

Chronic lymphocytic leukemia: recommendations for next-generation sequencing

Leucemia linfática crónica: recomendaciones para el estudio de secuenciación masiva

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Abstract

Introduction: The integration of laboratory and clinical technologies and knowledge is key to an accurate diagnosis of hematologic neoplasms. Over the past decade, next-generation sequencing (NGS) has enabled the molecular characterization of these diseases, identifying mutations, copy number variations, and recurrent fusion genes. This advancement has driven the update of recommendations for the diagnosis and management of patients with hematologic neoplasms. Recent classifications by the World Health Organization (WHO), the International Consensus Classification (ICC), and the European LeukemiaNet (ELN) highlight the relevance of genetic data, incorporating them into their diagnostic and risk stratification algorithms. In this context, the implementation of targeted NGS panels is recommended for their ability to simultaneously analyze multiple genetic alterations. **Objective:** Although NGS is available in many laboratories, its use is not yet standardized, and there is no clear consensus on the methodology or the genes to be evaluated for each pathology. **Material and method:** The Molecular Biology Group in Hematology (GBMH), in collaboration with a panel of experts, developed this consensus guide. **Results:** Genes of interest were compiled based on their clinical relevance and the scientific evidence. **Conclusions:** This guide aims to assist professionals in the use of targeted NGS panels in chronic lymphocytic leukemia.

Keywords: Chronic lymphocytic leukemia. Next generation sequencing. Consensus guidelines.

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Resumen

Introducción: La integración de tecnologías y conocimientos del laboratorio y la clínica es clave para un diagnóstico preciso de las neoplasias hematológicas. En la última década, la secuenciación masiva (NGS, next-generation sequencing) ha permitido caracterizar el perfil molecular de estas enfermedades, identificando mutaciones, variaciones en el número de copias y genes de fusión recurrentes. Este avance ha impulsado la actualización de las recomendaciones para el diagnóstico y el manejo de los pacientes con neoplasias hematológicas. Las clasificaciones recientes de la Organización Mundial de la Salud (OMS), la International Consensus Classification (ICC) y la European LeukemiaNet (ELN) destacan la relevancia de los datos genéticos, incorporándolos en sus algoritmos diagnósticos y de estratificación del riesgo. En este contexto, la implementación de paneles dirigidos de NGS se recomienda por su capacidad para analizar múltiples alteraciones genéticas.

Objetivo: Aunque la NGS está disponible en muchos laboratorios, su uso no está estandarizado y no hay consenso sobre la metodología ni los genes a evaluar en cada patología. **Material y método:** El Grupo de Biología Molecular en Hematología (GBMH), en colaboración con un panel de expertos, elaboró esta guía consensuada. **Resultados:** Se recogen los genes de interés según su relevancia clínica y evidencia científica. **Conclusiones:** Esta guía busca orientar a los profesionales sobre el uso de paneles dirigidos de NGS en la leucemia linfática crónica.

Palabras clave: Leucemia linfática crónica. Secuenciación masiva. Guías de consenso.

Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative neoplasm characterized by the clonal expansion of mature B cells in peripheral blood, secondary lymphoid tissues, and bone marrow.¹ CLL is the most common type of leukemia in adults in Western countries, with an incidence of 4-5 cases per 100,000 inhabitants per year, increasing to more than 30 in individuals older than 80 years.^{2,3} It is a disease with a heterogeneous clinical course; while some patients require rapid treatment, in others the disease remains indolent.

Over the past 10 years, major advances have been made in understanding the biology of CLL. Comprehensive studies using next-generation techniques, such as next-generation sequencing (NGS), have investigated the genomic, epigenomic, and transcriptomic features of the disease and have enabled identification of the most common somatic genetic alterations associated with disease pathogenesis.⁴⁻⁷ Thus, the genetic alterations that shaped the classic prognostic models in CLL – del(13q), del(11q)/*ATM*, del(17p)/*TP53*, and trisomy 12 – have rapidly expanded to include more than 60 genes and more than 12 recurrent structural variants with the potential to optimize risk stratification and develop new treatment algorithms for the clinical management of patients.

In particular, progress has been made in the study of molecular alterations associated with chemoresistance and progression to highly aggressive forms. However, knowledge regarding the clinical impact of most of these alterations remains limited, and current recommendations include, before initiating treatment, testing peripheral blood for del(17p), del(13q), del(11q), and

trisomy 12 using fluorescence in situ hybridization techniques, as well as analysis of the immunoglobulin heavy chain variable region gene (IGHV) and the *TP53* gene by sequencing techniques.^{3,8}

Relevant methodological aspects in chronic lymphocytic leukemia

Traditionally, assessment of IGHV mutational status or somatic hypermutation in CLL has been performed by Sanger sequencing, which provides a relatively simple, affordable, precise, and reliable result in most patients. Although this is the most standardized methodology, it has some limitations, as it may fail or yield results that are difficult to interpret.⁹ In this context, with the incorporation of NGS into laboratories, there is growing interest in changing methodology, and indeed, the new European Research Initiative on CLL (ERIC) guidelines include NGS in their recommendations.¹⁰ NGS has the potential not only to overcome some of the analytical limitations of Sanger sequencing but also to expand knowledge regarding the clinical and biological relevance of immunoglobulins in CLL, as it provides information on the subclonal and intratumoral architecture of the disease. However, interpretation of results obtained by NGS is complex and requires specialized bioinformatics tools, which currently limits standardization. Recently, the IgCaller algorithm has been developed, allowing reconstruction and characterization of IGHV rearrangements sequenced by NGS.¹¹ This algorithm can analyze results obtained from whole-genome sequencing and has already been incorporated into some commercial NGS panels for CLL that include the IGHV region.

Table 1. Genes to be included in next-generation sequencing (NGS) panels in chronic lymphocytic leukemia

Gene	Reference sequence	Regions to be studied	Clinical value			
			Diagnosis	Prognosis	Treatment	Hereditary predisposition
Mandatory genes <i>TP53</i> <i>IGH</i>	NM_000546.5	Complete coding region VDJ region		X X	X X	X
Genes associated with treatment resistance <i>BCL2</i> <i>BTK</i> <i>PLCG2</i>	NM_000633.2 NM_000061.2 NM_002661.5	Complete coding region Exons 14-16 Exons 17-30			X X X	
Recommended genes <i>ATM</i> <i>BIRC3</i> <i>IGL</i> <i>NOTCH1</i> <i>SF3B1</i>	NM_000051.4 NM_001165.5+ NM_017617.5 NM_012433.3	Complete coding region Complete coding region IGLV3-21-IGLJ region Exon 34 and 3' UTR region Exons 14-16, 18		X X X X X		
Optional genes <i>BRAF</i> <i>CHD2</i> <i>DDX3X</i> <i>EGR2</i> <i>FBXW7</i> <i>IKZF3</i> <i>KRAS</i> <i>MED12</i> <i>MYD88</i> <i>NFKBIE</i> <i>NRAS</i> <i>POT1</i> <i>RPS15</i> <i>SAMHD1</i> <i>SETD2</i> <i>SPEN</i> <i>XPO1</i>	NM_004333.6 NM_001271.4 NM_001356.5 NM_000399.5 NM_033632.3 NM_001257408 NM_004985.5 NM_005120.3 NM_002468.5 NM_004556.3 NM_002524.5 NM_015450.3 NM_001018.4 NM_015474.3 NM_014159.7 NM_015001.3 NM_003400.4	Exons 11, 15 Complete coding region Complete coding region Complete coding region Complete coding region Exon 4 Exons 2, 3 Complete coding region Complete coding region Complete coding region Exons 2, 3 Complete coding region Complete coding region Complete coding region Complete coding region Exon 11 Exons 15, 16		X X X X X X X X X X X X X X X X X		

Regarding *TP53*, the currently recommended techniques for mutation analysis are both Sanger sequencing and NGS, and testing should be performed before each line of treatment,¹² since, unlike IGHV, *TP53* mutational status is unstable and subject to disease dynamics. Of note, the sensitivity of Sanger sequencing is approximately 10-20%, which does not allow detection of subclonal alterations. NGS can achieve greater sensitivity, detecting alterations between 1% and 5%, depending on the technology used. Detection by NGS of low-frequency *TP53* mutations has been shown to have clinical impact in patients with CLL.¹³⁻¹⁵ Similarly, the clonal evolution dynamics of mutations in other recurrent CLL genes, such as *NOTCH1*, *SF3B1*, and *ATM*, may also have clinical implications.¹⁶

In this context, conventional approaches such as Sanger sequencing are being replaced by NGS techniques not only to increase sensitivity but also because

of their versatility in analyzing multiple genes simultaneously. In CLL, in addition to mutational analysis of IGHV and the entire coding region of *TP53*, analysis of other recurrently mutated genes is recommended. These genes of interest are detailed in **table 1** and have been agreed upon by CLL experts from the Molecular Biology in Hematology Group (GBMH) based on their clinical relevance and scientific evidence. Supplementary table 1 illustrates the frequency and relevance of all these genes in CLL.

The genes to be included in NGS panels for CLL have been classified into different categories according to the following criteria:

- Mandatory genes: those whose study is necessary for clinical decision-making according to current CLL treatment algorithms.^{3,8}
- Genes associated with treatment resistance: genes whose alterations constitute a proven mechanism of

resistance to targeted therapy; mutational testing is highly recommended in cases of suspected progression or lack of response during treatment with the corresponding inhibitors (see “Clinical utility of NGS panel studies in CLL”).

- Recommended genes: genes whose mutations have well-documented prognostic impact in the literature but have not yet been incorporated into routine clinical practice (see “Clinical utility of NGS panel studies in CLL”).
- Optional genes: genes with recurrent variants in CLL but lower levels of evidence regarding clinical impact. Given the low frequency of most of these alterations, their study is considered of interest for generating new knowledge and potentially confirming their clinical value (Supplementary Data).

Regarding copy-number variation analysis by NGS, the technology is not yet sufficiently standardized and does not currently replace conventional techniques such as fluorescence in situ hybridization, which remains the reference method for analyzing 13q, 11q, 17p, and chromosome 12 in CLL, although some commercial panels already include such analyses.

Clinical utility of NGS panel studies in chronic lymphocytic leukemia

Although only *TP53* and *IGHV* testing has been widely implemented in routine CLL clinical practice, other alterations with prognostic value have been described, notably those affecting *NOTCH1*, *SF3B1*, *ATM*, and *BIRC3*. Furthermore, with the advent of new therapies targeting BTK and BCL2 pathways (ibrutinib, acalabrutinib, zanubrutinib, venetoclax), mutations in *BTK*, *PLCG2*, and *BCL2* genes have been identified as predictive of resistance to these agents.

Clinical utility of *TP53* genetic alterations

Genetic alterations in *TP53* – del(17p) or mutations – result in loss of protein function and may appear at diagnosis or during disease evolution, often being selected after multiple lines of therapy. Patients with CLL and *TP53* alterations show poor response to standard chemoimmunotherapy and represent the most unfavorable prognostic factor in multivariate analyses, associated with clearly reduced progression-free survival (PFS) and overall survival (OS).¹⁷⁻¹⁹ New targeted therapies with BTK or BCL2 inhibitors have represented a major advance in the treatment of patients with *TP53* alterations and are now the treatment of choice (Spanish

CLL Group Guidelines). Currently, these high-risk patients (both in first-line and in relapsed/refractory settings) achieve favorable responses and longer survival times.²⁰⁻²³ In conclusion, *TP53* alterations represent both a prognostic and predictive factor and must always be analyzed before initiating any line of treatment.³

Clinical utility of *IGH* variable region mutational status

Current recommendations include determining *IGH* mutational status before initiating first-line treatment, as its value does not change during the disease course.^{3,8} *IGH* mutational status allows stratification of patients with CLL into 2 main groups: unmutated patients (*IGHV*-UM), with $\geq 98\%$ identity to the germline sequence, and mutated patients (*IGHV*-M), with $< 98\%$ identity. *IGH* status has prognostic value, with *IGH*-UM patients exhibiting a shorter time to first treatment.

In cases of transformation of CLL into high-grade lymphoma (Richter syndrome), analysis of *IGH* rearrangement in the biopsy specimen is recommended to determine whether the lymphoma is clonally related. In addition, *IGHV* status also has predictive value for treatment response.

Currently, chemoimmunotherapy is no longer a preferred option in most clinical guidelines, even in patients with mutated *IGH*, in whom targeted therapies – such as the time-limited combination of venetoclax and obinutuzumab – are considered preferable. Regarding other first-line treatments approved and recommended as preferred options by the Spanish CLL Group (ibrutinib, acalabrutinib, zanubrutinib, venetoclax-obinutuzumab, and ibrutinib-venetoclax),²⁴ differences in response duration have been observed according to *IGH* mutational status, particularly with fixed-duration treatment regimens. Specifically, *IGHV*-UM patients treated with venetoclax-obinutuzumab or with the combination of ibrutinib-venetoclax (as demonstrated in the GLOW study) show shorter response duration vs those with *IGHV*-M.²⁴⁻²⁶

Up to one-third of patients with CLL present stereotyped, nearly identical *IGHV* sequences and can be classified into subsets, some of which have prognostic value independent of mutational status. Currently, the European ERIC group recommends reporting at least those subsets with demonstrated prognostic value, such as subset #2 (*IGHV*-UM and *IGHV*-M, very poor prognosis) and subset #8 (*IGHV*-UM, very poor prognosis, high risk of transformation to Richter syndrome).¹⁰ Recently, mutation at codon R110 in the IGLV3-21 chain

has been described as identifying an aggressive CLL subgroup independent of IGHV mutational status, and all cases in subset #2 harbor this mutation.²⁷

Clinical utility of *BTK*, *PLCG2*, and *BCL2* mutations

Mutations in the *BTK*, *PLCG2*, and *BCL2* genes constitute mechanisms of resistance to the targeted agents ibrutinib, acalabrutinib, zanubrutinib, and venetoclax. For example, covalent *BTK* inhibitors such as ibrutinib, acalabrutinib, and zanubrutinib are susceptible to the C481S mutation, while alterations in *BTK* and *PLCG2* are recurrent in patients with CLL who progress after treatment with covalent *BTK* inhibitors such as ibrutinib and are not present in pretreatment samples.^{28,29} Furthermore, acquired *BTK* mutations distinct from those identified with ibrutinib have been described after treatment with noncovalent *BTK* inhibitors.³⁰ These include mutations affecting codons T474 and L528W, which confer resistance to pirtobrutinib. According to available evidence, in cases of suspected progression or nonresponse to ibrutinib, NGS analysis of exons 14-16 of *BTK* and exons 17-30 of *PLCG2* is recommended.

Similarly, *BCL2* alterations are particularly frequent in patients with CLL who progress after venetoclax treatment and are not present at diagnosis or before therapy initiation.^{31,32} In cases of suspected progression or nonresponse to venetoclax, complete sequencing of the coding region of the gene is recommended.

Clinical utility of other gene mutations: *ATM*, *BIRC3*, *NOTCH1*, and *SF3B1*

Other mutations have prognostic impact but have not yet been incorporated into routine clinical practice. The following genes have well-documented clinical relevance.

Of note, prognostic impact – particularly in terms of OS – has been established mainly in studies conducted in the context of chemoimmunotherapy. Their prognostic value in the era of targeted therapies is not yet fully defined and requires further validation.

ATM

The *ATM* gene is a target region of del(11q). Indeed, in 30-40% of patients with CLL, deletion is accompanied by mutation.³³ *ATM* mutations have been associated with shorter treatment-free survival and worse OS.^{33,34} Studies suggest that biallelic inactivation is the

alteration that truly impacts PFS and OS,³⁵ although conflicting reports exist,¹⁶ and larger series are needed to confirm these associations.

BIRC3

BIRC3 is a member of the inhibitor of apoptosis protein family (c-IAP2). Truncating mutations prevent degradation of *MAP3K14*, leading to noncanonical activation of the nuclear factor kappa B pathway. In CLL, *BIRC3* mutations are associated with IGHV-UM status, trisomy 12, and del(11q), the region where the gene is located.³⁶ Some studies have associated these mutations with shorter PFS and OS,^{36,37} although results are inconsistent.^{16,38,39} In addition, *BIRC3* mutations have been linked to chemotherapy refractoriness.³⁶⁻³⁸

NOTCH1

The most frequent mutation in the gene encoding the *NOTCH1* receptor (c.7541_7542delCT) affects the PEST domain, preventing proteasomal degradation and resulting in a truncated, constitutively active receptor. A similar effect is achieved by mutations in the 3' UTR domain, which occur less frequently.⁴⁰ *NOTCH1* alterations are associated with IGHV-UM status, trisomy 12, advanced stage, poorer survival, and increased risk of transformation to Richter syndrome.⁴¹⁻⁴³ Several studies have confirmed association with shorter time to first treatment and worse OS; however, its impact on PFS remains unclear.⁴³ Patients with mutated *NOTCH1* express lower CD20 levels and do not benefit from the addition of rituximab, although they may benefit from obinutuzumab in combination regimens.^{19,44} *NOTCH1* mutations have also been associated with measurable residual disease positivity in patients treated with venetoclax plus rituximab.^{45,46}

SF3B1

Mutations in the gene encoding the spliceosome component *SF3B1* affect a highly conserved region in the C-terminal domain, interfering with protein-RNA interaction. Several clinical studies have demonstrated the independent prognostic impact of *SF3B1* mutations on time to first treatment, PFS, and OS.^{43,47-50} An association with shorter time to first treatment has also been described in a cohort of Binet A patients with IGHV-UM.⁵¹

Prognostic indices

Currently, the most widely used prognostic index for classifying patients with CLL has been the International Prognostic Index for Chronic Lymphocytic Leukemia (CLL-IPI).⁵² This index predicts time to first treatment, PFS, and OS in patients treated with chemoimmunotherapy, with the 2 strongest prognostic factors being, in order, the presence of *TP53* alterations (deletions or mutations) and absence of IGHV mutations. However, most patients are currently treated with new BTK and BCL2 inhibitors. Some authors have developed predictive indices for patients receiving these agents, in which *TP53* alterations appear to retain their prognostic and predictive value, whereas the impact of IGHV mutational status remains inconclusive.⁵³

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Conflicts of interest

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Ethical considerations

Protection of human subjects and animals. The authors declare that no experiments on humans or animals were performed for this research.

Confidentiality, informed consent, and ethical approval. This study does not involve personal patient data, medical records, or biological samples, and does not require ethical approval. SAGER guidelines do not apply.

Declaration on the use of artificial intelligence. The authors declare that no generative artificial

intelligence was used in the writing or creation of the content of this manuscript.

Supplementary data

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