Brief report

Distinct gene expression patterns in chronic lymphocytic leukemia defined by usage of specific *VH* genes

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The mutation status and usage of specific *VH* genes such as *V3-21* and *V1-69* are potentially independent pathogenic and prognostic factors in chronic lymphocytic leukemia (CLL). To investigate the role of antigenic stimulation, we analyzed the expression of genes involved in B-cell receptor (BCR) signaling/activation, cell cycle, and apoptosis control in CLL using these specific *VH* genes compared to *VH* mutated (VH-MUT) and *VH* unmutated (VH-UM) CLL not using these *VH* genes. *V3-21* cases showed characteristic expression differences compared to VH-MUT (up: *ZAP70* [or *ZAP-70*]; down: *CCND2*, *P27*) and VH-UM (down: *PI3K*, *CCND2*, *P27*, *CDK4*, *BAX*) involving several BCR-related genes. Similarly, there was a marked difference between *VH* unmutated cases using the *V1-69* gene and VH-UM (up: *FOS*; down: *BLNK*, *SYK*, *CDK4*, *TP53*). Therefore, usage of specific *VH* genes appears to have a strong influence on the gene expression pattern pointing to antigen recognition and ongoing BCR stimulation as a pathogenic factor in these CLL subgroups. (Blood. 2006;107: 2090-2093)

Introduction

Chronic lymphocytic leukemia (CLL) with unmutated immunoglobulin variable heavy chain *(VH)* gene (VH-UM) displays a worse prognosis compared to *VH*-mutated CLL (VH-MUT).[1,2] Higher expression of *ZAP70* (or *ZAP-70*), a receptor-associated tyrosine kinase, was identified in VH-UM compared to VH-MUT CLL.[3,4] B-cell receptor (BCR) cross-linking on $ZAP70^{+}$ CLL cells led to increased tyrosine phosphorylation of p72 *(SYK)*, indicating an increased activation after BCR stimulation as a potential mechanism accounting for the clinical differences of the *VH* mutation subgroups.[5-7]

CLL subsets with highly restricted BCR structure were identified, indicating a role for specific antigens in CLL pathogenesis.[8-14] The *V1-69* gene is the most common unmutated *VH* gene in CLL and is associated with a restricted *VDJ* structure that is distinct from the repertoire of normal B cells.[13,14] Similarly, *V3-21* gene usage comprises cases with a highly specific BCR structure as evidenced by homologous CDR3 sequences and a restricted *VL* gene usage.[10] Moreover, these cases show a poor clinical outcome regardless of the *VH* mutation status,[11] indicating an independent pathogenic and prognostic role of specific *VH* gene rearrangements.

To investigate the role of antigenic stimulation in the pathogenesis of CLL subgroups defined by specific *VH* genes, we analyzed the quantitative expression of 26 genes with central roles in BCR signaling, B-cell activation, cell cycle, and apoptosis control in cases defined by *V3-21* and *V1-69* usage as compared to VH-MUT and VH-UM CLL not using these *VH* genes.

Study design

Peripheral blood samples from patients with untreated CLL diagnosed according to established criteria were included after informed consent was obtained in accordance with the Declaration of Helsinki. These studies have been approved by the institutional review board of the University of Ulm. A non–CD19-purified cohort of 69 cases constituted the following subgroups: *V3-21* usage, 16 cases (9 mutated, 7 unmutated); *V1-69*, 17 (all unmutated); VH-MUT (not using *V3-21*), 17; VH-UM (not using *V3-21* or *V1-69*), 19. A CD19-purified cohort included 53 cases (30 overlapping with the nonpurified cohort): 8 using *V3-21* (5 mutated, 3 unmutated); 12 *V1-69*, 12 were VH-MUT (not using *V3-21*); 21 VH-UM (not using *V3-21* or *V1-69*). Distribution of age, sex, Binet stages, and the high-risk aberrations del11q22-23 and del17p13 within the cohorts and subgroups is detailed in Supplemental Table S1 (see the Supplemental Tables link at the top of the online article, at the *Blood* website). Preliminary data of our group implicate a specific biology for CLL using *V3-23*.[15] However, these results were not reproduced independently and no specific *VDJ* configuration has been observed in these cases. Because of these uncertainties and to avoid potential interferences, we excluded *V3-23* cases from the study. Fluorescence in situ hybridization (FISH) analysis and *VDJ* sequencing were performed as described.[16,17] *VH* homology cut-off was 98%. CD19 purification was carried out using

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Table 1. Comparison of candidate gene expression between the subgroups VH-MUT, VH-UM, and *V3-21* and *V1-69* and VH-UM

Gene	VH-UM vs VH-MUT	*V3-21* vs VH-MUT	*V3-21* vs VH-UM	*V1-69* vs VH-UM
Non–CD19-purified cohort				
AKT	0.62 (0.45, 1.0)	0.84 (0.6, 1.2)	1.2 (0.8, 2.1)	0.83 (0.58, 1.3)
ATM	0.57 (0.33, 0.97)	0.66 (0.47, 0.93)	1.2 (0.69, 2.1)	0.81 (0.44, 1.4)
BAX	1.4 (1.0, 1.9)*	0.75 (0.44, 1.1)	0.55 (0.38, 0.73)*	0.76 (0.57, 1.0)
BLNK	1.0 (0.66, 1.5)	0.77 (0.49, 1.2)	0.8 (0.5, 1.2)	0.51 (0.32, 0.8)*
CCND1	0.61 (0.4, 0.94)	0.66 (0.43, 0.94)	1.0 (0.69, 1.6)	1.2 (0.72, 2.0)
CCND2	2.1 (1.3, 3.2)*	0.58 (0.4, 0.8)*	0.27 (0.17, 0.44)*	0.8 (0.47, 1.4)
CCND3	1.4 (0.91, 1.9)	0.7 (0.42, 1.2)	0.51 (0.32, 0.95)	0.72 (0.46, 1.0)
CDK4	1.6 (1.1, 3.3)*	0.69 (0.42, 1.5)	0.43 (0.27, 0.71)*	0.32 (0.17, 0.62)*
FADD	0.8 (0.55, 1.2)	1.1 (0.74, 1.5)	1.3 (0.88, 1.9)	1.1 (0.71, 1.7)
JAK3	1.2 (0.86, 1.7)	0.82 (0.56, 1.2)	0.66 (0.49, 0.87)	0.74 (0.57, 0.98)
LYN	1.1 (0.7, 1.8)	0.78 (0.56, 1.2)	0.73 (0.42, 1.2)	0.92 (0.58, 1.5)
MYC	1.0 (0.6, 2.0)	0.84 (0.46, 1.4)	0.78 (0.43, 1.4)	0.96 (0.46, 1.9)
NFKB	1.4 (1.0, 2.4)	0.87 (0.57, 1.1)	0.58 (0.34, 0.88)	1.3 (0.82, 2.0)
P27	0.98 (0.75, 1.2)	0.63 (0.5, 0.83)*	0.65 (0.51, 0.85)*	1.1 (0.94, 1.5)
PI3K	1.7 (1.2, 2.7)*	0.77 (0.57, 1.1)	0.44 (0.28, 0.66)*	0.7 (0.47, 1.1)
PLCG2	0.75 (0.53, 1.1)	0.7 (0.48, 0.98)	0.93 (0.67, 1.4)	0.78 (0.53, 1.1)
STAT6	0.95 (0.69, 1.4)	0.96 (0.64, 1.4)	0.98 (0.67, 1.4)	0.64 (0.44, 1.0)
SYK	1.1 (0.79, 1.4)	0.69 (0.36, 1.1)	0.63 (0.34, 1.0)	0.57 (0.45, 0.8)*
TP53	1.3 (0.9, 1.9)	0.77 (0.52, 1.2)	0.57 (0.4, 0.82)	0.49 (0.32, 0.8)*
TRAF3	0.64 (0.46, 0.95)	0.87 (0.62, 1.3)	1.4 (0.94, 2.1)	1.3 (0.77, 2.1)
CD19-purified cohort				
BCL2	0.78 (0.51, 1.2)	0.8 (0.29, 1.3)	1.0 (0.36, 1.8)	0.92 (0.51, 1.7)
BCLXL	0.68 (0.49, 0.96)	1 (0.45, 2.2)	1.4 (0.63, 3.2)	0.86 (0.59, 1.3)
E2F1	0.8 (0.24, 3.0)	0.77 (0.37, 1.6)	1.1 (0.58, 2.2)	1.1 (0.61, 1.6)
FOS	0.67 (0.43, 1.1)	1.2 (0.48, 5.4)	1.7 (0.56, 5.2)	3.7 (1.5, 9.8)*
LCK	0.92 (0.5, 2.1)	1.4 (0.66, 3.3)	1.3 (0.73, 2.9)	0.65 (0.38, 1.3)
ZAP70	5.8 (2.6, 14)*	9.3 (3.5, 25)*	1.5 (0.75, 3.2)	0.74 (0.36, 1.4)

Comparison of candidate gene expression between the subgroups VH-MUT, VH-UM, and *V3-21* (Kruskal-Wallis test for 3-group comparisons) and between *V1-69* and VH-UM (Wilcoxon rank-sum test statistics for pair-wise comparisons). The numbers for the non–CD19-purified cohort are VH-UM versus VH-MUT, 19 and 17, respectively; for *V3-21* versus VH-MUT, 16 versus 17; for *V3-21* versus VH-UM, 16 versus 19; and for *V1-69* versus VH-UM, 17 versus 19. For the CD19-purified cohort, the respective numbers are 21 versus 12, 8 versus 12, 8 versus 21, and 12 versus 21. Data presented are fold expression changes with the corresponding 95% CIs (lower and upper confidence limits).

*Significant expression differences after *P* adjustment.

magnetically activated cell sorting (MACS) CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Twenty-six candidate genes were analyzed (Table 1), of which *ZAP70*, *LCK*, *FOS*, *E2F1*, *BCL2*, and *BCLXL* showed an overexpression in the $CD19^-$ as compared to the $CD19^+$ fraction of patient (n = 3) or healthy donor (n = 3) samples. These genes were therefore analyzed in the CD19-purified cohort. RNA extraction, cDNA synthesis, and real-time quantitative polymerase chain reaction (RQ-PCR) were performed as described[18,19] with 3 housekeeping genes for expression normalization (*PGK1*, *LMNB1*, *ACTB*). Primers for SYBR Green detection were used or designed as described[19] and as shown in Table S2. We used a closed testing procedure[20] together with the step-down maxT method[21] using nonparametric test statistics with bootstrap estimated null distributions to analyze gene expression differences between VH-MUT, VH-UM, and *V3-21*. For the analysis of *VH* unmutated CLL with versus without usage of the *V1-69* gene adjusted *P* values were computed by the step-down maxT procedure using Wilcoxon rank-sum statistics. For both analyses, a global significance level of .025 was used to control an overall significance level of 5%. Hodges-Lehmann estimates of log2-transformed expression data served to compute fold-change estimates and their 95% confidence intervals (CIs). The purified and nonpurified cohorts were analyzed separately. All statistical computations were done using R, version 2.1.1.[22]

Results and discussion

In line with previous studies,[3,4] a higher *ZAP70* expression was identified in VH-UM as compared to VH-MUT cases (Table 1; Figure 1). In addition, VH-UM showed a characteristic overexpression of *PI3K* and *CCND2*, for both of which a BCR-dependent up-regulation has been demonstrated experimentally.[23,24] This finding is in line with Chen et al[5] demonstrating that *ZAP70* expression is associated with increased BCR signaling and reinforces the concept of ongoing BCR stimulation as a pathomechanism in VH-UM CLL.[5-7]

The *V3-21* cases under study showed the characteristic distribution into *VH* mutated and *VH* unmutated cases with a median *VH* homology of 97.86%; half of them exhibited the characteristic CDR3 region of 7 amino acids of close homology (Table S3). *V3-21* cases showed significantly higher *ZAP70* levels as compared to VH-MUT with similar expression levels in the different *V3-21* subsets (*VH* mutated/unmutated/7-amino acid CDR3; Table 1, Figure 1). *ZAP70* expression in *V3-21* cases was comparable to VH-UM cases, strengthening the role of *ZAP70* as a prognostic marker recognizing unfavorable CLL subgroups and pointing to a common pathomechanism involving differential BCR signaling in these subgroups as recently proposed for *VH* unmutated CLL.[6,7] However, marked differences occurred when comparing *V3-21* cases with VH-UM CLL including a characteristic down-regulation of several genes in *V3-21* cases (Table 1). The lower expression of *PI3K* and *CCND2* in *V3-21* cases implicates a down-regulation of these BCR target genes despite antigenic stimulation and points to alternative pathways. Indeed, the down-regulation of candidate genes such as *BAX* and *P27* (Table 1) suggests apoptosis impairment and reduced cell cycle

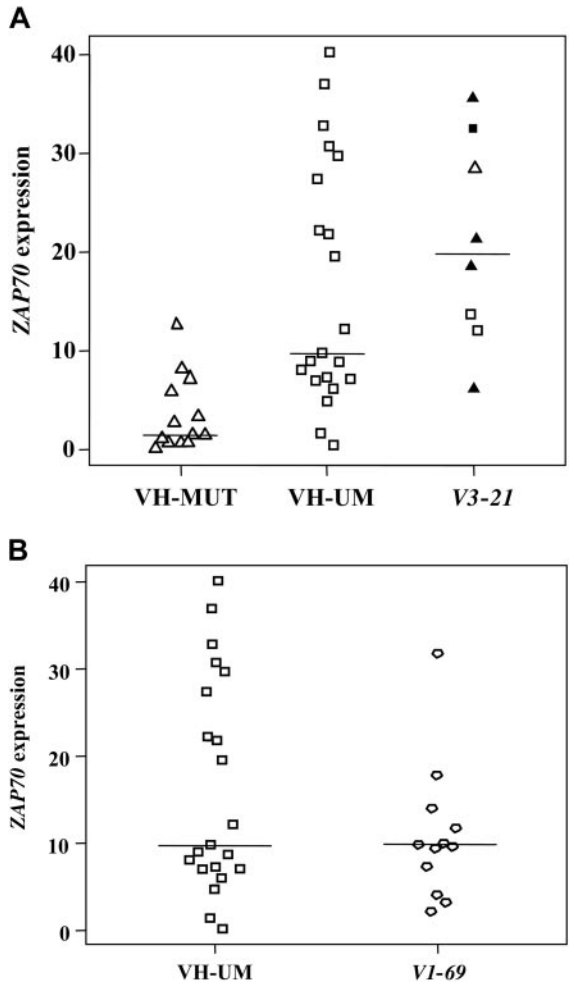

Figure 1. *ZAP70* expression. Results from the CD19-purified cohort. (A) VH-MUT, VH-UM, and *V3-21* cases; (B) VH-UM and *V1-69* cases. *V3-21* subgroup: □, *VH* unmutated; △, *VH* mutated cases; ▲ and ■, cases with the 7-amino acid CDR3.

inhibition as additional pathomechanisms in *V3-21* CLL, which is supported by a recent report.[25] When comparing the mutated versus unmutated *V3-21* subset, although restricted by low case numbers, no highly characteristic gene expression differences occurred (Table S4). Therefore, cases with *V3-21* usage appear to represent a rather homogeneous biologic group independently of the *VH* mutation status.

V1-69 cases under study showed the previously described CLL-specific *VDJ* configuration,[13,14] such as unmutated *VH*, long CDR3 lengths, and a biased usage of *JH6* (Table S5) indicating antigen-specificity of the BCR. When comparing these cases with *VH* unmutated cases not using *V1-69*, a number of differentially expressed genes was detected despite the concordant *VH* mutation status including the BCR-related genes *BLNK*, *SYK*, and *FOS* (Table 1). This finding points to a distinct biology of the *V1-69* subset among *VH* unmutated CLL, which might be explained by the involvement of a distinct antigen leading to antigen-specific BCR responses in *V1-69* CLL cells. Additionally, *V1-69* cases exhibited reduced levels of *TP53*, which was not due to a biased distribution of 17p cases (Table S1), and of *CDK4* implicating cell-cycle deregulation and apoptosis impairment in these cases.

The concept of antigen-specific BCR stimulation is also supported by the characteristic gene expression differences that occurred between the *V3-21* and *V1-69* subgroups (Table S6).

Generally, the *VH* gene-specific gene expression pattern identified supports the concept of antigen selection, as already implicated by the highly restricted *VDJ* features of these cells, and a persisting antigen-dependence of the CLL cells with ongoing BCR-specific stimulation as a pathomechanism in these CLL subgroups.[7-12] These findings strengthen the impact of *VH* gene usage on CLL pathogenesis and suggest a future role for epitope-specific therapeutic approaches in CLL.

Note added in proof. While this manuscript was under review, the results of high *ZAP70* expression in *V3-21* CLL could be confirmed by Kröber et al.[26]

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