Clinical implications of the molecular genetics of chronic lymphocytic leukemia

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ABSTRACT

Genetics and molecular genetics have contributed to clarify the biological bases of the clinical heterogeneity of chronic lymphocytic leukemia. In recent years, our knowledge of the molecular genetics of chronic lymphocytic leukemia has significantly broadened, offering potential new clinical implications. Mutations of *TP53* and *ATM* add prognostic information independently of fluorescence *in situ* hybridization cytogenetic stratification. In addition, next generation sequencing technologies have allowed previously unknown genomic alterations in chronic lymphocytic leukemia to be identified. Mutations of *NOTCH1*, *SF3B1* and *BIRC3* have been associated with short time to progression and survival. Each of these lesions recognizes a different distribution across different clinical phases and biological subgroups of the disease. The clinical implications of these molecular lesions are in some instances well established, such as in the case of patients with *TP53* disruption, who should be considered for alternative therapies/allogeneic stem cell transplant upfront, or in patients with *ATM* disruption, who are candidates to rituximab-based immunochemotherapy. On the contrary, *NOTCH1*, *SF3B1* and *BIRC3* mutations appear to have a specific significance, the clinical value of which is currently being validated, i.e. association to Richter syndrome transformation for *NOTCH1* mutations, and short progression-free survival after treatment for *SF3B1* mutations. Certainly, these new lesions have helped clarify the molecular bases of chronic lymphocytic leukemia aggressiveness beside *TP53* disruption. This review covers the recent advancements in our understanding of the molecular genetics of chronic lymphocytic leukemia and discusses how they are going to translate into clinical implications for patient management.

Genetic heterogeneity of chronic lymphocytic leukemia

The clinical course of chronic lymphocytic leukemia (CLL) is extremely heterogeneous. Accordingly, survival of patients with CLL ranges from less than 1-2 years to over 15 years.[1-15] The Rai and Binet clinical staging systems still remain the cornerstone for identifying CLL patients with advanced disease stages for whom treatment-free survival (TFS) and overall survival (OS) are usually short.[2,5,6] However, these staging systems do not provide risk stratification in early stage disease, that nowadays includes most cases of newly diagnosed CLL, and also fail to identify those patients who will develop chemorefractoriness.[11-15]

Understanding CLL genetics may help clarify the molecular bases of the clinical heterogeneity of this leukemia. In the 1990s, Juliusson *et al.*[16] applied conventional karyotype banding analysis to systematically assess the prevalence and prognostic impact of chromosomal abnormalities associated with CLL. Despite its technological limitations, this approach revealed that more than half of CLL patients had clonal chromosomal changes. Importantly, this pivotal analysis indicated that chromosomal abnormalities affect CLL outcome and served as a proof of concept to document that genetic alterations in CLL may be prognostically relevant in a hierarchical order.[16]

In 2000, this notion was unequivocally documented by the seminal study by Döhner *et al.*[17] that established interphase fluorescence *in situ* hybridization (FISH) analysis as a standard technique to evaluate cytogenetic lesions in CLL, detecting chromosomal abnormalities in over 80% of patients, thus overcoming the limited applicability and resolution of conventional karyotyping. By correlating FISH lesions with the course of the disease, a hierarchical model based on five risk categories was established. CLL cases harboring the 17p13 deletion independent of concomitant abnormalities (prevalence 7%) had the worst prognosis (median survival 32 months), followed by cases carrying the 11q22-q23 deletion (prevalence 18%, median survival 79 months), trisomy 12 (prevalence 16%, median survival 114 months), normal karyotype (prevalence 18%, median survival 111 months) and 13q14 deletion (prevalence 55%, median survival 133 months).[17]

Cytogenetic lesions, however, do not entirely explain the genetic basis of the clinical heterogeneity of CLL. Additional information has come from the detailed definition of the molecular correlates of CLL chromosomal aberrations. In fact, *TP53*, the tumor suppressor gene affected by 17p13 deletion, and *ATM*, the gene targeted by 11q22-q23 deletion, are not only deleted, but also recurrently mutated in CLL. Mutations of these genes, even in the absence of deletion, have a prognostic impact beside FISH cytogenetic stratification.[18-25]

In recent times, the improvements in next generation sequencing technologies have provided a novel opportunity to examine the CLL genome, and have allowed previously unknown genomic alterations to be identified, such as mutations of *NOTCH1* (neurogenic locus notch homolog protein 1), *SF3B1* (splicing factor 3B subunit 1) and *BIRC3* (baculoviral

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Figure 1. ***NOTCH1***, ***SF3B1***, ***BIRC3***, ***TP53*** **mutation type and distribution in CLL. Schematic representation of the human NOTCH1 (A), SF3B1 (B), BIRC3 (C), and TP53 (D) proteins, with their key functional domains. Color-coded symbols indicate the type and position of the mutations. Mutations are from the Novara CLL mutation database and from the COSMIC database (v. 61).**

IAP repeat-containing protein 3), that might translate into new biomarkers of potential clinical relevance.[26-33]

Pattern and distribution of genetic lesions affecting chronic lymphocytic leukemia outcome

Molecular characteristics of clinically relevant genetic lesions of chronic lymphocytic leukemia

Molecular defects of *TP53* and *ATM* are well-established genetic lesions carrying clinical relevance in CLL. The tumor suppressor gene *TP53* maps on the short arm of chromosome 17 (17p13) and codes for a central regulator of the DNA-damage-response pathway.[34] Activation of *TP53* leads to cell-cycle arrest, DNA repair, apoptosis, or senescence via both transcription-dependent and transcriptional-independent activities. Consistently, *TP53* plays a central role in mediating the pro-apoptotic and antiproliferative action of several DNA-damaging chemotherapeutic agents, including alkylators and purine analogs.[34]

In CLL, the *TP53* gene may be inactivated by deletion and/or somatic mutations.[17-21] Most cases with 17p13 deletion also carry *TP53* mutations on the second allele (~70%), while the remaining cases have a monoallelic 17p13 deletion in the absence of *TP53* mutations (~20%), or *TP53* mutations in the absence of 17p13 deletion (~10%).[35] In line with the genetic instability associated with defective DNA-damage checkpoints, *TP53* abnormalities frequently couple with complex cytogenetic abnormalities, particularly with unbalanced translocations.[21]

At the molecular level, approximately 75% of all mutations are missense substitutions, while the remaining lesions (~25%) are represented by truncating events, including frameshift insertions or deletions, non-sense substitutions and splice site mutations.[35,36] Most missense mutations are localized within exons 5-8, which encode the central DNA-binding domain of TP53, thus impairing DNA binding and target gene transactivation (Figure 1).[35]

The *ATM* gene is a member of the phosphatidylinositol-3 kinase (PT3K) gene family and 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 ionizing radiations and chemotherapeutic drugs.[37] *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 DNA-repair pathways and inducing apoptosis if the DNA damage cannot be repaired. Many of these effects are mediated by the activation of both TP53-dependent and TP53-independent cellular pathways.[37]

ATM is a large gene of 62 coding exons that maps on

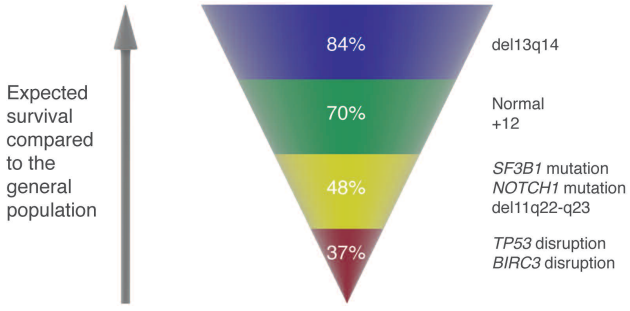

Figure 2. Expected survival of CLL patients stratified according to the integrated mutational and cytogenetic model and compared to the matched general population. Expected survival is calculated at ten years.

chromosome 11q22-q23, which is a minimally common deleted region in CLL. As for *TP53*, the *ATM* gene in CLL may be inactivated by both deletion and/or somatic mutations.[22,23] At the molecular level, *ATM* mutations consist in a mixture of missense substitutions distributed across the *ATM* coding sequence, with no hotspots.[22,23] Only a proportion of *ATM* mutated CLL (10%-20%) show a concomitant 11q22-q23 deletion. Similarly, only 20%-30% of CLL with 11q22-q23 deletion show *ATM* mutations, suggesting that the paradigm of tumor suppressor gene inactivation, i.e. deletion on one allele and mutation on the other allele, is not fully represented, or, alternatively, that additional tumor suppressor genes within the 11q22-q23 region are involved in CLL.[22-24,37-39]

Insights into the pathogenicity of *ATM* mutations in CLL are provided by the observation that two recurrent mutations (p.R2691C and p.P2699S) localize within functionally relevant sites, including the ATP-binding pocket of ATM, and critically impair ATM kinase activity.[25] *ATM* mutants with an impaired kinase activity sequester ATM wild-type proteins with a dominant negative effect and inhibit their activation in response to physical and chemical insults.[25]

Recent studies based on next generation sequencing have revealed new genes implicated in CLL and potentially carrying clinical relevance. *NOTCH1* encodes a transmembrane protein that acts as a ligand-activated transcription factor and regulates multiple target genes, including *MYC*, *TP53* and molecules of the NF-κB pathway.[40,41] In 2009, it was shown that the constitutive NOTCH signaling activation was implicated in CLL cell survival and apoptosis resistance. In an attempt to identify the underlying mechanism, the first identification of the NOTCH1 PEST domain mutation in CLL patients was reported.[42,43] At the molecular level, *NOTCH1* mutations in CLL are mainly represented by frameshift or non-sense events clustering within exon 34, and including the highly recurrent c.7544_7545delCT deletion (approx. 80%-95% of all mutations) (Figure 1).[26,27,31,32] *NOTCH1* mutations in CLL are selected to disrupt the C-terminal PEST domain of the protein, that is responsible for the proteosomal degradation of the activated form of NOTCH1. Indeed, truncation of the PEST domain is predicted to result in NOTCH1 impaired degradation, stabilization of the active NOTCH1, and deregulated NOTCH1 signaling.[44] Consistent with this notion, a number of cellular pathways, including those controlling cell metabolism and cell cycle progression, are deregulated in CLL harboring *NOTCH1* mutations. *NOTCH1* is preferentially targeted in specific biological groups of CLL.[27,45] In fact, *NOTCH1* mutations are significantly more common in CLL with unmutated immunoglobulin heavy variable (*IGHV*) genes and are enriched in CLL harboring +12, where they identify a distinct clinico-molecular subgroup characterized biologically by deregulated cell cycle and clinically by short survival.[26,27,31,32,46,47]

SF3B1 is a core component of the spliceosome, a complex of five small nuclear ribonucleoproteins involved in the splicing of precursor messenger RNA and in the formation of mature mRNA through the removal of introns in protein-encoding genes.[48-50] *SF3B1* mutations in CLL are almost always represented by missense substitutions affecting the HEAT domains of the SF3B1 protein and recurrently target five hotspots (codons 662, 666, 700, 704 and 742), with the K700E substitution accounting for approx. 40%-50% of all *SF3B1* mutations (Figure 1).[28-30] Among CLL genetic events, *SF3B1* lesions have shown a preferential, though not consistent, association with 11q22-q23 deletion and *ATM* mutations.[29,30] The functional consequences of *SF3B1* mutations in CLL are currently under scrutiny. The notion that *SF3B1* mutations in CLL are mainly missense substitutions targeting highly conserved regions of the protein suggests that they are selected to modify, rather than disrupt, SF3B1 functions.[51-53]

BIRC3 is a negative regulator of the MAP3K14 serin-treonine kinase, the pivotal activator of non-canonical NF-κB signaling.[54-57] *BIRC3* is also involved in maintaining wild-type TP53 levels by preventing NF-κB-mediated transcriptional and post-translational modifications of *MDM2* expression and function. Consistently, *BIRC3* knockdown contributes to cancer promotion through downregulation of the TP53 protein via MDM2.[58]

BIRC3 is recurrently disrupted in CLL by mutations, deletions or a combination of both.[33] *BIRC3* inactivating mutations are mainly represented by frameshift or non-sense substitutions causing the truncation of the C-terminal RING domain of the *BIRC3* protein (Figure 1), whose E3 ubiquitin ligase activity is required to prime MAP3K14 towards proteosomal degradation. On these bases, the functional consequence of *BIRC3* mutations in CLL is the constitutive activation of non-canonical NF-κB signaling, which is regarded as a mechanism of resistance to disease eradication in this leukemia.[33,58-63]

Though the sample size of the CLL cohorts so far investigated for these new genetic lesions is not powered to fully recapitulate all the correlations and anti-correlations between *NOTCH1*, *SF3B1* and *BIRC3* lesions with the other unfavorable genetic abnormalities, evidence from pivotal studies suggests that, at CLL diagnosis and first treatment, *NOTCH1* and *SF3B1* mutations tend to be mutually exclusive among themselves and with *TP53* abnormalities, thus representing alternative genetic mechanisms promoting high-risk disease.[26-30,64]

Molecular lesions at different clinical phases of chronic lymphocytic leukemia

CLL course may proceed through distinct clinical phases, ranging from a pre-malignant condition known as monoclonal B-cell lymphocytosis (MBL) to overt CLL, and even to transformation into an aggressive lymphoma known as Richter's syndrome (RS).[1,2]

Among the most recurrent genetic lesions, 13q14 dele-

tion and +12 are similarly represented in all the phases of the disease.[17,65-70] This observation is consistent with the notion that 13q14 deletion and +12 represent first step genetic abnormalities in CLL. All the other genetic events accumulate in the more advanced phases of the disease, suggesting that they represent second hit lesions that are progressively selected or acquired during the evolution of the clone.

As in other pre-malignant conditions, also MBL frequently harbor some of the genetic lesions that can be observed in the overt phases of the disease. Among these, 13q14 deletion is detected in MBL with a prevalence (~40%-50% of cases) that is similar to that observed in overt CLL.[65-69] What distinguishes MBL from CLL is the significantly lower rate (0%-3%) of secondary lesions that are known to associate with poor outcome in this leukemia, including 11q22-q23 deletion, 17p13 deletion and mutations of *TP53*, *NOTCH1*, *SF3B1* and *BIRC3*.[33,65-72]

Two categories of MBL exist.[73] One is represented by clinical MBL, which are detected in the context of a lymphocytosis investigated with laboratory techniques. The second category is represented by low count MBL, which are discovered while screening normal individuals of the general population for research purposes, and in whom the absolute number of lymphocytes is not increased.[73] While the impact of high-risk genetic lesions on clinical MBL survival is currently unknown, their occurrence associates with an increased rate of progression to overt CLL.[67,69] High-risk cytogenetic abnormalities have been occasionally described also in low count MBL, but the clinical implications of this observation are currently unknown.[66,68]

Three major clinical phases can be distingushed in overt CLL, including: i) newly diagnosed CLL; ii) progressive CLL; and iii) relapsed and fludarabine-refractory CLL.

Deletion of 17p13 occurs below 5% in newly diagnosed CLL.[11,14] Among progressive CLL, 17p13 deletion is observed in 8%-11% of cases at the time of first treatment, and increases up to ~30% in relapsed and fludarabine-refractory patients.[74-78] Mutations of *TP53* are found in ~5-10% of unselected newly diagnosed CLL, ~10% progressive CLL requiring first treatment, and ~40% relapsed and fludarabine-refractory cases.[78-80] By combining mutations and deletions, *TP53* disruption is observed in up to 10% of newly diagnosed CLL and more than 15% of progressive CLL requiring first treatment, and in 45% relapsed and fludarabine-refractory CLL, thus representing the most frequent lesion in this high-risk clinical condition (Table 1).[18-21,74-80]

Deletion of 11q22-q23 occurs in less than 10% newly diagnosed CLL, while its prevalence rises to ~20% at the time of first treatment and ~20% at the time of fludarabine-refractoriness.[11,14,74-77] *ATM* mutations have been shown in ~10-15% of newly diagnosed patients and in ~15% of progressive CLL requiring first treatment.[24-25,39] By combining mutations and deletions, genetic lesions of *ATM* occur in ~20% of diagnostic samples of CLL and in ~35% cases requiring first treatment.[24-25,39] These frequencies make *ATM* alterations the most common genetic lesion predicting poor outcome at CLL presentation and at the time of treatment requirement (Table 1).

NOTCH1, *SF3B1* and *BIRC3* mutations follow the same distribution across CLL clinical phases as other high-risk abnormalities. *NOTCH1* mutations characterize ~5%-10% newly diagnosed CLL, while their prevalence increases to 13%-20% in progressive CLL requiring first treatment and in relapsed cases.[26,27,31,32,64] *SF3B1* mutations recur in ~5-10% newly diagnosed CLL, in ~15% progressive CLL requiring first treatment and in ~20% relapsed and fludarabine-refractory patients.[28-30,64] Though occurring at low rates in newly diagnosed CLL (~5% of cases), *BIRC3* lesions are enriched among relapsed and fludarabine-refractory CLL (~25% of cases).[33] Due to the lack of information from clinical trials, the precise rate of occurrence at *BIRC3* lesions at the time of first treatment requirement still remains to be clarified (Table 1).

RS transformation is a very aggressive and an almost always lethal complication of CLL that combines the effects of both chemoresistance and rapid disease kinetics.[1] The genetics of RS strongly influences its clinical behavior. The high rate of *TP53* abnormalities (~60% of cases) accounts for the chemoresistant phenotype that is commonly observed in RS.[70] *NOTCH1* mutations and *MYC* network abnormalities are the second most frequent genetic lesions in RS, where they occur in ~30% of cases.[26] In RS, *NOTCH1* mutations are largely mutually exclusive with *MYC* oncogenic activation by translocation/amplification of the gene or by disruption of *MGA*, its negative regulator.[26,81] This finding is consistent with the observation that *NOTCH1* directly stimulates *MYC* transcription and suggests that activation of oncogenic *MYC* may be one common final pathway selected for tumorigenesis in ~60% RS.[26,81]

Lesions affecting *ATM*, *BIRC3* and *SF3B1*, that are otherwise frequent at the time of chemorefractory relapse, occur at low rates in RS, thus corroborating the notion that RS is molecularly distinct from chemorefractory progression without transformation.[29,33,70]

Clonal evolution of chronic lymphocytic leukemia

Though generally considered as a genetically stable dis-

Table 1. Clinical relevance of CLL genetic lesions.

	Diagnosis		First treatment	
	Prevalence %	Overall survival (years)	Prevalence (%)	Overall survival (years)
17p13 deletion[11,14,72,74,75]	4-5	5-7	8-10	1.5-2
TP53 mutation[19-21,77,78]	5-10	5-6	8-11	1.5-3
11q22-q23 deletion[11,14,72,74,75]	8-9	8-9	21-23	3-5
ATM mutation[24,25,39]	10-15	7-8	15	4
NOTCH1 mutation[26,27,29,30,32,62]	5-11	4-8	10	4.5
SF3B1 mutation[28-30,62]	6-9	4-9	17	4.5
BIRC3 disruption[33]	4	3.5	na	na

ease, CLL may undergo clonal evolution in a substantial fraction of cases. Comparison of the profile of cytogenetic lesions from primary and relapsed CLL samples has revealed differences in ~20-40% cases, illustrating the dynamic nature of clonal evolution in this leukemia.[70,82-91] The risk of developing new genetic lesions during the course of the disease is positively correlated with the duration of the follow up.[82,83] Additional factors contributing to clonal evolution in CLL are the *IGHV* mutation status and the selective pressure of treatments given during the disease history.[82,83] From a clinical standpoint, clonal evolution has been associated with poor outcome, treatment resistance and transformation.[70,82,83]

Based on conventional and FISH cytogenetic analysis, clonal evolution mainly consists in the development of 17p13 or 11q22-q23 deletions.[82,83] Mutation analysis of sequential samples has revealed that the development of new *TP53* mutations also contributes to clonal evolution, especially in chemorefractory patients and in patients complicated by RS.[18,70] Consistent with this observation, current guidelines recommend to repeatedly test for *TP53* lesions at treatment requirement also in cases that were previously wild-type.[2,92]

NOTCH1, *SF3B1* and *BIRC3* lesions may emerge during the course of CLL, thus expanding the spectrum of genetic events currently associated with clonal evolution.[93] Similar to *TP53* abnormalities and 11q22-q23 deletion, also the development of new *NOTCH1*, *SF3B1* and *BIRC3* lesions may occur at the time of shift to a more aggressive clinical phenotype. In this context, a fraction of *NOTCH1* mutations may develop at the time of transformation to RS.[26] Consistently, the acquisition of high-risk genetic lesions over time, including *NOTCH1*, *SF3B1* and *BIRC3* mutations, affects survival in a manner that is independent of modifications of other time-varying factors, such as patient age and disease stage.[93]

Open issues in the field of clonal evolution of CLL are: i) whether mutations detected from a certain timepoint onward were already present at subclonal levels in earlier disease phases and are thus subsequently selected (as demonstrated for a few cases), or whether they are acquired *de novo* during the course of the disease; and ii) whether the presence of small subclones harboring high-risk genetic lesions from the early phases of the disease may affect CLL outcome. A conclusive demonstration of the precise timing of mutations in CLL awaits studies aimed at tracking these lesions with high sensitivity techniques in sequential disease phases.

Clinical relevance of genetic lesions in chronic lymphocytic leukemia

Clinical relevance of TP53 abnormalities in chronic lymphocytic leukemia

TP53 abnormalities represent strong predictors of poor survival and refractoriness in CLL and, for these reasons, they have direct implications for the clinical management of this leukemia.

Among newly diagnosed CLL, patients harboring 17p13 deletion show a median overall survival (OS) of only 5-7 years.[17] Although the outcome of patients with 17p13 deletion is generally considered poor, it is important to underline that there is a small subgroup of cases, generally expressing mutated *IGHV* genes, who may exhibit a slowly progressive disease without treatment indications for years.[94]

At the time of treatment requirement, the outcome of patients with 17p13 deletion is almost always very poor. Patients with 17p13 deletion respond poorly (5% of complete response *vs.* ~50% in non-17p13 deleted CLL) to fludarabine-cyclophosphamide-rituximab (FCR), that is the most effective regimen available today for CLL. The poor response rate in 17p13 deleted patients translates into very short progression-free survival (PFS) (11.2 months *vs.* 51.8 months) and OS (38.1% at 36 months).[77] This is in line with the central role of the wild-type TP53 protein in priming CLL cells to apoptosis and in mediating the cytotoxic effect of DNA-damaging agents, including purine analogs.

Recently, by assessing the impact of CLL genetics on disease outcome, a number of prospective studies have consistently documented that *TP53* mutations, even in the absence of 17p13 deletion, represent a predictor of poor response to treatment and short survival in CLL. In the GCLLSG CLL4 trial (F *vs.* FC),[74] none of the *TP53* mutated CLL achieved a complete response, and the median PFS (23.3 *vs.* 62.2 months) and OS (29.2 *vs.* 84.6 months) were significantly shorter in patients with *TP53* mutations compared to *TP53* wild-type cases.[79] In the GCLLSG CLL8 trial (FC *vs.* FCR), the presence of *TP53* mutations associated with the lowest complete and overall response rates (6.9% *vs.* 36.4% and 62.1% *vs.* 95.3%, respectively), the shortest PFS (12.4 months *vs.* 45 months) and the shortest OS (39.3 months *vs.* not reached in all other patients) compared to *TP53* wild-type cases.[95] In the UK LRF CLL4 trial (chlorambucil *vs.* F *vs.* FC), *TP53* mutated patients showed a complete remission rate of only 5%, a 5-year PFS of 5%, and a 5-year OS of 20%.[80]

Although clinical trials have consistently shown a clear association between *TP53* mutations and poor outcome in CLL, some controversial issues still remain to be clarified in this field. Indeed, it is currently a matter of debate whether monoallelic *TP53* abnormalities have the same poor prognostic effect as biallelic *TP53* lesions, and whether the *TP53* mutation type and position in the protein might impact on patient oucome. In fact, in contrast to the GCLLSG CLL4 trial, cases from the LRF CLL4 trial harboring isolated *TP53* mutations or deletions showed a longer PFS and OS after treatment compared to cases harboring biallelic *TP53* disruption.[79,80] Also, in a retrospective study, patients harboring truncating *TP53* mutations or missense substitutions mapping outside the DNA binding domain seem to have a longer OS from diagnosis than cases harboring mutations within the DNA-binding motifs.[96]

On these bases, at least three main clinical implications need to be considered:[2,92] i) patients with *TP53* abnormalities should not be treated until disease progression, since they can occasionally experience a prolonged TFS; ii) alongside 17p13 deletion, *TP53* mutation analysis should be performed in all CLL patients before treatment initiation, since cases with *TP53* disruption should be considered for alternative therapies upfront (see below); iii) a thorough search for *TP53* mutations/deletions should be performed repeatedly before each line of therapy.

Chemorefractoriness is due to *TP53* disruption in ~40% of CLL patients failing treatment, but the molecular basis of this aggressive clinical phenotype remains unclear in a sizeable fraction of patients (~60%).[78] In order to optimize the early diagnosis of chemorefractory CLL, it is crucial to

understand the molecular basis of chemorefractoriness beyond *TP53* disruption. The new molecular lesions recently identified may shed some light on this (see below).

Clinical relevance of ATM abnormalities in chronic lymphocytic leukemia

Deletion of 11q22-q23 was historically associated to CLL progression, poor response to alkylator- and fludarabine-based chemotherapy and, ultimately, short survival.[17,74,76] The introduction of chemo-immunotherapy based regimens has changed the prognosis of patients with this genetic abnormality. In fact, in the GCLLSG CLL8 trial, treatment with FCR significantly improved the complete response rate in CLL patients with 11q22-q23 deletion compared to FC alone (51% *vs*. 15%, *P*<0.0001), making these patients more similar to standard-risk patients in terms of response and PFS.[77]

The prognostic impact of *ATM* mutations in CLL has been investigated in few studies, due to the complexity of the DNA sequencing procedure of such a large gene in the absence of well-defined mutational hotspots, and also because of the difficulty in discriminating somatic mutations and damaging germline mutations from single nucleotide polymorphisms. At CLL presentation, patients harboring *ATM* mutations have a statistically significant reduction in both OS (85 months) and TFS (40 months) compared to patients with wild-type *ATM* and *TP53* genes (217 months and 130 months, respectively).[22-25]

The impact of *ATM* mutations on response to treatment and chemorefractoriness is still an open issue. *In vitro*, CLL cells with mutations affecting either one or both *ATM* alleles show defective apoptosis in response to radiation and chemotherapy-induced DNA damage. On the contrary, 11q22-q23 deleted CLL with a normal residual *ATM* allele preserve the DNA damage response, suggesting that loss of a single *ATM* allele might not be sufficient to induce chemorefractoriness.[97] Consistent with these pre-clinical observations, in the UK LRF CLL4 trial, patients with both *ATM* mutation and 11q22-q23 deletion showed a significantly reduced PFS (median 7.4 months) compared to those with wild-type *ATM* (28.6 months), 11q22-q23 deletion alone (17.1 months), or *ATM* mutation alone (30.8 months). Consistently, OS for patients with biallelic *ATM* alterations was significantly reduced compared to those with wild-type *ATM* or *ATM* mutations alone (median 42.2 *vs*. 85.5 *vs*. 77.6 months, respectively).[39] On these bases, at least in the context of CLL treated with alkylating agents or purine analogs, only the co-occurrence of 11q22-q23 deletion and *ATM* mutation associated with poor outcome. Importantly, in the UK LRF CLL4 trial, the PFS of CLL harboring biallelic *ATM* lesions was similar to that of patients with *TP53* alterations.[39] Therefore, when using chemotherapy alone, *ATM* mutation could be one mechanism that accounts for a fraction of chemorefractory CLL in which no aberrations of *TP53* are detected.

In conclusion, *ATM* disruption represents an independent marker of poor prognosis in CLL patients, particularly if they are treated with chemotherapy regimens not containing rituximab. Since the addition of rituximab to intensive combination chemotherapy (i.e. FCR) leads to an improved outcome in CLL harboring 11q22-q23 deletion, this regimen represents the treatment of choice in clinically fit patients belonging to this genetic subgroup. Nonetheless, even in the immunochemotherapy era, 11q22-q23 deleted patients have a short PFS and, therefore, may be particularly suited for investigational agents combined with immunochemotherapy or maintenance strategies aimed at prolonging disease remission.[98]

Clinical relevance of novel molecular lesions

Beside their pathogenetic role, *NOTCH1*, *SF3B1* and *BIRC3* lesions may also represent new biomarkers for the identification of poor-risk CLL patients.

Retrospective studies have consistently shown the impact of *NOTCH1* and *SF3B1* mutations on newly diagnosed CLL outcome. *NOTCH1* mutated patients have a more rapidly progressive disease and a significantly shorter OS probability (21%-45% at 10 years) compared to *NOTCH1* wild-type cases (56%-66% at 10 years).[26,27,32] *SF3B1* mutated patients are characterized by a significantly shorter time to treatment requirement and a significantly shorter OS (10%-48% at 10 years) compared to wild type cases (60%-77% at 10 years).[28-30] In a retrospective analysis of newly diagnosed CLL, *BIRC3* disruption identifies patients with a poor survival (median OS 3 years) similar to that associated with *TP53* abnormalities.[33]

Data from prospective studies and clinical trials validate the clinical importance of *NOTCH1* and *SF3B1* mutations in CLL.[64,99] In a well-characterized population-based cohort of newly diagnosed CLL patients who were included in the Scandinavian Lymphoma Etiology study, the presence of *NOTCH1* or *SF3B1* mutations was strongly associated with poor outcome, both in terms of shorter time to treatment and decreased survival.[99] In fact, time to treatment was only 4.8 months in patients harboring *NOTCH1* mutations and 2.4 months in patients harboring *SF3B1* mutations. This higher propensity to progression translated into a short OS of 66 months in *NOTCH1* mutated patients and 63 months in *SF3B1* mutated patients, that was significantly reduced compared to that of wild-type cases.[99] In addition, *NOTCH1* and *SF3B1* mutations in this study had a similarly poor impact on prognosis as *TP53* aberrations.[99]

In the UK LRF CLL4 trial, patients harboring *NOTCH1* and *SF3B1* mutations have an OS (55 and 54 months, respectively) that is significantly shorter compared to wild-type patients (83 months) and longer than that of patients carrying *TP53* abnormalities (26 months).[64] Overall, these data document that, at the time of treatment requirement, patients with *NOTCH1* and *SF3B1* mutations display an outcome that is intermediate between the one marked by *TP53* abnormalities and the one characterizing wild-type cases. While both *NOTCH1* and *SF3B1* mutations are independent predictors of OS by multivariate analysis in this trial, only *SF3B1* mutations, but not *NOTCH1* mutations, significantly correlate with a short PFS.[64] These data point to chemoresistant progression as a potential reason for the poor outcome in *SF3B1* mutated patients, while *NOTCH1* mutations apparently have no impact on disease sensitivity to treatment. The short survival associated with *NOTCH1* mutations can be explained, at least in part, by a substantial risk (~50% at 15 years) of developing RS in patients harboring this genetic lesion.[100]

The GCLLSG is currently exploring the role of new mutations in CLL patients treated with first-line FC or FCR (CLL8 trial), as well as the role of alemtuzumab in overcoming *NOTCH1* and *SF3B1* alterations in relapsed/refractory patients (CLL2H trial). Preliminary

data from the GCLLSG CLL8 trial[77] indicate that both *SF3B1* and *NOTCH1* mutations represent independent predictors of short PFS after treatment with FCR.[101] In particular, in this trial, *NOTCH1* mutations appear to identify a subset of CLL patients that may not benefit from the addition of rituximab to FC.[101] Conversely, based on a preliminary analysis of the GCLLSG CLL2H trial,[75] patients harboring *NOTCH1* mutations may have a superior PFS after alemtuzumab treatment compared to *NOTCH1* wild-type cases, at least in the relapsed/refractory setting.[102]

Though information on the impact of *BIRC3* lesions on response to treatment is currently lacking, their enrichment among fludarabine-refractory CLL might suggest an association of these molecular defects with chemorefractory progression.[33]

The integration of the most recurrent and clinically relevant new molecular lesions into the backbone of the FISH hierarchical model has allowed a better understanding of the genetic basis of CLL heterogeneity and a significant improvement in patient prognostication.[93] According to a proposed model, four genetic groups of patients are hierarchically classified:[93] i) high-risk patients, harboring *TP53* and/or *BIRC3* abnormalities independent of co-occurring genetic lesions, that account for approximately 15%-20% newly diagnosed CLL and show a 10-year survival of 29%; ii) intermediate-risk patients, harboring *NOTCH1* and/or *SF3B1* mutations and/or del11q22-q23 in the absence of *BIRC3* and *TP53* abnormalities, that account for ~15%-20% newly diagnosed CLL and show a 10-year survival of 37%; iii) low-risk patients, harboring +12 or a normal genetics, that account for approximately 40% of newly diagnosed CLL and showed a 10-year survival of 57%; and iv) very low-risk patients, harboring del13q14 only in the absence of any additional abnormality, that account for ~20%-25% newly diagnosed CLL and a nearly normal life expectancy with a 10-year survival (69%) that did not significantly differ from a matched general population (Figure 2).[93]

Molecular genetics as a guide for choosing therapy

TP53 disruption is at present the only molecular marker that changes the therapeutic approach to CLL patients. The median OS of *TP53* disrupted CLL treated with intensive immunochemotherapy regimens (i.e. FCR) is within 2 to 3 years,[77,95] thus resembling the outcome of acute leukemias. As a consequence, the occurrence of *TP53* abnormalities represents a strong indication for treating patients with drugs with a *TP53*-independent mechanism of action and for performing an allogeneic stem cell transplant (SCT) consolidation in eligible cases.

The anti-CD52 monoclonal antibody alemtuzumab has so far been the single agent with the highest efficacy in CLL with 17p13 deletion. Nevertheless, few published trials dedicated to this subgroup of patients are available and approved therapeutic options remain limited.[75,103-112] At first-line, patients with *TP53* disruption and treated with alemtuzumab-based regimens can achieve a response rate of 60%-90%, a complete response rate of 20%-60% and a PFS of 10-17 months. In the context of relapsed/refractory disease, the response rate ranges from 30% to 80%, the complete response rate from 0% to 30% and the PFS from 3 to 12 months.[75,103-112]

The efficacy of alemtuzumab is increased by its combination with high-dose steroids. In the UK CLL206 trial, alemtuzumab combined to high-dose methylprednisolone was administered to 39 CLL with 17p13 deletion (17 untreated and 22 previously treated).[111] This combination resulted in a response rate of 85%, a complete response rate of 36%, a median PFS of 11.8 months and a median OS of 23.5 months. Based on these results, alemtuzumab combined to high-dose methylprednisolone represents the most effective cytoreductive therapy to be considered for fit patients with *TP53* disruption as a bridge to allogeneic SCT, which is still required because otherwise responses are of short duration.

Studies employing reduced intensity conditioning allogeneic SCT consolidation in chemorefractory or high-risk CLL have provided evidence of a graft-*versus*-leukemia effect and potential long-term control in 17p13 deleted patients.[113-116] On these bases, according to the EBMT guidelines, young CLL patients requiring treatment and harboring *TP53* abnormalities have an indication for allogeneic SCT.[117] In almost all studies addressing the issue of allogeneic SCT in CLL with *TP53* abnormalities, the disease status at the time of the transplant, namely the lack of remission and/or the presence of bulky lymphadenopathy, strongly predicts failure.[113-116] Consistently, the actual strategy for fit patients with *TP53* abnormalities is to induce the disease at least in partial remission to perform a reduced intensity conditioning allogeneic SCT.

Despite these advances, a number of unmet clinical needs remain in the setting of CLL patients requiring treatment and harboring *TP53* abnormalities, including: i) the design of alternative and safer approaches for remission induction, as remission rates with the current regimens are reached only in approximately one-third of patients with relatively high costs in terms of toxicity and infections; and ii) the design of alternative strategies for those patients who are not eligible to transplant procedures because of age and comorbidities.

Novel compounds that act through mechanisms completely different from chemotherapy may overcome the refractoriness induced by *TP53* abnormalities. Among fludarabine-refractory CLL with 17p13 deletion, ofatumumab, a new anti-CD20 antibody, led to a response rate of 41%; however, responses were of short duration.[118] Preliminary results obtained from ongoing clinical trials employing novel drugs interfering with the B-cell receptor signaling suggest that these novel agents may circumvent the negative impact of 17p13 deletion.[119,120]

Currently, data on genetic alterations of *NOTCH1*, *SF3B1* and *BIRC3* are not sufficient to substantiate a role, if any, for these mutations in guiding therapeutic choices. This issue still awaits clarification by the analysis of these new mutations in the context of multiple clinical trials.

Conclusions

In recent years, our understanding of the complexity of the molecular genetics of CLL has broadened profoundly and this has translated into important implications for an optimized management of patients. Indeed, a workup at diagnosis for *TP53* and *ATM* disruption, and *NOTCH1*, *SF3B1* and *BIRC3* mutations enables a more refined prognostic stratification of patients. None of these markers, however, represents *per se* an indication for early treatment, but prompts a closer clinical follow up. For patients

with disease progression, it is mandatory to perform *TP53* mutation analysis alongside 17p13 deletion, since cases with *TP53* disruption should be considered for alternative therapies upfront, including alemtuzumab plus steroids followed by an allogeneic SCT in young and fit patients. Although there are currently no treatment strategies for *TP53* disrupted CLL in the elderly, the use of low doses of alemtuzumab has proven feasible.[109] *ATM* disruption represents a strong indication for the use of the FCR scheme in clinically fit patients, whilst in elderly/frail patients with this lesion rituximab-based alternative approaches should be explored.

NOTCH1, *SF3B1* and *BIRC3* mutations at present do not guide therapeutic choices. However, they represent markers of short time to progression and survival, with potentially different clinical implications that need to be conclusively validated. *NOTCH1* mutations apparently do not impact on CLL chemosensitivity, but are associated to a substantial risk of RS transformation, particularly if associated to *TP53* disruption; therefore, a close follow up and early node biopsy is recommended in patients with this/these lesion(s). *SF3B1* mutations are associated to a chemoresistant progression of the disease after alkylating agents and/or fludarabine therapy. *BIRC3* mutations are also associated to a chemorefractory disease, although their impact on response to treatment has not yet been proven in the context of clinical trials. Thus, the molecular bases of CLL aggressiveness have been extended beyond *TP53* disruption. Due to the clonal evolution of the disease, a thorough search for *TP53* mutations/deletions should be repeatedly performed before each line of therapy in view of the clinical relevance of these abnormalities; we know that 11q22-q23 deletions can be also acquired over time and there is evidence of the acquisition of *NOTCH1* mutations. Finally, the efficacy of new drugs needs to be tested according to the presence of these molecular lesions for a future personalized medicine approach based on the genetic profile of CLL patients.

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