

Chapter 4

Recurrent Gene Mutations in CLL

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Abstract Next-generation sequencing of whole genomes and exomes in chronic lymphocytic leukemia (CLL) has provided the first comprehensive view of somatic mutations in this disease. Subsequent studies have characterized the oncogenic pathways and clinical implications of a number of these mutations. The global number of somatic mutations per case is lower than those described in solid tumors but is in agreement with previous estimates of less than one mutation per megabase in hematological neoplasms. The number and pattern of somatic mutations differ in tumors with unmutated and mutated *IGHV*, extending at the genomic level the clinical differences observed in these two CLL subtypes. One of the striking conclusions of these studies has been the marked genetic heterogeneity of the disease, with a relatively large number of genes recurrently mutated at low frequency and only a few genes mutated in up to 10–15 % of the patients. The mutated genes tend to cluster in different pathways that include NOTCH1 signaling, RNA splicing and processing machinery, innate inflammatory response, Wnt signaling, and DNA damage and cell cycle control, among others. These results highlight the molecular heterogeneity of CLL and may provide new biomarkers and potential therapeutic targets for the diagnosis and management of the disease.

Keywords Chronic lymphocytic leukemia • Next-generation sequencing • Somatic mutations • NOTCH1 • SF3B1 • MYD88

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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the proliferation and progressive accumulation of a peculiar population of mature CD5-positive B lymphocytes in bone marrow, blood, and lymphoid tissues [1, 2]. The disease exhibits a heterogeneous clinical course, ranging from an indolent evolution with a normal lifespan of the patients to a rapid progression of the disease and poor response to therapy that adversely impact on their survival. In some patients, the tumor cells may transform into an aggressive diffuse large B-cell lymphoma (DLBCL), a situation named Richter's syndrome (RS), which is associated with rapid progression of the clinical symptoms and a median survival of less than 1 year [3]. This different clinical behavior has been mainly related to two distinct molecular subtypes of the disease characterized by the presence of high or low numbers of somatic mutations in the variable region of the immunoglobulin genes (*IGHV*). These mutations are introduced by a process known as somatic hypermutation (SHM) that occurs physiologically in the germinal center of the lymphoid follicle as a mechanism to generate high affinity antibodies. The mutational status of *IGHV* reflects the origin of the disease in cells that have experienced the germinal center microenvironment or have developed outside this particular topographic site. The tumor cells of these two subtypes of CLL also have a particular epigenetic imprint related to the different putative cell of origin in naïve or memory B-cells [4]. As expected from the different clinical manifestations, CLL with mutated and unmutated *IGHV* also has important biological differences, but the mechanisms that lead to the different clinical behaviors are not well understood [1, 2].

In addition to the different cell of origin, the heterogeneous clinical evolution of the disease has been related to the presence of different chromosomal alterations in the tumor cells. Deletions of 11q22-q23 and 17p13 are associated with adverse outcome, whereas deletion of 13q14, the most common alteration, is associated with a favorable prognosis when present as an isolated aberration. Trisomy 12 is also common but its relationship to the evolution of the disease has been controversial in different studies [1, 2]. All these genomic aberrations are shared by both molecular subtypes of CLL, but high-risk alterations are more frequently found in CLL with unmutated *IGHV*. *MIR15a/MIR16A* and probably also *DLEU2* are the targets of 13q deletions, whereas *ATM* and *TP53* are inactivated by mutations in the remaining allele of the respective 11q and 17p deletions. The combination of genetic studies and *IGHV* mutational analysis are helpful tools to stratify the risk of patients with CLL. However, the heterogeneity of the clinical course and the different response to current therapies are not completely explained by these parameters, highlighting the need for more thorough molecular studies to clarify the oncogenic pathways of the disease.

The emergence of next-generation sequencing (NGS) technologies has offered higher throughput and greatly increased sensitivity, enabling the analysis of complete genomes, exomes (all annotated exons), or transcriptomes (all RNA transcripts) of individual cancers [5]. The most defining characteristic of NGS is massive parallelization, i.e., the ability to obtain millions of sequences in a single experiment. This allows the collection of sequence information at any nucleotide

position in the genome with high redundancy, which, in turn, allows the identification of genome changes in heterogeneous cancer samples and can discriminate the entire range of genomic alterations in a single experiment [5]. These technologies are being systematically applied to the analysis of cancer genomes and have started to provide the first view of the complex landscape of somatic mutations in different types of tumors [6]. Several subtypes of lymphoid neoplasms have been recently investigated with NGS, including CLL [7–10], hairy-cell leukemia (HCL) [11], follicular lymphoma (FL) [12], DLBCL [12–14], Burkitt lymphoma (BL) [15–17], and plasma cell myeloma (PCM) [18]. Although the number of cases examined in most of these tumors is still relatively low to draw definitive conclusions, the findings are relevant and provide new insights into the pathogenesis of the diseases with important clinical implications.

Patterns of Somatic Mutations in the Whole Genome of CLL Patients

The sequence of seven whole genomes (WG) and more than 200 whole exomes (WE) of CLL has been completed recently [7–10]. These studies have provided a first view of genes and pathways targeted by recurrent somatic mutations and have identified potential mechanisms contributing to the mutagenic process in the disease. The WG studies have shown that CLL genomes carry around 1,000 somatic mutations per tumor in non-repetitive regions of the genome (Fig. 4.1). This mutational load corresponds to an average of 0.9 mutations per megabase and 10–20 nonsynonymous mutations per case (range 2–76). The number of mutations is significantly higher in CLL with mutated (12.8 ± 0.7) than unmutated (10.6 ± 0.7) *IGHV* [9]. Comparing these findings with other neoplasms, CLL has a similar mutational load compared to acute myeloid leukemias, but it is lower than in DLBCL (3.2 mutations per Mb, range 5–135 per case) [12–14] or PCM (1.3 mutations per Mb and ~ 35 nonsynonymous mutations per case) [18].

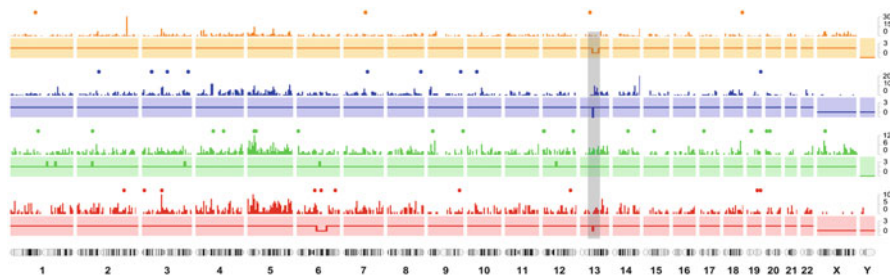


Fig. 4.1 Somatic mutations in the whole genome of four patients with chronic lymphocytic leukemia (CLL). For each case the density of mutations per 5-Mb window is represented with bars and protein-altering mutations by dots. The copy number alterations are represented by the solid bar and the shaded rectangle highlights the 13q14 deletion present in three of the four cases (with permission from Puente et al. [7])

The distribution of the nucleotide substitutions in cancer genome sequences differs among tumors. The analysis of these patterns suggests that certain changes are related to particular mutagenic mechanisms, such as tobacco carcinogens in respiratory tract tumors or ultraviolet light exposure in melanoma [19–21]. The most common change in CLL, as in other tumors, is the C>T transition in the context of CpG dinucleotides. These changes were slightly higher in CLL with unmutated *IGHV*, but the difference was not significant [7]. Puente et al. also detected a particular nucleotide substitution that was related to the molecular subtype of CLL. Thus, *IGHV*-mutated cases showed a significantly higher proportion of A>C/T>G mutations than cases with unmutated *IGHV* ($16 \pm 0.2\%$ vs. $6.2 \pm 0.1\%$). The base preceding the adenine in A-to-C transversions showed an overrepresentation of thymine when compared to the prevalence expected from its representation in non-repetitive sequences in the wild-type genome, and there were fewer A-to-C substitutions at GpA dinucleotides than would be expected by chance [7]. This finding was subsequently confirmed in the analysis of 105 whole exomes by Quesada et al. [9]. This difference between CLL subtypes (*IGHV*-mutated and -unmutated) might reflect the molecular mechanisms implicated in their respective development. The pattern and context of these A>C mutations are consistent with the frequent error introduced by DNA polymerase η (POLH) when it is recruited to repair DNA breaks. DNA polymerase η is highly expressed in the follicular germinal center cells, and its error-prone action contributes to create diversity in immunoglobulin genes. The action of DNA polymerase η does not require transcription, and therefore it may act broadly in the genome. Therefore, the differences observed between CLL with mutated and unmutated *IGHV* regarding A>C bias may be an additional imprint of the germinal center microenvironment reflecting the different cell of origin of the two subtypes of CLL.

The analysis of the distribution of the mutations in the genome has revealed that they do not occur randomly but seem to be distributed with a different frequency in particular regions. A recent study has shown that somatic mutations in CLL, as in other solid tumors, are related to the chromatin organization and are more frequent in heterochromatin regions of the genome, whereas the density diminishes in regions with open chromatin. The reasons for these differences are not clear but may include different access to mutagens or DNA repair mechanisms among others [22].

Repertoire of Somatic Mutations in Coding Regions

The analysis of somatic mutations in coding regions of cancer genomes has revealed that most tumor subtypes show very few genes recurrently mutated at relatively high frequencies, but they have a large spectrum of genes mutated at low frequency. On the contrary, some tumor subtypes have a predominant gene that is mutated in virtually all cases or in a very high number of them. In some tumors, this predominant gene carries exactly the same mutation, indicating the strong driver function in the oncogenesis of the cells. The two whole exome studies of large cohorts of CLL have revealed a very heterogeneous landscape of somatic

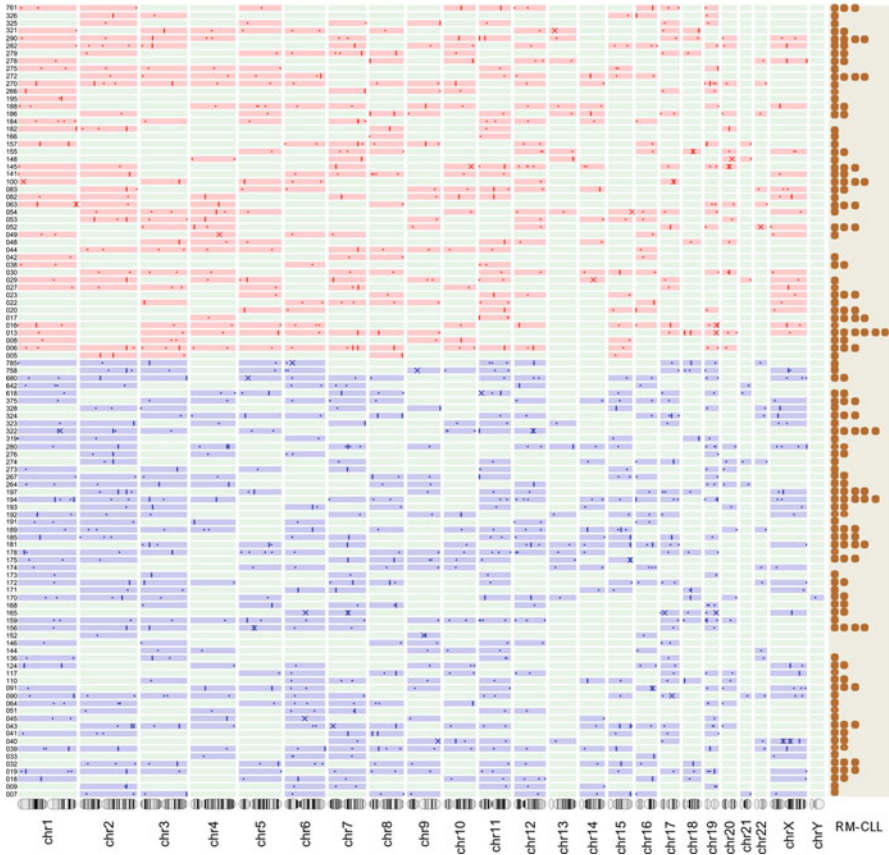


Fig. 4.2 Somatic mutations in the exome of CLL. Distribution and location of protein-coding mutations (*dots*), insertions, and deletions (*X*) in 60 CLL cases with mutated (*blue*) and 45 CLL cases with unmutated (*red*) *IGHV*. Recurrently mutated genes are highlighted with *vertical bars* and summarized for each individual with *orange dots* (with permission from Quesada et al. [9])

mutations, with only a few genes mutated in 10–15 % of the cases and a large number of genes mutated at lower frequencies (2–5 %) (Fig. 4.2). A similar pattern has been observed in DLBCL, FL, and PCM. On the contrary, HCL, Waldenström macroglobulinemia (WM), and to a lesser extent BL, have an opposite scenario, with a single mutated gene present in all or in a very high number of cases. Thus, the *BRAF* mutation V600E has been detected in all HCL [11, 23] and *MYD88* L256P in the vast majority of WM [24]. The frequency of the *MYD88* L265P mutation among patients with a family history of WM was 100 %, and the frequency among patients with sporadic cases was 86 % [24]. However, these mutations are not disease-specific, since they can be also found in other types of lymphomas. *MYD88* mutations have been reported in 9 % of gastric mucosa-associated lymphoid tissue lymphoma (MALT) and 29 % of activated B-cell subtype of DLBCL [25]. Similarly, BL carries

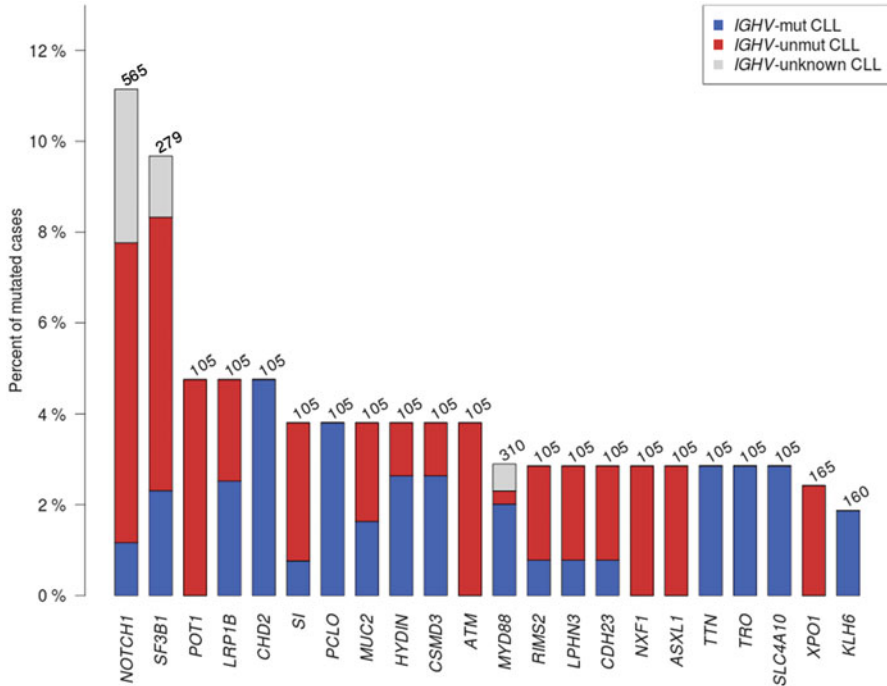


Fig. 4.3 Repertoire of mutations in CLL. Frequency of the most common somatic mutations in CLL according to the IGHV mutational status (data are taken from Quesada et al. [9])

somatic mutations in *ID3* in 60–70 % of the cases, whereas this gene is apparently not mutated in other lymphoid neoplasms [15–17].

The exome sequencing studies have shown the marked molecular heterogeneity of CLL, with more than 1,000 genes carrying somatic mutations expected to result in functional changes. However, the number of genes recurrently mutated in two or more patients is around 100, and most of them at frequencies below 3–5 % (Fig. 4.3; Table 4.1). One important finding is the different distribution of mutated genes in the two subtypes of the disease with mutated and unmutated *IGHV*. Some genes, such as *NOTCH1*, *SF3B1*, *XPO1*, and *POT1*, are mutated preferentially or exclusively in the group of CLL with unmutated *IGHV*. By contrast, *MYD88*, *CHD2*, or *KLHL6* occur in CLL with mutated *IGHV*, suggesting that the different clinical behavior of these two subtypes of CLL may be related to the activation of different molecular mechanisms (Fig. 4.3, Table 4.1) [7, 9]. Concordantly with the influence of the germinal center microenvironment in CLL with mutated *IGHV*, the mutations in some genes, such as *KLHL6*, bear the signature of the SHM machinery [7]. However, the extent to what this mechanism contributes to the mutational repertoire of CLL is not yet fully understood.

The functional clustering analysis of the mutated genes shows enrichment of genes in few pathways that tend to include one of the genes mutated at higher frequency

Table 4.1 Comparison of mutations in genome studies

	Wang et al. [10] ^a	Quesada et al. [9], and Puente et al. [7]	Fabbri et al. [8] ^b	
No. of mutations per megabase	0.7 ± 0.36	0.9		
No. of coding mutations (range)	20 (2–76)	14 (4–25)	10.4 (7–13)	
Mutation	Untreated (%)	Treated (%)	Untreated (%)	Untreated (%)
TP53	8	30	1	8
SF3B1	12	23	10	–
MYD88	10	9	3	2
ATM	5	17	4	–
FBXW7	0	10	–	–
NOTCH1	3	7	12	15
ZMYM3	5	3	–	–
DDX3X	0	10	2	–
MAPK1	0	7	–	–
POT1	–	–	5	–
LRP1B	–	–	5	–
CHD2	–	–	5	–
PLEKHG5	–	–	–	4
TGM7	–	–	–	4
BIRC3	–	–	–	4

^aMutation percentage between 61 chemotherapy-naïve and 30 chemo-treated CLL samples

^bIncludes the exome sequence of five patients and an extended validation series of 48 patients

together with several mutated genes at low frequency [9, 10]. These pathways include *NOTCH1* signaling (*NOTCH1*, *FBXW7*), mRNA splicing, processing, and transport (*SF3B1*, *U2AF2*, *SFRS1*, *XPO1*, *DDX3X*), innate inflammatory (*MYD88*, *TLR2*, *MAPK1*), DNA damage response and cell cycle control (*ATM*, *TP53*, *POT1*), and Wnt signaling [9, 10]. Interestingly, mutations in genes of these pathways also seem to be differentially represented in the two molecular subtypes of CLL. Mutations in genes of the *NOTCH1*, mRNA transport, and DNA damage response pathways are more common in CLL with unmutated *IGHV*, whereas mutations in the innate inflammatory pathway occur predominantly in *IGHV*-mutated CLL.

The molecular heterogeneity of CLL is further highlighted by the different incidence of the mutated genes in the two large CLL exome studies (Table 4.1, Fig. 4.4) [7, 9, 10]. The most common mutated genes in the ICGC study were *NOTCH1* (12 %) and *SF3B1* (10 %), followed by *POT1* (5 %), *CHD2* (5 %), and *LRP1B* (5 %), whereas in the Wang et al. study these were *TP53* (15 %), *SF3B1* (15 %), *MYD88* (10 %), and *ATM* (9 %). *NOTCH1* mutations in the latter study were only detected in 4 % of the cases, whereas *TP53* and *ATM* were found mutated in 1 % and 4 % of the cases, respectively, in the ICGC study. Intriguingly, the number of recurrent mutations observed in common in both studies represents only a very small fraction, underlining the complex molecular heterogeneity of the

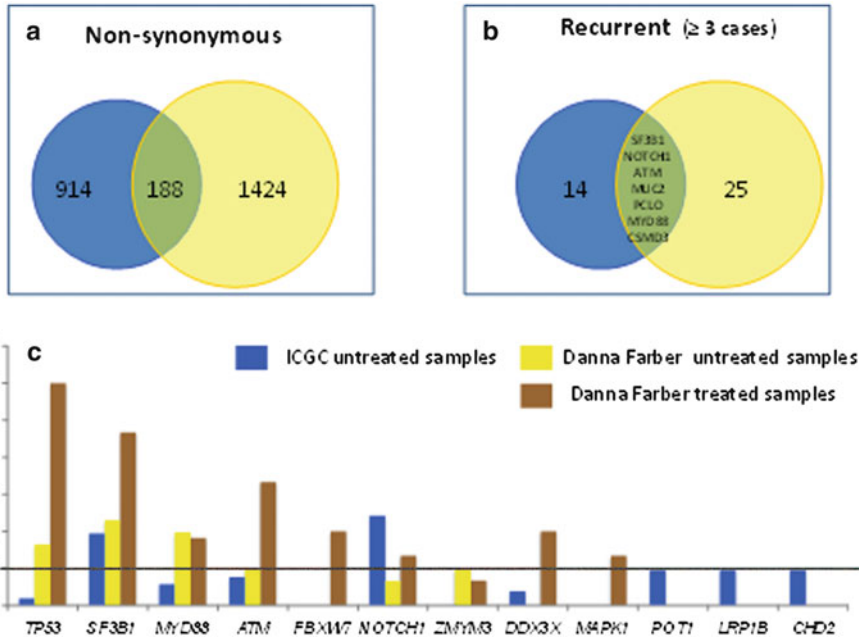


Fig. 4.4 Comparison of the mutations in two independent whole exome studies [9, 10]. *Blue* identifies mutations in the ICGC exome study [9], *yellow* and *brown* identify mutations in the Dana Farber exome study [10]. (a) Nonsynonymous mutations with commonly mutated genes found in both series. (b) Recurrent somatic mutations with commonly mutated genes identified in both series. (c) Frequency of most prevalent mutations in untreated and treated patients reported in both series

disease (Fig. 4.4). The comparison of the clinical features of these two series of patients reveals marked differences. Thus, all samples in the ICGC analysis were obtained from untreated patients whereas 33 % of the samples in the Wang et al. study were collected at relapse after previous treatments [10]. Similarly, the cohort from Wang et al. had higher numbers of patients with adverse prognostic parameters (advanced stage, 21 % vs. 8 %; adverse cytogenetic aberrations, 43 % vs. 15 %; high ZAP70 expression, 46 % vs. 29 %), and the patients were younger than in the ICGC cohort (median age 54 vs. 62 years). These findings suggest that the different distribution of mutated genes in CLL reflects the clinical and biological heterogeneity of the disease. The relatively low frequency of the mutations of all these genes is a real challenge to fully understand their implications in the pathogenesis of the disease.

The clinical and biological relevance of most of these mutated genes is still unknown since they have been identified for the first time in this disease or even in any type of cancer in these NGS-CLL studies. However, the functional implications and clinical impact of some of them, particularly *NOTCH1*, *SF3B1*, and *MYD88*, have been already evaluated in relatively large series of patients.

***NOTCH1* Mutations**

NOTCH1 encodes a class I transmembrane protein that serves as a ligand-activated transcription factor regulating cell differentiation, proliferation, and apoptosis. The NOTCH receptor family consists of four transmembrane proteins, which have an extracellular domain for ligand binding and an intracellular domain mediating signaling [26]. In resting conditions, the receptor is a heterodimeric complex composed of two fragments: the extracellular domain (N_{EC}), which acts as the receptor for ligands and is usually expressed on the surface of other cells, and a transmembrane and intracellular component (N_{TM}) that acts as the signaling mediator once it is released from the N_{EC} component by the activation of the receptor. These two fragments are stabilized by the heterodimerization domain (HD) composed of the C-terminus of the N_{EC} and the N-terminus of the N_{TM} fragments. The binding of the ligand to the N_{EC} component triggers an initial site-specific metalloproteinase-catalyzed proteolytic cleavage in the HD. This cleavage generates a truncated membrane-bound molecule. Intramembrane proteolysis of NOTCH by gamma-secretase releases the intracellular NOTCH domain (ICN), which translocates to the nucleus, resulting in the assembly of active transcription complexes that interact with the transcription factor CBF1/RBP-Jk, leading to derepression/activation of CBF1-dependent target genes. The C-terminus of the protein has a PEST domain (a sequence rich in proline (P), glutamate (E), serine (S), and threonine (T) residues), which limits the function of the activated receptor by targeting the protein for proteasome degradation via the FBXW7-SCF ubiquitin ligase complex. The phosphorylation of the PEST domain, which mediates this proteasome targeting, is triggered by the recruitment of the RNA polymerase II holoenzyme to the transcriptional complex and thus establishes a limiting termination mechanism to NOTCH signaling [27].

NOTCH1 activation has an important role in normal T-cell development. Somatic mutations targeting this protein have been identified in around 60 % of patients with T-cell acute lymphoblastic leukemia (T-ALL) [27]. Most mutations in this leukemia affect the extracellular HD and/or the C-terminal PEST domain. The biological significance of these two major types of mutations is different. The HD mutations are clustered in a “hot spot” spanning residues 1,574–1,622 of HD-N, and these generate a ligand-independent or hypersensitive active receptor which usually has a strong oncogenic potential, whereas mutations involving the C-terminal PEST domain generate a premature stop codon, resulting in a truncated and more stable protein that accumulates in the tumor cells. This truncated protein increases NOTCH1 concentration, but it seems to have a lower oncogenic potential since it is not able to fully transform T-cells in murine models. Therefore, NOTCH1-truncating mutations may cooperate with other oncogenic events in the full leukemic transformation of T-cells [27].

The vast majority of *NOTCH1* mutations detected in CLL occur in exon 34 at the TAD or PEST domains and usually generate a truncated and more stable protein that is then overexpressed in the cell (Table 4.2) [7, 8]. The most frequent mutation

Table 4.2 Frequency and type of mutations reported in *NOTCH1* in CLL patients

Mutation	<i>n</i>	Percentage %
P2515Rfs*4	128	81.0
L2482Ffs*2	5	3.2
Q2540*	2	1.3
Q2394*	2	1.3
p.Q2404*	2	1.3
Q2444*	1	0.6
Q2503*	1	0.6
P2437fs*36	1	0.6
P2162del122	1	0.6
K2182fs*61	1	0.6
S2342fs*13	1	0.6
P2415fs*82	1	0.6
A2464fs*14	1	0.6
S2471fs*1	1	0.6
T2478fs*6	1	0.6
L2336fs*19	1	0.6
E2268fs*86	1	0.6
S2330fs*25	1	0.6
Q2417insP	1	0.6
P2463fs*15	1	0.6
V1722M	1	0.6
G2459*	1	0.6
S2274fs	1	0.6
S2470fs	1	0.6
N2143fs	1	0.6
P2458fs	1	0.6

Data from [8, 10, 35, 37, 40]

in CLL is a 2 bp frameshift deletion in the PEST domain, p.P2515Rfs*4, that represents 85–90 % of all *NOTCH1* mutations in this disease (Table 4.2). Most other mutations have been described in single cases and only the mutations p.F2482Ffs*2, p.Q2540*, and p.Q2394* have been detected in more than one patient (2–4 %). One mutation in the HD, p.V1722M, was acquired in the Richter’s transformation of a CLL that already carried the p.P2515Rfs*4 [8]. The association of *NOTCH1* mutations in these two domains is relevant because they act synergistically up-regulating the *NOTCH1* signaling pathway and seem to be associated with a more aggressive disease [28]. A recent study has described the translocation dic(9;14)(q34;q32), fusing the 3’*IGH* with the 5’*NOTCH1*, resulting in a tenfold up-regulation of the *NOTCH1* mRNA. This translocation was also acquired in the Richter’s transformation of a CLL that already had the common p.F2482Ffs*2 mutation, suggesting that it could be involved in the progression of the disease [29]. The relevance of the activation of *NOTCH1* pathway in the pathogenesis of CLL has been highlighted by the finding of recurrent inactivating somatic mutations in *FBXW7* in four patients [10]. *FBXW7* is a ubiquitin ligase that targets several oncoproteins, *NOTCH1* among them, for proteasome degradation and it is

considered a tumor suppressor gene. Two of the mutations identified in CLL are known to activate NOTCH1 pathway in T-ALL [30].

The oncogenic potential of *NOTCH1* mutations in B-cells and their functional consequences in CLL are not well known yet. Initial studies showed that NOTCH1 and NOTCH2 signaling were constitutively active in CLL cells compared to normal B lymphocytes [31]. Stimulation of CLL cells by NOTCH ligands increased the activation of the NF κ B pathway and cell survival, whereas inhibition of NOTCH signaling accelerated the spontaneous apoptosis of CLL cells suggesting that NOTCH plays a role in sustaining CLL cell survival [31]. Gene expression profiling of *NOTCH1*-mutated CLL has revealed a large number of differentially expressed genes compared to *NOTCH1*-unmutated CLL [7]. This differential signature was significantly enriched in genes of the NOTCH1 signaling pathway and two metabolic pathways (oxidative phosphorylation and glycolysis/gluconeogenesis) that also underlie T-ALL with *NOTCH1* mutations [7]. These findings strongly suggest that *NOTCH1* mutations in CLL are functional and activate the downstream NOTCH pathway.

Several studies have now investigated *NOTCH1* mutations in different large series of CLL patients and have found a frequency that varies between 4 and 12 %. The reasons for these differences are not completely clear. A recent study reporting a low frequency (4.7 %) was conducted in a population-based cohort of CLL patients suggesting that, similarly to the low frequency of *TP53* mutations in the same group of patients, the higher frequency of *NOTCH1* mutations in other studies may be due to certain patient selection [32, 33]. However, a similar low ratio has been found in other non-population-based studies or even in patients with relapsed disease [34], whereas a frequency in the higher range (12 %) has been observed in a study of nonselected patients [7]. Some of these studies concentrate the mutational analysis only around the most common p.F2482Ffs*2 mutation whereas others cover the whole exon 34. Therefore, a combination of epidemiological and technical aspects may influence the reported differences in the frequency of *NOTCH1* mutations.

The clinical impact of *NOTCH1* mutations has been described in different series. These studies seem to identify a subgroup of patients with aggressive disease. *NOTCH1* mutations occur more frequently in CLL with unmutated *IGHV* (≈ 20 % vs. 3.5 %), and high expression of ZAP70 (≈ 30 % vs. 5 %) or CD38 (≈ 23 % vs. 5 %) [7, 8, 35–37]. The patients also present more advanced Binet and Rai stage and higher levels of LDH and $\beta 2$ -microglobulin [35–37]. *NOTCH1*-mutated CLL carries less frequently 13q deletions but trisomy 12 is significantly more common in these cases [9, 10, 34, 36, 37] particularly when this chromosomal alteration is the sole genetic aberration [38]. However, some studies have not found a significant association with trisomy 12 [32]. *NOTCH1*-mutated CLL with trisomy 12 is also enriched in cases with an unmutated *IGHV* status.

Given the association between *NOTCH1* mutations and parameters of aggressive disease it is not surprising that most studies have found that these patients have shorter overall survival (OS) and progression-free survival (PFS) than patients with CLL without *NOTCH1* mutations [35–37, 39, 40] (Fig. 4.5). However, whether the

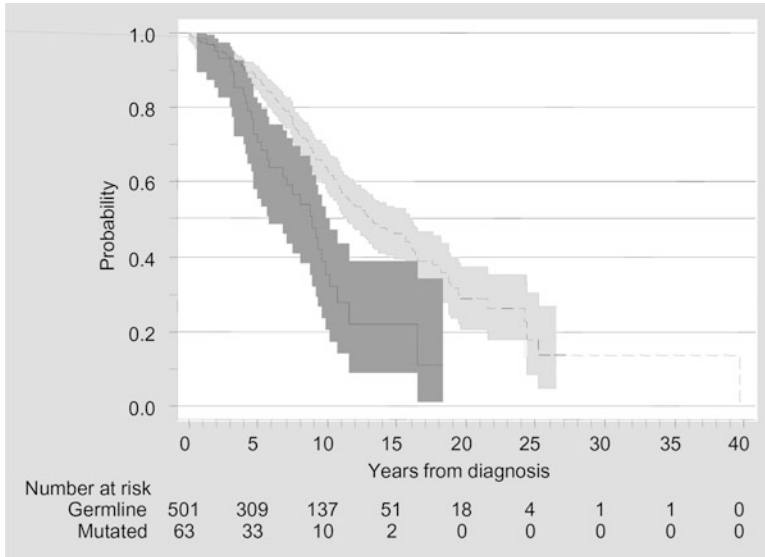


Fig. 4.5 Overall survival of CLL patients according to *NOTCH1* mutations. Overall survival in *NOTCH1*-mutated (solid line) and *NOTCH1*-unmutated (dashed line) CLL patients ($p < 0.001$). The 95 % confidence interval for each group of patients is depicted (with permission from Villamor et al. [37])

prognostic value of *NOTCH1* mutations is independent of other parameters associated with an aggressive disease, such as the *IGHV* mutational status, is not completely clear. Several studies have found that both *NOTCH1* mutations and the mutational status of the *IGHV* contribute independently to the shorter OS [35, 36]. However, other studies have not confirmed these findings [37, 40]. In this sense, Villamor et al. found a subgroup of patients with *NOTCH1*-mutated *IGHV*-mutated CLL that behaved as low-risk CLL with a long survival without requirement for treatment [37]. Similarly, the relationship between *NOTCH1* mutations and PFS does not seem to be independent of the *IGHV* mutational status [36].

NOTCH1 mutations also seem to have an impact on the requirement for and response to treatment. Patients with these mutations need therapy more frequently and earlier than patients with unmutated *NOTCH1* [35–37, 39]. However, the relationship between *NOTCH1* mutations and shorter time to treatment does not seem independent of the *IGHV* mutational status [37]. Refractoriness to treatment is significantly more frequent among *NOTCH1*-mutated than in unmutated patients [8, 37]. On the other hand, *NOTCH1* mutations do not seem to influence the ratio of complete or partial response to therapy [36, 37], but patients carrying these mutations reach a complete molecular response with negative minimal residual disease (MRD) less frequently [37]. The presence of *NOTCH1* mutations seems to also influence the evolution of the patients after reaching a CR to the first treatment. Thus, these patients had significantly shorter PFS after CR,

independently of the *IGHV* and the MRD status [37]. These findings suggest that *NOTCH1*-mutated CLL patients, particularly if young and fit, may be candidates for intensive or investigational treatments. However, more information is warranted from prospective clinical trials to define the real impact of *NOTCH1* mutations in CLL patients.

One of the most striking findings pertaining to *NOTCH1* mutations has been its association with the transformation to DLBCL (Richter's syndrome) (RS) [7, 8, 35, 37]. Patients with these mutations develop RS more frequently (23 % vs. 1.3 %) and more rapidly than those with *NOTCH1*-unmutated CLL [37]. At 10 years from diagnosis, the cumulative incidence of transformation to DLBCL was 6 % for *NOTCH1*-unmutated patients and 31 % for *NOTCH1*-mutated patients [37]. Interestingly, after adjusting for other variables associated with transformation, including high expression of CD38, trisomy 12, absence of del(13q), previous exposure to purine nucleoside analogues or anthracyclines, and unmutated *IGHV*, only *NOTCH1* mutation and *IGHV* SHM were independently associated with a higher risk of developing DLBCL [37]. In most CLL patients, the RS emerges from the same clone as the prior CLL, but in some patients, particularly with *IGHV*-unmutated CLL, the DLBCL may correspond to a second tumor clonally unrelated with the CLL. In patients with *NOTCH1* mutations, a clonal relationship with the previous CLL has been confirmed in several cases [7, 8]. On the other hand, *NOTCH1* mutations do not impact on the development of clonally unrelated DLBCL [39].

The timing of acquisition of *NOTCH1* mutations in the DLBCL transformation has been examined in paired samples in two studies [8, 37]. In most of the patients, the same *NOTCH1* mutation observed in the DLBCL was already present in the initial CLL, but in 31 % of the cases of one study [8], the mutation was not detected in the CLL component. Interestingly, samples from one patient harboring the mutation at diagnosis and progression were subjected to ultradeep NGS, which showed that this mutation occurred in 59 % of the sequencing reads obtained from the RS phase but was restricted to 5 % of the reads obtained at the time of CLL diagnosis [8]. These findings suggest that *NOTCH1* mutations may be present long before DLBCL transformation, and the clone carrying the mutation may be selected during the evolution of the disease.

Although the previous study supports the expansion of *NOTCH1*-mutated clones at the moment of Richter transformation, the timing of acquisition of these mutations in the evolution of the disease is not clear. A study of *NOTCH1* mutations in monoclonal B-cell lymphocytosis (MBL) found mutations in only two of 59 (3.3 %) cases [41]. The identification of these mutations in 4–12 % of CLL cases at diagnosis and in around 20 % at progression may suggest that these mutations are frequently acquired during the evolution of the disease [8, 35, 42]. However, this apparent increase in *NOTCH1* mutations with the progression of the disease may reflect the different proportion of *IGHV*-unmutated CLL in the evolution of the disease rather than a real acquisition of new mutations [42]. In this regard, the study by Rasi et al. on MBL [41] included 50 cases with mutated *IGHV* and only 2 *NOTCH1* mutations were observed in this group (4 %). This frequency is identical

to that found in CLL with mutated *IGHV* at diagnosis (8/206, 3.9 %) by the same group [35]. However, the analysis of only nine MBL with *IGHV*-unmutated CLL precludes any proper evaluation. Similarly, the proportion of *IGHV*-unmutated CLL at progression was 68 %, but only 33 % at diagnosis [35] supporting the idea that *IGHV*-unmutated CLL progresses to Richter syndrome more frequently than *IGHV*-mutated cases.

A recent study of *NOTCH1* mutations in sequential samples of 200 patients showed a relative stability of these mutations. The median interval between samples was 3.5 years (0.2–21.6 years) and a change in the status of *NOTCH1* mutations was observed only in three patients (1.5 %). One patient acquired a mutation after 9.5 years of stable disease, whereas in the remaining two patients the initial mutation detected at diagnosis was not found in the samples obtained at relapse 4 and 7 years later after having received two lines of treatment [37]. The putative disappearance of a *NOTCH1* mutation after treatment has been observed in another case [29]. Interestingly, using a more sensitive sequencing technique the presence of the *NOTCH1* mutation was detected in the negative samples of these three cases [37]. The identification of small subclones carrying the mutation may reflect the complex fluctuation of different tumor lines in the evolution of the disease. A recent study of sequential samples of three patients at different moments of the disease using NGS has identified different patterns of subclonal evolution of CLL, with many subclones present at very low frequencies evolving over the years [43]. Taken together, these results suggest that acquisition of *NOTCH1* mutation during the evolution of the disease, although possible, is an infrequent phenomenon. Further studies should clarify the relevance of the modulation of clones carrying *NOTCH1* mutations and whether real new clones acquiring the mutations may emerge during the evolution of the disease before transformation.

NOTCH1 Mutations in Other B-Cell Lymphoid Neoplasms

Interestingly, *NOTCH1* mutations in B-cell tumors do not seem to be limited to CLL. Recently, *NOTCH1* mutations have been reported in 12 % of primary mantle cell lymphoma (MCL) and in 2 of 10 MCL cell lines. Similar to CLL, 86 % of the mutations occurred in exon 34, and 8 of 16 detected mutations were p2514rfs*4. Patients with mutated *NOTCH1* had worse overall survival than patients with wild-type *NOTCH1*, and the impact on prognosis was significant and independent of the International Prognostic Index (IPI) and the histological subtype. Inhibition of this pathway in MCL cell lines reduced proliferation and induced apoptosis, supporting the role of *NOTCH1* mutations in the aggressive behavior of a subset of these lymphomas [44]. Intriguingly, *NOTCH2* but not *NOTCH1* mutations have been identified in splenic marginal zone lymphomas [45, 46], while *NOTCH2* mutations have not been detected in CLL.

Somatic Mutations in the Splicing and RNA Processing Machinery

A surprising finding in both large whole exome sequencing CLL studies was the relative frequent mutations in *SF3B1*, an element of the splicing machinery, that were found in 10–15 % of the cases, establishing this gene as one of the most commonly mutated in CLL, second only to *NOTCH1* [9] or *TP53* [10]. All mutations appeared to be heterozygous substitutions clustering in exons 12–15, a conserved region coding for several distinct amino acid residues within motifs 4–9 of its 22 HEAT repeats. A mutational hot spot has been detected at codon 700 (57 %) followed by codon 662 (11 %) and 666 (10 %) (Table 4.3). Interestingly, recurrent somatic mutations of *SF3B1* and other genes of the RNA splicing machinery had been found recently in patients with myelodysplastic syndromes (MDS), particularly in patients with refractory anemia with ring sideroblast (RARS) [47–49]. Intriguingly, no mutations in *SF3B1* were found in any other type of lymphoid neoplasms [9, 10].

Splicing is a pleiotropic mechanism necessary for cell functioning, and specific alterations in the splicing of oncogenes and tumor suppressors have been related to cancer development [50]. Splicing of messenger RNA is carried out by the spliceosome, a complex of five small nuclear ribonucleoproteins (snRNPs) (U1, U2, U4/U6, and U5). The assembly of the spliceosome occurs on each pre-mRNA, which contains specific sequences that drive and regulate this process. The crucial signal sequences are the splice donor site (5' end), the branch site (near the 3' end), and the splice acceptor site (3' end of the intron). The first step in this process is the recognition of the 5' donor site by the U1 snRNP followed by the recruitment of the U2 snRNP complex at the 3' branch. *SF3B1* is a component of this complex that allows the binding of the U2 snRNP to the branch point [51, 52]. The interaction between these complexes at the 5' and 3' ends leads to

Table 4.3 Somatic mutations described in *SF3B1* in CLL patients [9, 10, 36, 56]

Mutation	<i>n</i>	%
K700E	66	46
G742D, G	32	22.4
K666E, T, R, M, N	14	9.8
E662V, D, N	5	3.5
N626Y	4	2.8
G740E	3	2
R625L, H	3	2
Y623C	3	2
K741N	2	1.4
I704F, N	2	1.4
Other ^a	9	6.3

^aOther: R618Y, D663I, V701F, Q903R, T663I, D894G, V701F, Q669, Q670E

the removal of the corresponding intron with high fidelity [49]. Mutations affecting the splicing recognition sites, or elements of the spliceosome complexes, may cause abnormal transcription and various types of abnormal or alternative splicing events, causing altered outcomes of thousands of genes. These abnormalities include reduced transcription, exon skipping, intron retention, and cryptic splice site activation with truncated (or elongated) exons [49].

Consistent with the essential role of SF3B1 in maintaining appropriate gene expression patterns, the amino acid sequence of the protein shows a high level of phylogenetic conservation, especially in the regions that are affected by the somatic mutations found in CLL and MDS. Structurally, the SF3B1 protein has two well-defined regions: the N-terminal hydrophilic region, containing several protein-binding motifs, and the C-terminal region, which consists of 22 nonidentical HEAT repeats where all somatic alterations identified in CLL are located. A model of the C-terminal domain of the SF3B1 protein has shown that most mutations generate alterations on the inner surface of its structure defining a binding interface [9]. The mechanisms by which a mutant SF3B1 protein may facilitate a clonal expansion have not yet been elucidated. Using comparative analysis of exon arrays, Quesada et al. [9] uncovered a set of 184 genes with exons showing differential inclusion levels in *SF3B1* cells. Further, NGS of CLL transcriptomes uncovered a few transcripts with abnormal splicing junctions at 3' acceptor sites that were differentially expressed between *SF3B1*-mutated and -unmutated tumors. This finding is consistent with the function of SF3B1 ensuring the fidelity of the 3' branching site and, therefore, activation of cryptic 3' splice sites is the expected effect of altering SF3B1 function. These novel isoforms included truncated versions of *FOXPI*, encoding a member of the forkhead transcription factor group, whose altered expression has been linked to the pathogenesis of DLBCL [53].

SF3B1 mutations are detected more frequently in patients with advanced disease and with adverse biological features, such as elevated serum β 2-microglobulin and unmutated *IGHV* [9, 39]. These mutations have been associated with 11q deletions in one study [10] but not in others [9, 36, 54]. The presence of *SF3B1* mutations confers poor prognosis to the patients, with shorter time to disease progression and overall survival [9, 10, 54]. This relationship is independent of other prognostic factors such as clinical stage, CD38 or ZAP70 expression. However, the association with shorter overall survival was independent of the *IGHV* mutational status in one study [54] but not in another [9]. *SF3B1* mutations seem to be related to refractoriness to fludarabine treatment independently of *TP53* mutations, since they are more frequently found in refractory cases (17–30 %) than at diagnosis 5–10 % [36, 54]. Concordantly, *SF3B1* mutations were associated with reduced PFS in patients treated with fludarabine plus cyclophosphamide in a clinical trial with a median survival of 46 and 29.4 months for a wild type and mutant *SF3B1* allele, respectively (HR 2.08; 95 % CI 1.29–3.34, $p = 0.002$) [36]. On the other hand, Wang et al. found these mutations at a higher frequency in relapsed patients after treatment than in patients at diagnosis [10]. All these findings suggest that *SF3B1* mutations are associated with poor prognosis and confer refractoriness to fludarabine treatments.

In addition to *SF3B1*, exome studies have revealed mutations in different genes of the spliceosome subunits and RNA transport machinery. Ramsay et al. have identified 46 somatic mutations affecting 30 genes whose products are involved in RNA processing [55]. These genes were detected in 44 of 140 (31 %) patients studied by exome sequencing. Most of these mutations were predicted to have a functional effect and included several frameshifts, premature stop codons, or missed splicing sites. The mutated genes participate in several complexes of the RNA maturation process, particularly U2 complexes and export machinery. While most of these mutations are not likely to drive CLL progression, a second, less prevalent mutational hot spot was detected in genes coding for RNA transport factors. Thus, nine CLL patients, all of them in the *IGHV*-unmutated group, presented mutations in one of these factors. This suggests that the RNA transport pathway might provide novel targets for pharmacological intervention in a subset of the most aggressive CLL cases.

***MYD88* Mutations**

MYD88 was identified as a recurrently mutated gene at low frequency in the initial whole genome study of CLL [7]. This protein is a critical adaptor molecule of the interleukin-1 receptor/toll-like receptor (TLR) signaling pathway [30]. MyD88-deficient mice lose the ability to produce proinflammatory cytokines in response to a wide range of TLR ligands. MYD88 is recruited to the cytoplasmic portion of the TLRs and interacts with IRAK4 and IRAK1. Activated IRAK4 phosphorylates and activates IRAK1, which subsequently interacts with TNFR-associated factor-6 (TRAF6), causing the oligomerization and activation of TRAF6. The activation of this pathway finally results in activation of NFκB. Immunoprecipitation of MYD88 in tumor cells from CLL patients with mutations in this gene resulted in the co-immunoprecipitation of large amounts of IRAK1, suggesting a constitutive activation of this pathway. Consistent with this functional feature, CLL tumors containing *MYD88* mutations displayed an elevated activation of the downstream effectors STAT3 and NFκB p65 subunit. Stimulation of interleukin-1 receptor and the different TLRs in *MYD88*-mutated CLL cells induced the secretion of significantly higher amounts of interleukin-1 receptor antagonist, interleukin 6, and chemokine ligands 2, 3, and 4 (CCL2, CCL3, CCL4), when compared to *MYD88*-unmutated CLL [7]. The high production of these cytokines had been implicated in the recruitment of macrophages and T lymphocytes by CLL cells, creating a favorable niche for their survival [56–58].

Activating mutations of this gene were identified in 9 of 310 patients (2.7 %). Contrary to *NOTCH1* and *SF3B1*, virtually all these mutations occurred in CLL with mutated *IGHV* and low expression of ZAP70 and CD38. Interestingly, the age at diagnosis of almost all these patients was below 50 years, significantly younger than average age of diagnosis of patients with CLL (median age at diagnosis 65–70) [1, 59].

Other Mutations

Exome studies have revealed a long list of additional mutated genes at low frequencies that may play a role in the pathogenesis of the disease in small subsets of patients. The oncogenic function of most of these genes is unknown but their participation in mechanisms and pathways frequently altered in cancer suggest that they may also have an oncogenic potential in CLL. *Protection of telomeres 1 (POT1)* was mutated in 5 % of the patients, all of them with *IGHV*-unmutated CLL [9]. *POT1* is the first shelterin found mutated in any type of cancer. The role in telomere protection suggests that these mutations may contribute to the development of chromosomal aberrations in CLL, but the functional effect of these mutations is not known yet. Kelch-like protein 6 (*KLHL6*), implicated in the formation of the germinal center during cell maturation and B-cell antigen receptor (BCR) signal transduction, is mutated in 3 % of the CLL cases, and all of them have mutated *IGHV* [7]. *BIRC3*, an inhibitor of the noncanonical NF κ B pathway, has been found mutated in some cases of CLL [60]. These mutations are inactivating through frameshifts, premature stop codons, and deletions of the gene. They are more frequent in CLL refractory to chemotherapy (24 %) than at diagnosis (4 %). Interestingly, *BIRC3* mutations are mutually exclusive to *NOTCH1*, *SF3B1*, and *TP53* in cases refractory to chemotherapy, and patients with these mutations have a similar poor prognosis as the ones carrying *TP53* mutations. The fact that these relevant mutations were not detected in both whole exome studies emphasizes the molecular heterogeneity of the disease and suggests that capturing the complete landscape of somatic mutations in CLL may require larger studies of the whole genome or exome.

Conclusion

The initial genome sequencing studies in CLL have emphasized the high molecular heterogeneity of the disease and have identified a high number of genes and pathways that are altered in different subgroups of patients, suggesting that they may be relevant in the clinical and biological evolution of the disease. The initial functional and clinical studies of the most common mutations have already revealed the importance of these mutations and have delineated subgroups of patients with different prognosis and response to treatments. This information should provide new biomarkers and potential therapeutic targets to improve the diagnosis and management of the disease. However, the large number of genes mutated at low frequency may have also an important role in the particular dynamic of the disease in patients carrying these mutations. The understanding and translation to the clinic of all this knowledge is challenging and will require further studies integrating genomic studies in well annotated groups of patients.

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