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Human Cancer Biology

Immune Thrombocytopenia in Patients with Chronic Lymphocytic Leukemia Is Associated with Stereotyped B-cell Receptors

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Abstract

Purpose: To assess biologic features related to the development of immune thrombocytopenia (ITP) in patients with chronic lymphocytic leukemia (CLL).

Experimental Design: We retrospectively analyzed 463 patients with CLL with available immunoglobulin heavy-chain variable (IGHV) gene status and B-cell receptor (BCR) configuration [heavy-chain complementary-determining region 3 (HCDR3)], of whom thirty-six developed ITP, according to previously defined criteria. Most of them had available cytogenetic analysis.

Results: We observed a significant association between ITP occurrence and IGHV unmutated gene status ($P = 0.0001$), unfavorable cytogenetic lesions ($P = 0.005$), and stereotyped HCDR3 ($P = 0.006$). The more frequent stereotyped HCDR3 subsets were #1 (IGHV1-5-7/IGHD6-19/IGHJ4; 16 of 16 unmutated) and #7 (IGHV1-69 or IGHV3-30/IGHD3-3/IGHJ6; 13 of 13 unmutated), both being significantly more represented among patients developing ITP ($P = 0.003$ and $P = 0.001$, respectively). Moreover, restricting the analysis to unmutated patients, subset #7 confirmed its independent significant association with the occurrence of ITP ($P = 0.013$). Both unmutated IGHV mutational status, del(11)(q23) and stereotyped BCR were significantly associated with shorter time to ITP development ($P < 0.0001$, $P = 0.02$, and $P = 0.005$, respectively) than other patients.

Conclusion: Our data suggest that patients with CLL and peculiar BCR conformations are at higher risk of developing secondary ITP and that stereotyped BCR may be involved in the pathogenesis of this complication. Clin Cancer Res; 18(7); 1870–8. ©2012 AACR.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of monoclonal B lymphocyte with a distinct phenotype (CD5+, CD23+, CD22−, and low level of surface Ig) in peripheral lymphoid organs, bone marrow, and peripheral blood (1, 2). The clinical outcome of patients with CLL is widely heterogeneous and frequently associated with cellular and molecular markers and/or specific genomic alterations. In particular, patients with CLL can display somatic mutations on the immunoglobulin heavy-chain variable (IGHV) gene, which correlate with a favorable prognosis, whereas unmutated IGHV patients generally have a worse clinical outcome. It has been reported that more than 20% of patients with CLL exhibit closely homologous (’stereotyped’) heavy-chain complementary-determining region 3 (HCDR3) sequences. This finding has suggested that clones sharing stereotyped BCRs may expand due to the stimulation by a restricted set of epitopes and that antigenic driving may play an important role in the pathogenesis of the disease (2–6). In addition, some of the most represented stereotyped subsets were distinguished by a peculiar clinical outcome (2–4, 6), a distinct cytogenetic profile (4, 7, 8), or a higher risk of transformation in Richter syndrome (9).

The clinical course of patients with CLL is frequently complicated by autoimmune phenomena leading to cytopenias (autoimmune cytopenias (AIC)), mainly represented by autoimmune hemolytic anemia and/or immune thrombocytopenia (ITP; refs. 10–16). The risk of AIC occurrence in the course of CLL has been reported to be higher for patients with poor prognostic variables (i.e., high blood

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

C. Visco and F. Maura contributed equally to this work.

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lymphocyte count, rapid blood lymphocyte doubling time, increased serum β2 microglobulin level, high expression of Zeta-chain-associated protein kinase 70 (ZAP-70) and CD38, and unmutated IGHV gene, which also confers a more aggressive clinical behavior. These studies have suggested an important role for the B-cell receptor (BCR) in the pathogenesis of autoimmunity in the course of the disease. Our present analysis revealed that patients with chronic lymphocytic leukemia (CLL) and ITP were not only of the unmutated subtype but also carried stereotyped IGHV repertoire. Patients with CLL and ITP had a 1-in-2 chance of carrying a stereotyped heavy-chain complementary-determining region 3, which was restricted to subset #1 and #7 in the majority of cases. Our findings strongly support a role for BCR triggering by specific antigens in the pathogenesis of this immune complication.

**Translational Relevance**

Autoimmune hemolytic anemia and immune thrombocytopenia (ITP) are the more frequently observed autoimmune complications in patients with chronic lymphocytic leukemia. It has been shown that both these complications occur significantly more frequently among patients with the unmutated immunoglobulin heavy-chain variable (IGHV) gene, which also confers a more aggressive clinical behavior. These studies have suggested an important role for the B-cell receptor (BCR) in the pathogenesis of autoimmunity in the course of the disease. Our present analysis revealed that patients with chronic lymphocytic leukemia (CLL) and ITP were not only of the unmutated subtype but also carried stereotyped IGHV repertoire. Patients with CLL and ITP had a 1-in-2 chance of carrying a stereotyped heavy-chain complementary-determining region 3, which was restricted to subset #1 and #7 in the majority of cases. Our findings strongly support a role for BCR triggering by specific antigens in the pathogenesis of this immune complication.
response to platelet transfusion (in patients without known refractoriness to platelet concentrates), and/or a rapid (<1 week) response to high-dose intravenous Ig (IVIg). Lack of response to platelet transfusion was defined as the failure to obtain satisfactory responses in terms of bleeding or platelet number to 2 or more platelet transfusions. For patients whose ITP was diagnosed at the time of CLL presentation we used the same diagnostic criteria, except for the rapid fall of the platelet count, which could not be established in patients presenting with no data on their previous platelet count.

Identification of stereotyped subsets

A stereotype cluster label was assigned to HCDR3 sequences by means of pairwise alignment with known stereotyped sequences available from different publicly available databases (3–6). In agreement with established procedures, a primary filter excluding pairs of sequences whose length differed more than 3 amino acids was applied. Then, sequences sharing more than 60% identity and less than 3 gaps in resulting alignment were considered as stereotyped (4, 6, 32). Such analysis was conducted with the global alignment algorithm (33) with BLOSUM62 as similarity matrix (34) under the conductor (http://www.bioconductor.org/packages/2.7/bioc/html/Biostrings.html).

Statistical analyses

All contingency analyses were conducted by the Fisher exact test. Bonferroni correction was used to adjust significance for multiple testing comparisons. Quantitative variables were compared with the nonparametric Mann–Whitney U test. The association with overall survival was tested using the Kaplan–Meier estimator and log-rank test with the standard normal asymptotic distribution. The time of ITP was defined as the time from CLL diagnosis to the date of ITP occurrence. The competing effect of death on the relationship between time to ITP and each of the considered group was modeled by proportional hazards of competing risks using the crv function of cmprsk package in R. Cox proportional hazard was used to conduct multivariate analysis. A P value less than 0.05 was considered significant for all statistical calculations. All the analyses were conducted with appropriate functions in R software (www.r-project.org).

Results

Patients’ characteristics and ITP occurrence

Productive IGHV-D-J rearranged sequences were identified in all the 463 patients included in the study (Supplementary Fig. S1 for details). On the basis of the 98% sequence identity criteria, 197 of 463 patients (42.5%) were unmutated. Patients with ZAP-70 and CD38 positivity were 197 of 463 patients (42.5%) and 131 of 463 (31.9%), respectively. Del(13)(q14), trisomy 12, del(11)(q23), del(17)(p13), and normal karyotype were found in 113 of 325 (34.8%), 45 of 325 (13.8%), 36 of 325 (11.1%), 19 of 325 (5.8%), and 112 of 325 (34.5%), respectively. Our population appeared representative of a nonselected CLL series as biologic variables showed the expected impact on survival curves (Supplementary Fig. S2).

According to our definition, the diagnosis of ITP was confirmed in 36 (7.7%) of 463 patients. The median time to ITP development was 32 months (range, 0–102). Seven patients developed ITP concomitantly to CLL diagnosis. Thirty-two (89%) patients required specific therapy for ITP. Concomitant hemolytic anemia (Evans syndrome) was observed in 10 of them. Biologic and molecular features of patients developing or not ITP are summarized in Table 1.

ITP occurrence was significantly associated with unmutated IGHV (28 of 36; 77.7%; P < 0.0001) and ZAP-70 positivity (20 of 26; 76.9%; P = 0.014; see Table 1). On the basis of available FISH data, we found that among unfavorable cytogenetic deletions, that is, del(11)(q23) and/or del(17)(p13), only del(11) retained statistical significant association with ITP occurrence (P = 0.02; Table 1). Of note, among 45 patients with trisomy 12 only one (2.3%) developed ITP. Among clinical variables, neither age, gender, nor Binet stage at CLL diagnosis were significantly associated with ITP development.

IGHV-D-J gene usage

The IGHV gene usage in patients with CLL developing ITP is reported in Table 2. We found that ITP was more frequently observed in patients expressing VH2 (15 of 36; 41.7%) or VH3 families (14 of 36; 38.9%). We found a higher but not significant prevalence of ITP in IGHV1-69 cases than other IGHV families across the whole data set (8 of 67; 12% vs. 28 of 396; 7%). Considering IGHD genes usage, we observed that among 41 patients with IGHD6-19, 8 (19.5%) developed ITP. This prevalence was significantly higher than non–IGHD6-19 patients (28 of 422; 6.6%; P = 0.009).

HCDR3 subsets

Overall, stereotyped HCDR3 sequences were identified in 133 of 463 patients (28.7%), 92 (69.2%) of whom had unmutated configuration (P < 0.0001). The most represented stereotyped subsets were: #1 (IGHV1-5-7/IGHD6-19/IGHJ4; 16 cases), #2 (IGHV3-21; 16 cases), #7 (IGHV1-69 or IGHV3-30/IGHD3-3/IGHJ6; 13 cases), #3 (IGHV1-69 and IGHV4-30/IGHD2-2/IGHJ6; 10 cases), #4 (IGHV4-34; 9 cases), and #9 (IGHV1-69/IGHD3-3/IGHJ6; 8 cases).

Stereotyped HCDR3 sequences were significantly more prevalent in patients with ITP (18 of 36; 50%) than patients without this complication (115 of 427; 27%; P = 0.006; Table 1), a finding probably related to the higher prevalence of unmaturated IGHV among stereotyped HCDR3 patients and to the fact that all patients with stereotyped HCDR3 and ITP had unmaturated IGHV gene.

A Cox proportional hazard model was built including all variables that resulted significantly associated to a higher risk of ITP development in univariate analysis (Fig. 1). These included ZAP-70, IGHV mutational status, cytogenetic
features, and HCDR3 sequence results. As shown in Supplementary Table S1, IGHV unmutated was the only variable retaining an independent association with ITP development ($P = 0.03$; Supplementary Table S1).

### Stereotyped patients and ITP

The majority of stereotyped patients with ITP (10 of 18; 56%) were characterized either by subset #1 (5 of 18; 27.8%) or subset #7 (5 of 18; 27.8%; Table 2). When considering the whole HCDR3 sequences distribution in our series, 31% (5 of 16) of patients with subset #1 and 38.5% (5 of 13) of patients with subset #7 developed ITP. Conversely, the stereotyped subsets #2 and #3 rarely developed ITP (Table 2), and none of the 9 patients with subset #4 developed ITP ($P = 0.9$, $P = 0.8$, and $P = 0.3$, respectively).

The risk of developing ITP was significantly higher in patients with subsets #1 and #7 than in all other patients ($P = 0.003$ and $P = 0.001$, respectively), also after restricting the analysis to unmutated patients (Fig. 2A and B). Subsets #1 and #7 were also characterized by a shorter time to ITP development than all other patients ($P = 0.0076$ and $P < 0.0001$, respectively; Fig. 3A and B). To avoid the bias represented by the association between ITP development and unmutated status (all patients with subset #1 and #7 had unmutated status), we conducted a multivariate analysis including the 3 variables. Subset #7 ($P = 0.01$), #1 ($P = 0.05$), and unmutated status ($P = 0.0002$) were independently associated to a higher risk of ITP development. Finally, comparing subsets #1 and #7 with patients showing the same IGHV gene usage but without homologous HCDR3, the correlation with ITP development was still significant (data not shown). For further clarification, a direct comparison of cumulative incidence of ITP for (i) unmutated with subset #1 or #7; (ii) unmutated stereotyped neither #1 or #7; (iii) unmutated nonstereotyped; and (iv) mutated is shown in Fig. 3C, confirming the independent additive contribution of each variable in the risk of ITP development.

### Discussion

In the present study, we investigated the IGHV profiles of a large series of patients with CLL, comparing them according to the occurrence of ITP. Our analysis revealed that patients with CLL and ITP carried stereotyped IGHV repertoire significantly more frequently than other patients with CLL without this complication (50% vs. 27%, $P = 0.006$). Patients with CLL and ITP had a 1-in-2 chance (18 of 36) of carrying a stereotyped HCDR3. Inter-CLL homology was even more striking in the unmutated group of patients with ITP, with 64% of cases (18 of 28) belonging to a stereotyped subset. Furthermore, IGHV sequences were restricted to subset #1 and #7 in nearly 30% of them. Given the evidence that the BCR of one third of CLL displays nearly identical or highly HCDR3 regions (6, 22), the finding that half of patients with CLL and ITP expressed stereotyped BCR strongly supports a role for BCR triggering by specific antigens in the pathogenesis of this immune complication.

Overall, we identified 133 patients with stereotyped HCDR3, representing 28.7% of our patients, which resembles what previously reported by others (3–6). Confirming previous findings, the occurrence of ITP in our series was significantly associated with unmutated IGHV and ZAP-70 positivity (15, 17, 23, 35). A significant group of our patients had available FISH results (325 of 463) which made us possible to investigate on a large scale for the first time the impact of known chromosomal aberrations on ITP occurrence showing that unfavorable lesions, particularly del(11)(q23), were associated with the development of this complication.

![Table 1. Clinical and biologic characteristics of patients with CLL developing or not ITP](image-url)

<table>
<thead>
<tr>
<th></th>
<th>With ITP (36)</th>
<th>Without ITP (427)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range), y</td>
<td>67 (39.7–83.8)</td>
<td>68 (31–93)</td>
<td>NS$^a$</td>
</tr>
<tr>
<td>Median follow-up (range), mo</td>
<td>53 (4.8–120)</td>
<td>50.4 (1–120)</td>
<td>NS$^a$</td>
</tr>
<tr>
<td>Male</td>
<td>27/36 (75%)</td>
<td>278/427 (65%)</td>
<td>NS</td>
</tr>
<tr>
<td>CD38 &gt;50%</td>
<td>14/32 (44%)</td>
<td>117/379 (30.7%)</td>
<td>NS</td>
</tr>
<tr>
<td>ZAP-70 &gt;20%</td>
<td>20/26 (77%)</td>
<td>164/317 (52%)</td>
<td>0.014</td>
</tr>
<tr>
<td>No cytogenetic aberrations$^b$</td>
<td>5/22 (23%)</td>
<td>107/303 (35.3%)</td>
<td>NS</td>
</tr>
<tr>
<td>Del(13)(q14)</td>
<td>7/22 (32%)</td>
<td>106/303 (35%)</td>
<td>NS</td>
</tr>
<tr>
<td>Trisomy 12</td>
<td>1/22 (4.5%)</td>
<td>44/303 (14.5%)</td>
<td>NS</td>
</tr>
<tr>
<td>Trisomy 11</td>
<td>6/22 (27.3%)</td>
<td>30/303 (10%)</td>
<td>0.024</td>
</tr>
<tr>
<td>Del(17)(p13)</td>
<td>3/22 (13.6%)</td>
<td>16/303 (5.2%)</td>
<td>NS</td>
</tr>
<tr>
<td>Unmutated IGHV</td>
<td>28/36 (78%)</td>
<td>169/427 (39.5%)</td>
<td>&lt;0.0001c</td>
</tr>
<tr>
<td>Stereotyped HCDR</td>
<td>18/36 (50%)</td>
<td>115/427 (27%)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

NOTE: $P$ values are calculated by the Fisher exact test.

$^a$P value calculated by Mann-Whitney U test.

$^b$According to hierarchical classification.

$^c$Significance retained after Bonferroni multiple testing correction.
Differently from aggressive B-cell malignancies, where the BCR is constitutively activated by somatically acquired genetic lesions (36), in CLL and other indolent lymphomas the BCR is stimulated by external foreign or autoantigens that provide proliferative and antiapoptotic signals to the B cells. In line with this, HCDR3 restrictions are rare in most aggressive B-cell lymphomas, as in normal B cells, suggesting that they may originate from random B cells (37). Instead, CLL B-cell development might be influenced by antigen recognition through a stereotyped BCR, as might be the case of lymphomas of mucosa-associated lymphoid tissues, that can express stereotyped BCR with strong HCDR3 homology to rheumatoid factors, or mantle cell lymphomas, that can exhibit somatic hypermutation patterns in IGHV genes that are typical of receptors that have undergone selection by antigen (38). However, it is still unclear whether antigen involvement is restricted to the malignant transformation phase, or whether the putative antigen(s) may continuously trigger the CLL clone (39, 40). Our study points to the occurrence of a stereotyped response of CLL B cells to an antigen(s) but does not clarify the association between stereotyped malignant B cells and the autoreactive nonmalignant B cells. If antigen binding on clonal B cells is important, this is not because of direct antibody production, but immune response to the antigen should be mediated by other cells in the microenvironment, and this is the case of T cells. Given that the course of CLL is typically characterized by profound immunosuppression,

<table>
<thead>
<tr>
<th>Patients code</th>
<th>Age/gender</th>
<th>% IGHV mutation</th>
<th>IGHV mutational status</th>
<th>VH</th>
<th>DH</th>
<th>JH</th>
<th>Subset</th>
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<tbody>
<tr>
<td>MI-VI408</td>
<td>63.9/M</td>
<td>99.59</td>
<td>UM</td>
<td>IGHV5-a</td>
<td>IGHV6-19</td>
<td>IGHJ4</td>
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<td>MI-VI401</td>
<td>67.1/M</td>
<td>99.63</td>
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<td>IGHV6-19</td>
<td>IGHJ4</td>
<td>1</td>
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<tr>
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<td>100.00</td>
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<td>IGHJ4</td>
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<td>100.00</td>
<td>UM</td>
<td>IGHV5-51</td>
<td>IGHV6-19</td>
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<td>100.00</td>
<td>UM</td>
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<tr>
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<td>98.65</td>
<td>UM</td>
<td>IGHV3-21</td>
<td>IGHV5-9</td>
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<td>2</td>
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<tr>
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<td>64.8/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV1-69</td>
<td>IGHV2-2</td>
<td>IGHJ6</td>
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<tr>
<td>MI-V1123</td>
<td>39.7/M</td>
<td>100.00</td>
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<td>IGHV1-69</td>
<td>IGHV3-3</td>
<td>IGHJ6</td>
<td>7</td>
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<tr>
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<td>100.00</td>
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<tr>
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<td>100.00</td>
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<td>MI-V1237</td>
<td>77.8/M</td>
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<td>UM</td>
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<td>IGHV1-2</td>
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<td>IGHV3-3</td>
<td>IGHJ3</td>
<td>N41</td>
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<td>88.88</td>
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<td>IGHV3-7</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
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<tr>
<td>MI-V150</td>
<td>72.7/M</td>
<td>92</td>
<td>M</td>
<td>IGHV6-01</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
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<td>94.80</td>
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<td>IGHV1-3</td>
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<td>NS</td>
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<td>94.85</td>
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<td>95.14</td>
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<td>IGHV1-3</td>
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<td>NS</td>
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<td>95.49</td>
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<tr>
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<td>97.92</td>
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<tr>
<td>MI-V1385</td>
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<td>97.95</td>
<td>M</td>
<td>IGHV3-23</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V113</td>
<td>56.7/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV1-69</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V1150</td>
<td>81.5/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV2-7</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V1162</td>
<td>58.4/F</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV2-5</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V1281</td>
<td>67.6/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV1-69</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V1329</td>
<td>69.2/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV3-48</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V1334</td>
<td>73.0/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV3-33</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V1343</td>
<td>60.6/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV4-39</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V1380</td>
<td>60.5/F</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV1-2</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
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<tr>
<td>MI-V1411</td>
<td>83.8/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV3-33</td>
<td>IGHV1-3</td>
<td>IGHJ6</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V176</td>
<td>66.5/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV3-15</td>
<td>IGHV1-3</td>
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Abbreviations: M, mutated; NS, not stereotyped HCDR3; UM, unmutated.
with T-cell function impairment and altered immune surveillance, both the microenvironment and cell-to-cell interactions are likely to be implicated in the emergence of nonneoplastic autoreactive B and T cells (12, 39, 41–44). Murine and human studies on autoimmune hemolytic anemia have shown that autoreactive T-helper (TH) cells are critical for the induction of the autoimmune phenomena (21). These autoreactive TH cells could be induced by the pathologic autoantigen presentation mediated via CLL cells, that could function as autoantigen-presenting cells, triggering the TH cell–mediated autoantibody production against platelet antigens by normal B lymphocytes. The antibodies causing ITP in patients with CLL are most often polyclonal high-affinity IgG directed against the platelet surface antigen GpIIb/IIIa (21). Although the mechanisms that result in the production of these pathologic antibodies are believed to be similar to those proposed for autoimmune hemolytic anemia, this has not yet been shown.

The more frequently observed IGHV genes among patients with ITP and CLL was IGHV1-69 (22%), reflecting the high prevalence of this gene in patients with CLL and its frequent association with unmutated status. A bias toward the VH1 gene family was observed in patients with ITP and CLL (15 of 36; 41.6%) compared with patients without ITP (103 of 427; 24.1%; \( P = 0.01 \)), confirming previous findings (15). The IGHV3 subgroup as a whole, which is the most frequently used subgroup in CLL, was also frequent among patients with ITP (39%), being equally represented by genes associated to bad prognosis (IGHV3-21 or IGHV3-23) or to indolent clinical course (IGHV3-72, IGHV3-30; ref. 45). Compared with patients

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**Figure 1.** ITP development risk. A, IGHV mutational status. B, ZAP-70 expression. C, cytogenetic aberration evaluated by FISH [not unfavorable: del(13)(q14), trisomy 12, and normal vs. unfavorable: del(11)(q23) and del(17)(p13)]. D, CD38 expression.

**Figure 2.** ITP development risk in CLL. Analysis of ITP development risk related to stereotyped subsets. A, subset #1. B, subset #7.
with CLL of other series (6, 46), our study confirmed the low frequency of the IGHV3-21 gene in patients with CLL of our geographic area, representing 3.88% of cases. Only one patient with CLL and ITP of our series (2.8%) had this particular IGHV gene usage. Similarly to our findings in patients with ITP, the IGHV3 family was the more prevalent among patients with CLL and autoimmune hemolytic anemia (66% of cases) in another report, with similar distribution of favorable and unfavorable genes (24). The IGHV3 genes are characterized by their unique property of binding certain superantigens (e.g., staphylococcal protein A; ref. 47). The evidence in our study of a high frequency of stereotyped IGHV3 sequences in patients with CLL developing ITP is unusual, as other large series (6) reported a lower chance of carrying a stereotyped HCDR3 for CLL cases expressing IGHV3 family. This finding might be indicative, at least for some cases, of selection by superantigens through HCDR3-based recognition.

The VH4 family, which is usually found in 20% of CLL irrespective of the mutational status (48, 49), was found in 24% (94 of 427) of patients without ITP but in only 8% (3 of 36) of patients with ITP ($P = 0.056$). Interestingly, cases with IGHV4-34 were not found in our patients with ITP, although this was the second most represented gene in our CLL series (37 of 463; 8%). IGHV4-34 gene is known to encode for antibodies that are intrinsically autoreactive and can recognize antigenic determinants of the I/i blood group antigen (5, 6). Whereas IGHV4-34 antibodies are infrequent in the sera of healthy individuals, the IGHV4-34 gene is very frequent in the repertoire of peripheral B cells (50), suggesting an anergic status of these cells. As previously suggested (5), the highly recurrent hypermutated status of this BCR subset, possibly contributing to the lower responsiveness of these cells to BCR antigenic stimulation, may contribute to the low frequency of autoimmune phenomena we found in this group of mutated anergic CLL B cells.

Interestingly, we found that IGHV4-34 gene usage was significantly associated with ITP development. The evidence that among IGDH6-19 patients who developed ITP, 5 of 7 (72%) belonged to subset #1 further strengthens the potential role of HCDR3 in ITP development.

Our results will need to be validated in independent or prospective cohorts of patients with CLL. A prospective registry of incidental newly diagnosed patients with CLL from our geographic region (CLL Veneto) is ongoing at our Institutions and will have the aim of independently confirm our findings. However, the strong association between ITP occurrence and stereotyped BCRs in our series of patients with CLL suggests that distinct antigen-binding sites on CLL B cell could facilitate the development of autoimmune phenomena in the course of the disease.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** C. Visco, F. Maura, G. Tuana, L. Agnelli

**Writing, review, and/or revision of the manuscript:** C. Visco, F. Maura, G. Tuana, L. Agnelli, A. Neri, F. Redeghiero, A. Cortelezzi

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Figure 3. ITP development risk in CLL. A, subset #1 and (B) #7 patients were compared with patients with unmutated IGHV mutational status for time to ITP development. C, unmutated subset #1 and #7 patients were compared with IGHV unmutated stereotyped non-subset #1 or #7, unmutated (UM) nonstereotyped, and mutated (M) IGHV patients.
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Study supervision: C. Visco, F. Maura, A. Neri, F. Rodeghiero, A. Cortelezzi

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