IGHV gene mutation at heart of CLL treatment

IGHV mutation analysis should be top of mind when acquiring prognostic and potentially therapeutic information in chronic lymphocytic leukemia, said Curtis A. Hanson, MD, in a CAP TODAY webinar made possible by a special educational grant from Diaceutics. Dr. Hanson, professor of laboratory medicine and pathology, Mayo Clinic College of Medicine, explained the structure and function of IGHV, the mutation assay, and the clinical value of mutation status. Here is an edited transcript of what he said.

May 2019—Chronic lymphocytic leukemia is a neoplasm of small mature B-cells and the most common leukemia diagnosed in adults. Median age of diagnosis is 70 years, but there is a surprisingly large percentage of patients, about 10 percent, who are younger than 55, and it's not uncommon now to occasionally see CLL patients, about two percent, in their 40s.

Dr. Hanson

Although the overall survival rate is good, there is a lot of variability in patients with CLL, ranging from indolent and slowly progressive to others who have a more progressive course that requires aggressive treatment. That has been the holy grail in CLL: trying to identify those who will develop aggressive disease and treating them with appropriate therapy.

The first important step was developing a large number of prognostic markers that were useful in establishing risk categories. The next step, which has taken place over the past few years, is that new therapies for progressive disease in CLL are expanding, with improvements seen in overall survival, even for patients whom we previously would have put into a high-risk group.

Pathologists see CLL patients at several points in the course of their disease. The first step in the CLL evaluation process is to suspect it or recognize it. That might be because of a peripheral lymphocytosis, adenopathy, organomegaly, or, not uncommonly, an incidental pathology finding. The second step is to make the diagnosis, which is dependent not only on flow cytometric immunophenotyping but also, from a surgical pathology and hematopathology perspective, on tissue biopsies.

Step three is to subclassify it, making sure we obtain the right prognostic information, which will drive therapy, with the primary findings now from a pathology and laboratory perspective being the mutation status of the immunoglobulin heavy-chain variable region gene (*IGHV*) and CLL FISH studies.

During patient follow-up (step four), we need to be able to detect residual disease, and minimal residual disease studies by flow cytometry are the current standard. Finally, when we move into disease progression, the most important assays now center around *TP53* mutation, by FISH and sequencing.

The historic staging systems in CLL have been the Rai (modified) and Binet systems. And the key immunophenotype is CD5 co-expression with a variety of pan-B cell antigens—CD19, CD20, and CD23.

CD5 is also a characteristic finding in mantle cell lymphoma, which we can confirm with genetic studies or immunostains for CCND1. Importantly, 10 to 20 percent of marginal zone and lymphoplasmacytic lymphomas may also express CD5 and, if not considered, can easily be confused and misdiagnosed as CLL when those two disorders involve the peripheral blood and bone marrow.

When we do encounter immunophenotypes that are not classic for CLL, it's critical that we recommend to our clinicians that they consider a lymph node or other tissue biopsy so we can be sure we will have the right diagnosis, as opposed to arbitrarily pushing everything into a CLL diagnosis.

The classification scheme for patients who have a CLL-like immunophenotype is simple. Two criteria are needed: knowing what the absolute clonal B-cell count is, and the presence or absence of adenopathy. For those who have greater than 5,000 clonal B-cells, it's simple: Regardless of adenopathy status, it's CLL. For those who have less than 5,000 B-cells and no adenopathy, it's monoclonal B-cell lymphocytosis. With adenopathy, it's small lymphocytic lymphoma.

The development of prognostic markers has been one of the two biggest improvements in CLL. Unmutated *IGHV* gene is a molecular marker associated with poorer prognosis and shorter survival (mean OS = 95 months). Forty to 50 percent of patients will have the unmutated *IGHV* gene. The remainder are mutated; the prognosis is good and the mean overall survival is 293 months.

It's important also to look for *TP53* point mutations. This is related to, but separate from, looking for *P53* deletions by FISH. The incidence at diagnosis is five to 10 percent.

CLL FISH studies cannot be used to diagnose CLL because the anomalies—13q, trisomy 12, 11q, and 17p (p53)—can be seen in other lymphoproliferative disorders.

To discuss the clinical value of *IGHV* mutation status, I'm going to begin with an early history. It was known that the Rai and Binet staging systems did not recognize the biological diversity of CLL or predict response to modern therapy. Today the most important prognostic system in CLL is the CLL International Prognostic Index, or CLL-IPI. But before the CLL-IPI, a simple prognostic system had been proposed based on only two markers: FISH testing and *IGHV* mutation status, with patients separated into three risk groups. So it was recognized early on that the *IGHV* gene was going to be an important prognostic marker.

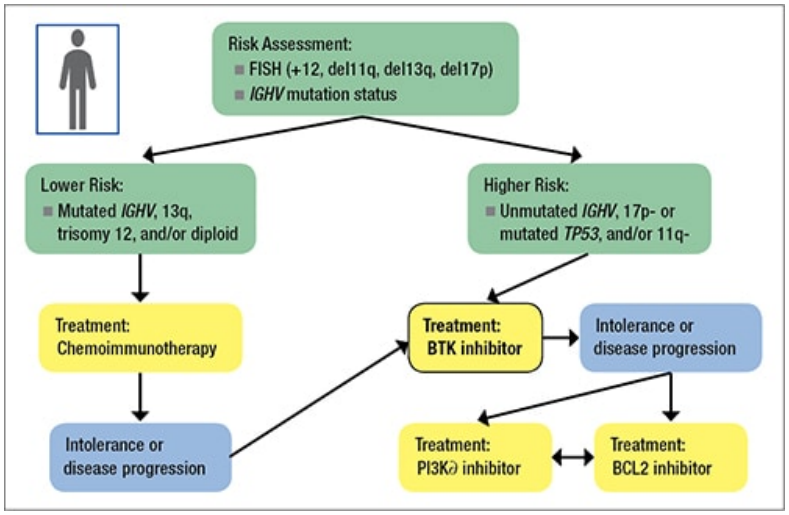

Fig. 1. Typical application of "risk" in a CLL patient

The flow-based prognostic markers—CD38, ZAP70, and CD49d—were easy to do and provided a lot of information but have not stood up over time as independent markers and are not required, in my view, in the evaluation of CLL patients today. There is also a variety of serum-based prognostic markers: soluble CD23, thymidine kinase, and β2-microglobulin. In several studies performed over time, β2-microglobulin has retained its independent prognostic value.

Let's walk through how to apply that typical application of risk and how it might define therapy in CLL patients. (**Fig. 1**).

You start with a FISH panel and determine the *IGHV* mutation status. From there, you move into the mutated or lower-risk versus unmutated or higher-risk category. If lower risk, the treatment (if indicated) will typically begin with traditional chemoimmunotherapy. If higher risk, the patient will likely receive the relatively new Bruton tyrosine kinase, or BTK, inhibitors. Now there are also other small-molecule inhibitors available, such as the PI3Kα and BCL2 inhibitors, for the small percentage of patients who might be intolerant of the BTK inhibitor or whose disease progresses.

Pathologists need to be aware of patients with (del)17p and/or *TP53* mutations. Both are associated with poor outcomes and relatively resistant to standard chemotherapy and chemoimmunotherapy regimens. Patients with these mutations fare much better when treated with small-molecule inhibitors of BTK, phosphatidylinositol 3-kinase, or BCL2. Progression-free survival and overall survival of CLL patients with a (del)17p or a *TP53* mutation but without (del)17p are similar. Both (del)17p by FISH and *TP53* mutation by sequencing have prognostic and predictive value and should guide therapeutic decisions.

In our laboratory, where we do a lot of *TP53* mutation analyses, the test is looking at exons four to nine, which covers more than 90 percent of the described pathologic mutations. The key is that sufficient clonal B-cells—at least 25 percent—are needed to get the analytical sensitivity out of the assay. We do it by Sanger sequencing, and we do a pre-sort of the peripheral blood clonal B-cells in advance to enrich for relatively pure CD19+ B-cells, so we can be sure we have the right mix of cells to do the assay correctly.

Let's talk about the antibody structure and function of *IGHV* because it's important to understand how recombination occurs and what is meant by variable mutations. Antibodies are composed of a Fab region (variable fragment antigen binding) and an Fc region (constant fragment crystallizable). The Fab region is composed of one heavy and one light chain, and each of those chains has one variable region and one constant region.

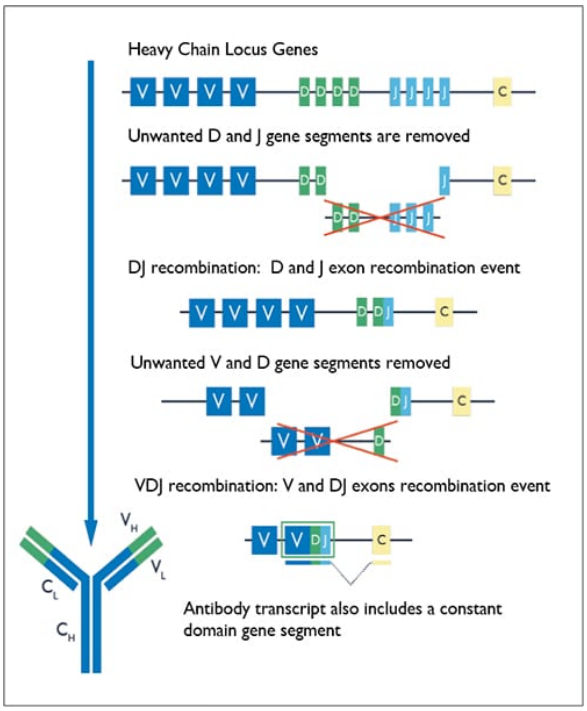

Fig. 2. *IGHV* H and L chain regions drive antigen specificity. The *IGHV* gene is found on chromosome 14 and encoded in several gene segments. Multiple copies of these segments—called V, D, and J segments—exist and are tandemly rearranged. In normal B-cell maturation, chromosomal recombination of the V, D, and J segments form the V region of the Ig H and L chains.

Variable regions have seven amino acid segments: three hypervariable as well as complementarity determining regions (CDR) and four framework (FR) regions. The CDRs help constitute how antigens are recognized, whereas the framework regions provide the support for proper folding and orientation of the CDRs.

Many will have seen variations of VDJ (variable, diversity, joining) recombination maps that occur within the heavy chain of the immunoglobulin. (**Fig. 2**). That is what drives antigen specificity as we pull together the various combinations of variable, diverse, and joining region genes. The *IGHV* gene is found on chromosome 14 and is encoded in gene segments. In normal B-cell maturation, chromosomal recombination of the V, D, and J segments come together to form that variable region of the immunoglobulin heavy and light chains, which is a separate pathway but using this same kind of process. Each developing B-cell will assemble a unique Ig V region by this somatic V, D, and J gene segment recombination together with hypervariable changes.

Antigen affinity is increased by the somatic hypermutation that occurs after that B-cell comes into contact with an antigen in the follicular germinal center. Somatic hypermutation introduces random nucleotide changes into the V genes, which leads to B-cells that express immunoglobulins with a high degree of antigen specificity. Since CLL originates from a single lymphoid cell, each daughter cell that occurs off that original cell will reflect that same *IGHV* rearrangement of the V, D, and J regions together with these hypervariable, somatic mutation changes.

The *IGHV* mutation assay can be done by Sanger sequencing or next-generation sequencing. With Sanger, only one sample can be run at a time. NGS technology is the best approach and represents a significant improvement over Sanger. It allows for batch analysis and simultaneous identification of the clonal *IGH* rearrangement, the tumor-specific rearrangement sequence, and, importantly for this assay, determination of the somatic mutation percentage.

To determine *IGHV* mutation status, blood or bone marrow can be used. In our laboratory, EDTA is preferred by far but ACD is an acceptable specimen. RNA is extracted and converted to cDNA using reverse transcription. PCR amplifies the *IGH* gene rearrangements with multiplex primers that span the leader, all V and D segments, and a portion of the J segment. Those sequence data are then analyzed to identify the *IGHV* rearrangement and the unique sequence, and results are compared to a germline *IGHV* database. The percent identity of the tumor *IGHV* to the closest germline sequence is calculated.

According to National Comprehensive Cancer Network and International Working Group on CLL guidelines, rearrangements with a mutation frequency of greater than or equal to two percent are mutated and considered a good prognosis, and rearrangements with a mutation frequency of less than two percent are unmutated and considered as having a poor prognosis. This information should be in the report, of course, but we as pathologists need to think beyond that: What do our clinicians need to know in terms of prognosis and how can this information be translated into other information to inform treatment decisions?

The interpretation is dependent on having enough clonal cells to amplify the clonal *IGH* gene rearrangement. We use five percent of total lymphocytes, by flow, to make sure we have enough clonal cells for a reliable, specific, and sensitive assay, and the prognostic significance of *IGHV* mutation status can be determined only if we can find a single, functional *IGH* rearrangement—functional meaning the sequence implies that it can go on and form an intact immunoglobulin molecule.

One of the advantages of having a lot of experience is being able to recognize when there are problems in interpretation. Here are two such problems: You may find more than one functional rearrangement, or you may find a nonfunctional rearrangement. In both situations, these are findings of uncertain significance and thus the *IGHV* status cannot be determined.

When the mutation status is at or near the two percent cutoff, interpret with caution, particularly if the entire *IGHV* could not be sequenced because of the use of framework region 1 V region primers. While two percent sounds like a perfect objective number, we all know there are degrees of subjectivity when the results get close to the cutoff number.

The CAP now has a proficiency testing program for *IGHV*. Sequence analysis of the *IGHV* gene is used to determine the somatic hypermutation status. Any sequencing method can be used, and one would submit the V-gene allele, percent similarity, and mutation status.

Now for the clinical value of *IGHV* gene mutation status. CLL suffered for a long time because there were too many prognostic markers. Individually they were informative, but the large number of markers became confusing and probably slowed progress.

Diagnostic lab tests	Recommendation
At diagnosis	
CBC and Diff	Always
Blood flow study	Always
Before treatment	
Bone marrow study	When clinically indicated
Serum chemistries, immunoglobulins, DAT	Always
CLL FISH for 13q-, 11q-,17p-, +12	Always
Chromosome karyotype	Not generally indicated
TP53 mutation	Always
***IGHV* mutation status**	**Always**
Serum β2-microglobulin	Desirable

Fig. 3. *IGHV* recommended by International Working Group on Chronic Lymphocytic Leukemia

But we have learned a lot about all these prognostic markers in the past five or so years. Clinicians wanted a simpler prognostic risk categorization as the first step, which has led to a more appropriate understanding of these new therapies that came out at the same time. Along that line, ZAP70 and CD38 early on were thought to be possible surrogates for *IGHV*. The thinking was, "Everybody can do a flow assay. Doing the *IGHV* status is hard. Maybe we can use these flow-based assays instead."

Various studies have looked at the concordance between CD38 and ZAP70 expression, using flow cytometry, and *IGHV* mutation status. And although there is a general concordance, there is not sufficient equivalence such that either marker can be used in place of *IGHV* analysis. And with these surrogate markers, there is too much variability in assigning consistent cutoff percentage expression. The conclusion is that neither marker can be used as a replacement for *IGHV* mutation status in determining prognosis and subsequent treatment. Studies done at Mayo Clinic have also confirmed these findings. It's time to move away from these surrogate-type markers to *IGHV*, which has clear clinical value.

The International Working Group on CLL is the group that subsequently drove the CLL International Prognostic Index and, in my view, the development of prognostic markers and how to use them, and now how to use new CLL therapies. The IWCLL guidelines, updated in 2018, say that *IGHV* mutation status should always be required before treatment in the baseline evaluation of patients with CLL (**Fig. 3**).

The CLL-IPI was a study of more than 3,400 treatment-naive patients from world-renowned CLL cancer centers in five countries. A subsequent validation was done of 838 patients from Mayo Clinic. A large set of prognostic markers were looked at and only five were found to have any value for use in a composite score: (del)17p FISH, *IGHV*, β2-microglobulin, clinical stage, and age.

Variable	Adverse factor	Grading
TP53 (17p)	Deleted and/or mutated	4
IGHV	Unmutated	2
B2M (mg/L)	> 3.5	2
Clinical stage	Binet B/C or Rai I-IV	1
Age	> 65	1
Prognostic score		**0–10**

Risk group	Score
Low	0–1
Intermediate	2–3
High	4–6
Very high	7–10

Fig. 4. CLL-IPI summary: *IGHV* is critical

Based on these five parameters, the group came up with four risk groups: low, intermediate, high, and very high. The clinical application is not only improved staging, but also for testing novel therapeutics in high- or very high-risk groups. **Fig. 4** is a summary of those parameters and the prognostic scoring system that was developed. The only way to get to a seven to 10 score—that is, the very high category—is to have a *TP53* abnormality. Conversely, the only way to be in a low-risk group is to be age 65 or older with none of the above factors, or having a Rai stage zero and none of the above. The Mayo Clinic validation data from that CLL-IPI study separate patients into these four categories. You can also see that a score of two for the unmutated *IGHV* status is critical to have in order to move into high or very high status. As pathologists we need to understand what its role is, and thus its inclusion in the workup of patients with CLL, in particular those patients being considered for therapy or showing signs of progressive disease.

A clinical colleague of mine approaches the risk categories in this way: If a patient falls into a low-risk group, he would typically do nothing, and that's an important clinical statement. He also would not typically treat those who are in the intermediate-risk category unless they're symptomatic. He would likely treat those in the high-risk group unless they're asymptomatic, and that's where we get into the decision: chemoimmunotherapy with fludarabine and rituximab or targeted therapy for BTK with the small-molecule inhibitor ibrutinib. Most important, if the patient is in the very high-risk category, it's important to treat in experimental protocols or with ibrutinib or other small-molecule inhibitors.

To sum up, CLL FISH testing for prognosis is well established and well understood. More important than FISH for prognostic determination at this time, however, with the exception of (del)17p, is *IGHV* status. *IGHV* needs to be included in the initial comprehensive workup and assessment of CLL patients because its status can help clinicians make better treatment decisions.

The full webinar is available at www.captodayonline.com.