Commentary

Molecular Analysis of Human Immunoglobulin Heavy Chain Variable Genes (IgVH) in Normal and Malignant B Cells

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Antigen binding to the membrane-bound immunoglobulin (Ig) receptor is crucial for clonal survival during B cell development and antigenic stimulation of mature B cells.⁴¹ Together with co-stimulatory signals (eg, CD40 and cytokines) it controls B cell proliferation and differentiation.³⁷ In the past, molecular analysis of Ig genes has been used mostly to prove clonality of a malignant lymphoproliferation. A detailed analysis, however, reveals that much more can be learned because clonal development, selection processes by antigen, and Ig variable gene (IgV) hypermutation leave a fingerprint of individual clonal development and current activities.

The quality of antigen binding is determined primarily by recombination events during ontogeny involving VH, D, and JH genes and, later in the development, VL and JL genes as well as subsequent somatic hypermutation of IgVH and IgVL genes during antigenic responses of mature B cells.⁴⁹ Signaling by the Ig receptor eventually rescues pre-B cells and proliferating mature B cells from apoptotic cell death. It thereby forms the basis of a life-long recurring selection process that is reflected by the Ig receptor repertoire and molecular composition of the Ig receptors in B lymphocyte populations of individuals at different ages. The molecular recombination of VH, D, and JH genes in developing B lymphocytes is probably a random process that would lead to a random use of functional VH genes as determined by their frequency in seven different VH gene families.⁹ However, positive or negative selection due to differences in antibody binding efficiency or antigen availability lead to relative over- or underrepresentation of distinctive VH families and VH genes in the receptor repertoire of fetal B lymphocytes. Subsequent antigenic exposure to environmental, infectious, or self antigens on an individual and genetic basis further influences clonal sizes of mature B cell families by usage and selection of distinct VH genes, thereby affecting their frequencies in the adult B cell population. Furthermore, recent results of single cell analysis in IgM⁺/

CD5⁺ and IgM⁺/CD5⁻ adult populations suggest that VH genes and VH, D, and JH recombinations are affected differently by antigen-induced selection processes in CD5⁺ and CD5⁻ B lymphocytes.⁵

The mechanism of somatic hypermutation of IgV genes is still not completely understood. Functionally, it represents the molecular basis of affinity maturation of naive B cell populations after antigenic exposure and interactions. On a molecular basis it consists of a site-specific hypermutability linked to the transcription of IgV genes acting from the 5' of the IgV gene promotor to around 1.5 kb downstream. The constant region exon, which is separated from the variable region by several kilobases of intron, is not affected. Mutations consisting mainly of point mutations accumulate within the antigen-binding complementary determining regions (CDR) in a nonrandom pattern. Rarely, duplications and deletions are also found.^{15,57}

The somatic hypermutation process is dependent on B cell differentiation and maturation. Pregerminal center B cells usually exhibit unmutated germline receptors. Antigen-activated proliferating germinal center B cells show high mutation rates and evidence of ongoing mutations because evolutionary trees of receptor mutations can be demonstrated by single cell analysis of reactive germinal centers³² as well as by receptor cloning in the follicular lymphoma.⁵⁸ Postgerminal center memory B cells show mutated but stable receptors, as demonstrated by single-cell analysis of reactive follicles indicating that the hypermutation process is normally concentrated in, if not restricted to, the germinal center area and shuts off when cells leave the germinal center microenvironment.³³ However, it is not clear whether the extrafollicular antigen-driven proliferation may under certain (and so far unknown) circumstances also result in further mutation

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and selection at a lower rate or whether further mutations depend on re-entry of memory B cells into the germinal center microenvironment.⁵¹ Early memory B cells exhibit lower mutation rates than mature plasma cells. CD5⁺ IgM⁺ peripheral B cells, which are thought to be involved mainly in nongerminal center, non-T cell-dependent immune reactions, may accumulate somatic mutations in their IgV regions. Continuous ongoing mutations resulting in microheterogeneity of the clonal receptor repertoire are found in extrafollicular marginal zone B cell lymphoma and diffuse large B cell lymphoma obviously independent of the germinal center microenvironment, showing at least in malignant B cell populations the possibility that the proliferation-associated hypermutation process may be still active independent of the germinal center microenvironment (see below). What kind of molecular process turns the hypermutation machinery off or on is so far not known. Interestingly, at least one other gene in nonneoplastic memory B cells, the *bcl-6* gene, has been identified recently and shows promoter-dependent hypermutability of the first exon paralleling and almost identical to the hypermutation of Ig variable genes with respect to mutation frequency and involved B cell subpopulations.⁵²

This discussion suggests that a more refined molecular analysis of Ig receptors in malignant lymphoma is a rewarding tool because it promises insights into the individual history and functional behavior of neoplastic clones, especially if findings are evaluated with a functionally oriented classification, eg, the R.E.A.L. classification.¹⁷ Furthermore, such analysis might allow better understanding of enigmatic and less characterized lymphoma entities, eg, the primary effusion lymphomas (PEL) reported in this issue.⁴⁰

IgVH Gene Analysis Is Helpful in Understanding Malignant B Cell Development

Molecular analysis of Ig genes in malignant lymphoma over the last decade yielded findings consistent with normal B cell development and suggested normal counterparts of lymphoma cells, but it has also yielded differences and astonishing findings, hard to understand at the present time. The analysis may be focused on several questions as discussed below.

Biased Usage of VH Genes?

With respect to peripheral blood B cells, it has recently been shown that a small number of VH genes are expressed by a majority of human CD5⁺ and CD5⁻ B cells. Nine VH family members, comprising 18% of functional VH genes, were expressed by more than 50% of peripheral blood B lymphocytes. The usage of normally underrepresented germline VH genes in malignant lymphoma would be an interesting finding because some of these genes, even in their germline configuration, are known to code for autoantibodies recognizing defined autoantigens. In this respect, VH4-34/DP 63 is interesting

because it encodes for an antibody that recognizes autologous determinants of red blood cells, the L/i antigens.^{44,45} This receptor has been found to be overrepresented in peripheral blood B cells.⁴² However, a more detailed analysis by Brezinschek et al⁵ found it significantly more often than expected only in unmutated populations, whereas it was underrepresented in mutated populations, implying that it may be less frequently involved in immune responses to exogenous antigens or even that B cells expressing this receptor might be deleted during the process of antigen-driven differentiation. The very frequent occurrence of this receptor in various lymphomas¹⁶ ranging from lymphomas with typically unmutated Ig genes such as CLL³⁹ or mantle cell lymphomas to those with very highly mutated receptors such as diffuse large B cell lymphomas of nodal²⁰ or extranodal localization¹⁶ may be important; it invites the hypothesis that certain VH genes and their respective autoantigen binding may have a role in lymphoma pathogenesis. The number of analyzed cases is still too low to draw statistically significant conclusions for most VH genes, but it would be important and interesting to know whether lymphomas related to distinct localizations or autoimmune dysfunctions differ in their relative frequency of VH gene usage (eg, by comparing gastric marginal zone B lymphoma of MALT type with other extranodal marginal zone B cell lymphomas).

It is therefore of interest to see that for the PELs reported by Matolcsy,⁴⁰ random usage of VH genes appears to be highly suggestive: no Ig receptor has been used twice among the seven investigated cases and three of the germline genes used (VH4-39, VH1-18, and VH3-30.3) are among the nine most frequently used VH genes in the normal peripheral blood B lymphocyte repertoire.

Is Somatic Hypermutation of IgHV Genes Present?

Somatic hypermutation of IgV genes in normal B cell populations is strongly correlated to B cell differentiation.³ The comparison of IgV gene patterns in lymphoma B cell populations has to be interpreted on the level of differentiation, reached by the suggested normal counterpart of the lymphoma B cell population. Reported findings for some lymphomas (summarized in Table 1) clearly correspond to normal B cell development.

Neoplastic B cells of chronic lymphocytic leukemia and mantle cell lymphoma do not show considerable somatic mutation similar to their normal counterparts of pre-germinal center B lymphocytes.²³ Rare mutated cases of mantle cell lymphoma could correspond to the population of mutated CD5⁺ peripheral blood B cells showing a low mutation frequency. Somatic hypermutation of Ig variable genes is found at the expected frequency in plasma cell tumors such as multiple myeloma⁵³ and also in PEL cells⁴⁰ compared to mutation frequencies found in normal bone marrow plasma cells. The same is true for low grade marginal zone B cell lymphoma of MALT type and even splenic marginal zone

Table 1. VH Gene Analysis in Normal and Malignant Human B Cells

Cell type	Immunophenotype	Mutation frequency (%)	Microheterogeneity (ongoing mutations)	Reference No.
Normal B cell				
Naive	slgM+/slgD+, CD5+	0–0.3	no	5, 28
Naive	slgM+/slgD+, CD5–	0–1.5	no	5, 12
GC	slgM+/slgD–, CD38+	0.3–4.3	yes	32, 35
Memory	slgM+/slgD–, CD38–	3.9	no	28, 29
Plasma cell	slg–, CD38+	8	no	11
B cell lymphoma				
B-CLL	slgM+/–slgD+/–, CD5+	0	no	39
MCL	slgM+/slgD+, CD5+	0–2.2	yes/no	10, 23, 30
Burkitt's (endemic)	slgM+/slgD–	5–15	yes	27, 55
Burkitt's (sporadic)	slgM+/slgD–	0.3–5.6	yes	27, 55
FL	slgM+/slgD–	10.5	yes	58
Nodal DLBL	slgM+/slgD–	9.9	yes	31
Extranodal DLBL	slgM+/slgD–	11.5	yes	16
MZBC of MALT-type	slgM+/slgD–, CD38–	4–4.5	yes	46, 47
Splenic lymphoma	slgM+/slgD–, CD38–	4.2	no	59
PEL	slg–, CD38+	7.7	yes/no	40
Multiple myeloma	slg–, CD38+	8.9	no	56

slg: surface immunoglobulin; MCL: mantle cell lymphoma; FL: follicular lymphoma; DLL: diffuse large cell lymphoma; MALT: mucosa-associated lymphoid tissue; DLBL: diffuse large cell B cell lymphoma; MZBC: marginal zone B cell; PEL: primary effusion lymphoma.

B cell lymphoma, which frequently show an average of about 4% point mutations similar to normal postgerminal center memory B cells. Findings differing from their normal counterparts are found in follicular lymphoma. Single cell picking of reactive germinal centers usually shows only moderate amounts of somatic hypermutation in the range found also in postgerminal memory B cells.³² Early hypermutated germinal center lymphocytes show highly hypermutated cells at low frequencies that are lost during further differentiation by apoptosis.³⁸

Follicular lymphoma, the counterpart of normal germinal center reaction, and diffuse large cell lymphomas of nodal and extranodal origin exhibit extremely high degrees of somatic hypermutation in the range of 10–12%. In follicular lymphoma, this amount of mutation is achieved despite the fact that lymphoma cells proliferate less than their normal counterparts, suggesting a continuous accumulation of mutations by the ongoing hypermutation. Tumor cells arrested in the site where somatic hypermutation and isotypes are occurring, namely the follicular microenvironment, therefore are still subject to these processes and are influenced further by persisting antigen challenge resulting in more ongoing mutations, as already suggested by Bahler et al,¹ by the specific defect of apoptotic cell death in follicular lymphoma due to bcl-2 protein hyperexpression.

Antigen Selection?

Somatic hypermutation leading to single base substitutions may either lead to replacement of an amino acid (R) or remain silent (S) if the resulting codon encodes for an identical amino acid. The accumulation of replacement mutations in CDRs has been interpreted as indicative of exogenous antigen-driven affinity maturation and qualified as positive selection. The accumulation of S mutations above an expected random value is qualified as negative selection and interpreted as an indication for the

preservation of primary antibody structures in the antigen-driven selection process, especially if these mutations are more numerous in CDRs than in framework regions. Random mutation will be found if the hypermutation mechanism is acting or has acted but, due to neoplastic alterations, the response to antigen binding is not reflected by changes in population size due to loss of surface Ig defects in receptor-mediated signaling, lack of apoptosis, or combined effects. Evidence of positive or negative selection has been found in some lymphomas such as low grade marginal zone B cell lymphoma of MALT type in the stomach^{46,47} (but not in salivary gland²), in many diffuse large B cell lymphomas of the stomach,¹⁶ and in multiple myeloma.⁵⁰ The finding of antigen-driven selection processes in multiple myeloma and also PEL representing tumors with lack of Ig expression at the cell surface could indicate a transformation process in a postgerminal center mature B cell that normally lacks the hypermutation mechanism.

To calculate R to S mutations, the probability model described by Chang and Casali⁸ is widely used. This model is based on the assumption that the frequency of random mutations in the FR or CDR is size-dependent. Size distribution of FR:CDR is 0.77:0.23. In antigen-mediated selection, as the antigen response progresses, all mutations in the CDR (not only R mutations) increase to nearly equal distributions in FR and CDR.^{21,25} The increase in mutation rate and the relative increase in R mutations in CDR should probably be calculated independently to get a clearer view on selection processes as done by Hallas et al.¹⁶

A more direct hint to antigen selection can be evaluated if lymphomas use the same germline gene and if somatic mutations are found at identical nucleotides resulting in identical amino acid substitutions. This has been found in diffuse large B cell lymphomas of the stomach, where the DP54 germline gene appears to be overrepresented. The probability for observed mutations

in identical nucleotides was calculated below 0.0001%, offering the possibility of mutations selected by an identical antigen during lymphomagenesis.¹⁶

For many marginal zone B cell lymphomas of nongastric origin as well as high grade lymphomas, eg, Burkitt's lymphoma, random distribution of mutations is found, reflecting the activity of the hypermutation machinery without obvious effects on clonal selection or propagation. This is especially interesting in relation to the finding of ongoing mutations leading to microheterogeneity (see below).

Ongoing Mutation: Clonal Microheterogeneity?

Ongoing mutation leading to clonal microheterogeneity has been linked to the germinal center microenvironment in normal immune reactions. In malignant lymphomas it is also clearly demonstrated without obvious link to germinal centers in marginal zone B cell lymphomas of MALT type, splenic marginal zone lymphoma, and diffuse large B cell lymphoma of nodal and extranodal origin. The implication of this unusual finding, also evident in one of the PELs reported in this issue, is still not well understood because the mechanism of somatic hypermutation has to be further elucidated. However, the resulting microheterogeneity is also present during lymphoma progression (if low- and high-grade components of one tumor clone are compared) and in response to lymphoma treatment and relapse, as recently shown in diffuse large B cell lymphomas.⁴³ This finding offers new approaches to investigate clonal mechanisms in lymphoma progression and may even have clinical importance.

Are Germinal Centers Pathogenic Hot Spots of Lymphoma Development?

With respect to the broad range of human B cell lymphomas and Hodgkin's disease, it is noteworthy that analysis of the Ig variable gene and the hypermutation mechanism suggests that most lymphoma entities, and therefore most lymphomas, represent germinal center or post-germinal center stages. This implies that somatic hypermutation of Ig variable genes, necessary as it is for the affinity maturation of immune reactions, also raises the danger of lymphoma development as recently discussed.²⁶

For many of these lymphomas, crucial transforming events are linked to the Ig locus on chromosome 14 where the normal process of V-D-J recombination and isotype switching occurs. However, recombination events leading to chromosomal translocations may further imply that any oncogene within these translocations may come under the control of the Ig promotor/enhancer and would be prone to somatic hypermutation as a direct result of the mutation process occurring at the endogenous Ig locus. The link between promotor proximity and mutation targeting may explain mutations in *c-myc* genes translocated to the Ig locus in Burkitt's lymphoma.⁴ However, not only point mutations but also somatic hypermu-

tation within germinal centers, leading to structural alterations of the Ig variable genes, may promote translocations as in the case of follicular lymphoma, endemic Burkitt's lymphoma, and certain translocations in diffuse large B-cell lymphomas, eg, t(3;14), involving the *bcl-6* gene.^{6,14,24,36}

In this respect, non-Ig genes linked to transcriptional initiation in mutating normal germinal center B cells may also be permissible in the hypermutation process as recently found for the *bcl-6* gene, which is heavily mutated in normal memory B cells but not mutated in naive human B lymphocytes.⁵²

The necessity of a DNA repair mechanism involved in microdeletion through error-prone repair¹³ or by correcting the unmutated complementary to the mutated base⁷ and their defects offers another phase of the link between somatic hypermutation of Ig variable genes and lymphoma development.

Conclusion

The molecular analysis of IgV genes is an important tool for understanding the pathogenesis of neoplastic changes in B cell tumors. The interpretation of results has to be linked to the stage of differentiation of neoplastic B cells related to their normal counterparts. It is obvious that so far, too few tumors have been analyzed thoroughly to support conclusions to some of the discussed questions. The implication of these findings in disease progression, treatment response, and even vaccine development is evident and should be used in clinical management of lymphoma patients.^{18,19,34,48,54}

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