Variable heavy-chain gene analysis of follicular lymphomas: subclone selection rather than clonal evolution over time
Aarts, W.M.; Bende, R.J.; Bossenbroek, J.G.; Pals, S.T.; van Noesel, C.J.M.

Published in: Blood

DOI: 10.1182/blood.V98.1.238

Citation for published version (APA):
To investigate B-cell receptor evolution in follicular lymphomas (FLs), immunoglobulin variable heavy chain (VH) gene regions of 3 FLs were analyzed at different time points. One FL with a high somatic mutation load and intraclonal VH gene diversity was investigated in situ. VH gene transcripts were amplified and sequenced from samples of approximately 50 tumor cells isolated from frozen tissue sections by laser microdissection. Interestingly, the mutation pattern of the prevalent subclone in the relapse biopsy was virtually identical to that of a subclone isolated by microdissection from the presentation biopsy 9 years earlier. In a second FL, proof was obtained that the subclone that dominated the relapse sample had already been present in the initial biopsy. The finding that subclones found in the relapses of these FLs had not evolved over time but were preexistent, challenges the concept of antigen-driven B-cell receptor evolution during disease course.

© 2001 by The American Society of Hematology

Variable heavy-chain gene analysis of follicular lymphomas: subclone selection rather than clonal evolution over time

Wilhelmina M. Aarts, Richard J. Bende, Janneke G. Bossenbroek, Steven T. Pals, and Carel J. M. van Noesel

Study design

Patient material

Fresh tissue of FLs 3, 6, and 8 and their respective relapse samples were obtained from surgically removed lymph nodes from the departments of pathology of the Academic Medical Center, Amsterdam, the Westinde Hospital, The Hague, and of the Leiden University Medical Center, the Netherlands. FL 3 was first diagnosed in 1993 (FL 3-'93) and relapsed in 1995 (FL 3-'95). From patient 6, FL tissue was available from 1994 (FL 6-'94) and from 1998 (FL 6-'98). FL 8 was established in 1983 (FL 8-'83) and relapsed in 1992 (FL 8-'92). Clinical data have been described previously.

Microdissection of samples

Microdissection was performed with a laser-microbeam system (PALM GmbH, Bernried, Germany). Samples of approximately 50 cells were dissected from a 10-µm unstained frozen tissue section from FL 8-'83, “catapulted” into 3 µL complementary DNA (cDNA) reaction mixture, and stored on ice until cDNA synthesis.

cDNA synthesis

RNA of bulk material was isolated from frozen tissue sections and cDNA was synthesized as described.14 From microdissected samples, cDNA was synthesized without prior RNA isolation. Samples were incubated with the cDNA reaction mixture as described in a total volume of 20 µL. After incubating for 15 minutes at 37°C, the enzyme was inactivated during 10 minutes at 95°C. Next, 20 µL water was added.

Amplification of the VH gene

cDNA reaction mixture (1 µL) was used in a 25-µL polymerase chain reaction (PCR) volume by using a forward primer specific for the leader of the VH3 gene family in combination with a reverse primer specific for Cμ (Cμ1: 5'-CGTATCCGACGGGAAATTCTC-3') or Cγ.14 Next, a nested PCR was performed using 2.5 µL of the first PCR product in a 25-µL reaction. A VH3 primer that anneals in the FR1 region (VH3FR, 5'-TCCCTGAGACTCTCTTCTG-3') was combined with the appropriate reverse primer, either Cμ, or Cγ.14 PCR conditions were the same as those described for the CDR3-specific PCR.

© 2001 by The American Society of Hematology
The second time point (FL 8-'92) 9 years later consisted of IgG-expressing tumor cells only that contained 35 mutations compared with V3-23, 30 of which were shared with the IgM- and IgG-sequences of the 1983 sample (Figure 1A). At both time points high intraclonal variation was observed, generally believed indicative of ongoing somatic hypermutation.

The subclones present in FL 8-'83 were studied in more detail by dissecting samples of approximately 50 cells from the 20 neoplastic follicles. Of each sample, the V_H-C_m and V_H-C_y gene transcripts were amplified and sequenced. All PCR and sequence reactions were carried out in duplicate, and “consensus” sequences thus obtained of each sample were compared. In general, we found a random distribution of subclones over the follicles without obvious subclone dominance within individual tumor follicles (data not shown). Significant intraclonal sequence variation was found (ie, 2.7 and 3.1 somatic mutations per immunoglobulin sequence) compared with the IgM- or IgG-derived consensus sequences as derived from crude tissue analyses, respectively (Table 1). Interestingly, we noticed that the IgM-derived sequences from samples 2, 3a, 3b, and 4a, as well as the IgG-derived sequences from samples 2 and 9a from FL 8-'83 clearly differed from the consensus 8-'83 sequences but were almost identical to the mutation pattern of the IgG+ subclone that dominated the 8-'92 sample (Figure 1A). The only difference was a replacement mutation at amino acid position 30 present in FL 8-'92 but not found in any of the sequences of FL 8-'83 (Figure 1A). This close resemblance and the fact that we found significant intraclonal variation makes it very likely that the dominant subclone of 8-'92 was already present in the presentation biopsy. The findings suggest that over time subclone selection occurred instead of evolution of a subclone by continued somatic hypermutation in combination with BCR selection.

On the basis of this finding, we assayed the initial biopsies of FLs 3 and 6 for the presence of the subclones that dominated their respective relapses. For this purpose, we designed time–specific PCR primers of which critical 3'–position(s) matched solely with the sequences of the relapse populations. In FL 3, we had previously found a decrease in the number of somatic mutations over time and successive mutation patterns that were not in favor of clonal evolution. Now, we obtained a PCR product from cDNA of FL 3-93 with 3-95–specific primers. Sequencing of this product indeed proved that the clone that dominated the relapse 3-95 had already been present in the initial biopsy 3-93, most likely at a very low frequency (Figure 1B). The fact that we found this subclone with an identical mutation pattern at both time points is not in support of ongoing somatic hypermutation. In FL 6, the PCR approach was not successful (data not shown). Interestingly, in this case, the number of somatic mutations had increased over time, from 44 to 50 mutations compared with the germline.

Table 1. Diversity of subclones found in follicular lymphoma 8-'83

<table>
<thead>
<tr>
<th>Isotype</th>
<th>No. of clone sequences</th>
<th>Total no. of intraclonal nucleotide differences*</th>
<th>Intraclonal variation (no. of mutations/clone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-83 IgM</td>
<td>22</td>
<td>59</td>
<td>2.7</td>
</tr>
<tr>
<td>8-83 IgG</td>
<td>20</td>
<td>61</td>
<td>3.1</td>
</tr>
<tr>
<td>8-92 IgG</td>
<td>6§</td>
<td>8</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*IgM, immunoglobulin M; IgG, immunoglobulin G.

§Clones were not obtained by microdissection of samples, but bacterial clones were made from the VH3–polymerase chain reaction product derived from 8-'92 complementary DNA.10
gene, whereas the successive mutation patterns were also potentially compatible with clonal evolution.

Thus, we provided evidence for selective outgrowth of minor subclones in the course of FL disease. Previously, the gradual overgrowth by an already major subclone, without alteration of the consensus mutation pattern, has been documented. Matolcsy et al described a diffuse large B-cell lymphoma (DLBL) that evolved from a FL. The mutation patterns of the FL and its DLBL relapse were different and more suggestive of subclone selection than of clonal evolution. However, the investigators were not able to demonstrate, by PCR with subclone-specific primers, the presence of the DLBL clonotype in the FL.

On the basis of observed mutation patterns, intraclonal variation and genealogic relationships between tumor subclones in FL, a role for antigen-receptor ligands in lymphomagenesis has been proposed. However, the evidence we obtained for subclone selection rather than clonal evolution questions a role for BCR ligands in the growth of at least some FLs. Interestingly, Ottensmeier et al recently described a FL subclone with a stop codon in the functionally rearranged V<sub>H</sub> gene. Among other clones, this subclone was also found in the relapse sample 10 months later, suggesting that BCR expression was not essential for the propagation of this FL. In conclusion, we think it is worth considering that the expansion of FLs is independent of the quality of the BCR but is determined by various other genetic alterations that give selective growth advantage during disease course.

References