MINIREVIEW

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The clinical implications of gene mutations in chronic lymphocytic leukaemia

Davide Rossi*[,1] and Gianluca Gaidano[2]

[1]Department of Hematology, Oncology Institute of Southern Switzerland and Institute of Oncology Research, 6500 Bellinzona, Switzerland and [2]Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, 28100 Novara, Italy

Chronic lymphocytic leukaemia (CLL) is a molecularly heterogeneous disease as revealed by recent genomic studies. Among genetic lesions that are recurrent in CLL, few clinically validated prognostic markers, such as *TP53* mutations and 17p deletion, are available for the use in clinical practice to guide treatment decisions. Recently, several novel molecular markers have been identified in CLL. Though these mutations have not yet gained the qualification of predictive factors for treatment tailoring, they have shown to be promising to refine the prognostic stratification of patients. The introduction of targeted drugs is changing the genetics of CLL, and has disclosed the acquisition of previously unexpected drug resistant mutations in signalling pathway genes. Ultra-deep next generation sequencing has allowed to reach deep levels of resolution of the genetic portrait of CLL providing a precise definition of its subclonal genetic architecture. This approach has shown that small subclones harbouring drug resistant mutations anticipate the development of a chemorefractory phenotype. Here we review the recent advances in the definition of the genomic landscape of CLL and the ongoing research to characterise the clinical implications of old and new molecular lesions in the setting of both conventional chemo-immunotherapy and targeted drugs.

Chronic lymphocytic leukaemia (CLL) is an indolent B-cell malignancy characterised by the accumulation of mature CD5+/CD19+/CD23+ lymphocytes with weak surface expression of a monoclonal immunoglobulin (Müller-Hermelink *et al*, 2008). The clinical course of CLL ranges from a very indolent condition, with a nearly normal life expectancy, to rapidly progressive leading to early death. Asymptomatic patients are managed with watch and wait until the development of symptoms that result from cytopenias, adenopathy, or splenomegaly, as outlined by the 2008 International Workshop on CLL (Hallek *et al*, 2008).

An extensive array of effective options are available when treatment is required, though the disease still remains incurable. Combination chemo-immunotherapy with fludarabine, cyclophosphamide and rituximab (FCR) is the most effective regimen for young and fit patients, as it yields excellent long-term results. As CLL often affects elderly individuals, more tolerable therapeutic approaches have been successfully applied, such as chemo-immunotherapy combining chlorambucil with an anti-CD20 antibody (rituximab, obinutuzumab, ofatumumab), and bendamustine-based regimens (Ghia and Hallek, 2014). Most recently, therapies targeting the B-cell-receptor (BCR) signalling pathway, such as ibrutinib and idelalisib, have shown high-response rates and tolerability (Byrd *et al*, 2014).

CLL pathogenesis might be viewed as a cooperation between microenvironmental mechanisms and tumour genetics. The chronic lymphocytic leukaemia (CLL) genome carries ~2000 molecular lesions, including ~20 non-synonymous mutations and ~5 gross structural abnormalities Puente *et al*, 2015). At variance with other indolent B-cell lymphoproliferative disorders, where one predominant gene is molecularly altered in virtually all cases, CLL has a heterogeneous genetic profile and no unifying lesions have been so far identified. Recurrent balanced translocations, which commonly occur in many types of mature B-cell tumours, are extremely rare in CLL, being limited to the t(14;18) translocation involving the *BCL2* genes in ~2% of unselected cases (Cavazzini *et al*, 2008). Few molecular alterations recur at a frequency >5% in CLL, whereas a large number of biologically and clinically uncharacterised genes are mutated at lower frequencies (Landau *et al*, 2015; Puente *et al*, 2015). The most recurrent molecular lesions of CLL point to the deregulation of

*Correspondence: Dr D Rossi; E-mail: davide.rossi@eoc.ch

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cellular programs of clinical importance, namely: (i) apoptosis; (ii) DNA damage response; and (iii) cell signalling (Figure 1). Though the pathogenic implications of CLL genetic lesions has not been fully characterised in most instances, their usefulness as prognostic biomarkers (i.e. biomarkers that provide information on the likely outcome of CLL), or predictive biomarkers (i.e. biomarkers that provide information on the likely benefit from a specific CLL treatment) has been investigated to a certain extent.

APOPTOTIC CHECKPOINT DEREGULATION

Deletion 13q14 is the most frequent genetic lesion of CLL occurring in 50–60% of cases. The minimal deleted region on 13q14 contains the *miR15A* and mi*R16A* microRNAs (Calin *et al*, 2002). In normal cells, *miR15A* and *miR16A* inhibit the expression of multiple genes, including *BCL2*, the cyclins *CCND1* and *CCND3*, and cyclin-dependent kinase 6 (*CDK6*) (Klein *et al*, 2010). Deletion of *miR15A* and *miR16A* abrogates this inhibitory effect, favours the constitutive survival and cycling of tumour B-cells, and causes CLL in mouse models. In a relevant fraction (~25%) of CLL patients, deletion of 13q14 occurs in the absence of any concomitant driver genetic lesion. Patients harbouring solely 13q14 deletion have an excellent clinical outcome with a progression rate of <1% per year and an expected survival only slightly lower than that of the general population (Rossi *et al*, 2013). *BCL2* is one of the genes that are upregulated in CLL as a consequence of *miR15A*/mi*R16A* deletion. Consistent with the central contribution of *BCL2* activation in the pathogenesis of CLL, selective inhibition of BCL2 through the BH3 mimetic venetoclax results into high-response rates in relapsed or refractory patients, including those harbouring high-risk genetic abnormalities (Roberts *et al*, 2016).

DNA DAMAGE RESPONSE DEREGULATION

TP53 codes for a central regulator of the DNA-damage response pathway and, when functional, triggers CLL cell apoptosis in response to chemotherapy. *TP53* may be disrupted in CLL by deletions, mutations or a combination of both. Mutations represent the most frequent form of *TP53* inactivation in CLL and are frequently (~70% of the cases) accompanied by the loss of the second allele through 17p13 deletion (Rossi *et al*, 2009). The frequency of mutations lacking 17p13 deletion varies among different studies depending on patient cohort and the methodology used, but in general it represents ~30% of all *TP53* defects, whereas sole 17p13 deletion with the absence of *TP53* mutation are less frequent (~10% of all *TP53* defects). The high proportion of *TP53* mutations in the absence of 17p13 deletion may be in part attributed to the presence of two *TP53* mutations on individual alleles in CLL cells. In some patients, neutral loss of heterozygosity (uniparental disomy) of the *TP53* locus copy number was described, which results in duplication of the mutant allele. The direct clinical implication of these molecular observations is that, in order to perform a comprehensive evaluation of the *TP53* gene status in CLL, it is recommended to assess both the presence of chromosome 17p13 deletion by FISH, which is the more sensitive approach for deletion assessment, and of *TP53* mutations by gene sequencing (Pospisilova *et al*, 2012). At diagnosis, the incidence of *TP53* abnormalities has been reported to be 4–8%. As disease progresses, the incidence rises to 10–12% at the time of first line treatment, 40% in fludarabine-refractory CLL, and 50–60% in Richter syndrome.

The clinical importance of *TP53* abnormalities in CLL is tightly linked to the poor prognosis marked by this genetic lesion and its close association with chemorefractoriness, as documented by a number of observational studies and prospective trials led in both the chemotherapy and immunochemotherapy era. Among newly diagnosed CLL, patients harbouring *TP53* abnormalities have the worst outcome, with an estimated median overall survival (OS) of 3–5 years (~30% of cases are alive at 10 years, accounting for a ~70% reduction of the expected survival compared to the general population) (Rossi *et al*, 2013). However, in situations without treatment indication, *TP53* abnormalities should not be routinely tested, as they might turn a 'watch and wait' strategy into a 'watch and worry' situation for the patient without any immediate therapeutic consequences. Indeed, it is important to stress that

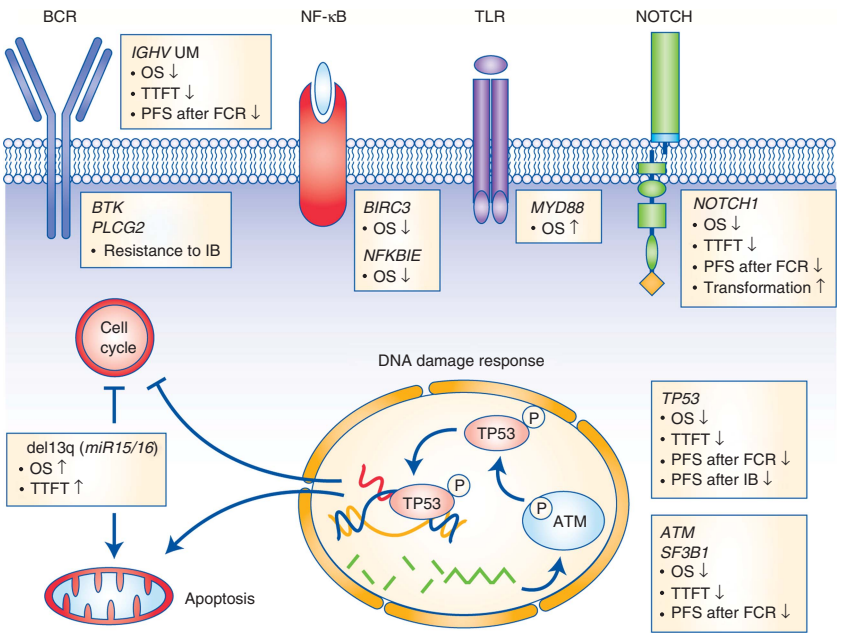

Figure 1. Significantly mutated genes and pathways in CLL. Cellular programs that are affected by the most recurrent molecular lesions are represented. Boxes show the genes that are recurrently mutated in each cellular programme and the clinical implications of gene mutations. Arrows indicate the positive or negative prognostic impact of the genetic lesions.

there is a small subgroup of patients with 17p13 deletion (and mostly mutated *IGHV* genes) who may exhibit stable disease for years without treatment indications.

The outcome of patients with *TP53* abnormalities and need for treatment is poor if treated with chemo/chemoimmuno-therapy, as they will very rarely achieve complete response. Such poor response to chemo +/− immunotherapy translates into an estimated progression free survival of 1 year or less and an OS in the range of 2–3 years from the time of front-line treatment (Zenz *et al*, 2010; Stilgenbauer *et al*, 2014). The Bruton tyrosine kinase (BTK) inhibitor ibrutinib as a single agent or combined to rituximab induces a response rate >80%, and a progression free survival >80% after 2 years of follow-up in untreated patients with *TP53* abnormalities (Farooqui *et al*, 2015). Similar proportions of response and progression free survival rates are observed with the PI3Kδ inhibitor idelalisib combined to rituximab (Furman *et al*, 2014). Therefore, it is recommended that patients with *TP53* deletion/mutation are treated with novel inhibitors (ibrutinib, idelalisib and rituximab) (Eichhorst *et al*, 2015). Although these results appear significantly better than every previous historical control in CLL with *TP53* abnormalities, ibrutinib and idelalisib-rituximab *per se* do not promise long-lasting remissions, especially in the setting of relapsed disease. Indeed, *TP53* defects predispose to the development of complex karyotype, which in turn marks genetic instability and favors the accumulation of resistance mutations. Consistently, complex karyotype is a powerful predictor of outcomes for ibrutinib-treated patients (Thompson *et al*, 2015). Therefore, allogeneic stem cell transplantation should still be offered and discussed in patients with CLL harbouring *TP53* defects, a complex karyotype, sufficient physical fitness and an available donor (Dreger *et al*, 2014).

Given their value as predictive biomarkers of resistance to chemo +/− immunotherapy, current guidelines recommend to test 17p13 deletion and *TP53* mutations in CLL patients requiring therapy (Hallek *et al*, 2008; Pospisilova *et al*, 2012; Eichhorst *et al*, 2015; Zelenetz *et al*, 2015). Because leukaemic clones may evolve, FISH for 17p13 deletion and *TP53* mutation analyses should be repeated at each disease progression requiring treatment. Sanger sequencing is the currently recommended approach for *TP53* mutation analysis. However, due to its limited sensitivity, conventional Sanger sequencing misclassifies as wild type those CLL cases harbouring *TP53* mutations of low-clonal abundance (<10% of the alleles). Such small *TP53* mutated subclones occur in a significant fraction of CLL, have the same unfavourable prognostic impact as clonal *TP53* defects, and anticipate the development of a chemorefractory phenotype among CLL patients requiring treatment (Landau *et al*, 2013; Rossi *et al*, 2014; Landau *et al*, 2015). Thanks to its high sensitivity (down to 1–0.1%), deep next generation sequencing is capable of detecting these minor, but clinically relevant, *TP53* mutated subclones. Therefore, deep next generation sequencing should be considered as a useful tool for a comprehensive assessment of *TP53* disruption in CLL.

The *ATM* gene encodes a nuclear serine/threonine kinase whose activity is induced by chromosomal double-strand breaks that arise endogenously or after exposure to DNA-damaging agents, including chemotherapeutic drugs. *ATM* protects the integrity of the genome by regulating the cell-cycle arrest at G1/S and G2/M to prevent processing of damaged DNA, and by activating the DNA-repair pathways or, alternatively, inducing apoptosis if the DNA damage cannot be repaired. As for *TP53*, the *ATM* gene is inactivated in CLL by both deletion and/or somatic mutations, which result in impaired DNA damage responses. Deletion of 11q22-23 always includes *ATM* and occurs in <10% newly diagnosed CLL, whereas its prevalence rises to ~20% at the time of first treatment. Deletion 11q22-23 co-occurs with *ATM* mutations in 30–40% of patients. *ATM* mutations generally consist in a mixture of missense substitutions distributed across the *ATM* coding sequence, with no clear hotspots, and have been observed in ~10–15% of newly diagnosed patients and in ~15% of progressive CLL requiring first treatment. By combining mutations and deletions, genetic lesions of *ATM* occur in ~20% of diagnostic samples of CLL and in ~35% cases requiring first treatment (Skowronska *et al*, 2012).

From a clinical perspective, the presence of *ATM* deletion at the time of CLL presentation identifies a group of patients with intermediate-risk disease (~40% of cases are alive at 10 years, accounting for a ~50% reduction of the expected survival compared to the general population) (Rossi *et al*, 2013). Among CLL requiring treatment, the presence of 11q22-23 deletion alone or combined to *ATM* mutations associates with poor response to chemotherapy. The addition of rituximab to chemotherapy significantly improved the outcome of CLL patients harbouring *ATM* lesions. However, even among CLL treated with FCR, 11q22-23 deletion still remains an adverse factor that identifies a group of patients with intermediate-risk disease that are projected to progress in a relatively short time (Stilgenbauer *et al*, 2014).

SF3B1 is a core component of the mRNA splicing machinery. *SF3B1* mutations occur with a prevalence that ranges from 7 to 10% of unselected CLL, are enriched in cases harbouring unmutated *IGHV* genes, and tend to co-occur with *ATM* deletion or mutation. *SF3B1* mutations in CLL are generally represented by missense nucleotide changes that recurrently target hotspots (codons 662, 666, 700, 704, 742), with a single amino-acid substitution (K700E) accounting for ~50% of all *SF3B1* mutations (Quesada *et al*, 2011; Rossi *et al*, 2011; Wang *et al*, 2011).

Though the role of *SF3B1* mutations in CLL pathogenesis still remain largely unclear, the recent notion that the spliceosome participates in the *ATM*-induced DNA damage response and the observation that *SF3B1* mutations are associated with a defective response to DNA damage, suggest that *SF3B1* mutations may have a similar role as *ATM* abnormalities in deregulating the DNA damage programme (Te Raa *et al*, 2015). Consistently, similar to *ATM* defects, also the presence of *SF3B1* mutations at the time of CLL presentation identifies a group of patients with intermediate-risk disease (40% of cases are alive at 10 years, accounting for a ~50% reduction of the expected survival compared to the general population) (Rossi *et al*, 2013). Among CLL requiring treatment, *SF3B1* mutations can potentially help refining prognostication of treatment relapse, though they do not represent a predictive biomarker for treatment tailoring. Indeed, the *SF3B1* status does not impact on the chance of achieving responses to chemo +/− immunotherapy, though patients harbouring *SF3B1* mutations show a shorter PFS than *SF3B1* wild type cases (Stilgenbauer *et al*, 2014).

SIGNALLING PATHWAY DEREGULATION

At variance with other B-cell tumours, genes encoding for components of the BCR signalling machinery are not usually targeted by somatic mutations in unselected CLL. The introduction of targeted drugs inhibiting the BCR signalling is changing the genetics of the disease, and has disclosed the acquisition of previously unexpected drug resistant mutations in the BCR pathway genes, including mutations affecting the *BTK* binding site of ibrutinib or gain-of-function mutations in *PLCG2* (Woyach *et al*, 2014). Ibrutinib resistant mutations of the BCR pathway are not detectable before ibrutinib exposure, thus indicating that they are biologically irrelevant in the absence of selective pressures imposed by the drug (Famà *et al*, 2014). From a clinical standpoint, *BTK* and *PLCG2* mutation analysis may be of help in the assessment of patients that progress under ibrutinib therapy.

The load of somatic hypermutation of the rearranged immunoglobulin heavy-variable genes (*IGHV*) genes, which is associated with antigen binding capacity and functions of the BCR, is one of the most important prognostic biomarkers in CLL. At the time of presentation, CLL cases carrying mutated *IGHV* genes (~60% of patients) experience an indolent course compared with patients carrying unmutated *IGHV* genes (Hamblin *et al*, 1999). At the time of treatment requirement, CLL patients harbouring mutated *IGHV* genes, but lacking both *TP53* and *ATM* defects (~30% of cases), have a high chance of achieving a durable remission after FCR (Rossi *et al*, 2015). Therefore, FCR is currently the best option in physically fit patients with progressive CLL whose disease has a low-risk molecular profile (mutated *IGHV* genes without *TP53* and *ATM* defects). Because the *IGHV* mutation status is a powerful biomarker for the prediction of duration of response after FCR, its assessment is recommended by the current guidelines in patients requiring therapy (Eichhorst *et al*, 2015).

The NOTCH receptor genes encode a family of heterodimeric transmembrane proteins (NOTCH1 to NOTCH4) that function as ligand-activated transcription factors. When the NOTCH receptors interact with their ligands through the extracellular subunit, two consecutive proteolytic cleavages of the NOTCH proteins are initiated and lead to pathway activation. Upon activation, the cleaved intracellular portion of the NOTCH receptors (ICN) translocates into the nucleus where it recruits a transcriptional complex that modifies the expression of a number of target genes, including *MYC* and NF-κB signalling components. The most prominent mechanism of NOTCH signal suppression is operated through its PEST domain of the ICN, which is recognised by the FBXW7 ubiquitin protein ligase and directed towards proteasomal degradation.

NOTCH1 mutations characterise ~15% of unselected CLL and are represented by frameshift or non-sense events clustering within exon 34, including the highly recurrent c.7544_7545delCT deletion (~80% of all mutations), as well as by non-coding mutations affecting the 3′ UTR region of *NOTCH1*, which cause aberrant splicing events resulting in a deletion of the last 158 coding bases of exon 34 (Puente *et al*, 2015). *NOTCH1* mutations in CLL are selected to disrupt the PEST domain of the protein, resulting in NOTCH1 impaired degradation, stabilization of the active ICN, and deregulated NOTCH signalling. *NOTCH1* is preferentially targeted in specific biological groups of CLL. In fact, *NOTCH1* mutations are significantly more common in CLL with unmutated *IGHV* genes, and are enriched in CLL harbouring +12 (Puente *et al*, 2015).

The clinical implication of *NOTCH1* mutations affecting exon 34 has been clarified to a certain extent. Conversely, though mutations in the 3′ UTR of the gene seem to behave similarly to exon 34 mutations, their role as prognostic biomarker needs further validation. At the time of CLL presentation, the presence of *NOTCH1* mutations in exon 34 identifies a group of patients with intermediate-risk disease (~40% of cases are alive at 10 years, accounting for a ~50% reduction of the expected survival compared to the general population) and those in whom CLL is more likely to transform into RS (cumulative incidence of transformation at 10 years of ~50%) (Rossi *et al*, 2012, 2013). Among CLL requiring treatment, cases harbouring *NOTCH1* mutations in exon 34 seem not to benefit from the addition of an anti-CD20 monoclonal antibody to chemotherapy. Indeed, among CLL harbouring *NOTCH1* mutations, treatment with FCR does not result into the expected increase in minimal residual disease response nor into an improvement in PFS or OS compared to treatment with the sole FC (Stilgenbauer *et al*, 2014). *NOTCH1* mutations in exon 34 associate with low CD20 levels in CLL and are responsible for a dysregulation of HDAC-mediated epigenetic repression of CD20 expression, which may explain the lower sensitivity to anti-CD20 treatment of CLL harbouring *NOTCH1* mutations (Pozzo *et al*, 2015).

In CLL, NF-κB signalling is generally upregulated through specific interactions between protective microenvironmental niches and CLL cells. At least in a fraction of cases, CLL gain active NF-κB signalling by mutating NF-κB genes. The non-canonical NF-κB pathway is engaged by CD40 and BAFF receptors. Upon receptor binding, the TRAF3/MAP3K14-TRAF2/BIRC3 negative regulatory complex of non-canonical NF-κB signalling is disrupted, allowing the cytoplasmic release and stabilization of MAP3K14, the central activating kinase of non-canonical NF-κB signalling. The stabilised MAP3K14 activates the IKKα kinase, which in turns directly phosphorylates NF-κB/p100, inducing partial proteolysis of p100 to p52 by the proteasome. The p52 protein dimerises with RelB to translocate into the nucleus, where it regulates gene transcription.

The Baculoviral IAP repeat containing 3 (*BIRC3*) gene, which cooperates in the TRAF3/MAP3K14-TRAF2/BIRC3 negative regulatory complex of non-canonical NF-κB signalling, is mutated in ~2% of unselected CLL (Rossi *et al*, 2013). At the biochemical level, *BIRC3* mutations cause the truncation of the C-terminal RING domain of the BIRC3 protein, whose E3 ubiquitin ligase activity is essential for switching off MAP3K14 through proteosomal degradation, thus leading to constitutive non-canonical NF-κB activation. From a clinical standpoint, *BIRC3* mutations identify a genetic subgroup of cases characterised by poor risk disease (Rossi *et al*, 2013).

NF-κB comprises a small family of transcription factors, including the NF-κB/Rel members RelA, RelB, c-Rel, NF-κB1, and NF-κB2. These proteins are kept inactive by cytoplasmic association with the IκB inhibitory proteins. The NF-κB inhibitor epsilon (NFKBIE) belongs to the IκB inhibitory protein family and counteracts NF-κB activation via cytoplasmic retention of the Rel proteins. The *NFKBIE* gene is affected by a recurrent 4 bp deletion in ~5% unselected CLL. *NFKBIE* mutation results in protein truncation, reduced inhibitory interaction with the Rel transcription factor, and enhanced NF-κB activation (Mansouri *et al*, 2015). Though the precise clinical implication of *NFKBIE* mutations remains to be clarified, their enrichment among CLL presenting in advanced stage suggests that they might be involved in disease progression.

In B-cells, Toll-like receptors are central to the BCR-independent response to antigens by sensing a variety of pathogen-associated molecular patterns derived from bacteria, viruses, and fungi. Adaptor proteins, including the myeloid differentiation factor 88 (MYD88), are essential for initiating Toll-like receptors signalling. MYD88 has a modular structure with a death domain (DD) at the N terminus, and a Toll-IL-1 receptor (TIR) domain at the C terminus. The TIR domain of MYD88 is crucial for signal transduction as it mediates contacts with the intracellular TIR domains of the TLRs upon signalling activation. The DD domain allows oligomerization of the active MYD88 and its interaction with the respective DD of the serine-threonine kinases IRAK1-4, thus resulting in a multimeric complex. This complex propagates the signal and leads to activation of a series of cascades and transcription factors, such as NF-κB, AP-1 and STAT3. Most *MYD88* mutations in CLL are represented by the L265P missense substitution, which affects the evolutionarily conserved beta-beta loop of the TIR domain of MYD88, suggesting that it has been selected to change the structure of MYD88 and to allow spontaneous homodimerization and recruitment of IRAK1 and IRAK4. Consistently, in B-cell tumours, mutant *MYD88* results in uncontrolled formation of the MYD88/IRAK complex, which translates into the recruitment of TRAF6, constitutive phosphorylation of TAK1 and, ultimately, the elevation of NF-κB activity and cytokine secretion. *MYD88* gene mutations occur in ~3% of unselected CLL, whereas they are enriched in a specific

clinical subgroup of patients characterised by young age at presentation, mutated *IGHV* genes and expected survival similar to that of the age–sex matched normal population (Martínez-Trillos *et al*, 2014).

CONCLUSION

The use of genetic prognostic markers is not recommended in patients that are not in need of treatment. In the era of personalised medicine, the challenges for the treatment of patients with CLL will involve correctly matching therapies to the unique genetic composition of each individual tumour. Predictive biomarkers that are recommended by the guidelines and that should be routinely applied in the clinical practice for treatment tailoring are: (i) *IGHV* mutations, that mark durable remission after FCR; and (ii) *TP53* defects, that mark resistance to imunochemotherapy and represent an indication to targeted agents (i.e. ibrutinib or idelalisib plus rituximab). *NOTCH1* mutation status, that associates with resistance to anti-CD20 monoclonal antibody, as well as complex karyotype, *BTK* and *PLCG2* mutations, that associate with resistance to ibrutinib, might represent novel predictive biomarkers. However, their value needs to be confirmed before they can be used in clinical routine.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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