively low BCR signaling strength in MBL may offer

a plausible explanation for less aggressive expansion than in CLL. Our data suggest that CLL risk loci likewise cannot account for the different clinical behavior between the two conditions, and lend further support to the conclusion that the prevalence and type of genetic pathogenic changes are indistinguishable between MBL and low-risk CLL.[18,41] However, the observation of subclonal genetic CLL driver mutations in MBL supports a scenario of gradual clonal expansion driven by moderate autonomous BCR signaling, possibly leading to a certain degree of genetic instability that facilitates gradual acquisition of CLL driver mutations. This conclusion supports the notion that MBL and CLL represent a spectrum of the same biological condition, in which the cumulative stimuli from constitutive immune signaling through the BCR together with timing and type of eventually acquired genetic drivers govern the clonal dynamics from long-term balance between expansion and apoptosis over gradual expansion to clinically aggressive CLL.

Disclosures

No conflicts of interest to disclose.

Contributions

EQ, MTK, JAE, and CAMvB performed the BCR analysis and functional testing. JHS-Y, DP, VN, RALdG, DAS, JK, and RU-P performed genetic testing and bioinformatic analyses. AA and KS analyzed CLL subsets. HES-B, AK-H, and KZ recruited probands. JSPV, MAN, and HJ supervised data acquisition. HV designed the study.

Funding

This study was funded by the German Cancer Aid Foundation (Deutsche Krebshilfe) 108935. MAN and JHS-Y are funded by Fondecyt 1230298 (ANID, Chile). RU-P is funded by MAG2095 (MINEDUC, Chile). JHS-Y is funded by Doctorado Becas Chile (2016-72170683)

Data-sharing statement

SNP array datasets generated during this study are available from the corresponding author on reasonable request. Whole exome sequencing datasets generated during this study are available in the DRYAD repository at: https://datadryad.org/stash/dataset/doi:10.5061/dryad.np5hqbzwz.

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