

EDITORIAL

Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: updated ERIC recommendations

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IMMUNOGENETIC ANALYSIS IN CLL: BIOLOGICAL AND CLINICAL RELEVANCE

Pivotal studies from the 1990s offered the first pieces of evidence for the role of immunoglobulin (IG) receptors and antigen stimulation in the natural history of chronic lymphocytic leukemia (CLL). These studies revealed the pronounced skewing of the IG gene repertoire in CLL, strongly implying a role for antigen selection in disease development.^{1–4} In addition, they showed that the presence and load of somatic hypermutation (SHM) within the rearranged IG heavy variable (IGHV) genes of the clonotypic B-cell receptor (BcR) IG dichotomizes CLL into two broad categories: the first includes cases with no or limited SHM ('unmutated' CLL, U-CLL), while the second includes cases with a significant SHM load ('mutated' CLL, M-CLL).^{1,3} Of note, within the clinical arena, IGHV mutation burden enabled the accurate prediction of the clinical course of the disease based on the level of SHM within the expressed IGHV genes.^{5,6} It has subsequently been shown that U-CLL is markedly different from M-CLL not only regarding the clinical course, but also in terms of biological features: the former is associated with adverse prognostic genomic aberrations, increased BcR signaling capacity, shorter time to progression and an overall inferior outcome compared to M-CLL.⁷

In hindsight, these studies represent true landmarks in the understanding of CLL, forming the cornerstone for an immunologically oriented view of CLL ontogeny and evolution. Indeed, rather than focusing only on genomic lesions, as is the norm for most cancers including almost all other lymphoid malignancies, these studies highlighted the critical role of the BcR IG, thus opening new directions for research into CLL-microenvironment interactions that have led to a more comprehensive appreciation of the biological heterogeneity of CLL.^{8–10}

An important milestone in the timeline of CLL-focused immunogenetic research was the observation that one-third of CLL patients carry (quasi)identical, also referred to as stereotyped BcR IGs, and can be classified into subsets defined by distinctive sequence motifs within the IG variable heavy complementarity-determining region 3 (VH CDR3).^{11–13} Mounting evidence suggests that patients assigned to the same subset have similar biological features (for example, signaling capacity^{14,15} and genomic aberration profiles^{16–19}) and clinical course, often differing significantly from other patients within the same SHM category.^{20–22} On these grounds, it has been proposed that patient classification based on BcR IG stereotypy can supersede the more generic categorization into U-CLL versus M-CLL, at least for major subsets. For instance, stereotyped subset #2 (IGHV3-21/IGLV3-21) has emerged as a prototype of aggressive disease independently of the SHM load.²³

Additional evidence for the importance of immunogenetic analysis in CLL was provided by three recent independent studies demonstrating that the SHM status of the IGHV genes was

associated with a particular clinical response to chemoimmunotherapy regimens in both clinical trials and general practice, such as the FCR (fludarabine/cyclophosphamide/rituximab) combination. More specifically, fit M-CLL patients treated with FCR (especially those lacking del(17p) and/or del(11q)) experienced more prolonged responses, delayed progression and a significant improvement in overall survival compared to U-CLL patients who received the same treatment.^{24–26} Determining the SHM level is therefore important, not only for general assessment of the disease course in CLL, but also for guiding treatment decisions: put simply, it is not only a prognostic test, but also a predictive test for the use of certain therapies, such as FCR. This will likely have important implications in the near future due to the emergence of novel treatments and will impact the clinical management of patients with CLL.

SETTING STANDARDS FOR IMMUNOGENETIC ANALYSIS IN CLL: PAST AND ONGOING ERIC INITIATIVES

ERIC, the European Research Initiative on CLL (www.ericll.org) has been at the forefront of immunogenetic research in CLL. The first ERIC recommendations for the determination of IGHV gene mutational status in CLL were published in 2007.²⁷ They were subsequently complemented by instructions detailing how to handle analytically challenging cases or cases difficult to categorize.²⁸ In addition, since 2007, ERIC provides the community with an expert online forum (www.ericll.org/services/) to discuss general queries on IG gene sequence interpretation in CLL, or to analyze and provide advice about actual IG gene rearrangement sequences that can be difficult to interpret in everyday practice. More recently, ERIC participated in the development of the ARResT/AssignSubsets bioinformatics tool, which is freely available online, and enables one to robustly and easily determine whether a particular IGHV-IGHD-IGHJ gene rearrangement belongs to one of the so-called 'major' (that is, largest) CLL stereotyped subsets.²⁹

To improve the quality of IG gene sequence analysis in CLL within the international research community, ERIC has organized educational workshops on the topic (2009–2016: Uppsala, Paris, Thessaloniki, Stresa, Brno, Uppsala). Furthermore, it has established the IG Network with the main objective of harmonizing IG gene sequence analysis in CLL to ensure that results are reliable and comparable among different laboratories, hence promoting optimal patient care (www.ericll.org/pages/networks/ignetwork). In this context, very recently, ERIC also started periodic (biannual) rounds of certification of IG gene sequence analysis in CLL to disseminate expertise in reliable IG sequence analysis (www.ericll.org/pages/ighv-certification). Thirty-seven diagnostic laboratories from 17 different countries in Europe and Australia have already participated in the first certification round and passed this test.

UPDATE OF THE ERIC RECOMMENDATIONS FOR IMMUNOGENETIC ANALYSIS IN CLL

Capitalizing on many years of active involvement in this field combined with the experience gained from the activities mentioned above, we recently critically discussed all aspects of

Table 1. Technical considerations for determination of the IGHV somatic hypermutation status of clonotypic IGHV-IGHD-IGHJ gene rearrangements in CLL

Item	Recommendations	Remarks
<i>Material</i>		
Anticoagulants	EDTA (or CPT)	
Cells/tissue	Blood, bone marrow, tissue biopsy	Purification of B cells usually not necessary unless low fraction of leukemic cells
Nucleic acid	gDNA or cDNA	cDNA useful when mutations within the IGHJ gene impair amplification
<i>Production of template for sequencing</i>		
Primers	5': leader 3': IGHJ or IGHC	VH FR1, VH FR2 and VH FR3 primers are not acceptable IGHC primers (on cDNA) useful when mutations within IGHJ gene impair amplification
Amplification	Multiplex PCR	individual PCR reactions (for each 5' primer) may be useful when more than one rearrangement found
Detection of IGH rearrangement	GeneScan or PAGE electrophoresis	Agarose gel electrophoresis strongly discouraged (lack of resolution)
Cloning	Not necessary	Except in rare circumstances (more than one rearrangement not isolated by simplex PCR)
<i>Sequencing</i>		
Methodology	Direct, both strands	Both strands mandatory for high-quality sequence
Sequence alignment	IMGT/V-QUEST (www.imgt.org)	Adjustable parameters: (1) search for insertions/deletions; (2) number of accepted <i>D</i> genes
IGHV identity (%)	Automatic or adjusted	Adjusted: use option 'search for insertions/deletions' when low % identity
Stereotypic subset identification	ARResT/AssignSubsets (bat.infospire.org/arrest/ericll.org/pages/services/tool)	Applicable for the current 19 major BcR stereotyped subsets in CLL ^a

Abbreviations: BcR, B-cell receptor; cDNA, complementary DNA; CLL, chronic lymphocytic leukemia; CPT, citrate/pyridoxal 5'-phosphate/Tris; EDTA, ethylenediaminetetraacetic acid; gDNA, genomic DNA; PAGE, polyacrylamide gel electrophoresis. ^aAgathangelidis and colleagues.⁷

Table 2. Reporting IGHV gene somatic hypermutation status in CLL

Item	Recommendations
<i>Standard cases</i>	
Methodology	Report type of: primers, ^a PCR product analysis, sequencing method, bioinformatics tools
Gene identification	IGHV, IGHD, IGHJ genes and alleles; IGHD may be difficult to precisely identify (due to deletions and/or SHM)
Productive rearrangement	Mutational status determined only for productive rearrangements; if unproductive, mention reasons (out-of-frame junction, stop codon)
IGHV gene: % of nucleotide identity to germ line	Classification: U-CLL ≥ 98%; M-CLL < 98%; borderline CLL when 97–97.9%
Subset identification	For subsets with well-established prognostic value (subsets #1, #2, #4 and #8)
<i>Difficult cases (frequency^b)</i>	
Double rearrangements (10.5%)	
Productive+non-productive concordant status (7.8%)	Same as standard cases (mutational status defined by the productive rearrangement)
Productive+non-productive discordant status	
Productive U+non-productive M (0.4%)	Mutational status not determined
Productive M+non-productive U (0.2%)	Consider as M-CLL
Double productive	
Concordant status (1.3%)	Same as standard cases
Discordant status (0.7%)	Mutational status not determined
Multiple (more than two) productive rearrangements ^c	Mutational status not determined (unless it can be performed on sorted B-cell clones and predominant clones are easily identified)
Single unproductive rearrangement (0.6%)	Mutational status not determined (after failure of alternative PCR attempts)
Missing anchors (C104/W118) (0.4%)	Mutational status possible if evidence for IG expression on leukemic cells and/or preserved G-X-G motif in VH FR4

Abbreviations: CLL, chronic lymphocytic leukemia; IG, immunoglobulin; M-CLL, mutated CLL; U-CLL, unmutated CLL. ^aLeader primers are the only recommended option. That said, in rare cases when leader primers are unsuccessful at providing a product that can be sequenced and VH FR1 primers are used (discouraged for the determination of SHM status), the report should indicate that the use of VH FR1 primers might underestimate the total number of IGHV somatic hypermutations as a part of the VH domain is missing. ^bAll frequencies according to Langerak *et al.*²⁸ ^cCases with two or more B-cell clones.³²

immunogenetics for diagnostic purposes in CLL, bearing in mind new knowledge available, and reached a consensus on the essential requirements necessary for reliable and reproducible IG gene analysis and interpretation of CLL-specific IGHV-IGHD-IGHJ gene rearrangements. The updated ERIC recommendations are listed in Tables 1 and 2. In the sections that follow, we will focus on the most critical revisions and additions to the original recommendations from 2007.²⁷

Primers

Only the use of leader primers allows for the amplification of the entire sequence of the rearranged IGHV gene, thus enabling the true and complete level of SHM to be determined, and is the recommended choice. The use of primers annealing to the VH FR1 or even more downstream parts of the VH domain (for example, the VH FR2 or VH FR3) is not acceptable for determining the SHM status: in these cases, an insufficient portion of the IG gene rearrangement will be amplified, thus potentially obscuring or preventing the true level of SHM from being determined since the total number of IGHV SHMs may be underestimated. That said, in rare cases when leader primers are unsuccessful at providing a product that can be sequenced and VH FR1 primers are used (not generally recommended), the report should indicate that the use of VH FR1 primers might underestimate the total number of IGHV SHMs.

Sequence alignment and annotation

1. Alignment tool and database. The user sequence is analyzed using IMGTV-QUEST, the analysis tool for rearranged nucleotide sequences of IMGT, the international ImMunoGeneTics information system (www.imgt.org). IMGTV-QUEST (up to 50 per batch online) provides the identification of the closest germ line V and J genes and alleles, and comprises the results of the integrated IMGT/JunctionAnalysis for the detailed analysis of the junction and the identification of the D genes and alleles for IGH gene rearrangements.
2. Nomenclature. The IMGTV-QUEST reference directory against which the user sequences are analyzed includes the sequences of the germ line IMGT V, D and J genes and alleles, approved by the Human Gene Nomenclature Committee, endorsed by NCBI and at the origin of Immunoinformatics (journal.frontiersin.org/article/10.3389/fimmu.2014.00022/full).

IMGTV-QUEST terms and standards for CLL sequence analysis interpretation are detailed in Supplementary Material.

Reporting the results of immunogenetic analysis in CLL

As for every diagnostic test, rigorous standards and meticulous attention to details are of utmost importance. Therefore, every laboratory report should:

1. List the identified IGHV, IGHD and IGHJ genes and alleles. Occasionally, reliable IGHD gene assignment is difficult or impossible, especially in rearrangements with short VH CDR3 sequences and/or due to multiple SHM; this should be clearly stated in the report ('IGHD gene and allele assignment: not possible'), but without raising any concern or flagging as an important detail.
2. State whether the particular IGHV-IGHD-IGHJ gene rearrangement is productive or unproductive. Essential criteria for considering a rearrangement as productive include the utilization of a functional IGHV gene and an in-frame VDJ junction bearing the anchors of the VH CDR3, namely cysteine C104 (2nd-CYS) and tryptophan W118 (J-TRP) (the latter part of the W-G-X-G motif of the J-REGION in VH FR4).

For unproductive rearrangements, the underlying reason (for example, out-of-frame junction or stop codon) should be mentioned. Before reporting an unproductive rearrangement as a definitive result in clinical routine, one should take particular attention to carefully review the flow cytometry result for the presence of surface immunoglobulin, as well as the sequence at the nucleotide level and the chromatogram, and make additional attempts on either the same or different starting material using the same or different primer sets to exclude technical flaws during the initial amplification, sequencing or immunoinformatic analytical steps.

3. Specify the percentage of identity of the identified rearranged IGHV gene and allele to its closest germ line counterpart.
4. State whether the identified productive IG gene rearrangement leads to membership of a major stereotyped subset. Considering ever-increasing evidence about the clinical implications of BcR IG stereotypy, at least for certain major stereotyped subsets,^{10–12} the IG Network advises everyone to analyze the clonal IGHV-IGHD-IGHJ gene rearrangement for possible membership in aggressive (major) subsets. Particular attention should be given to the identification of subset #2 (IGHV3-21/IGL3-21) as most cases belonging to this subset would be otherwise reported as M-CLL, given the presence of a moderate level of SHM, usually leading to an identity to the germ line below the 98% cutoff. It is now well established that patients belonging to subset #2 have an inferior outcome, similar to U-CLL patients and patients with *TP53* aberrations.¹² Membership in other major stereotyped subsets can also be explicitly reported, namely the aggressive #1 (IGHV clan I genes/IGKV1 (D)-39) and #8 (IGHV4-39/IGKV1(D)-39), the latter also associated with the highest risk for Richter's transformation among all CLL, as well as subset #4 (IGHV4-34) that includes a subgroup of patients with a particularly indolent form of CLL and rarely in need of treatment, which might be relevant for both patients and physicians alike.¹⁰ Membership to any of these subsets can be investigated using the ARResT/Assign-Subsets tool, whereby users submit their FASTA sequences and a report is generated with IMGTV-QUEST results and subset assignment along with an assignment confidence level ranging from 'borderline' to 'extreme'. Users are urged to use caution and their best judgement, or ask for advice, especially for clinical use of, for example, 'borderline'- or 'low'-confidence assignments. The tool together with comprehensive background and instructions are available at <http://tools.bat.infospire.org/arrest/assignsubsets/>.
5. Provide a concise overall assessment of the clinical implications of the immunogenetic results; several examples and templates are provided as Supplementary Material. The following issues merit special consideration:
 - i. Cases with a single unproductive rearrangement, apparent double rearrangements or lacking VH CDR3 anchors (overall frequency: ~3% of cases).²⁸ In these instances, the report should include a relevant statement, depending on the particular type of 'issue', following the published ERIC recommendations for reliable interpretation of problematic cases^{28,30} and/or by consulting the online ERIC Review Board (www.ericll.org/services/).
 - ii. Cases with IGHV gene rearrangements with 'borderline' identity to the closest germ line counterpart. Classification into the M-CLL or U-CLL category should always be based on the established 98% cutoff value for identity to the germ line (M-CLL: < 98%; U-CLL: 98–100%). That notwithstanding, for cases close to the cutoff (for example, 97–97.99%), caution is warranted regarding the precise prognostic implications³¹ and a statement to this effect should be included in the report (for example, 'Following

the 98% cutoff value for discriminating between IG-mutated or IG-unmutated CLL cases, the SHM status of this particular rearranged IGHV gene is borderline. The clinical implications remain to be elucidated¹).

CONCLUDING REMARKS

The BcR IG sequence is the ideal clonal marker for CLL: it is present from the birth of every CLL clone and in contrast to other markers, most notably genomic aberrations, remains stable over time and unaffected by disease evolution. Recent evidence has reinforced the important prognostic/predictive value of immunogenetic analysis in CLL, while also highlighting the need to move from the mere determination of IGHV gene mutational status to a more comprehensive assessment of the IGHV-IGHD-IGHJ gene rearrangement including stereotyped subset assignment, at least in for certain cases.

ERIC has pioneered the adoption of good practices and will continue to strive to ensure reliable immunogenetic analysis in CLL for the benefit of patients. That said, this topic is far from being exhausted, especially due to the advent of novel analytical approaches, that is, high-throughput sequencing, necessitating further efforts for standardization, currently an unmet need and the focus of an ongoing initiative within ERIC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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