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# The Immunoglobulin genes and Chronic Lymphocytic Leukemia (CLL)

*Review based on the doctoral thesis*

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## ABSTRACT



The somatic hypermutation (SMH) status of the immunoglobulin (Ig) $V_H$ genes can divide chronic lymphocytic leukemia (CLL) into two prognostic subsets, with mutated $V_H$ genes display superior survival compared to unmutated cases. Biased $V_H$ gene usage has also been reported in CLL which may reflect antigen selection. In a $V_H$ gene analysis of 265 CLL cases we confirmed the prognostic impact of the $V_H$ mutation status and found preferential $V_H$ gene usage in both the mutated and unmutated subset. Interestingly, CLL cases rearranging one particular $V_H$ gene, $V_H$3-21, displayed poor outcome despite that two-thirds showed mutated $V_H$ genes. Many of the $V_H$3-21 utilizing cases expressed λ light chains, rearranged a $V_λ$2-14 gene, and had homologous complementarity determining region 3s (CDR3s), implying recognition of a common antigen epitope. We thus believe that the cases rearranging the $VH_3$-21 gene comprises an additional CLL entity. We further analyzed the $V_H$ gene rearrangements and, specifically, the heavy chain CDR3 sequences in 346 CLL cases to investigate the role of antigens in CLL. We identified six new subgroups with similar HCDR3 features and restricted VL gene usage as in the $V_H$3-21-using group. Our data indicate a limited number of antigen recognition sites in these subgroups and give further evidence for antigen selection in the development of CLL. Different mutational cutoffs have been used to distinguish mutated CLL in addition to the 2% cutoff. Using three levels of somatic mutations we divided 323 CLLs into subsets with divergent survival (<2%, 2–5% and >5% mutations). This division revealed a low-mutated subgroup (2–5%) with inferior outcome that would have been masked using the traditional 2% cutoff. A 1513A/C polymorphism in the $P2X_7$ receptor gene was reported to be more frequent in CLL, but no difference in genotype frequencies was revealed in our 170 CLL cases and

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200 controls. However, CLL cases with the 1513AC genotype showed superior survival than 1513AA cases and this was in particular confined to CLL with mutated VH genes. In summary, we could define new prognostic subgroups in CLL using Ig gene rearrangement analysis. This also allowed us to gain insights in the biology and potential role of antigen involvement in the pathogenesis of CLL.

*Chronic lymphocytic leukemia*

B-cell chronic lymphocytic leukemia (CLL) is the most common adult leukemia in Western countries; approximately 400–500 cases are diagnosed annually in Sweden. CLL is characterized by a malignant B-cell proliferation with surface expression of CD5 and CD23, and low levels of surface immunoglobulin (Ig) (1). CLL is a heterogeneous disease, where some patients survive for many years with minimal or without treatment, whereas others die rapidly of their disease. CLL is more common in men than women with a median age of diagnosis of approximately 65 years.

In the clinic two prognostic scoring systems are used, Rai and Binet, but except for these there are few good clinical prognostic markers that can predict survival in CLL at an early stage of the disease (2,3). However, molecular genetic studies are providing new insights in this disease. In CLL, no single large genetic aberration has been indicated in all cases, although a number of aberrations are evident in up to 80% of cases (4). The most common of these are deletions at 13q14, 11q22–q23 (the ATM gene), 17p13 (the p53 gene) and 6q21 and trisomy of chromosome 12. These aberrations can define risk groups, where poor risk groups include cases with del(11q), del(17p) and trisomy 12, while del(13q) is associated with better prognosis (4). The somatic hypermutation status of the immunoglobulin variable heavy chain (IgVH) genes has been proven to be a powerful prognostic indicator in chronic lymphocytic leukemia (CLL), since cases with mutated VH genes show significantly longer survival than unmutated CLL cases (5,6). The analysis of the Ig genes was thus suggested as a new important prognostic tool in CLL (5–8).

*The immunoglobulin genes*

During an immune response the B-cell can secrete large quantities of Ig which are a key element of the secondary immune reaction. The Ig molecule consists of two identical heavy chains and two identical light chains (κ or λ) and is expressed on the surface of all mature B-cells. The heavy and light chains each consist of a variable (V) region which is responsible for antigen binding and a constant (C) region which decides the effector function. The Ig heavy chain (IgH) locus is located on chromosome 14q32 and the two immunoglobulin light chain (IgL) loci on chromosome 2 (κ) and 22 (λ). The IgH locus is estimated to be 1100 kb long and consists of clusters of $V_H$, diversity (D), joining ($J_H$) and constant (C) germline gene segments. The IgH locus consists of approx. 95 $V_H$ gene segments, of which 51 represent functional $V_H$ genes and the remaining non-functional pseudo genes (9). The $V_H$ genes can be grouped into seven $V_H$ gene families ($V_H1$–$V_H7$) based on at least 80% sequence homology within the $V_H$ gene family. The IgL loci consists of $V_L$ and

$J_L$ gene segments, but in the IgL loci no D genes have been identified. There are 36–40 functional $V_\kappa$ and 29–33 functional $V_\lambda$ genes (10,11).

One of the most important stages in the B lymphocyte development process is the rearrangement of the IgH/IgL genes. The ordered rearrangement of the Ig genes initially occurs during the transition from the pro B-cell to the pre B-cell stage and involves rearrangement of the IgH locus. During this process, one D and one $J_H$ gene segment are initially recombined and thereafter one VH segment is joined to the previously formed $DJ_H$ complex. This $V_HDJ_H$ rearrangement will then form the V region of the heavy chain Ig molecule. The heavy chain Ig is expressed on the surface in conjunction with a surrogate light chain to form the pre B-cell receptor (pre-BCR) which is essential for continued survival of the immature B-cell (12). The transition from the pre B-cell stage to the immature B-cell stage is marked by a cessation of the IgH gene rearrangement process and activation of the IgL gene recombination. Joining of a $V_\kappa$ gene to a $J_\kappa$ gene occurs initially at the Igk locus and if the rearrangement is successful it will be expressed along with the heavy chain Ig as the B-cell receptor (BCR) on the cell surface. This event marks the transition to the mature B-cell stage with the cell expressing IgM and IgD. If a $V_\kappa J_\kappa$ recombination does not render a functional κ light chain, an additional IgL rearrangement (the second Igk locus or the Igλ locus) event may be initiated to rescue the cell from elimination (13, 14).

The large number of combinatorial events which can occur in the rearrangement of the IgH and IgL loci is the first stage in generating the large antibody diversity seen in the immune system. During the joining of the different gene segments random nucleotides may be inserted and base-pairs may be deleted in the junctions, for example the D-$J_H$ and $V_H$-D junction, which further generates diversity in the antibody repertoire. Just considering the combinatorial events of the heavy and light chain gene segments there are over 1.6 million possible combinations which is further amplified via random nucleotide insertions or deletions (15). This is estimated from the following calculation; the possible $V_H/D/J_H$ gene combinations are $51V_H$ genes $\times$ 27 D genes $\times$ 6 $J_H$ genes = 8,262 and together with a particular $V_L$ gene rearrangement, for example a $V_\kappa J_\kappa$, this will render $8262 \times (40\times5) = 1.6$ million potential combinations.

*Somatic hypermutation of the Immunoglobulin genes*

Recognition of the functional Ig molecule with its specific antigen initiates a process of SHM which primarily occurs in the germinal center (GC) environment of lymph nodes and follows antigen recognition and co-stimulation by helper T-cells (16). Although mainly seen in the secondary lymphoid tissues such as the lymph nodes GCs have also been reported in other sites such as sites of inflammation in autoimmune disease (17, 18). The SHM mechanism involves targeted mutations, mainly point mutations but also insertions and deletions, downstream of the V gene promoter of the IgH and IgL loci. SHM occurs at rates of $10^{-5}$ to $10^{-3}$ mutations per base pair per generation (1 million fold higher than spontaneous mutations) (19). The SHM process recognizes certain nucleotide motifs preferentially with a bias for RGYW (R =A/G, Y=C/T and W=A/T) and its inverse repeat WRCY (20), and

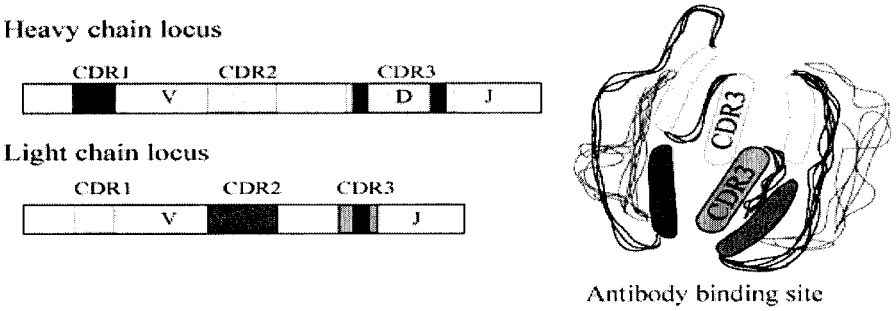


*Fig. 1.* Rearranged variable region of the Immunoglobulin genes showing complementarity determining regions (CDR) 1, 2 and 3. The CDR's come together to form the antigen binding site in the Immunoglobulin molecule. V=variable, D=diversity and J=joining.

recently a new motif has been proposed utilizing a DGYW/WRCH motif where D=A/G/T and H=T/C/A (21).

The SHMs are particularly focused in six regions called complementarity determining regions (CDRs) of which there are three in each V gene rearrangement. The CDRs are hypervariable regions and come together to form the antigen binding site of the Ig molecule (Figure 1). The heavy chain CDR3 is considered the most variable region between different Ig as it spans the $V_HDJ_H$ junction and contributes to the most diversity in the antibody binding site (22). Mutations in CDRs may either lead to amino acid replacements (R) and possibly changes in the affinity for the specific antigen or silent (S) mutations which will leave the amino acid unchanged. Successive rounds of SHM therefore lead to production and selection of cells with higher affinity for the initiating antigen than the original B-cell. In the normal human repertoire 40% of B-cells show evidence of SHM and express the characteristic memory marker, CD27 (23).

*The Ig genes and SHM in B-cell neoplasms*

B-cell leukemias/lymphomas arising from the clonal expansion of a single B-cell which has passed the pre B-cell stage will have rearranged IgH and IgL genes unique to that cell and therefore the IgH/IgL rearrangements could be used as specific tumor markers. Furthermore, the presence or absence of SHM will indicate the differentiation stage of the malignant cell at transformation. This analysis is primarily carried out on the VH region of the IgH gene rearrangements (24). Currently, a cut off of 98% or greater homology to the germline genes is utilized to delineate SHM to rule out polymorphisms and Taq polymerase errors (25). For example, most mantle cell lymphomas are unmutated indicating that the transformation occurred in the pre-GC stage (26), whereas all multiple myelomas show evidence of extensive somatic hypermutation corresponding to a derivation from a terminally differentiated plasma cells (27). Clonal development within a tumor may also be traced by analysis of the Ig gene rearrangement in subclones. Evidence of ongoing SHM may

be seen, for instance in follicular lymphoma (28–30), the GC B-cell like form of diffuse large cell lymphoma (31–33), Burkitts lymphoma (34, 35) and splenic marginal zone lymphoma (36). These latter forms of lymphomas derive from GC B-cells, which is in contrast to multiple myeloma and the activated B-cell like form of diffuse large B-cell lymphoma which do not show signs of ongoing SHM and therefore originate from a more differentiated, post-GC B-cell (33,37).

Until recently, the cell of origin of CLL was thought to be a naïve pre-GC cell with no evidence of SHM (38–40). However, analysis of the $V_H$ gene mutation status in CLL has demonstrated that roughly half of the patients show evidence of SHM in the $V_H$ region indicating passage through a GC reaction while the remaining patients show germline configuration of their $V_H$ genes (5, 6, 41, 42). This has seen great interest in the field as this division was shown to be diagnostically useful with patients with unmutated $V_H$ genes in the tumor clone had having a clinically worse prognosis than patients with somatically hypermutated $V_H$ genes (5–8). Thus, the division of CLL by SHM status was suggested as a good prognostic marker in this disease which in general shows a heterogeneous clinical spectrum.

Differences in the SHM status may indicate different subpopulation origins, however immunophenotype and expression profiling has indicated a very similar overall phenotype to memory cells for both the unmutated and mutated CLL cases (43–46). CLL cells with unmutated $V_H$ genes may differentiate through a T-cell independent mechanism independent of the GC. CLL cells with mutated $V_H$ genes, which in general have a low mutational load, may differentiate through a GC reaction or through a GC pathway outside the lymph node, which are characterized by a low mutation load and home to a similar environment to the unmutated cells. One possible environment which has been postulated is the marginal zone which is known to contain both Ig unmutated and mutated B-cells (47–50). Lately, the SHM in mutated CLLs has been shown to be representative of the normal SHM process since the $V_H$ gene sequences display characteristic targeting of the SHM hotspots (51).

*V gene utilization in lymphoproliferative disease*

An overrepresentation of certain VH genes has been shown in different B-cell malignancies and in autoreactive B-cells in autoimmune diseases (26, 27, 38, 41, 42, 52–54). In CLL, the $V_H$1-69 and $V_H$4-34 genes have been found preferentially expressed in several studies (5, 8, 38, 41, 42, 55, 56). Over utilization of specific $V_H$ genes have also been demonstrated in other B cell tumors, such as salivary gland MALT lymphoma ($V_H$1-69) (57), multiple myeloma ($V_H$1-69, $V_H$3-9, $V_H$3-23, and $V_H$3-30) (27), mantle cell lymphoma ($V_H$3-21 and $V_H$4-34) (26, 53) and nodal marginal zone B-cell lymphoma (NMZL) ($V_H$1-69 and $V_H$4-34) (54).

In CLL, differences are also evident within the Ig unmutated and mutated CLL groups. In the mutated group, restricted usage of the $V_H$3-07 gene and the $V_H$4-34 gene has been demonstrated (5, 41). The latter V gene has been associated with both self and non-self antigens as well as has been found overrepresented in both autoimmune disease and different lymphomas (58–62). In the unmutated CLL group,

biased usage of the $V_H$1-69 gene has been reported by a number of groups with $V_H$1-69 representing a large proportion of the unmutated $V_H$ gene rearrangements (5, 42, 56, 63, 64). In addition to frequent usage, the $V_H$1-69$^+$ CLL rearrangements show unusual molecular characteristics such as preferential rearrangement of certain D and J genes and a CDR3 longer than normally seen in CLL rearrangements not utilizing the $V_H$1-69 gene (56, 63). Frequent use of one allele (51p1) is another feature of these rearrangements (38, 39, 63). A recent paper has also shown that the features of the $V_H$1-69 rearrangements in CLL are distinctly different from that seen in the normal elderly population (64). The finding of preferential $V_H$, D and $J_H$ gene usage as well as longer than average CDR3s in $V_H$1-69$^+$ CLL has led to the speculation of a possible antigen component involved in the development of CLL with the hypothesis that BCRs encoded by specific $V_HDJ_H$ combinations could generate Ig molecules with affinity to similar antigenic epitopes.

*A $P2X_7$ receptor polymorphism and CLL*

$P2X_7$ is one of seven members of the P2X family of nucleotide receptors. It functions as a ligand (ATP-adenosine triphosphate) gated ion channel expressed on the plasma membrane and is expressed on many cells in the immune system, such as lymphocytes, macrophages and dendritic cells, as well as smooth muscle cells, fibroblasts, neurons and epithelial cells. $P2X_7$ is a bifunctional receptor: at tonic low level stimulation of ATP it acts as an ion channel, and may provide growth and survival stimulation, while at longer chronic stimulation of ATP it forms a large pore which allows molecules of large size (<900Da) to pass through and triggers apoptosis (65–67). In both normal B-cells and T-cells a central role for $P2X_7$ appears to be in apoptosis; however its growth stimulatory properties have been less well defined, but transfection of $P2X_7$ cDNA has been shown to result in increased proliferation of lymphocytes (68).

$P2X_7$ has been indicated to be of importance for development and progression of CLL in two recent reports (69, 70). A single nucleotide polymorphism (SNP) at nucleotide position 1513 (1513 A→C) was shown to result in an amino acid change from glutamic acid to alanine in the carboxyl terminal tail and resulted in a loss of function in the receptor in normal B-cells and CLL cells (70, 71). Individuals with the 1513AA genotype had normal function while individuals with the 1513AC genotype have a 50% loss of receptor function and 1513CC individuals have an almost complete loss of function. While affecting the function, the 1513AC genotype does not appear to affect the surface expression of the receptor (71). Interestingly, the 1513AC genotype has been reported to be associated with increased incidence in CLL patients (42%) compared to healthy controls (11%) (70). Furthermore, expression of the P2X7 receptor in CLL has been associated with a more aggressive disease which showed higher expression than indolent cases (69).

*CLL utilizing the Ig gene $V_H$3-21*

We initially performed $V_H$ gene analysis in 119 CLL patients to investigate the SHM

status as well as the $V_H$ gene usage with the Ig unmutated group comprised 69 cases (58%) and the mutated group 50 cases (42%) (72). There was an overrepresentation of certain $V_H$ genes with the $V_H1$ family gene, $V_H1$-69, and the $V_H3$ family gene, $V_H3$-21, representing 15.7% and 11.2% of the $V_H$ genes amplified, respectively. In accordance with previous studies, the $V_H1$-69$^+$ cases were almost exclusively represented in the Ig unmutated group (5, 42, 56, 63, 64). As expected, the CLL cases utilizing the $V_H1$-69 gene showed poor survival as all of them belonged to the unmutated group, but surprisingly the cases utilizing a mutated $V_H3$-21 gene also had a poor overall survival (median 63 months). In order to further characterize the $V_H3$-21 group, we extended this study to 265 CLL cases and identified 31 $V_H3$-21 cases with 21 displaying mutated and ten unmutated $V_H$ genes (73). We could confirm the worse prognosis of the mutated $V_H3$-21$^+$ cases (n=21) with a median survival of 72 months and showed that the whole $V_H3$-21 group, including both mutated and unmutated, had a short median survival of 83 months (Figure 2). From a clinical point of view this group is interesting as they display a poor overall survival, particularly considering that two thirds of the cases are mutated and, therefore, should have a good prognosis. We therefore conclude that $V_H3$-21-utilizing cases do not fit the pos-

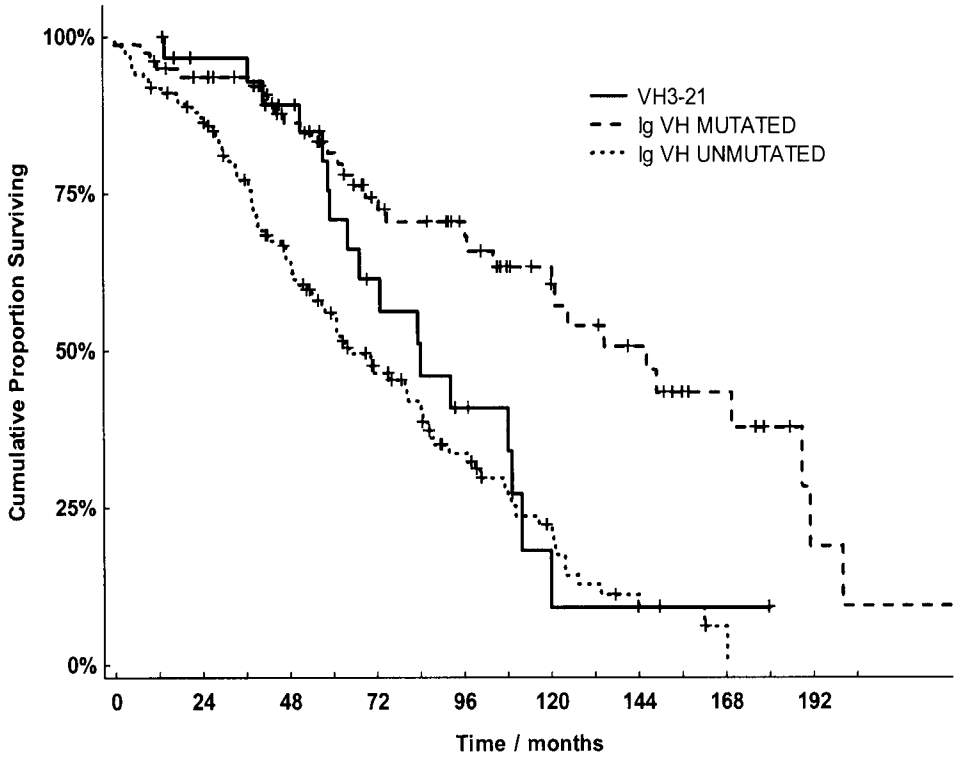


*Fig. 2.* Survival analysis of 265 CLL cases displaying Ig $V_H$ unmutated (median survival 70 months), Ig $V_H$ mutated (median survival 146 months) and $V_H3$-21 cases (median survival 83 months).

tulated prognostic classification of mutated and unmutated CLL and that $V_H$3-21 gene usage defines a group with worse survival irrespective of the SHM status.

At the molecular level the $V_H$3-21$^+$ cases showed unusual characteristics such as short CDR3s (8 codons) compared to the remaining rearrangements (15 codons) and they also displayed highly homologous CDR3s, seven cases showed identical CDR3s with a conserved amino acid motif (ARDANGMDV) and a further five cases showed only one amino acid difference from these seven cases. A restricted λ expression was revealed in both unmutated and mutated $V_H$3-21$^+$ cases (28 cases express λ and 3 κ on immunophenotype), therefore, we sequenced the light chain gene rearrangements and found that 24 out of 28 λ expressing cases rearranged the same $V_λ$ gene, $V_λ$2-14. Considering the importance of the CDRs in the antigen binding site and in particular the CDR3, our results of restricted CDR3 features and predominant $V_λ$2-14 rearrangements indicated that the $V_H$3-21$^+$ cases showed signs of a common antigen recognition site. The $V_H$3-21 gene has been shown to be utilized in certain autoantibodies produced in rheumatoid arthritis, Sjögren's syndrome and primary biliary cirrhosis and, similarly to the $V_H$1-69 gene, an association with autoreactivity specificities has been shown for this VH gene (60, 74, 75). However, the $V_H$3-21/λ2-14 antibody specificity and its role in the development of CLL have yet to be defined. In the Scandinavian population they represent approximately 11% of CLL cases, but they have also been identified in a large German CLL cohort (76) and in a cohort of Australian CLL patients (3 VH3-21 gene rearrangements out of 100 CLL cases analyzed, own unpublished data).

*The potential role of antigens in CLL*

There exists several lines of evidence indicating that an antigen component is involved in CLL development, such as the overrepresentation of $V_H$1-69 gene rearrangements in CLL and the auto- and polyreactive specificities associated with $V_H$1-69 usage (41, 42, 63, 77, 78) as well as the recent findings of similar CDR3s in the $V_H$3-21$^+$ cases. However, a definitive antigenic identification has remained elusive.

In order to investigate antigen involvement we characterized 407 $V_H$ gene rearrangements in 346 CLL (79). We carried out alignment analysis of the HCDR3 sequences in all 368 functional rearrangements to study the region which is considered to contribute to the most variability between Ig binding sites as it spans the $V_H$/DJ/$_H$ junctions. From this analysis we identified seven groups (51 cases) with similar HCDR3s with three or more cases in each group (79). The largest group contained 22 cases utilizing the $V_H$3-21 gene with very high homology between the HCDR3s as discussed previously. The six additional groups consisted of 29 cases which had within each group high similarities between their HCDR3s, i.e. similar $V_H$, D and $J_H$ gene usage, utilization of the same D reading frame and similar lengths of the HCDR3s. Additionally, many of these groups displayed similarities in the junctional regions. For example, four cases rearranged the $V_H$1-69 gene together with a D3-16 and a $J_H$3 gene, where the D3-16 gene was used in the same reading

frame and the CDR3 (18 amino acids) was of identical length in all cases. The $V_H$/D junction consisted of two amino acids and was identical in all rearrangements while four out of five amino acids were identical in the D/$J_H$ junction (Figure 3). We also performed VL gene rearrangement amplification and sequencing in the six new groups with homologous HCDR3s and could show highly restricted VL gene use within the subgroups, as have found in the $V_H$3-21$^+$ cases. In the four cases rearranging the $V_H$1-69/D3-16/$J_H$3 genes, all rearranged a $V_\kappa$A27 gene. To investigate if the preferential rearrangement of certain $V_L$ genes was restricted to cases with homologous rearrangements, we analyzed further CLL cases utilizing the same $V_H$ gene as in the homologous groups ($V_H$1-69, $V_H$4-39 and $V_H$1-2) but without homology between their HCDR3s. A variety of different $V_L$ genes were found rearranged in these cases, for example in the $V_H$1-69 utilizing cases 14 non-homologous CLL cases rearranged ten different $V_L$ genes and restriction was only seen in the two $V_H$1-69 homologous groups. These data indeed indicate that preferential $V_L$ gene rearrangement was restricted to CLL cases with homologous HCDR3s.

The probability of the same $V_H$/D/$J_H$ rearrangement occurring twice is in the order of 1/8262 and occurring with one particular light chain rearrangement is less than 1/1.6 million therefore these findings are unlikely to be a random finding. Further support was given by an analysis of 227 HCDR3 from the public databases that showed only homology between two sequences as well as the reported finding of no duplicate Ig rearrangements in about 10,000 Ig sequences obtained from normal tonsils (80–82). Previous studies have identified the same $V_H$1-69/D3-3/$J_H$6 (63) and $V_H$4-39/D6-13/$J_H$5 (83, 84) combinations in CLL with similar amino acid motifs in the HCDR3s, and, additionally, we found four $V_H$1-69/D3-16/$J_H$3 rearrangements from CLL cases in the public databases which were highly homologous to our $V_H$1-69 group cases. All of these latter findings strengthen our data of groups with restricted rearrangements and homologous HCDR3s in CLL. Our study reveals indirect signs of antigen selection in subsets of CLL with multiple cases showing both highly homologous $V_H$ and $V_L$ rearrangements, thus suggesting a limited number of antigens involved in the selection of these BCRs.

A recently identified $V_H$4-39/VκO2 utilizing IgG$^+$ CLL subgroup, that displayed similar HCDR3s to our $V_H$4-39 group, has been shown to have comparable antigen binding sites to antibodies reacting towards bacterial carbohydrates and certain autoantigens (84). An antibody termed SMI, which utilizes the $V_H$1-69/VκA27 genes and has been isolated from a CLL patient, shows reactivity to a variety of self antigens such as human Ig, myoglobulin thyroglobulin, actin and ssDNA (85). Furthermore, an anticardiolipin antibody was identified from the public database with a similar HCDR3 to the $V_H$1-69/D3-16/$J_H$3 rearrangements in our study (GenBank accession no AAL67508).These groups with highly homologous HCDR3 coupled to restricted VL gene usage were confined mainly to the Ig unmutated group with poor prognosis. Considering the recent data showing more BCR signaling, as measured by syk phosphorylation, in unmutated cases than mutated cases (86), this may indicate that a limited number of antigens may signal through the BCR and play a

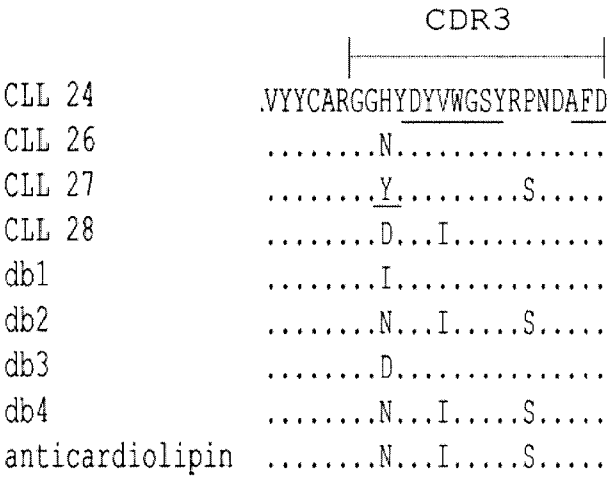


*Fig. 3*. Amino acid alignment of 4 CLL $V_H$1-69 CDR3 rearrangements and an additional 4 cases from the public databases (db1-4). Also shown is the CDR3 from an anticardiolipin antibody. A dot indicated homology with the first sequence.

more important role in tumor progression of the unmutated cases. Further studies of BCR signaling within these specific subgroups and the antigenic specificity may shed light on these issues.

*Somatic hypermutation in CLL*

The VH unmutated and mutated groups in CLL display differential prognosis in terms of overall survival, which could be confirmed in both of our initial studies (87). Additionally, this division indicates that the original B-cells may have undergone different biological pathways during their differentiation before transformation. The classification of SHM currently utilizes a border of 2% to delineate CLL cells which have or have not undergone the process of SHM (5, 6, 88–90). This border was originally set to rule out polymorphisms and Taq polymerase error; however the true biological border is unknown. Recent reports have suggested other borders as the best prognostic indicator such as 4% or 5% (91, 92). We investigated the mutation border at various levels in 323 CLL cases and in our cohort the best statistical discriminator of overall survival was the division into three groups based on <2%, 2–5% and >5% mutations and the median survival for these three groups was 72 months, 97 months and 150 months, respectively. This resulted in division of the Ig mutated group into two subsets with the low-mutated (2–5%) cases displaying worse survival compared to the high-mutated (>5%) cases. Thus, the low-mutated (2–5%) cases represent a group with poor survival which would have been masked using the 2% cutoff level. The poor prognostic $V_H$3-21 cases contained mainly low-mutated cases and therefore could be incorporated into the low-mutated group which fits the poor prognosis associated with both of these subsets. Only two $V_H$3-

$21^+$ cases showed >5% mutations and one of these cases displayed poor overall survival of 63 months.

The utilization of $V_H$ gene sequencing in the routine diagnostic setting has not been readily accepted as it is considered technically difficult and expensive to carry out, therefore surrogate markers such as the protein tyrosine phosphatase, ZAP-70 which can be analyzed by flow cytometry or immunochemistry have been proposed (93, 94). Initial studies showed a high correlation between the VH mutation status and ZAP-70 expression, where CLL cases with unmutated VH genes showed higher expression of ZAP-70, than mutated cases, however, in larger studies discordant result were evident especially in the Ig mutated group (93–96). These possible divergent results may underlie a different clinical course for certain patients such as the low mutated (2–5%) CLL cases. Subdivision of CLL into more than two prognostic groups may thus be useful in evaluation of ZAP-70.

The reason for the diverse prognosis between the CLL groups with different mutational frequencies is unknown. The degree of SHM in the cell may reflect the number of rounds of divisions the cells have undergone in the GC which is supported by a correlation with telomere length and degree of mutations (97). Therefore, it is possible that mutated cells which have divided more frequently during the GC reaction (highly mutated, >5%) may be more anergic/resting post GC when they transform to CLL and therefore represent more benign tumor cells. Alternatively, poor risk genetic aberrations such as p53 mutation/deletions may underlie the worse prognosis in this low mutated (2–5%) CLL subset.

*Polymorphism in the P2X7 receptor*

The loss of function SNP (1513 A→C) of the P2X7 receptor gene was analyzed in a cohort of 170 CLL cases as well as in 200 healthy age-matched controls (98). The 1513 AC genotype showed a frequency of 23% and 21% in the CLL cohort and control population, respectively, whereas the 1513CC genotype was represented in <1% of CLL cases and 5% of controls, respectively. This study did not find any association between increased prevalence of the 1513 polymorphism and development of CLL, which is in contrast with the report by Wiley *et al* that showed an increased incidence of this SNP in CLL (70). This difference could be explained by difference in patient groups included, since the study by Wiley *et al* included familial CLL cases which may have resulted in an overrepresentation of the 1513AC genotype in their study.

In this study the overall survival was found to be significantly different for CLL cases with the 1513AA vs. the 1513AC genotype; the median survival for cases with the 1513AC (n=35) genotype was 104 months compared to 72 months in cases with the 1513AA genotype (98). When the Ig unmutated and mutated CLL cases were associated with $P2X_7$ genotype data no difference in overall survival was demonstrated between the Ig unmutated with 1513AA and the 1513AC genotypes. However, in the mutated group the 1513AC genotype showed significantly different survival compared to the 1513AA genotype; the Ig mutated 1513AA cases showed

a median survival of 98 months compared to 151 months in Ig mutated 1513AC cases. The poor prognostic group utilizing the $V_H$3-21 gene did not influence the results when excluded from this study.

The reason for the better survival associated with the 1513AC genotype in the Ig mutated cases is currently unknown. Wiley et al has shown that CLL cells with the 1513AC allele have a decreased susceptibility to undergo apoptosis and they may therefore have a prolonged survival *in vivo*, which seems in contrast to the better survival seen in the Ig mutated 1513AC individuals in our study. However, the bifunctional role of the $P2X_7$ receptor in lymphocyte proliferation and in apoptosis makes it difficult to predict which mechanism is playing a more important part. Alternatively, CLL cells are sensitive to their microenvironment (99) and reduced function of the $P2X_7$ receptor in the T-cells and/or dendritic cells may therefore influence the survival of the CLL cells. The CLL cells with mutated VH genes may be more dependent of T-cell interactions after traversing the GC and more susceptible to cells in the microenvironment, whereas CLL cells with unmutated VH genes may develop through a T-independent pathway and therefore may be less susceptible to microenvironment changes. Future studies are required to investigate the mechanisms involved in CLL.

Currently, a number of reports have been published which do not support this association of the $P2X_7$ polymorphism with overall survival (100–102). No association was found between the $P2X_7$ polymorphism and sensitivity to fludarabine, Bcl–2 expression and overall survival in a study by Starcyznski *et al*. (101). Furthermore, a study by Zhang *et al* study could not reveal any association between the $P2X_7$ polymorphism, overall survival and $V_H$ gene status, while Nuckel *et al* found no association with treatment free survival (100, 102). However, a number of important differences exist between the studies which may explain the divergent results. The CLL cases from our study are mainly from referral centers which would tend to deal with more aggressive cases, while the materials in the Zhang *et al* study were collected mainly from non-referral centers and contain more benign cases (102). The median survival for Ig mutated cases in our cohort was 111 months (<10yrs) whereas in the report from Zhang *et al* their Ig mutated group had a median survival of 25 years (102). Also, the report from Starcyznski *et al* and Nuckel *et al* did not show the Ig mutated division of the 1513AC genotype in survival, which was the strongest finding in our report (100, 101). An analysis on a larger CLL cohort will hopefully resolve the issue of the importance of the $P2X_7$ polymorphism in CLL prognosis.

*Concluding remarks*

The SHM status in CLL is a powerful prognostic indicator of survival in this clinically heterogeneous disease, however; additional prognostic information could be obtained with individual $V_H$ genes such as $V_H$3-21. The $V_H3\text{-}21^+$ cases define a new prognostic group in CLL with poor prognosis irrespective of their SHM status (72, 73). Furthermore, the overrepresentation of certain $V_H$ genes such as $V_H$3-21 and

$V_H$1-69, and presence of seven groups with homologous HCDR3s combined with restricted $V_L$ usage are highly indicative of a role for antigen involvement in CLL pathogenesis (79). Although the Ig $V_H$ mutational status is a good prognostic indicator division of the Ig mutated groups is additionally possible indicating further heterogeneity in this group of CLL cases. A polymorphism in the $P2X_7$ receptor influences clinical outcome through a currently unknown mechanism in VH mutated CLL cases (98). Further studies are warranted to assess the true impact of this receptor and the 1513A–>C polymorphism on outcome in CLL. The true biological nature of the differing prognosis between the Ig unmutated and mutated CLL cases is still relatively unknown and future studies will be necessary on well defined CLL cohorts to tease out these differences.

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