Stereotyped B-Cell Receptor Is an Independent Risk Factor of Chronic Lymphocytic Leukemia Transformation to Richter Syndrome

Davide Rossi,1 Valeria Spina,1 Michaela Cerri,1 Silvia Rasi,1 Clara Deambrogi,1 Lorenzo De Paoli,1 Luca Laurenti,2 Rossana Maffei,3 Francesco Forconi,8 Francesco Bertoni,5 Emanuele Zucca,5 Claudio Agostinelli,6 Antonello Cabras,7 Marco Lucioni,8 Maurizio Martini,2 Michele Magni,7 Silvia Deaglio,9 Marco Ladetto,9 Josep F. Nomdedeu,10 Caroline Besson,11 Antonio Ramponi,1 Vincenzo Canzonieri,12 Marco Paulli,8 Roberto Marasco,3 Luigi M. Larocca,2 Antonino Carbone,7 Stefano A. Pileri,6 Valter Gattei,12 and Gianluca Gaidano1

Abstract

Purpose: Few biological prognosticators are useful for prediction of Richter syndrome (RS), representing the transformation of chronic lymphocytic leukemia (CLL) to aggressive lymphoma. Stereotyped B-cell receptors (BCR) may have prognostic effect in CLL progression. We tested the prognostic effect of stereotyped BCR for predicting RS transformation.

Experimental Design: The prevalence of stereotyped BCR was compared in RS (n = 69) versus nontransformed CLL (n = 714) by a case-control analysis. Subsequently, the effect of stereotyped BCR at CLL diagnosis on risk of RS transformation was actuarially assessed in a consecutive CLL series (n = 753).

Results: RS (n = 69) displayed a higher prevalence of stereotyped BCR (P < 0.001) compared with nontransformed CLL. The actuarial risk of RS transformation was significantly higher in CLL carrying stereotyped BCR (P < 0.001). Among BCR subsets most represented in CLL, subset 8 using IGHV4-39/IGHD6-13/IGHJ5 carried the highest risk of RS transformation [hazard ratio (HR), 24.50; P < 0.001]. Multivariate analysis selected stereotyped BCR (HR, 3.33; P = 0.001) and IGHV4-39 usage (HR, 4.03; P = 0.004) as independent predictors of RS transformation. The combination of IGHV4-39 usage and stereotyped BCR in the same patient identified CLL with a very high risk of RS transformation (5-year risk, 68.7%). The risk carried by stereotyped BCR and IGHV4-39 usage was specific for RS transformation and had no effect on CLL progression without transformation.

Conclusions: Analysis of BCR features may help identify CLL patients at risk of RS. A close monitoring and a careful biopsy policy may help early recognition of RS in CLL patients using stereotyped BCR, particularly if combined with IGHV4-39.

Richter syndrome (RS) represents the clinicopathologic transformation of chronic lymphocytic leukemia (CLL) to aggressive lymphoma, mainly occurring as diffuse large B-cell lymphoma (DLBCL; refs. 1, 2). The risk of CLL transformation to RS is substantial, ranging from 3% to 16% (1–6).

The pathogenetic mechanisms underlying CLL transformation to RS are poorly understood (6–12). In CLL, immunogenetic findings suggest that antigen stimulation may be involved in disease development (13, 14). In fact, nonrandom combinations of specific immunoglobulin heavy chain (IGH) V-D-J

Authors’ Affiliations:1 Amedeo Avogadro University of Eastern Piedmont, Novara, Italy; 2 Catholic University of the Sacred Heart, Rome, Italy; 3 University of Modena and Reggio Emilia, Modena, Italy; 4 University of Siena-AOUS, Siena, Italy; 5 Seragnoli Institute, University of Bologna, Bologna, Italy; 6 Instituto Nazionale Tumori, Milan, Italy; 7 University of Pavia, Pavia, Italy; 8 University of Turin, Turin, Italy; 9 Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; 10 Hôpital Bicêtre, Paris, France; and 11 Centro di Riferimento Oncologico, Aviano, Italy

Received 12/18/08; revised 3/30/09; accepted 4/1/09; published OnlineFirst 6/9/09.

Grant support: PRIN 2006, Rome, Italy; Progetto Affari, Fondazione CRT, Torino, Italy; Ricerca Sanitaria Finalizzata, Regione Piemonte, Torino, Italy; Novara-AIL Onlus, Novara, Italy; Associazione italiana per la ricerca sul cancro, Milan, Italy; Siena-AIL, Siena, Italy; Ricerca Finalizzata I.R.C.C.S. e Alleanza contro il cancro, Rome, Italy; AIL Venezia, Pramaggiore, Italy; Ricerca Scientifica Applicata, Trieste, Italy; Oncosuisse grant OCS-02296-08-2008, Switzerland. V. Spina has been supported by a fellowship from Associazione Franca Capurro per Novara Onlus, Novara, Italy.

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

Requests for reprints: Davide Rossi, Division of Hematology, Department of Clinical and Experimental Medicine and BRMA, Amedeo Avogadro University of Eastern Piedmont, Via S. Mauro 10, 28100 Novara, Italy. Phone: 39-321-660663; Fax: 39-321-620421; E-mail: rossidav@med.unipmn.it.

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doi:10.1158/1078-0432.CCR-08-3266
Translational Relevance

Richter syndrome (RS) represents the transformation of chronic lymphocytic leukemia (CLL) to aggressive lymphoma. Because disease extent influences RS outcome, prompt recognition of RS may be clinically useful and may be improved by availability of biological predictors. Usage of stereotyped B-cell receptors (BCR) is a specific biological feature of CLL. We document that, at CLL diagnosis, stereotyped BCR usage is an independent risk factor of subsequent transformation to RS. Among subsets defined by stereotyped BCR, a specific subset using the IGHV4-39 gene carries a 24-fold increased risk of RS transformation. Combination of IGHV4-39 usage and stereotyped BCR in the same patient defines a very high risk of RS transformation (5-year risk, 68.7%). Analysis of BCR features may help identify CLL patients at risk of RS. A close monitoring and a careful biopsy policy may help early recognition of RS in CLL patients using stereotyped BCR, particularly if combined with IGHV4-39.

Genes lead to structural similarity of the B-cell receptor (BCR) in a significant fraction of CLL (15–25). The heavy chain complementarity determining region 3 (HCDR3) of these CLL cases is termed as stereotyped. The similarity of the BCR from various CLL patients suggests that the antigens these receptors bind to are similar and potentially relevant to disease pathogenesis (13, 14, 26).

In contrast to CLL, data addressing immunogenetic clues of antigen stimulation in RS are scant. High prevalence of unmutated IGHV genes, biased IGHV gene usage, and some degree of stereotyped HCDR3 have been observed in small RS series (6, 27–32). However, a comprehensive characterization of the role of stereotyped HCDR3 in RS development and RS prediction is lacking.

From a clinical standpoint, recent findings suggest that prompt recognition of RS transformation may be clinically useful because disease extent is known to influence outcome of RS patients (5). This clinical observation mandates the identification of biological predictors of RS transformation that may be investigated already at the time of CLL diagnosis. Despite the abundance of biological markers available for predicting CLL progression, only few have been shown to be useful for RS prediction (6, 11).

In this study, we investigated a large cohort of RS for HCDR3 clustering to (a) assess the role of BCR in RS and (b) verify whether stereotyped HCDR3 at diagnosis may help in the identification of CLL subgroups at high risk of subsequent RS transformation.

Materials and Methods

Patients. The study was based on a multiinstitutional consecutive series of 753 CLL with typical sIg low/CD5+/CD23+ phenotype and Matutes score of ≥3 (ref. 33; Supplementary Table S1), of which 39 had transformed to RS and 714 never developed clinical evidence of RS (median follow-up of the whole series, 41.1 months; median time to RS transformation, 23.0 months; median follow-up of CLL nontransformed to RS, 42.0 months). Thirty additional cases of RS were also collected. All RS cases (n = 69) had a previous or concomitant diagnosis of CLL that was defined according to International Workshop on Chronic Lymphocytic Leukaemia-National Cancer Institute (IWCLL-NCI) guidelines and was confirmed by a Matutes score of ≥3 in all cases (33–35). RS diagnosis was based on histology of lymph node or extranodal tissue excisional biopsies. All RS cases were classified as DLBCL according to the WHO Classification of Tumors of the Hematopoietic and Lymphoid Tissues (36). In all 69 RS cases, the tissue used for molecular studies was the biotic tissue used for RS diagnosis.

Study design. The design of the study was divided into three steps. The first step of the study consisted of a case-control analysis comparing prevalence of HCDR3 clustering in cases of RS (n = 69) versus CLL (n = 714) of the multiinstitutional consecutive series that were known not to have transformed to RS. IGHV-D-J gene usage, mutation status, and HCDR3 clustering of the nontransformed CLL used as control group were representative of the disease (Supplementary Tables S2, S3, S4, and S5).

The second step of the study consisted of an actuarial assessment of the effect of stereotyped HCDR3 at CLL diagnosis on the risk of subsequent transformation to RS. For this purpose, stereotyped HCDR3 used at CLL diagnosis by CLL leukemic cells were considered for the analysis. A multiinstitutional CLL cohort of 753 consecutive CLL, of which 39 had transformed to RS, was used for the actuarial study.

The third step of the study aimed at verifying whether HCDR3 subsets observed in RS also occurred in a consecutive series of de novo DLBCL (n = 63).

Patients provided informed consent in accordance with local institutional review board requirements and Declaration of Helsinki. The study was approved by the local institutional review board.

Analysis of IGHV-D-J, IGKV-, and IGLV- rearrangements. Rearrangements of IGHV-D-J and IGKV-J light chain genes were amplified with family-specific primers that hybridize to sequences in the IGHV, IGKV (IGKV) and IGKV (IGLV) leader region, and framework region 1 (FR1) or FR2 in conjunction with the corresponding IGKJ, IGKJ, or IGKJ primers (37). PCR products were directly sequenced with the ABI PRISM BigDye Terminator v1.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems) using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were aligned to ImMunoGeneTics (IMGT) database (Supplementary Table S6), and the Murray database (n = 1029; Supplementary Table S6), and the Murray database (n = 530; ref. 22).

For HCDR3-driven clustering, all in-frame IGHV-D-J rearrangements were converted into amino acid sequences and aligned according to the putative HCDR3 amino acid sequences using the multiple sequence alignment software ClustalW (1.83). Subsets identified as having stereotyped HCDR3 amino acid sequences were those characterized by an alignment score of ≥60 and an amino acid identity of ≥60%. Subsets that had not been previously described and that were composed of only two HCDR3s were termed as provisional and considered only if they shared junction residues, used the same IGHJ-J gene, or displayed a restricted light chain usage.

Subset nomenclature was according to Murray et al. (22). For the purpose of this study, subsets not previously included in the Murray nomenclature system were assigned an alphabet letter.

EBV analysis. Analysis of EBV infection was done by PCR and/or in situ hybridization of EBER transcripts, as previously reported (37).
Interphase fluorescence in situ hybridization. Probes used for fluorescence in situ hybridization (FISH) analysis were LSI13 and LSD135319 for detection of del13q14, CEPI2 for detection of aneuploidy of chromosome 12, LSIp53 for detection of del17p13, LSIATM for detection of del11q22-q23, and LSI IGH dual-color break-apart rearrangement probe for detection of translocations involving 14q32 (Vysis). A BCL-3 split signal probe (Dako) was used for detecting BCL-3 rearrangements. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole and antifade, and signals were visualized using an Olympus BX51 microscope (Olympus Italia). For each probe, at least 500 interphase cells with well-delineated fluorescent spots were examined.

Statistical analysis. Categorical variables were compared by χ² test and Fisher’s exact test when appropriate. Date of transformation was defined as the date of the biopsy, showing that RS transformation occurred. Time-to-RS transformation was measured from date of CLL diagnosis to date of transformation, death, or last follow-up. Time to progression was measured from date of CLL diagnosis to date of progression to symptomatic disease according to IWCLL-NCl guidelines (34, 35), death, or last follow-up. Survival analysis was done by Kaplan-Meier method, using log-rank statistics to test for significant associations (40). Cox analysis was used to build a multivariate model (41). All statistical tests were two-sided. Statistical significance was defined as P value of <0.05. The analysis was done with Statistical Package for the Social Sciences software v.16.0.

Results

Clinical, morphologic, and phenotypic features of RS cases. Male/female ratio of the 69 RS patients was 1.8:1. Median age at RS diagnosis was 65 years (25th to 75th, 58-73 years). Sites of diagnostic biopsies documenting RS transformation included lymph node (58 of 69, 84.0%), gastrointestinal tract (3 of 69, 4.3%), spleen (2 of 69, 2.8%), nasopharynx (2 of 69, 2.8%), skin (2 of 69, 2.8%), tonsil (1 of 69, 1.4%), and thyroid (1 of 69, 1.4%). All RS cases were classified as DLBCL according to WHO classification criteria (36). CD10 was expressed in 3 of 52 (5.8%) cases, MUM1 in 34 of 48 (70.8%), and BCL6 in 10 of 53 (18.9%). According to immunohistochemistry expression pattern (42), the overwhelming majority of RS (45 of 49, 91.8%) were classified as nongerminal center, and only 4 of 49 (8.2%) RS were classified as germinal center B-cell type. Median Ki-67 index was 52% (25th to 75th, 35-77%). EBV infection was documented in 1 of 62 (1.6%) RS cases. Clonal relationship between CLL and RS was documented in 42 of 48 (87.5%) assessable patients.

RS are characterized by high prevalence of stereotyped HCDR3. Immunogenetic features of RS (n = 69) were compared with those of nontransformed CLL (n = 714). Sixty-nine in frame IGHV-D-J rearrangements were obtained from the transformed phase of 69 RS patients. Prevalence of IGHV homology of ≥98% was higher in RS (48 of 69, 69.6%) than in nontransformed CLL (276 of 714, 38.7%; P < 0.001). Usage of IGHV, IGHD, and IGHE genes in RS and nontransformed CLL is reported in Supplementary Tables S2, S3, and S4. IGHV4-39 was the gene most frequently used by RS (all RS cases, 6 of 69, 8.6%; IGHV unmutated RS, 6 of 48, 12.5%; IGHV mutated RS, 0 of 21; Supplementary Table S2).

Thirty-four of 69 (49.3%) RS displayed stereotyped HCDR3 (Supplementary Table S7). Prevalence of stereotyped HCDR3 was significantly higher in RS compared with nontransformed CLL when considering all cases (RS, 34 of 69, 49.3% versus nontransformed CLL, 152 of 714, 21.3%; P < 0.001), cases with IGHV homology of ≥98% (RS, 28 of 48, 58.3% versus nontransformed CLL, 95 of 276, 34.4%; P = 0.002) and cases with IGHV homology of <98% (RS, 6 of 21, 28.6% versus nontransformed CLL, 57 of 438, 13.0%; P = 0.043; Supplementary Tables S7 and S8).

Stereotyped HCDR3 at CLL diagnosis is an independent predictor of RS transformation. Results of the case-control analysis prompted investigation of stereotyped HCDR3 as a risk factor for RS transformation. The effect on risk of RS transformation carried by stereotyped HCDR3 at CLL diagnosis was actuarily assessed in a cohort of 753 CLL. After a median follow-up of 41.1 months, 39 of 753 CLL had transformed to RS. By univariate analysis, CLL using stereotyped HCDR3 carried a significantly higher risk of RS transformation (events/n, 21/174; 5-year risk, 14.4%) compared with CLL without stereotyped HCDR3 (events/n, 18/579; 5-year risk, 4.0%; P < 0.001; Table 1; Fig. 1A).

In CLL, stereotyped HCDR3 preferentially associate with unmutated IGHV genes (21, 22). On these bases, we assessed the risk of RS transformation of IGHV unmutated CLL after stratification for stereotyped HCDR3 usage. CLL with stereotyped HCDR3 and IGHV homology of ≥98% showed a significantly higher risk of transformation (events/n, 18/113; 5-year risk, 19.2%) compared with CLL with IGHV homology of ≥98% but without stereotyped HCDR3 (events/n, 10/191; 5-year risk, 6.7%; P = 0.003; Fig. 1B). This observation shows that stereotyped HCDR3 is not a surrogate of IGHV homology for RS prediction.

Concerning IGHV gene usage, univariate analysis documented a significantly shorter time to transformation in CLL using IGHV4-39 (events/n, 6/20; 5-year risk, 35.4%) compared with CLL using other IGHV genes (events/n, 33/733; 5-year risk, 5.6%; P < 0.001; Table 1; Fig. 1C). Other variables associated with RS transformation are reported in Table 1.

We then tested the independent predictive value for RS transformation of stereotyped HCDR3 and IGHV4-39 usage. Multivariate analysis selected stereotyped HCDR3 at CLL diagnosis [hazard ratio (HR), 3.33; 95% confidence interval (95% CI), 1.63-6.80; P = 0.001] and IGHV4-39 usage (HR, 4.03; 95% CI, 1.57-10.32; P = 0.004), along with CD38 expression (HR, 3.34; 95% CI, 1.61-6.92; P = 0.001), as independent predictors of RS transformation (Table 2).

<p>| Table 1. Variables at CLL diagnosis predicting RS transformation by univariate analysis |
|-----------------------------------------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th><strong>Events/n</strong></th>
<th><strong>5-y risk</strong></th>
<th><strong>SE</strong></th>
<th><strong>P</strong></th>
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</thead>
<tbody>
<tr>
<td>Rai stage 0-II</td>
<td>28/664</td>
<td>5.0%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Rai stage III-IV</td>
<td>11/89</td>
<td>17.4%</td>
<td>5.2%</td>
</tr>
<tr>
<td>CD38, &lt;30%</td>
<td>12/445</td>
<td>3.1%</td>
<td>1.1%</td>
</tr>
<tr>
<td>CD38, &gt;30%</td>
<td>23/190</td>
<td>15.5%</td>
<td>3.3%</td>
</tr>
<tr>
<td>IGHV homology, &lt;98%</td>
<td>11/449</td>
<td>3.1%</td>
<td>1.0%</td>
</tr>
<tr>
<td>IGHV homology, ≥98%</td>
<td>28/304</td>
<td>11.4%</td>
<td>2.3%</td>
</tr>
<tr>
<td>No IGHV4-39</td>
<td>33/733</td>
<td>5.6%</td>
<td>1.1%</td>
</tr>
<tr>
<td>IGHV4-39</td>
<td>6/20</td>
<td>35.4%</td>
<td>13.5%</td>
</tr>
<tr>
<td>No stereotyped HCDR3</td>
<td>18/579</td>
<td>4.0%</td>
<td>1.0%</td>
</tr>
<tr>
<td>Stereotyped HCDR3</td>
<td>21/174</td>
<td>14.4%</td>
<td>3.4%</td>
</tr>
</tbody>
</table>

Abbreviations: Events/n, number of events over number of patients; 5-y risk, 5-y risk of transformation calculated by Kaplan-Meier method; P, P value calculated by log-rank test.
Variables not associated with RS transformation in multivariate analysis included *IGHV* homology (\( P = 0.166 \)) and Rai stage (\( P = 0.087 \)).

**Stereotyped HCDR3 using *IGHV*4-39 identifies a subgroup of CLL at very high risk of RS transformation.** The prevalence of stereotypic HCDR3 was stratified according to *IGHV* gene usage in RS and nontransformed CLL. The HCDR3 of all RS using *IGHV*4-39 (six of six, 100%) entered a subset compared with only 2 of 14 (14.3%) HCDR3 of nontransformed CLL using *IGHV*4-39 (\( P = 0.001 \); Supplementary Tables S7 and S8).

The observation that all RS using *IGHV*4-39 carried stereotyped HCDR3 prompted investigation of the interaction between *IGHV*4-39 usage and stereotyped HCDR3 in the model. Multivariate Cox analysis selected the interaction between *IGHV*4-39 usage and stereotyped HCDR3 at CLL diagnosis as the strongest independent predictor of RS transformation (HR, 4.76; 95% CI, 1.76-12.88; \( P = 0.002 \); Table 2).

Accordingly, bivariate analysis segregated three risk categories (Fig. 2): (a) CLL using both *IGHV*4-39 and stereotyped HCDR3 represented the disease category with the highest risk of transformation (events/n, 6/8; 5-year risk, 68.7%); (b) CLL using stereotyped HCDR3 but without *IGHV*4-39 showed an intermediate risk of transformation (events/n, 15/178; 5-year risk, 9.9%); and (c) CLL using a nonstereotyped HCDR3, independent of *IGHV*4-39 usage, showed the lowest risk of RS transformation (Fig. 1).

**Table 2. Multivariate analysis for RS transformation**

<table>
<thead>
<tr>
<th>Biological and clinical variables*</th>
<th>HR</th>
<th>95% CI</th>
<th>( P )</th>
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<tr>
<td><em>IGHV</em>4-39</td>
<td>4.03</td>
<td>1.57-10.32</td>
<td>0.004</td>
</tr>
<tr>
<td>Stereotyped HCDR3</td>
<td>3.33</td>
<td>1.63-6.80</td>
<td>0.001</td>
</tr>
<tr>
<td>CD38 expression ( \geq 30% )</td>
<td>3.34</td>
<td>1.61-6.92</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Interaction analysis**

<table>
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<tr>
<th>Interactions</th>
<th>HR</th>
<th>95% CI</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IGHV</em>4-39 stereotyped HCDR3</td>
<td>4.76</td>
<td>1.76-12.88</td>
<td>0.002</td>
</tr>
<tr>
<td>HCDR3 interaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stereotyped HCDR3</td>
<td>3.11</td>
<td>1.50-6.48</td>
<td>0.002</td>
</tr>
<tr>
<td>CD38 expression ( \geq 30% )</td>
<td>3.16</td>
<td>1.50-6.64</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Abbreviation: \( P \) value calculated by Cox analysis.

*Covariates at CLL diagnosis that entered the analysis were *IGHV*4-39 usage versus no *IGHV*4-39 usage, stereotyped HCDR3 versus no stereotyped HCDR3, *IGHV* gene homology \( \geq 98\% \) versus *IGHV* gene homology \(<98\%\), CD38 expression \( \geq 30\% \) versus CD38 expression \(<30\%\), and Rai stage 0-II versus Rai stage III-IV.

*Covariates at CLL diagnosis that entered the analysis were *IGHV*4-39 usage versus no *IGHV*4-39 usage, stereotyped HCDR3 versus no stereotyped HCDR3, *IGHV* gene homology \( \geq 98\% \) versus *IGHV* gene homology \(<98\%\), CD38 expression \( \geq 30\% \) versus CD38 expression \(<30\%\), and Rai stage 0-II versus Rai stage III-IV, and *IGHV*4-39 stereotyped HCDR3 interaction.

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**Fig. 1.** Survival curves for cumulative probability of transformation to RS according to stereotyped HCDR3 and *IGHV*4-39 usage. A. CLL using stereotyped HCDR3 displays a significantly higher risk of RS transformation (5-y risk, 14.4%) compared with CLL without stereotyped HCDR3 (5-y risk, 4.0%; \( P = 0.001 \)). B. CLL with stereotyped HCDR3 and *IGHV* homology \( \geq 98\% \) show a significantly higher risk of transformation (5-y risk, 19.2%) compared with CLL with *IGHV* homology of \(<98\%\) but without stereotyped HCDR3 (5-y risk, 6.7%; \( P = 0.003 \)). C. CLL using *IGHV*4-39 harbor a significantly higher risk of transformation (5-y risk, 35.4%) compared with CLL using other *IGHV* genes (5-y risk, 8.6%; \( P < 0.001 \)).
IGHV4-39 (seven of eight cases) consisted by cases belonging to HCDR3 IGHV4-39 using both Results of the bivariate analysis prompted transformation (events/n, 5/7; 5-year risk, 64.3%; SE, 11.1%; SE, 10.5%; HR, 2.86; P = 0.306), subset 3, subset 4, and subset 9 (5-year risk, 0 and P > 0.05 in all instances) did not display an increased risk of RS transformation compared with CLL without stereotyped HCDR3 (Supplementary Fig. S1).

The risk carried by stereotyped HCDR3 and by subset 8 at CLL diagnosis is specific for RS transformation and has no effect on other types of CLL progression. Because transformation to RS and progression to symptomatic disease according to IWCLL-NCl guidelines are distinct events in CLL (34, 35), we also evaluated the effect of stereotyped HCDR3 and of IGHV4-39 usage on risk of CLL progression without transformation. Time to progression did not differ between CLL with stereotyped HCDR3 and CLL without stereotyped HCDR3 after adjustment for IGHV homology of ≥98% or <98% (P = 0.934 and P = 0.415, respectively). Also, time to progression did not differ between CLL harboring IGHV4-39 and CLL without IGHV4-39 (P = 0.711).

We then compared the effect of specific HCDR3 subsets on risk of transformation to RS and on risk of progression without transformation. Subset 8 using IGHV4-39 emerged as a strong predictor of RS transformation (HR, 24.50; P < 0.001) but did not predict CLL progression (P = 0.081). Conversely, subset 2 using IGHV3-21, which, as expected, was a risk factor for CLL progression (HR, 2.81; P = 0.002; refs. 14, 43), did not predict RS transformation (P = 0.306). Finally, subset 4 using IGHV4-34 and considered a benign subset (20), predicted neither RS transformation (P = 0.985) nor CLL progression (P = 0.089).

Recurrent cytogenetic findings associate with RS transformation in CLL harboring stereotyped HCDR3. FISH karyotype of the CLL phase was available in 596 cases, including 30 cases transformed to RS. The distribution of FISH abnormalities was assessed in the HCDR3 subsets most represented in our series. CLL belonging to subset 8, which used IGHV4-39 and displayed the highest risk of RS transformation, carried a recurrent cytogenetic pattern represented by +12 as the sole cytogenetic abnormality in four of five (80.0%) cases, all of which had transformed to RS. None of the subset 8 CLL with +12 showed translocation of the IGH locus or translocation of BCL-3. Conversely, CLL belonging to subset 4, which used IGHV4-34 and displayed no risk of RS transformation, carried a favorable FISH karyotype (del13q14) in all cases (n = 7).

Stereotyped HCDR3 of RS are not observed in de novo DLBCL. To define whether stereotyped HCDR3 observed in RS occur also in primary aggressive B-cell non–Hodgkin's
lymphoma, we tested 63 cases of de novo DLBCL for HCDR3 clustering. Overall, stereotyped HCDR3 observed in RS occurred only sporadically in de novo DLBCL. In particular, (a) the HCDR3 of a single case of de novo DLBCL entered a RS subset, namely subset 7 (Supplementary Table S9); (b) none of the de novo DLBCL carried a stereotyped HCDR3 belonging to subset 8 using IGHV4-39; and (c) none of the de novo DLBCL clustered with another de novo DLBCL, whereas 12 of 69 (17.3%) RS clustered with another RS (P = 0.001). These data suggest that HCDR3 clustering observed in RS is exceptional in de novo DLBCL.

**Discussion**

The rationale of the study was 2-fold: (a) assess the role of BCR in RS and (b) verify whether stereotyped HCDR3 at diagnosis may help in the identification of CLL subgroups at high risk of subsequent RS transformation. We document that (a) RS carry stereotyped HCDR3 at a very high frequency, (b) stereotyped HCDR3 at CLL diagnosis is an independent risk factor of RS transformation, and (c) the risk of RS transformation depends upon specific subsets. In particular, HCDR3 subset 8 identifies CLL with a very high risk of transformation.

In CLL, the finding of stereotyped HCDR3 in 20% to 25% of cases coupled to the binding of specific antigens by some stereotyped HCDR3 have led to imply the role of antigen stimulation in leukemogenesis (13–26). In the context of RS, this hypothesis is further reinforced by (a) the frequency (~50%) of stereotyped HCDR3, significantly higher than in nontransformed CLL; (b) the preferential usage of IGHV4-39 gene; and (c) the overrepresentation of a specific HCDR3 subset (subset 8) using the IGHV4-39/D6-13/15 rearrangement. Notably, our review of all IGHV-D-J rearrangements used by RS and reported in the literature also documents (a) frequent usage of IGHV4-39 (all RS cases, 7 of 55, 12.7%; IGHV unmutated RS, 7/47, 15.2%) with a prevalence that is similar to that observed in our cohort (P = 0.467 when comparing all RS from our series versus all RS from the literature; P = 0.703 when comparing IGHV unmutated RS from our series versus IGHV unmutated RS from the literature) and (b) stereotyped HCDR3 belonging to subset 8 in a fraction of cases (27–32). CLL using HCDR3 subset 8 have been shown to bind (auto)antigens associated with apoptosis and oxidation (44).

Biased usage of stereotyped HCDR3 and subset 8 in RS is confirmed also when an independent CLL cohort, previously published by Murray et al. (22), is used as control group. Prevalence of stereotyped HCDR3 is higher in RS compared with the CLL cohort of Murray et al. (22) when considering all cases (P < 0.001), cases with IGHV homology of ≥98% (P < 0.001), and cases with IGHV homology of <98% (P = 0.051). Also, HCDR3 subset 8 is the sole HCDR3 subset preferentially used by RS compared with the CLL cohort of Murray et al. (ref. 22; P < 0.001).

Our data show that stereotyped HCDR3 does not represent a surrogate of IGHV homology of ≥98% when considering RS transformation as an end point. In fact, CLL with stereotyped HCDR3 and IGHV homology of ≥98% showed a significantly higher risk of transformation compared with CLL with IGHV homology of ≥98% but without stereotyped HCDR3. Also, stereotyped HCDR3 is selected as an independent predictor of RS transformation in multivariate analysis after adjusting for IGHV homology of ≥98% and other potentially confounding covariates. Finally, the risk of RS transformation associated with the most represented subsets, including HCDR3 subset 8, is not affected by IGHV homology.

The effect of stereotyped HCDR3 usage on CLL progression and survival is heterogeneous and may depend upon specific subsets (21). Such heterogeneity is observed also in the setting of RS transformation. In fact, some HCDR3 subsets, including subset 8, are associated with RS transformation, whereas other HCDR3 subsets, including the benign subset 4 (21), display a negligible risk of RS.

The spectrum of HCDR3 subsets that are at risk of RS transformation apparently differs from the spectrum of HCDR3 subsets that are at risk of progression. In fact, HCDR3 subset 8 using IGHV4-39 is a strong predictor of RS transformation but has no significant effect on CLL progression. In contrast, subset 2 using IGHV3-21, which is a well-known risk factor of CLL progression (15, 43), failed to predict RS transformation. These observations reinforce the notion that biological and clinical risk factors of disease transformation to RS differ from risk factors of disease progression according to IWCLL-NCI guidelines (6).

The current definition of RS does not take into account the clonal relationship of RS with the original CLL clone (1, 2, 34–36). Accordingly, our study included clonally related RS, as well as RS for whom clonal relationship is unknown, and clonally unrelated RS. Subgroup analysis documented that IGHV4-39 usage and stereotypic HCDR3 are predictive of transformation also when the model is applied separately to clonally related RS and to RS for whom clonal relationship is unknown (data not shown). Due to the low number of cases (n = 6), no conclusion can be drawn from the subanalysis of clonally unrelated RS.

The heterogeneous outcome of specific HCDR3 subsets may depend upon their genetic profile (45). In the context of RS transformation, HCDR3 may identify CLL subsets with specific genetic features that increase the risk of RS. In our series, CLL belonging to HCDR3 subset 8 showed the highest risk of RS transformation and displayed the highest prevalence of +12 as the sole cytogenetic abnormality. The association between +12 and HCDR3 subset 8 has also been described in an independent CLL series that, however, did not include RS (45). Several lines of evidence suggest the involvement of +12 in RS transformation. First, RS frequently harbor +12 (1, 8, 46, 47). Second, +12 in the absence of del13q14 at diagnosis identifies a CLL subgroup at risk of RS transformation (6). Third, in our series, +12 (HR, 4.33; 95% CI, 1.33-14.08; P = 0.015) and del17p13 (HR, 4.89; 95% CI, 1.43-16.73; P = 0.011) were the sole FISH lesions associated with a significantly increased risk of subsequent transformation to RS.

Although a previous report linked usage of IGHV4-39 subset 8 with t(14;19) translocation in CLL, in our series BCL-3 translocation was not detected in any IGHV4-39 subset 8 CLL (48). This discrepancy might be due to the fact that our study included only CLL with a typical slg low/CD5+/CD23+ phenotype and a Matutes score of >3 (33), whereas most CLLs harboring t(14;19) translocation have an atypical phenotype (48–50).

Only few biological markers available for predicting CLL progression have been shown to be useful for RS prediction (6, 11). HCDR3 subset 8 and more in general HCDR3 subset usage may be appropriate biological markers for RS prediction.
because (a) these markers are independent predictors of RS transformation after adjusting for other biological and clinical risk factors and importantly (b) virtually all CLL using HCDR3 subset 8 ultimately transform to RS.

The identification of CLL patients belonging to HCDR3 subset 8 may be feasible whenever IGHV-D-J usage is known. In this specific context, knowledge of IGHV-D-J usage may surrogate cluster analysis for the identification of CLL belonging to subset 8. In fact, in the database used for this study and comprising 3204 CLL IGHV-D-J rearrangements, usage of IGHV4-39/D6-13/J5 identifies CLL belonging to subset 8 with a sensitivity of 100% and a specificity of 100% (not shown).

Disease extent is known to influence outcome of RS patients (5). Also, RS treatment requires dedicated treatment approaches, because regimens designed for CLL have been shown to be of little benefit for this disease (1–5, 51–53).

These observations suggest that prompt recognition of RS transformation may be clinically useful. Although at present it would be premature to use BCR features for tailoring treatment, a close monitoring and a careful biopsy policy may be of help for early recognition of RS transformation in patients carrying stereotyped HCDR3, particularly if belonging to subset 8.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Kostas Stamatopoulos, G. Papanicolaou Hospital, Thessaloniki, Greece, for critically reading the manuscript.

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Davide Rossi, Valeria Spina, Michaela Cerri, et al.


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