Expression of Mutated IGHV3-23 Genes in Chronic Lymphocytic Leukemia Identifies a Disease Subset with Peculiar Clinical and Biological Features

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Abstract

Purpose: B-cell chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease whose outcome can be foreseen by investigating the mutational status of immunoglobulin heavy chain variable (IGHV) genes. Moreover, a different prognosis was reported for CLL expressing specific IGHV genes in the context or not of stereotyped B-cell receptors. Here we investigated novel associations between usage of specific IGHV genes and clinical features in CLL.

Experimental Design: Among 1,426 CLL-specific IG-rearrangements, stereotyped B-cell receptor clusters never utilized the IGHV3-23 gene. Given this notion, this study was aimed at characterizing the IGHV3-23 gene in CLL, and identifying the properties of IGHV3-23–expressing CLL.

Results: IGHV3-23 was the second most frequently used (134 of 1,426) and usually mutated (M; 109 of 134) IGHV gene in our CLL series. In the vast majority of M IGHV3-23 sequences, the configuration of the 13 amino acids involved in superantigen recognition was consistent with superantigen binding. Clinically, M IGHV3-23 CLL had shorter time-to-treatment than other M non–IGHV3-23 CLL, and multivariate analyses selected IGHV3-23 gene usage, Rai staging, and chromosomal abnormalities as independent prognosticators for M CLL. Compared with M non–IGHV3-23 CLL, the gene expression profile of M IGHV3-23 CLL was deprived in genes, including the growth/tumor suppressor genes PDCD4, TIA1, and RASSF5, whose downregulation is under control of miR-15a and miR-16-1. Accordingly, relatively higher levels of miR-15a and miR-16-1 were found in M IGHV3-23 compared with M non–IGHV3-23 CLL.


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Chronic lymphocytic leukemia (CLL) is a heterogeneous disease whose clinical course can be foreseen by the presence of mutated (M) or unmutated (UM) immunoglobulin heavy chain variable (IGHV) genes (1). Although CLL with UM IGHV genes usually has a worse prognosis, great heterogeneity is still documented in the context of CLL subgroups expressing either UM or M IGHV genes (1).

Analyses of IGHV gene usage have revealed a biased repertoire occurring in CLL versus normal B cells, and in UM versus M CLL (1, 2). Moreover, a discrete fraction of CLL expresses stereotyped combinations of IGHV genes, complementarity-determining region-3 (HCDR3), and IGV light chains (3–7). These observations, coupled with an IGHV mutation profile often consistent with antigen-driven
Survival of patients with B-cell chronic lymphocytic leukemia (CLL) can be predicted by analysis of mutations in immunoglobulin heavy chain variable (IGHV) genes, as well as by the expression of specific IGHV genes or stereotyped B-cell receptors, also irrespective of IGHV mutational status. Here we documented that IGHV3-23, one of the most frequently used and usually mutated (M) IGHV gene in CLL, is constantly absent from stereotyped B-cell receptor clusters, retains the capacity to bind superantigens, and behaves as an independent negative prognosticator for M CLL.

When compared with other M CLL, the gene expression profile of M IGHV3-23 CLL seems deprived in growth/tumor suppressor genes, whose downregulation is controlled by miR-15a and miR-16-1, which in turn are expressed at higher levels in M IGHV3-23 CLL. Altogether, expression of the IGHV3-23 gene marks a CLL subset with distinct clinical-biological features, and may help to refine the prognostic assessment of M CLL.

**Materials and Methods**

**Characterization of CLL patients.** The present study included peripheral blood samples from 1,398 unselected CLL patients (median age 64.0 y; range, 32-97) collected in the context of a previous study (7). Patients provided informed consent in accordance with local Institutional Review Board requirements and the declaration of Helsinki. IGHV-diversity(D)-joining(J) rearrangements, IGHV gene mutational status, and HCDR3-driven clustering were reported elsewhere (7). The 2% cutoff was chosen to discriminate UM versus M CLL. CLL cases were also characterized for Rai stage at diagnosis; abnormalities involving chromosomes 11, 12, 13, and 17 by interphase fluorescence in-situ hybridization; and CD38 and ZAP-70 expression by flow cytometry. For CD38 and ZAP-70, the cutoffs of 30% and 20% of positive cells were chosen to discriminate between CD38low and CD38high, or ZAP-70low and ZAP-70high, respectively (7, 26, 27).

**Gene expression profiling analysis and data mining tools.** Procedures for purification of neoplastic and normal B cells, as well as preparation of purified dye-labeled amino-allyl RNA (aRNA) were previously described (25). Gene expression profiling (GEP) was done by a dual-labeling strategy using Cy3-labeled aRNA from pooled normal peripheral blood B cells of healthy donors as common reference, and Cy5-labeled aRNA from purified CLL cells as tester, hybridized to the Whole Human Genome (4 × 44 K) oligo microarray platform (Agilent Technologies); slides were analyzed by Agilent Microarray Scanner using the Agilent Feature Extraction Software (Agilent Technologies).

Supervised analyses were done using a modified version of the original significance analysis of microarrays (25, 28). GEP results were visualized by hierarchical clustering applying Ward’s method with Euclidean distance (29). The biological functions of differentially expressed genes were investigated by gene-ontology tree machine (30) and gene set enrichment analysis (31). Details may be found in Supplementary Materials and Methods.
Quantitative-real-time PCR. Expression of genes was evaluated by quantitative real-time PCR, using SYBR Green dye–containing reaction buffer (Applied Biosystems) for quantitation. Details and primer sequences may be found in Supplementary Materials and Methods.

Expression of miR-15a, miR-16-1, and the control RNU6B microRNA in M IGHV3-23 and M non–IGHV3-23 CLL cells was assessed using a standard TaqMan MicroRNA assay kit (Applied Biosystems) according to the manufacturer’s instructions and as described (32). Fold-change between classes was calculated as reported (33).

Statistical analysis. Time-to-first-treatment (TTT) intervals were available for 617 patients, in which CLL diagnosis was made according to the recently published guidelines (34, 35). TTT comparisons were made using Kaplan-Meier plots and log-rank test. The Cox proportional hazard regression model was used to assess the independent effect of covariates, treated as dichotomous on TTT, with a backward procedure for selecting significant variables.

Results

Molecular characterization of IGHV3-23 genes in CLL. A total of 1,426 in-frame IGHV-D-J rearrangements were sequenced in 1,398 patients, and analyzed by a HCDR3-driven clustering. The number and the molecular features of clusters with stereotyped B-cell receptors were extensively described elsewhere (7), and were in keeping with other reports (5–7, 10, 11). By comparing IGHV gene usage with the distribution of IGHV genes in stereotyped B-cell receptor clusters, we observed that IGHV3-23 was totally absent in confirmed clusters (i.e., with at least three sequences per cluster), despite being the second most frequently used IGHV gene (134 of 1,426; Fig. 1). Such distribution was significantly skewed ($P < 0.0001$), compared with the distribution of IGHV genes belonging to stereotyped B-cell receptor clusters observed in our series (7). IGHV3-23 was predominantly mutated (UM/M, 25/109), and expressed either IGK or IGL (data available for 81 cases; IGK/IGL, 56/25) light chains (Supplementary Table S1).

Amino acid configuration of M IGHV3-23 genes in CLL. Complete amino acid sequences of M IGHV3-23 genes were available in 100 of 109 cases. No shared and/or recurrent amino acids were found in either HCDR or heavy-chain framework (HFR) regions (data not shown). Thirteen amino acid positions, located either in the HFR1 (three positions), HCDR2 (one position), or HFR3 (nine positions), were investigated because they were involved in superantigen (e.g., Staphylococcus Aureus protein A and HIV-gp120) recognition by IGHV3 subgroup genes (Supplementary Fig. S1A; refs. 13, 14). With the only exclusion of a few specific permissive amino acid changes (Supplementary Fig. S1B), the germline configuration at these positions should be maintained to allow superantigen recognition and binding with IGHV3-expressing IG (13, 14). When M IGHV3-23 sequences from our CLL series were checked for amino acid changes involving these 13 positions, 68 of 100 had either zero or a single nonpermissive amino acid change, with 2 nonpermissive amino acid changes being found in additional 19 sequences (Supplementary Table S1).

Clinical features of M IGHV3-23 CLL. Of 1,398 patients, TTT intervals were available for 617 cases. In this cohort, along with the IGHV gene mutational status (UM/M, 241/376), information regarding the following prognosticators was also available: (a) Rai staging (stage 0, $n = 331$; stage 1, $n = 150$; stage 2, $n = 89$; stage 3, $n = 10$; stage 4, $n = 37$); (b) CD38 and ZAP-70 expression status (CD38$^{\text{low}}$, $n = 492$; CD38$^{\text{high}}$, $n = 125$; ZAP-70$^{\text{low}}$, $n = 371$; ZAP-70$^{\text{high}}$, $n = 246$); and (c) karyotype abnormalities as detected by fluorescence in-situ hybridization (13q-, $n = 186$; +12, $n = 71$; 11q-, $n = 54$; 17p-, $n = 49$; normal karyotype, $n = 257$). All the reported variables were proven to be significant predictors for TTT (Supplementary Table S2 and Fig. S2).

Fig. 1. Usage of IGHV genes and chance to belong to clusters of homologous HCDR3. Expression of the 10 most frequently used IGHV genes in the series of 1,426 IG rearrangements. For each IGHV gene, histograms report the total number of IG rearrangements (closed histograms), the number of IG rearrangements not included in confirmed clusters of homologous HCDR3 (open histograms), and the number of IG rearrangements included in confirmed clusters of homologous HCDR3 (dotted histograms).
in keeping with our previous studies and those of other groups (1, 7, 12, 27, 36). Conversely, expression of IGHV3-23 (54 cases; UM/M, 11/43) failed to have prognostic relevance in this series (Supplementary Table S2).

Because IGHV3-23 genes, as expressed by CLL cells, were preferentially mutated (Supplementary Table S1), we investigated the clinical behavior of M IGHV3-23 CLL (43 cases) in the context of a CLL cohort with M IGHV gene rearrangements (376 cases; Supplementary Table S2 and Fig. 2). The median TTT of the 43 M IGHV3-23 CLL patients (73 months) was significantly shorter than the median TTT of 326 M non–IGHV3-23 CLL (253 months; \( P = 0.0082 \); Fig. 2). Such a difference also held true when 7 non–6 months; Fig. 2; refs. 7, 10, 12) were included in the M IGHV3-23 CLL (Supplementary Table S3). Multivariate Cox proportional hazards analysis showed that IGHV3-23 (Supplementary Table S3). Multivariate Cox proportional hazards analysis showed that IGHV3-23 (median TTT, 6 months; Fig. 2; refs. 7, 10, 12) were included in the M non–IGHV3-23 CLL group (333 cases; \( P = 0.0153 \); Supplementary Fig. S3). No differences in terms of TTT were found by comparing 22 M IGHV3-23 CLL, whose B-cell receptors bore 0 to 1 nonpermissive amino acid changes in the 13 positions involved in superantigen binding (13, 14), with 9 M IGHV3-23 CLL whose B-cell receptor bore >2 nonpermissive amino acid changes (not shown).

The independent prognostic value of IGHV3-23 expression in M CLL was also evaluated. In this regard, all the investigated prognosticators and IGHV3-23 usage correlated with an increased risk of progressive disease by univariate analysis both in the presence and in the absence of CLL cases expressing the IGHV3-21/IGLV3-21 gene combination (median TTT, 6 months; Fig. 2; refs. 7, 10, 12). Notably, the distribution of prognosticators was not different in M IGHV3-23 cases compared with the remaining M non–IGHV3-23 CLL (Supplementary Table S3). Multivariate Cox proportional hazards analysis showed that IGHV3-23 usage, Rai stage, and the fluorescence in situ hybridization group were independent markers of disease progression for the whole cohort of M CLL (Table 1, model 1). Superimposable results were obtained by excluding the seven M CLL expressing IGHV3-21/IGLV3-21 gene combination from the analysis (Table 1, model 2; refs. 7, 10, 12).

**Differential GEP of M IGHV3-23 CLL.** The clinical results prompted us to characterize the GEP of M IGHV3-23 CLL cells. By comparing 5 M IGHV3-23 and 22 M non–IGHV3-23 CLL (Supplementary Table S4), 212 transcripts were selected by bioinformatics analysis, 108 upregulated and 104 downregulated in M IGHV3-23 CLL (Supplementary Table S5). A hierarchical clustering generated using these 212 best-correlated transcripts clearly split the 5 M IGHV3-23 from the 22 M non–IGHV3-23 CLL (Fig. 3A).

To elucidate the biological functions of genes representing the signature of M IGHV3-23 CLL, a gene-ontology tree machine analysis (30) of the 212 differentially expressed transcripts revealed an overrepresentation of GO classes related to apoptosis (Supplementary Table S6), always containing a set of genes (PDCD4, TIA1, and RASSF5) constantly downregulated in M IGHV3-23 CLL. Significantly lower levels of PDCD4, TIA1, and RASSF5 were detected by quantitative real-time PCR experiments in M IGHV3-23 (15 cases) compared with M non–IGHV3-23 (35 cases) CLL cells (Fig. 3B).

**Involvement of mir-15a and mir-16-1 in defining the differential GEP of M IGHV3-23 CLL.** PDCD4 and TIA1 were among the genes downregulated in leukemic cells transfected with mir-15a and mir-16-1, as well as in CLL cells expressing high levels of these two microRNAs (37). By extending this observation, we found that of the 212 transcripts representing the signature of M IGHV3-23 versus M non–IGHV3-23 CLL, as many as 42 genes (40 downregulated and 2 upregulated) were genes whose expression was modulated upon mir-15a and mir-16-1 transfection (37). Notably, 41 of the 42 genes showed the same type of deregulation (39 downregulated and 2 upregulated; Supplementary Table S5; \( r = 0.8549; P < 0.0001 \)) previously reported to be the result of mir-15a and mir-16-1 transfection (37). To further investigate the relationship between microRNA expression and GEP of M IGHV3-23 CLL, the 212 transcripts representing the expression signature of M IGHV3-23 CLL were tested with gene set enrichment analysis, focusing on the gene sets that are grouped for sharing the same DNA binding motifs, including the binding motifs of microRNAs (31). According to this analysis, the gene set containing genes under control of mir-15a and mir-16-1 presented the lowest nominal \( P \) value (nominal \( P < 0.0001 \); False Discovery Rate \( q \)-value = 0.016; Table S7). In the context of this gene set, M IGHV3-23 samples were characterized by a significant enrichment in downregulated genes, including PDCD4, TIA1, and RASSF5 (Fig. 4). Altogether, these data suggest an involvement of mir-15a and mir-16-1 in defining the differential GEP of M IGHV3-23 CLL.

To prove this suggestion, the relative levels of mir-15a and mir-16-1 were quantified by a quantitative real-time PCR analysis (Fig. 2). Kaplan-Meier curves comparing TTT of patients affected by CLL expressing IGHV3-23 and M IGHV mutational status (M IGHV3-23 CLL), or M IGHV mutational status in the context of non–IGHV3-23 IG rearrangements (other M CLL), and by M IGHV3-23/IGLV3-21 CLL. The number of patients (pts) included in each group is reported in parenthesis; the reported \( P \) value refers to log-rank test.
Table 1. Multivariate Cox regression analyses of TTT

<table>
<thead>
<tr>
<th>Model 1 (M IGHV, 376 patients)¹</th>
<th>HR (95% CI)*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rai stage (≥1 versus 0)</td>
<td>4.39 (2.94-6.56)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FISH group (17p- or 11q- or +12 versus normal or 13q-)</td>
<td>4.15 (2.74-6.27)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IGHV3-23 usage</td>
<td>1.75 (1.06-2.90)</td>
<td>0.029</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model 2 (M IGHV, 369 patients)²</th>
<th>HR (95% CI)*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rai stage (≥1 versus 0)</td>
<td>4.12 (2.74-6.19)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FISH group (17p- or 11q- or +12 versus normal or 13q-)</td>
<td>4.45 (2.91-6.80)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IGHV3-23 usage</td>
<td>1.89 (1.14-3.13)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

NOTE: Multivariate Cox regression analyses of TTT were done by including the following covariates: Rai stage, FISH group, IGHV3-23 usage, CD38, and ZAP-70. For each variable, the selected cutoff points or the compared categories are indicated.

HR, hazard ratio; 95% CI, 95% confidence interval; FISH, fluorescence in-situ hybridization.

*Based on the final model after backward selection of covariates.

¹376 CLL all with M IGHV rearrangements.
²369 CLL all with M IGHV rearrangements (7 M IGHV3-21/IGLV3-21 CLL were excluded).

PCR approach in M IGHV3-23 CLL (15 cases), M non–IGHV3-23 CLL (35 cases), and normal B cells purified from 15 healthy donors (Fig. 5). By considering the whole cohort of CLL samples, irrespective of IGHV usage, we confirmed the overall lower expression levels of both miR-15a and miR-16-1 in CLL compared with normal B cells from 15 healthy donors (P = 0.0023 and P < 0.0001 respectively; data not shown; refs. 24, 37). However, when CLLs were split into M IGHV3-23 and M non-IGHV3-23–expressing cases, M IGHV3-23 were found to express significantly higher levels of both miR-15a and miR-16-1 compared with M non-IGHV3-23 cases (Fig. 5). Notably, although miR-15a levels, as expressed by M IGHV3-23 CLL, seemed not significantly different compared with those of normal B cells, both M IGHV3-23 and M non–IGHV3-23 CLL displayed significantly lower expression levels of miR-16-1 compared with normal B cells (Fig. 5).

In keeping with these findings, no difference was found in the distribution of patients with 13q14 deletion between M IGHV3-23 CLL (13q14 deletion in 15 of 43 cases) and M non–IGHV3-23 CLL (13q14 deletion in 156 of 333 cases; P = 0.19) from our series. By considering the cases used for microRNA expression experiments (data available in 47 of 50 cases), 8 of 15 M IGHV3-23 CLL bore the 13q14 deletion in >20% of nuclei, against 19 of 32 cases in the group of M non–IGHV3-23 CLL (P = 0.94; Supplementary Table S8). In both series, three cases had biallelic 13q14 deletions in a fraction of nuclei, whereas in a single case biallelic 13q14 deletion was detected in the majority of nuclei (Supplementary Table S8).

Discussion

The first observation of the present study is that the IGHV3-23 gene, one of the most frequently used IGHV gene in CLL, is constantly absent from clusters of stereotyped B-cell receptors (7). These data are in keeping with the two largest studies published so far, together investigating approximately 3,500 CLL, in which none of the clusters with at least three sequences per cluster included IGHV3-23 sequences (5, 6).

The absence of IGHV3-23 from clusters of stereotyped B-cell receptors can be theoretically explained by considering the high mutational load of the B-cell receptors in IGHV3-23–expressing CLL, which could make difficult the identification of sequence similarities by cluster analyses. In keeping with this observation, the vast majority of IGHV3-23 CLL from our series and those of others (5–7) had >2% mismatch variations from germline sequences. However, with the only exception of IGHV3-21 genes, the mutational load of IGHV3-23 rearrangements in our series was not higher than that of other M IGHV genes, either included or not included in clusters of stereotyped B-cell receptors (11) (3–7, 9, 10, 12). As an alternative explanation, IGHV3-23–expressing B-cell receptors might be selected through non–CDR-based recognition mechanisms, e.g., through interactions with superantigens, a general feature of B-cell receptors expressing IGHV subgroup genes (13, 14). Indeed, the vast majority of IGHV3-23 sequences of our series carried none or only one nonpermissive mutation in the 13 amino acid positions involved in superantigen recognition and binding (13, 14), thus virtually retaining the capacity to mediate superantigen interactions. This feature was also shared by other non–IGHV3-23 sequences from the IGHV3 subgroup (12), as well as by IGHV4-34, another usually M IGHV gene involved in superantigen binding via residues located outside the conventional antigen-binding sites (5, 13, 14, 38, 39).

Our study points to the worse prognosis of M IGHV3-23 compared with other M non–IGHV3-23 CLL. According

to our results, M IGHV3-23 CLL displayed shorter TTT intervals compared with M non–IGHV3-23 CLL. More importantly, IGHV3-23 usage retained an independent prognostic value, along with other known prognosticators (Rai stage and fluorescence in-situ hybridization group), as TTT predictor in the context of M CLL. A trend toward a worse clinical behavior of M IGHV3-23 CLL patients was also observed by comparing overall survival data, without reaching the statistical significance ($P = 0.08$) allegedly due to a relatively low number of events in our patient cohort (6 of 43 and 24 of 326 deaths in the M IGHV3-23 and M non–IGHV3-23 CLL cohorts, respectively).12

Although IGHV gene mutational status still remains a relevant prognosticator in CLL, other features of B-cell receptors have been identified as additional markers capable of refining the prognostic assessment of CLL patients, often involving genes of the IGHV3 subgroup. Expression of IGHV3-23 itself was preliminarily indicated as a marker for CLL with worse prognosis (40). Moreover, expression of IGHV3-21, associated or not with a specific homologous HCDR3 fragment and with the IGLV3-21 light chain, has been shown to identify a CLL subset with poor outcome (3, 7, 12). On the other hand, the IGHV3-72 gene was shown to be expressed by CLL with a particularly stable and benign clinical course (11). According to the results presented here, we suggest including the expression of IGHV3-23 genes as an additional marker further refining the prognostic assessment of M CLL.

In an attempt to explain the peculiar clinical behavior of M IGHV3-23 CLL, we compared the GEP of CLL cells belonging to this class with that of M non–IGHV3-23 CLL. Among the genes downregulated in M IGHV3-23 CLL we focused on PDCD4, TIA1, and RASSF5 for several reasons: (a) PDCD4 is a tumor suppressor gene frequently downregulated in tumors, and it inhibits neoplastic transformation and tumor invasion (41); (b) TIA1 is a proapoptotic factor that regulates gene expression by binding to RNA (42); and (c) RASSF5, a gene found inactivated in a variety of cancers, belongs to a family of RAS-binding proteins and acts as a growth/tumor suppressor gene upon binding to microtubules (43, 44). Notably, an altered expression pattern of microtubule genes, with a significant upregulation of TUBB2A ($P = 0.0286$; Supplementary Table S5) was also found in M IGHV3-23 CLL. Overexpression of TUBB2A was shown to be associated per se with a highly aggressive phenotype in other nonhemopoietic tumors (45).

To explore the putative mechanism(s) for the downregulation of tumor suppressor/proapoptotic genes in M IGHV3-23 CLL, experiments aimed at verifying promoter hypermethylation of PDCD4, RASSF5, and TIA1 (46) failed to detect a significantly different methylation pattern in any of the analyzed CpG-rich regions between M IGHV3-23 and M non–IGHV3-23 CLL.11

MicroRNAs are a new class of regulatory molecules, whose function is chiefly to target specific mRNAs leading to their degradation or inhibition of translation, thus diminishing the specific protein level (16, 17). In the present study, our interest in microRNAs, and in particular in miR-15a and miR-16-1, stemmed from the following findings: (a) as many as 39 of the 109 transcripts found to be downregulated in M IGHV3-23 CLL cells were previously

Fig. 3. IGHV3-23–associated GEP in CLL. A, hierarchical clustering of the 212 transcripts found to be differentially expressed between CLL samples expressing M IGHV3-23 and M non–IGHV3-23 genes (red and blue bar under the horizontal dendrogram, respectively) by supervised analyses. The color scale identifies relative gene expression changes normalized by the standard deviation of 1 with 0 representing the mean expression level of a given gene. B, validation of GEP results by quantitative real-time PCR. Three genes found differentially expressed between M IGHV3-23 and M non–IGHV3-23 CLL samples were assayed by quantitative real-time PCR. Data represent mean ± SE, as obtained in 15 M IGHV3-23 and 35 non–IGHV3-23; $P$ values (Student’s t-test) for each gene are shown.

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shown to be similarly downregulated in leukemic cells transfected with miR-15a and miR-16-1, as well as in CLL cells expressing high levels of these two microRNAs (37); and (b) gene set enrichment analysis selected the gene set containing genes under control of miR-15a and miR-16-1 as a gene set particularly rich in genes which turned out to be downregulated in the M IGHV3-23 CLL class, including PDCD4, TIA1, and RASSF5.

The miR-15a and miR-16-1 microRNAs belong to the same cluster and reside at chromosome 13q14.3, a region frequently deleted in CLL (47); deletions and/or mutations of germline sequences are among the reasons determining their downregulation in CLL (22–24). In our series, expression of both miR-15a and miR-16-1 was significantly downregulated in CLL cells, regardless of IGHV gene usage, as compared with normal B cells. However, M IGHV3-23 CLL cells expressed levels of both microRNAs which were significantly higher than those found in CLL cells expressing M non–IGHV3-23 genes. Although miR-15a and miR-16-1 levels remain overall lower than those found in normal B cells, this observation is not against a causal role of these microRNAs in determining the worse prognosis of M IGHV3-23 CLL. In fact, it is a general feature of microRNAs to act as micromanagers in the fine tuning of gene expression; as a result, relatively low modulation in microRNA copy number can lead to major phenotypic consequences (48, 49). Moreover, the notion that miR-15a and miR-16-1 are part of a microRNA signature identifying CLL cases characterized by a more aggressive clinical course (22) is again consistent with our findings.

The higher expression levels of miR-15a and miR-16-1 in M IGHV3-23 CLL could be explained following at least two lines of reasoning. First, M IGHV3-23 CLL might represent a homogeneous CLL group in which the reported inactivation of miR-15a and miR-16-1 does not occur, or occurs less strikingly than in other CLL (22, 24). In this regard, although 13q14 deletions were also documented in M IGHV3-23 CLL from our series, no concurrent information is available regarding the mutational status of the two microRNA genes and/or the contribution of clusters similar to the miR-15a/16-1 cluster (41, 49) in determining the total amount of miR-15a and miR-16-1 found in M IGHV3-23 CLL. Second, the levels of miR-15a and miR-16-1, as expressed by M IGHV3-23 CLL cells, could be the result of a regulation driven by various stimuli, including triggering of receptors involved in CLL-microenvironment interactions (50). Although formal proofs for modulation of microRNA expression by external stimuli are still lacking, it has been recently shown that expression of specific microRNAs significantly associates with ZAP-70 expression or with a M or UM IGHV mutational status (22, 24). According to this hypothesis, miR-15a and miR-16-1 expression levels, downregulated as part of the neoplastic transformation process, could have been subsequently remodelled in M IGHV3-23 CLL cells because of engagement of cell surface receptors by particular stimuli, such as interactions of specific superantigens with their corresponding B-cell receptors. The underrepresentation of ZAP-70 in M IGHV3-23 CLL also suggests pathogenetic mechanism(s) outside the canonical B-cell receptor stimulation for this disease subset.

Altogether, our study indicates that: (a) M IGHV3-23 CLL never belongs to clusters of stereotyped B-cell receptors; (b) M IGHV3-23-expressing B-cell receptors potentially

Fig. 4. Gene set enriched analysis in M IGHV3-23 versus M non–IGHV3-23 CLL. By utilizing the 212 genes representing the differential GEP signature of M IGHV3-23 CLL for a gene set enriched analysis, the “TGCTGCT, miR-15a and miR-16-1” gene set (11 genes in the gene set) turned out to be significantly enriched in downregulated genes in the M IGHV3-23 CLL subgroup. A, gene set enriched analysis plots for the “TGCTGCT, miR-15a and miR-16-1” gene set; shown is a plot of the Enrichment Score (ES) versus the gene list index; a description of the gene set enriched analysis algorithm is given in Subramanian et al. (31). B, corresponding heat-map showing relative expression of the gene members belonging to the “TGCTGCT, miR-15a and miR-16-1” gene set across M IGHV3-23 (5 cases) and M non–IGHV3-23 (22 cases) CLL.
retain the capability to bind superantigens; (c) M IGHV3-23 CLL has a worse prognosis than other M CLLs; (d) M IGHV3-23 CLL displays a distinct GEP consistent with the observed clinical behavior; and (e) the differential GEP of M IGHV3-23 CLL could at least in part be due to a relative overexpression of miR-15a and miR-16-1. It is tempting to speculate that M IGHV3-23 CLL cells, through triggering of their B-cell receptors, e.g., by superantigens, might modulate the expression of certain microRNAs (e.g., miR-15a and miR-16-1), which in turn could lead to downregulation of specific growth/tumor suppressor genes, thus providing the molecular basis for the worse prognosis of this CLL subset. Experimental studies are ongoing to show this hypothesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest to disclose.

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