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); 1–9. ©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
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.

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; refs. 10, 13–17). Despite CLL cells can produce autoreactive antibodies in vivo and after stimulation in vitro (18–20), more than 90% of the AIC occurring during CLL are due to polyclonal antibodies produced by nonmalignant B-cell clones. The loss of immune tolerance determined by T-cell dysfunction and/or a pathologic autoantigen presentation by CLL cell have been related to the pathogenesis of AIC (13, 21). However, there is strong evidence that the unmutated IGHV of the B-cell receptor (BCR) favors the occurrence of AIC, suggesting that CLL B cells may have a central role in the development of AIC. Neoplastic B cells with unmutated configuration are in fact characterized by polyreactive receptors that can bind multiple antigens and are more prone to interactions with surrounding T and B cells, like antibody producing nonneoplastic B cells (15, 17, 22–26).

On the basis of these considerations, we analyzed the IGHV repertoire of a large series of patients with CLL with secondary ITP, and we evaluated the occurrence of BCR stereotyped profiles. We showed that peculiar BCR configurations are frequently associated to ITP development in the course of CLL.

Materials and Methods

Patients’ selection and clinical characteristics

In the present study, we investigated 463 newly diagnosed patients with CLL who were referred to 2 major Institutions from northern Italy: Ospedale San Bortolo, Vicenza, and Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, Milano, Italy. Patients were selected according to the availability of follow-up data and material at diagnosis to carry out the genetic and molecular analyses. It was required that available samples were collected at CLL presentation or within 1 year from diagnosis, provided that none of the patients had received cytotoxic treatment. Patients included in the study were diagnosed between January 2000 and January 2011. All patients met the CLL diagnostic criteria of National Cancer Institute Working Group (27).

The median age was 68 years (range, 31–93 years), with a male/female ratio of 0.55. Binet stage was A in 79%, B in 13%, and C in 8%. Median follow-up from diagnosis of CLL was 51 months (range, 1–120) Overall, 54% of patients had cytotoxic treatment directed to CLL during follow-up. Regimens administered were in line with standard induction treatments at our Institution and consisted of alkylating and/or fludarabine regimens, chemoimmunotherapy or alemtuzumab.

Phenotypical, molecular, and genetic analysis

ZAP-70 and CD38 expression were assessed by cytofluorimetric analysis on peripheral blood samples or bone marrow aspirates in 343 and 411 patients, respectively, as previously described (28). A cutoff point of 20% and 30% of positive cells was used to discriminate ZAP-70 and CD38 positive from negative patients, respectively. To assess mutational status, RNA was obtained from peripheral blood or bone marrow specimens. Sequences were aligned to IMGT and analyzed with IMGT/VQUEST software. Sequences differing more than 2% from the corresponding germ line gene were considered mutated (M), as opposite to unmutated cases (UM, refs. 4, 29, 30). Cytogenetic abnormalities involving deletions at chromosomes 11q23, 13q14, and 17p13 and trisomy 12 were evaluated by FISH in 325 patients as previously described (31).

Definition of ITP

Following our previously reported criteria (15), the diagnosis of ITP was based on as follows: (i) an otherwise unexplained rapid (<2 weeks) and severe fall (at least half of the initial level and below 100 × 10^9/L) of the platelet count; (ii) a normal or augmented number of megakaryocytes in the bone marrow; (iii) no or limited (not palpable) splenomegaly, and (iv) no cytotoxic treatment in the last month. Any other common cause of thrombocytopenia, such as pseudothrombocytopenia, disseminate intravascular coagulation, thrombotic thrombocytopenic purpura, HIV and HCV infections, acute infections, as well as heparin treatment were ruled out by clinical and laboratory analyses, together with peripheral blood smear examination. Drug-induced thrombocytopenia was excluded on the basis of the lack of any temporal relationship to new drugs.

Patients with Binet C stage and extensive bone marrow involvement, where the interpretation of the number of megakaryocytes is sometimes problematic, represented 13.8% of patients with ITP. In those cases, we considered essential requisites for the diagnosis of ITP, the lack of
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 Biostrings package of Bioconductor (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 statistical significance values being calculated according to 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 crr function of cmpskr 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 frequently observed in patients expressing IGHV1-21 (15 of 36; 41.7%) or IGHV3-21 (14 of 36; 38.9%). In the ITP group, we observed that among unmutated pairs, the 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 for 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 unmutated IGHV among stereotyped HCDR3 patients and to the fact that all patients with stereotyped HCDR3 and ITP had unmutated 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.

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**Table 1. Clinical and biologic characteristics of patients with CLL developing or not ITP**

<table>
<thead>
<tr>
<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>
</tr>
<tr>
<td>Median follow-up (range), mo</td>
<td>53 (4.8–120)</td>
<td>50.4 (1–120)</td>
</tr>
<tr>
<td>Male</td>
<td>27/36 (75%)</td>
<td>278/427 (65%)</td>
</tr>
<tr>
<td>CD38 &gt;30%</td>
<td>14/32 (44%)</td>
<td>117/379 (30.7%)</td>
</tr>
<tr>
<td>ZAP-70 &gt;20%</td>
<td>20/26 (77%)</td>
<td>164/317 (52%)</td>
</tr>
<tr>
<td>No cytogenetic aberrationsb</td>
<td>5/22 (23%)</td>
<td>107/303 (35.3%)</td>
</tr>
<tr>
<td>Del(13)(q14)</td>
<td>7/22 (32%)</td>
<td>106/303 (35%)</td>
</tr>
<tr>
<td>Trisomy 12</td>
<td>1/22 (4.5%)</td>
<td>44/303 (14.5%)</td>
</tr>
<tr>
<td>Del(11)(q23)</td>
<td>6/22 (27.3%)</td>
<td>30/303 (10%)</td>
</tr>
<tr>
<td>Del(17)(p13)</td>
<td>3/22 (13.6%)</td>
<td>16/303 (5.2%)</td>
</tr>
<tr>
<td>Unmutated IGHV</td>
<td>28/36 (78%)</td>
<td>169/427 (39.5%)</td>
</tr>
<tr>
<td>Stereotyped HCDR</td>
<td>18/36 (50%)</td>
<td>115/427 (27%)</td>
</tr>
</tbody>
</table>

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

* $P$ value calculated by Mann–Whitney U test.

bAccording to hierarchical classification.

cSignificance 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 2. IGHV/IGHD/IGHJ gene usage and stereotyped HCDR3 of 36 patients with CLL and ITP complication

<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-V1408</td>
<td>63.9/M</td>
<td>99.59</td>
<td>UM</td>
<td>IGHV5-a</td>
<td>IGHD6-19</td>
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<td>IGHJ4</td>
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<td>IGHV1-18</td>
<td>IGHD6-19</td>
<td>IGHJ4</td>
<td>1</td>
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<td>100.00</td>
<td>UM</td>
<td>IGHV5-51</td>
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<td>MI-V1382</td>
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<td>IGHJ4</td>
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<tr>
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<td>98.65</td>
<td>UM</td>
<td>IGHV3-21</td>
<td>IGHD5-5</td>
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<td>100.00</td>
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<td>IGHD3-3</td>
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<td>100.00</td>
<td>UM</td>
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<td>65.6/F</td>
<td>100.00</td>
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<td>IGHD3-22</td>
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<tr>
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<td>IGHD5-5</td>
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<td>92</td>
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<td>94.85</td>
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<tr>
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<td>95.14</td>
<td>M</td>
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<td>IGHD3-16</td>
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<td>95.49</td>
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<td>IGHD5-24</td>
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<td>97.92</td>
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<td>IGHV4-59</td>
<td>IGHD3-22</td>
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<td>IGHJ4</td>
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<td>IGHD6-19</td>
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<td>100.00</td>
<td>UM</td>
<td>IGHV3-7</td>
<td>IGHD7-27</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>IGHD3-22</td>
<td>IGHJ3</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>IGHD3-10</td>
<td>IGHJ5</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>IGHD6-19</td>
<td>IGHJ3</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>IGHD3-3</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>IGHD3-22</td>
<td>IGHJ3</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>IGHD3-10</td>
<td>IGHJ4</td>
<td>NS</td>
</tr>
<tr>
<td>MI-V1411</td>
<td>83.8/M</td>
<td>100.00</td>
<td>UM</td>
<td>IGHV3-33</td>
<td>IGHD2-21</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>IGHD2-15</td>
<td>IGHJ3</td>
<td>NS</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 T H cell–mediated autoantibody production 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

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 \(IGHD6-19\) gene usage was significantly associated with ITP development. The evidence that among \(IGHD6-19\) 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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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Visco, F. Maura, M. Lionetti, S. Fabris, E. Novella, G. Reda, W. Barcellini, L. Baldini
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. Rodeghiero, A. Cortelezzi

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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