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IGHV Mutational Status Testing in Chronic Lymphocytic Leukemia

Jennifer Crombie, MD and **Matthew S. Davids, MD, MMSc**

Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

Abstract

As the therapeutic landscape for chronic lymphocytic leukemia (CLL) continues to expand, biological predictors of response to therapy are becoming increasingly important. One such predictive biomarker is the mutational status of the variable region of the immunoglobulin heavy chain (*IGHV*) gene, which is a powerful predictor of duration of response and overall survival with chemoimmunotherapy (CIT). As this test may influence choice of therapy between CIT and novel agents, it is critical that providers understand how mutational status is determined and the limitations of testing. Here, we describe the details of *IGHV* mutational status testing, highlighting the appropriate way to interpret this information and best apply it to the care of patients with CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common hematologic malignancy in the Western world, with approximately 19,000 new cases each year in the United States.[1] The disease is remarkably heterogeneous, with some patients never requiring treatment and others having rapidly progressive disease despite maximal therapy. The two best established CLL prognostic markers are fluorescence in situ hybridization (FISH) and the mutational status of the variable region of the immunoglobulin heavy chain (*IGHV*) gene.[2,3] Here, we focus on *IGHV* mutational status as a key prognostic factor that can help to delineate differences in disease outcomes, and increasingly can inform treatment choices.

IGHV Testing in the Clinic

In 1999, two landmark papers were published which identified the importance of somatic mutations in *IGHV* genes as a prognostic marker in CLL.[4,5] In both studies, mutated *IGHV* correlated with marked improvement in both progression free survival (PFS) and overall survival (OS). Since then, several other studies have validated these results. A recent meta-analysis confirmed an improved PFS for mutated *IGHV* patients, with a range 9.2 to 18.9 years, compared to unmutated patients, with a range of 1 to 5 years.[6] Similarly, OS was found to range from 17.9 to 25.8 years in mutated patients, compared to 3.2 to 10 years in unmutated patients.

Corresponding Author and Reprint Requests: Matthew S. Davids, MD, MMSc, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215, Office Phone: (617)-632-6331, Office Fax: 617-582-9104, Matthew_Davids@dfci.harvard.edu.

In the majority of cases, favorable cytogenetics track with mutated *IGHV* mutational status and unfavorable cytogenetics track with unmutated *IGHV* mutational status. Interestingly, in the event of discordant prognostic features, there is evidence that mutational status may have a superior ability to predict overall survival.[7,8] Recently, an international prognostic index score that includes *IGHV* mutation status, along with age, clinical stage, mutations of the tumor suppressor gene *TP53*, and serum beta-2 microglobulin level, was also found to predict time to first treatment and OS in CLL.[9–11] In this weighted model, the relative risk of *IGHV* mutational status is twice as much as age and clinical stage, and is second only to *TP53* mutational status in importance. More simplified prognostic scores, consisting of only *IGHV* and *TP53* mutational status, have similarly been shown to predict survival in patients with CLL.[12]

In recent years, the value of *IGHV* mutational status has become most clear in its ability to predict the durability of response to chemoimmunotherapy (CIT). FCR300, the original phase II study of fludarabine, cyclophosphamide and rituximab (FCR) for initial therapy of patients with CLL, demonstrated a PFS of 53.9% for patients with *IGHV* mutated disease compared to 8.7% in unmutated patients after 12.8 years of follow up.[13] The PFS curves for mutated *IGHV* patients also plateaued, suggesting sustained, long-term remissions, and even cure in a subset of these patients. In the subsequent phase III study, CLL8, the German CLL Study Group showed that *IGHV* unmutated status, along with *TP53* mutations and the presence of deletion 17p, had the strongest negative prognostic impact on PFS and OS.[14,15] Additionally, after nearly six years of follow up, more than 83% of *IGHV* mutated patients were still alive, and median OS was not reached, suggesting that as in FCR300, patients with *IGHV* mutated CLL benefit significantly from CIT with long-term disease control.

While patients with *IGHV* unmutated CLL have inferior duration of response to CIT, there does not appear to be a difference in response duration for these patients when treated with recently approved novel agents that target the B cell receptor (BCR) pathway kinases, albeit with shorter follow-up. In fact, responses may actually be more rapid in patients with unmutated *IGHV*, perhaps due to the greater reliance of such cells on tonic BCR signaling.[16] For example, a study of the Bruton's Tyrosine Kinase (BTK) inhibitor ibrutinib for frontline treatment of CLL reported a 40% rate of complete remission (CR) in patients with unmutated disease, compared to only 6% of patients with mutated disease.[17] This discrepancy does not mean that the drug was more effective in unmutated patients; rather, CR in CLL requires complete clearance of lymphocytosis, and patients with unmutated CLL tend to have faster resolution of the lymphocyte redistribution that is characteristic of this class of drugs compared to mutated patients where prolonged lymphocytosis often delays achievement of CR, even though the patients are responding well clinically to treatment.[16] Importantly, recently reported data on the 5-year follow-up of patients treated with ibrutinib showed equivalent PFS for mutated and unmutated patients, a remarkable finding that has not been observed in trials of CIT in CLL.[18] Similarly, other targeted therapies, including the PI3K-delta inhibitor idelalisib and the BCL-2 antagonist venetoclax achieve equivalently high response rates and PFS for both *IGHV* mutated and unmutated CLL.[19,20]

Despite the clear clinical observations regarding the prognostic importance of *IGHV* mutational status, the mechanism underlying this observation remains incompletely

understood. One hypothesis is that *IGHV* unmutated cells are more prone to undergo apoptosis, and that faster kinetics of CLL cell growth rather than greater resistance to cell death may be responsible for the inferior response durability with traditional CIT.[21] Additionally, whole exome sequencing efforts of CLL samples have identified an increased frequency of driver mutations in unmutated as compared to mutated *IGHV* CLL.[22]

Given the prognostic implications of *IGHV* mutational status and the potential of this test to influence treatment, it is important to understand how mutational status is determined and limitations of this testing.

Biology of the Immunoglobulin Heavy Chain Genes

The adaptive immune system is designed to provide dynamic protection against a wide array of potential pathogens. During normal B-cell maturation, chromosomal recombination of the V (variable), D (diversity) and J (junctional) segments form the V region of the heavy and light immunoglobulin chains. These recombination events account for the tremendous immunologic diversity that is fundamental to the humoral immune response. The addition or removal of nucleotides at the junctions of these segments creates further sequence complexity. While the entire variable region influences immunoglobulin function, three complementarity-determining regions (CDRs) contribute most to antibody specificity. CDR1 and CDR2 are located within the V segment, and CDR3, the most variable region, includes some of the V, all of the D and part of the J region (Figure 1).[23,24]

When a mature B-cell comes into contact with an antigen, further diversification takes place through a process of somatic hypermutation, typically within the germinal center. Somatic hypermutation introduces random nucleotide changes into the V genes, selecting for B-cells that produce immunoglobulins with the highest degree of selectivity, a process known as affinity maturation. Cells that fail to go through the affinity maturation process subsequently undergo apoptosis. As germline genes used to encode the V region have been mapped, it is possible to determine whether the malignant B-cell clone has undergone somatic hypermutation.

What specimens to Test

As lymphocytes are present in both peripheral blood and bone marrow, either source can be used for testing. Samples should be collected in tubes containing either ethylenediaminetetraacetic acid (EDTA) or trisodium citrate/pyridoxal 5'-phosphate/Tris (CPT). Heparin should be avoided, as it has the potential to interfere with DNA polymerase and reverse transcriptase.[25] A minimum of 3 mL of peripheral blood or 1 mL of bone marrow is typically required.

Technical Aspects and Limitations of *IGHV* Testing

Given the importance of *IGHV* mutation status on prognostication, investigators of the European Research Initiative on CLL (ERIC) developed a consensus recommendation on the minimum requirements for reliable and reproducible *IGHV* mutational testing.[25] Currently, *IGHV* mutational status is determined by amplifying the *IGHV* transcript by polymerase

chain reaction (PCR), sequencing the gene through Sanger sequencing, and comparing the transcript to known germline genes available in immunoglobulin databases.[25,26]

According to ERIC recommendations, both complementary DNA (cDNA) and genomic DNA (gDNA) are suitable for analysis. cDNA has the advantage of preferentially identifying functional rearrangements. However, the use of gDNA avoids the need for reverse transcription and can be more easily performed on stored samples. Either leader primers, which allow for sequencing of the entire *IGHV* region, or primers specific to framework-region 1 (FR1), can be used. While FR1 primers require approximation of the percentage of identity to the closest germline gene, they are more efficient than leader primers. If a borderline result is obtained, the lab can utilize both primers for a more comprehensive evaluation.

The percentage of identity is calculated using the ratio of the number of mutations within the *IGHV* region and the length in nucleotides of the most homologous germline *IGHV* gene found in immunoglobulin databases, including international immunogenetics information (IMGT), V-Base, and GenBank. Insertions and deletions are counted as one mutation, irrespective of the number of extra or missing nucleotides. Also, if an FR1 primer is used, the 5' nucleotides equal to the primer length are excluded.

Given the different techniques available for *IGHV* testing, there is a potential for discrepant results across institutions. There is also a risk that a transcript other than the primary clonal transcript will be amplified and that certain primers miss subclones. Framework-region primers also do not yield a full-length transcript, and can potentially lead to an inaccurate calculation of percent similarity to germline. In addition, there are differences in the immunoglobulin databases, consisting of germline immunoglobulin genes and polymorphisms, as well as variations in the software programs used to calculate the percentage of nucleotide mutations.

More recently, targeted exome or genome next generation sequencing (NGS) has emerged as an alternative method to reliably sequence the entire V-D-J sequence, including the highly variable CDR3.[27] Comparisons of NGS-based testing to traditional methods suggests that NGS may provide more accurate results. In addition, NGS allows for identification of multiple productive clonally unrelated *IGHV* rearrangements, which were observed in 24% of patients with CLL in one study.[28]

Application of Testing

Mutation rates of ≥2% difference from germline are considered mutated, while unmutated disease has a <2% mutation rate. The 98% homology cutoff was chosen to exclude potential polymorphic variant sequences.[4,5] It is important that providers be mindful that this is a mathematical cutoff, especially when interpreting borderline mutational values. Identification of more than one clone, where one is mutated while the other is unmutated, also appears to have an intermediate prognosis as compared to mutated alone or unmutated alone.[28,29] In the event of discordant clones, additional prognostic markers can also help

guide interpretation and prognostication. For example, concurrent ZAP70 overexpression or 11q deletion by FISH are more likely to be associated with unmutated disease.[30,31]

It should be noted that unlike FISH testing, which can change over time through a process of clonal evolution, *IGHV* mutational status is generally considered to be a stable prognostic marker, and as such serial testing is generally not required. However, changes in mutational status have been reported, possibly due to the emergence of subclones.[32] Since *IGHV* mutational status provides useful prognostic information that can influence counseling or patient follow up, we generally send it soon after a diagnosis of CLL is made. However, *IGHV* status is not typically a major factor in the decision of when to initiate therapy. Rather, initiation of therapy continues to be guided by the 2008 International Workshop on CLL (IWCLL) criteria, in which only patients with advanced stage or symptomatic disease warrant therapy.[33] *IGHV* mutational status can influence choice of therapy, with mutated *IGHV* patients being better candidates for chemoimmunotherapy, while unmutated *IGHV* patients are more likely to benefit from a novel agent based approach. Ongoing prospective studies will provide further evidence for the utility of this approach.

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Summary Table

What is the test?

IGHV mutational testing assesses the percentage of sequence variability between the V region of the immunoglobulin heavy chain gene in a clonal CLL population compared to the homologous germline V region sequence.

How is it measured?

IGHV mutational status is determined by amplifying the expressed, clonal *IGHV* transcript by PCR, sequencing the gene through Sanger sequencing and comparing the transcript to known germline sequence.

What are the normal range values?

Mutated *IGVH* is classified as ≥2% mutated when compared to germline. Unmutated disease is <2% mutated compared to germline.

What conditions or types of conditions is it used for?

IGHV testing is a useful prognostic tool for patients with CLL. *IGHV* status may also have prognostic implications in other lymphomas, such as follicular and mantle cell lymphoma.[34,35]

What tests are helpful to do with it for a more complete picture?

In addition to *IGHV* testing, other prognostic testing such as FISH cytogenetics should be performed for patients with CLL. FISH can identify chromosomal abnormalities which may have prognostic and treatment implications. For example, deletions of chromosome 17p or 11q are associated with an adverse clinical outcome, while deletions of 13q, suggest a more favorable clinical course.[2]

How does it impact treatment?

Although *IGHV* mutation status is a powerful prognostic tool, the decision to initiate therapy continues to be based on the 2008 IWCLL guidelines, in which only patients with advanced or symptomatic disease should be treated. However, *IGHV* status can facilitate counseling and appropriate follow-up intervals. *IGHV* status may also influence what type of therapy is started in the frontline setting, as CLL patients with *IGHV* mutated disease can have highly durable responses to CIT. In contrast, patients with unmutated *IGHV* should be considered for novel targeted therapies, which appear to be equally efficacious irrespective of *IGHV* status.

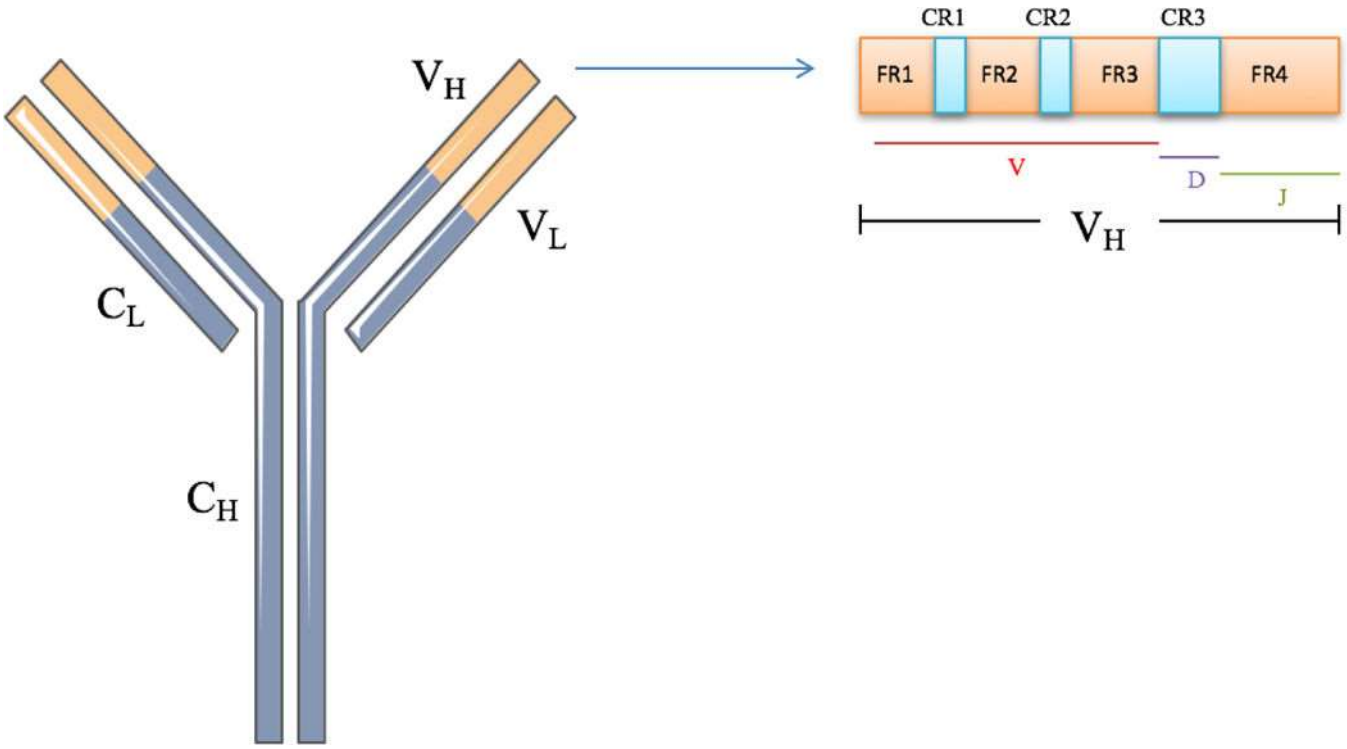

Figure 1.

Schematic of an immunoglobulin molecule. The variable regions for the heavy (V_H) and light (V_L) are depicted in orange. The constant regions of the heavy (C_H) and light chain (C_L) are depicted in purple. Within the V_H region, consisting of V, D, and J segments, are the framework regions (FR1-4) and the complementarity-determining regions (CDR1-3).