DISTINCT GENE EXPRESSION PATTERNS IN CHRONIC LYMPHOCYTIC LEUKEMIA
DEFINED BY USAGE OF SPECIFIC VH GENES

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Abstract

The mutation status and usage of specific VH genes such as V3-21 and V1-69 are potentially independent pathogenic and prognostic factors in CLL. To investigate the role of antigenic stimulation, we analyzed the expression of genes involved in B-cell receptor 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: 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.
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 ZAP-70, a receptor associated tyrosine kinase, was identified in VH-UM compared to VH-MUT CLL (3, 4). B-cell receptor (BCR) crosslinking on ZAP-70 positive 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.
Patients and Methods

Peripheral blood samples from patients with untreated CLL diagnosed according to established criteria were included after informed consent. 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 non-purified 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, gender, Binet stages, and the high risk aberrations del 11q22-23 and del 17p13 within the cohorts and subgroups is detailed in suppl. table 1. 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. Due these uncertainties and to avoid potential interferences, we excluded V3-23 cases from the study. FISH analysis and VDJ sequencing were performed as described (16, 17). VH homology cut-off was 98%. CD19 purification was carried out using MACS CD19 MicroBeads (Miltenyi, Germany). 26 candidate genes were analyzed (table 1), of which ZAP-70, LCK, FOS, E2F1, BCL2, and BCL-XL showed an overexpression in the CD19-negative as compared to the CD19-positive 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 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 suppl. table 2). 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 vs. 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 0.025 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. The purified and non-purified cohort 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 ZAP-70 expression was identified in VH-UM as compared to VH-MUT cases (table 1 and figure 1). In addition, VH-UM showed a characteristic overexpression of PI3K and CCND2, for both of which a BCR dependant up-regulation has been demonstrated experimentally (23, 24). This finding is in line with Chen et al. (5) demonstrating that ZAP-70 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 (aa) of close homology (suppl. table 3). V3-21 cases showed significantly higher ZAP-70 levels as compared to VH-MUT with similar expression levels in the different V3-21 subsets (VH mutated/-unmutated/7aa CDR3) (table 1, figure 1). ZAP-70 expression in V3-21 cases was comparable to VH-UM cases strengthening the role of ZAP-70 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 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 occured (suppl. table 4). 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 (suppl. table 5) 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 (suppl. table 1), 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 (suppl. table 6).

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-dependance 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.
### Tables and Figures

**Table 1:** Comparison of candidate gene expression between the subgroups VH-MUT, VH-UM, and V3-21 (Kruskal-Wallis test for three-group comparisons), and between V1-69 and VH-UM (Wilcoxon rank-sum test statistics for pairwise comparisons). Fold expression changes are shown with the corresponding 95% confidence intervals (LCL/UCL: lower/upper confidence limit). Significant expression differences after p adjustment are indicated (*) and highlighted in bold.

<table>
<thead>
<tr>
<th>Gene</th>
<th>VH-UM vs. VH-MUT</th>
<th>V3-21 vs. VH-MUT</th>
<th>V3-21 vs. VH-UM</th>
<th>V1-69 vs. VH-UM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC (LCL, UCL)</td>
<td>FC (LCL, UCL)</td>
<td>FC (LCL, UCL)</td>
<td>FC (LCL, UCL)</td>
</tr>
<tr>
<td></td>
<td>(n=19 vs. 17)</td>
<td>(n=16 vs. 17)</td>
<td>(n=16 vs. 19)</td>
<td>(n=17 vs. 19)</td>
</tr>
<tr>
<td>AKT</td>
<td>0.62 (0.45,1.0)</td>
<td>0.84 (0.6,1.2)</td>
<td>1.2 (0.8,2.1)</td>
<td>0.83 (0.58,1.3)</td>
</tr>
<tr>
<td>ATM</td>
<td>0.57 (0.33,0.97)</td>
<td>0.66 (0.47,0.93)</td>
<td>1.2 (0.69,2.1)</td>
<td>0.81 (0.44,1.4)</td>
</tr>
<tr>
<td>BAX</td>
<td><strong>1.4 (1.0,1.9)</strong> *</td>
<td>0.75 (0.44,1.1)</td>
<td><strong>0.55 (0.38,0.73)</strong> *</td>
<td>0.76 (0.57,1.0)</td>
</tr>
<tr>
<td>BLNK</td>
<td>1.0 (0.66,1.5)</td>
<td>0.77 (0.49,1.2)</td>
<td>0.8 (0.5,1.2)</td>
<td><strong>0.51 (0.32,0.8)</strong> *</td>
</tr>
<tr>
<td>CCND1</td>
<td>0.61 (0.4,0.94)</td>
<td>0.66 (0.43,0.94)</td>
<td>1.0 (0.69,1.6)</td>
<td>1.2 (0.72,2.0)</td>
</tr>
<tr>
<td>CCND2</td>
<td><strong>2.1 (1.3,3.2)</strong> *</td>
<td><strong>0.58 (0.4,0.8)</strong> *</td>
<td><strong>0.27 (0.17,0.44)</strong> *</td>
<td>0.8 (0.47,1.4)</td>
</tr>
<tr>
<td>CCND3</td>
<td>1.4 (0.91,1.9)</td>
<td>0.7 (0.42,1.2)</td>
<td>0.51 (0.32,0.95)</td>
<td>0.72 (0.46,1.0)</td>
</tr>
<tr>
<td>CDK4</td>
<td><strong>1.6 (1.1,3.3)</strong> *</td>
<td>0.69 (0.42,1.5)</td>
<td><strong>0.43 (0.27,0.71)</strong> *</td>
<td><strong>0.32 (0.17,0.62)</strong> *</td>
</tr>
<tr>
<td>FADD</td>
<td>0.8 (0.55,1.2)</td>
<td>1.1 (0.74,1.5)</td>
<td>1.3 (0.88,1.9)</td>
<td>1.1 (0.71,1.7)</td>
</tr>
<tr>
<td>JAK3</td>
<td>1.2 (0.86,1.7)</td>
<td>0.82 (0.56,1.2)</td>
<td>0.66 (0.49,0.87)</td>
<td>0.74 (0.57,0.98)</td>
</tr>
<tr>
<td>LYN</td>
<td>1.1 (0.7,1.8)</td>
<td>0.78 (0.56,1.2)</td>
<td>0.73 (0.42,1.2)</td>
<td>0.92 (0.58,1.5)</td>
</tr>
<tr>
<td>MYC</td>
<td>1.0 (0.6,2)</td>
<td>0.84 (0.46,1.4)</td>
<td>0.78 (0.43,1.4)</td>
<td>0.96 (0.46,1.9)</td>
</tr>
<tr>
<td>NFKB</td>
<td>1.4 (1.2,4)</td>
<td>0.87 (0.57,1.1)</td>
<td>0.58 (0.34,0.88)</td>
<td>1.3 (0.82,2.0)</td>
</tr>
<tr>
<td>p27</td>
<td>0.98 (0.75,1.2)</td>
<td><strong>0.63 (0.5,0.83)</strong> *</td>
<td><strong>0.65 (0.51,0.85)</strong> *</td>
<td>1.1 (0.94,1.5)</td>
</tr>
<tr>
<td>PI3K</td>
<td><strong>1.7 (1.2,2.7)</strong> *</td>
<td>0.77 (0.57,1.1)</td>
<td><strong>0.44 (0.28,0.66)</strong> *</td>
<td>0.7 (0.47,1.1)</td>
</tr>
<tr>
<td>PLCG2</td>
<td>0.75 (0.53,1.1)</td>
<td>0.7 (0.48,0.98)</td>
<td>0.93 (0.67,1.4)</td>
<td>0.78 (0.53,1.1)</td>
</tr>
<tr>
<td>STAT6</td>
<td>0.95 (0.69,1.4)</td>
<td>0.96 (0.64,1.4)</td>
<td>0.98 (0.67,1.4)</td>
<td>0.64 (0.44,1)</td>
</tr>
<tr>
<td>SYK</td>
<td>1.1 (0.79,1.4)</td>
<td>0.69 (0.36,1.1)</td>
<td>0.63 (0.34,1)</td>
<td><strong>0.57 (0.45,0.8)</strong> *</td>
</tr>
<tr>
<td>TP53</td>
<td>1.3 (0.9,1.9)</td>
<td>0.77 (0.52,1.2)</td>
<td>0.57 (0.4,0.82)</td>
<td><strong>0.49 (0.32,0.8)</strong> *</td>
</tr>
<tr>
<td>TRAF3</td>
<td>0.64 (0.46,0.95)</td>
<td>0.87 (0.62,1.3)</td>
<td>1.4 (0.94,2.1)</td>
<td>1.3 (0.77,2.1)</td>
</tr>
</tbody>
</table>

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**Figure 1:** ZAP-70 expression of a) VH-MUT, VH-UM and V3-21 cases, b) VH-UM and V1-69 cases.

The results confer to the CD19 purified cohort. V3-21 subgroup: rectangles indicate VH unmutated, triangles VH mutated cases, filled symbols indicate cases with the 7aa CDR3.

a)
References


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