Cell Oncol. (2015) 38:93–109
DOI 10.1007/s13402-014-0215-3

REVIEW

Molecular basis of chronic lymphocytic leukemia diagnosis and prognosis

Mohammad Shahjahani · Javad Mohammadiasl · Fatemeh Noroozi · Mohammad Seghatoleslami · Saeid Shahrabi · Fakhredin Saba · Najmaldin Saki

Accepted: 23 December 2014 / Published online: 7 January 2015

Abstract
Backgrounds Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in adults and is characterized by a clonal accumulation of mature apoptosis-resistant neoplastic cells. It is also a heterogeneous disease with a variable clinical outcome. Here, we present a review of currently known (epi)genetic alterations that are related to the etiology, progression and chemo-refractoriness of CLL. Relevant literature was identified through a PubMed search (1994–2014) of English-language papers using the terms CLL, signaling pathway, cytogenetic abnormality, somatic mutation, epigenetic alteration and micro-RNA.
Results CLL is characterized by the presence of gross chromosomal abnormalities, epigenetic alterations, micro-RNA expression alterations, immunoglobulin heavy chain gene mutations and other genetic lesions. The expression of unmutated immunoglobulin heavy chain variable region (IGHV) genes, ZAP-70 and CD38 proteins, the occurrence of chromosomal abnormalities such as 17p and 11q deletions and mutations of the *NOTCH1, SF3B1* and *BIRC3* genes have been associated with a poor prognosis. In addition, mutations in tumor suppressor genes, such as *TP53* and *ATM,* have been associated with refractoriness to conventional chemotherapeutic agents. Micro-RNA expression alterations and aberrant methylation patterns in genes that are specifically deregulated in CLL, including the *BCL-2*, *TCL1* and *ZAP-70* genes, have also been encountered and linked to distinct clinical parameters.
Conclusions Specific chromosomal abnormalities and gene mutations may serve as diagnostic and prognostic indicators for disease progression and survival. The identification of these anomalies by state-of-the-art molecular (cyto)genetic techniques such as fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), single nucleotide polymorphism (SNP) microarray-based genomic profiling and next-generation sequencing (NGS) can be of paramount help for the clinical management of these patients, including optimal treatment design. The efficacy of novel therapeutics should to be tested according to the presence of these molecular lesions in CLL patients.

Keywords CLL · Signaling pathways · Cytogenetic abnormality · Somatic mutation · Epigenetic alteration · Micro-RNA

M. Shahjahani · F. Saba
Department of Hematology and Blood Banking, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

J. Mohammadiasl
Department of Medical Genetics, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

F. Noroozi · M. Seghatoleslami · N. Saki (✉)
Health Research Institute, Research Center of Thalassemia & Hemoglobinopathy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
e-mail: najmaldinsaki@gmail.com

S. Shahrabi
Department of Biochemistry and Hematology, Faculty of Medicine, Semnan University of Medical Sciences, Semnan, Iran

1 Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults, accounting for approximately 30 % of all leukemia cases in European and North American countries with an incidence of 3–5 cases per 100,000 [1–3]. CLL is rare under 45 years of age, and its prevalence increases with age. The median age of patients at diagnosis is 70 years and only 10–15 % of the patients are diagnosed under 50 years of age [4, 5]. CLL is characterized by a clonal expansion of mature

non-functional B-cells with a high expression of CD5, CD19, CD20 and CD23 and a low expression of surface immunoglobulins IgM, IgD, and CD79a compared to normal B-cells [6–8]. These malignant B-CLL cells represent over 99 % of peripheral blood mononuclear cells (PBMCs) in CLL patients. Approximately 2–5 % of CLL patients show a T-cell phenotype, and these patients have a less favorable prognosis than patients with a B-CLL phenotype [9]. CLL develops through increased proliferation of immature lymphocytes in lymphoid organs, which results from an increased expression of anti-apoptotic BCL-2 family proteins [10]. As a result, CLL cells can survive for months (unlike normal cells, which only survive for a few days), thereby decreasing the number of normal lymphocytes and inducing immunodeficiency [11].

CLL is a heterogeneous disease that, based on its clinical course and response to treatment, can be divided into indolent and aggressive forms [12, 13]. In case of an indolent form, the disease does usually not progress to a severe form, and the patient may survive for years without treatment [14]. In case of an aggressive form, the cell number may quickly double and, as a result, the disease may be fatal within a relatively short period of time [13, 15].

Currently several biomarkers are being used as CLL prognosticators, including elevated protein levels (e.g. TCL-1, ZAP-70, CD38), elevated RNA levels (e.g. CLLU1, LPL, miRNAs), gene mutations (e.g. *TP53*, *SF3B1, BIRC3, NOTCH1*) and epigenetic changes [16, 17]. Prognostic serum markers that can be used to predict the survival and response to treatment include increased lactate dehydrogenase (LD) levels, which are associated with a poor prognosis and a likelihood of progressing to Richter's syndrome, increased thymidine kinase (TK) levels, which are associated with aggressive disease, and unmutated immunoglobulin heavy chain variable region (IGHV) genes, which are associated with a high risk for genomic aberrations [16, 18–20]. Evaluation of the *IGHV* mutation status and FISH are among the most reliable molecular tools used in routine diagnostics to date to detect clinically relevant genetic aberrations, including 11q-, 13q-, 17p- and + 12 [21, 22]. The detection of these aberrations can be useful for the clinical management and proper treatment of CLL patients [23]. Only patients with progressive CLL require treatment, and this treatment results in an increased survival. A number of drugs that is currently used for CLL treatment is listed in Table 1, including fludarabine and cyclophosphamide. Fludarabine is a purine analog inducing the P53-dependent apoptotic pathway [11]. In addition to these conventional chemotherapeutic drugs, monoclonal antibodies, cell cycle inhibiting drugs and cell death inducing drugs, as well as immune modulating drugs (Table 1), are increasingly being used in the treatment of CLL [26, 27]. These drugs can be used in conjunction with fludarabine, cyclophosphamide and rituximab to enhance its efficacy and, thus, to improve patient survival [11].

Activation of cellular signaling pathways, such as the NOTCH1, WNT, TLR/IL-1R, BCR and JAK/STAT pathways, has frequently been observed in CLL patients. Most of these pathways are involved in cellular proliferation, differentiation and survival, and in determining cell fate. Mutations in factors participating in these pathways may cause increased cellular proliferation and survival rates, as also resistance to apoptosis due to an altered expression of e.g. downstream cell cycle regulatory proteins [31, 32]. The mode of action of each of these signaling pathways is depicted in Fig. 1.

Inhibitory agents (or activators) can be used to revert the apoptotic imbalance. For example, ABT 737 (a BCL-2 antagonist) can induce apoptosis in B-CLL cells, whereas DHMEQ (a NF-κB inhibitor) can induce expression changes in other genes involved in this pathway, including *c-IAP, BFL-1, BCL-XL* and *c-FLIP*, resulting in an increased therapeutic efficacy of fludarabine [33]. Nutlin mimics the molecular structure of P53 and inhibits the binding of MDM2 to it, thereby inducing apoptosis in CLL cells and eliciting synergistic effects with genotoxic drugs [34, 35]. Cell cycle inhibitors such as rapamycin and roscovitine can also induce apoptosis in CLL cells [12]. In spite of all this knowledge gathered, currently available treatment strategies have remained unsatisfactory, and further information on the pathogenesis of CLL is needed to increase the options.

By using advanced molecular (cyto)genetic techniques, our knowledge on the molecular events underlying CLL development has improved. While cytogenetic banding analysis allows the identification of less than 50 % of the (relevant) genetic abnormalities, fluorescence in situ hybridization (FISH) allows the detection of ~80 % of these abnormalities [36, 37]. In addition, comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) microarray-based CGH (array CGH) analyses have revealed additional abnormalities. An advantage of the latter techniques is that they do not require proliferating cells [11, 38, 39]. More recently, also next-generation sequencing (NGS) techniques have contributed to a better knowledge of the genetic abnormalities present in CLL cells, including their heterogeneity [40, 41]. As yet, however, these latter abnormalities cannot be used to reliably predict disease progression [42].

In this review we focus on recent (epi)genetic findings in CLL, including microenvironmental factors, and on the evaluation of their role in disease etiology and progression, patient survival and response to treatment, including recently developed therapeutic options and their efficacies.

2 Microenvironment and drug resistance

The bone marrow (BM) entails niches that provide specific physiological environments for hematopoietic stem cells (HSCs) and other non-hematopoietic stem cells, such as

Table 1 Drugs used in CLL treatment, their targets and mechanisms of action

Class	Agent	Target	Mechanism of action	References
Tyrosine kinase inhibitors	Fostamatinib	SYK	Inhibits SYK phosphorylation and its enzymatic activity	[16, 24, 25]
	Idelalisib (CAL-101)	PI3K-δ	Inhibits constitutive PI3K signaling and signaling derived from CD40, TNF-α, fibronectin, and BCR, leading to suppression of AKT activation	[16, 24, 25]
	Ibrutinib (PCI-32765)	BTK	Inhibits BTK phosphorylation and its enzymatic activity	[16, 24, 25]
Monoclonal antibodies	Obinutuzumab (GA101)	CD20	-	[26]
	Rituximab	CD20	Is effective in patients with trisomy 12 in combination with fludarabine and cyclophosphamide	[11, 27, 28]
	Alemtuzumab	CD52	Is effective in patients with del(17p) or *TP53* mutations in combination with methylprednisolone	[12, 27, 28]
	Dacetuzumab	CD40	-	[26]
	Lucatumumab	CD40	-	[26]
	Mapatumumab	TRAIL-R1	-	[26]
	Blinatumomab	CD3/CD19	-	[26]
	mAb 37.1	CD37	-	[26]
Inducers of cell death	ABT-199	BH3 mimetic	Reduced inhibition of BCL-XL thereby inducing cell death	[26, 27]
	ABT-263 (Navitoclax)	BCL-2	Reduced inhibition of BCL-XL thereby inducing cell death	[26, 27]
	ABT 737	BCL-2	New drug to restore unbalanced apoptosis	[12]
	Obatoclax	Pan-BCL-2 family	BCL-2 antagonists	[26]
	oblimersen	BCL-2	New therapeutic to restore unbalanced apoptosis	[12]
	Fludarabine	Purine analog	Induces P53-dependent apoptosis	[11]
	Cyclosphophamide	Purine analog	Induces P53-dependent apoptosis	[11]
	Rapamycin	-	Inhibits the cell cycle	[11]
	Nultlin	P53 mimetic	Mimics the structure of P53 and binds to MDM2	[11]
	dehydroxymethylepoxyquinomicin	NF-κB	Can enhance the effect of fludarabine	[11]
Immune modulatory drugs	Lenalidomide	TNF-α, IL-7, VEGF	Inhibist cytokines	[27]
miRNAs inhibitors drugs	Antagomirs	miRNAs	Probably forming a duplex: (miRNA/antagomir) that induces the degradation of the targeted miRNA	[29]
	AMOs anti-miRs	miRNAs	Produce an ASO-miRNA double stranded complex, leading to non-specific endonuclease cleavage of the targeted miRNA	[29, 30]
	LNA anti-miRs	miRNAs	Same as the AMO	[29, 30]
	MTg-AMOs	miRNAs	Same as the AMO, and enables silencing of multiple target miRNAs	[29, 30]
	MicroRNA sponges	miRNAs	Results in increased expression of the miRNA's native targets by competing with the native targets of miRNAs,	[29]
Cyclin-dependent kinase inhibitors	Flavopiridol	CDK	Inhibits cyclin-dependent kinase and induces apoptosis	[11, 26]
	Dinaciclib	CDK 1,2,5,9	Inhibits cyclin-dependent kinase and induces apoptosis	[26]
	SNS-032	CDK 2,7,9	Inhibits cyclin-dependent kinase and induces apoptosis	[26]

BCR B-cell receptor, *BTK* Bruton tyrosine kinase, *SYK* spleen tyrosine kinase, *NF-κB* nuclear factor kappa B, *AMO* anti-miRNA oligonucleotides, *LNA* locked nucleic acid, *ASO* Antisense oligonucleotides, *MTg-AMO* multiple-target anti-miRNA antisense oligodeoxyribonucleotide, *CDK* cyclin-dependent kinase

mesenchymal stem cells (MSCs), that regulate their survival, maintenance and proliferation [43]. CLL cells in the BM interact with different types of cells, such as MSCs, monocyte-derived nurse-like cells (NLCs) and T-cells, collectively referred to as the "microenvironment" [44, 45]. Many factors produced by the CLL microenvironment are involved in its

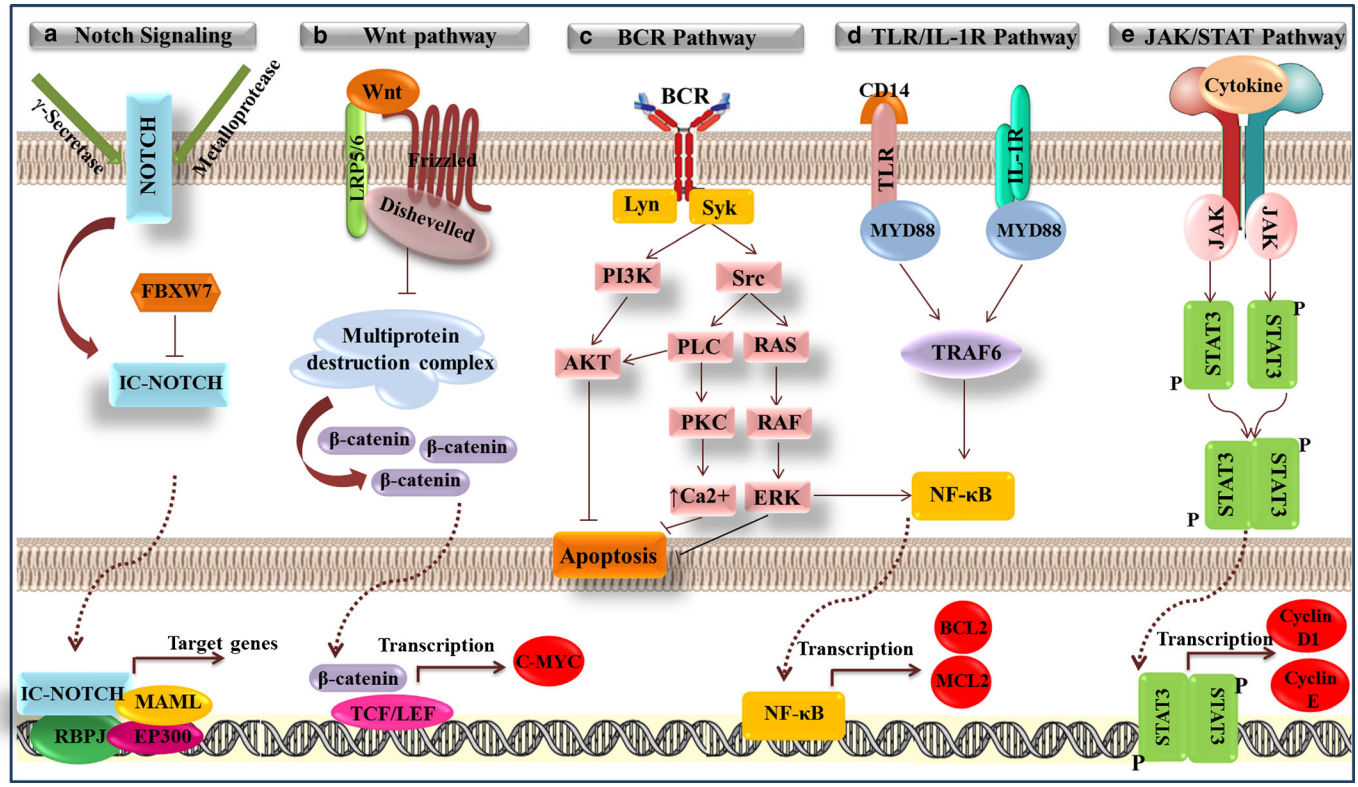

Fig. 1 Signaling pathways in CLL cells. **a**. NOTCH signaling is initiated by a series of proteolytic cleavages that lead to the release of the intracellular domain (IC-NOTCH) from the membrane to the nucleus. In the nucleus, IC-NOTCH recruits MAML, RBPJ and EP300, forming a complex that drives the transcription of its target genes. **b**. Binding of Wnt to Frizzled and LPR5/6 results in phosphorylation of β-catenin, release from its multi-protein complex and translocation to the nucleus, where it forms a protein complex that drives the transcription of target genes such as c-MYC. **c**. Increased activity of the BCR signaling pathway leads to activation of MAPK and NF-κB pathways followed by phosphorylation of the PI3K and Src kinase and subsequent transcription of the anti-apoptotic BCL-2 and MCL-2 protein coding genes, which results in inhibition of apoptosis and increased proliferation and survival of CLL cells. **d**. After activation of TLR/IL-1R, the MYD88 adapter protein becomes phosphorylated, leading to activation of downstream kinases and proteins, including TRAF6 and NF-κB. Increased NF-κB activation leads to inhibition of apoptosis and increased CLL cell survival. **e**. The JAK/STAT pathway is associated with cytokines, and its persistent activity results in STAT3 phosphorylation and an increased transcription of genes encoding cell cycle proteins, including Cyclin D1 and Cyclin E, which leads to increased CLL cell proliferation. *BCR* B-cell receptor, *TLR/IL-1R* toll-like receptor/interleukin-1 receptor, *JAK/STAT* Janus kinases/signal transducers and activators of transcription, *MAPK* mitogen-activated protein kinase, *NF-κB* nuclear factor κB, *MCL-2* myeloid cell leukemia-2

homing and trafficking, and it has been well-established that the microenvironment plays an important role in the pathogenesis of CLL [46–48].

B-cell surface receptor (BCR) stimulating cytokines, chemokines and adhesion molecules produced by the microenvironment in BM, lymph nodes and spleen play an important role in the accumulation, growth, survival and drug resistance of CLL cells [49]. CXCL12 and CXCL13 are chemokines that are constantly produced by MSCs and NLCs, and they can attract CLL cells that express high levels of its related receptors CXCR4 and CXCR5 and, by doing so, they can regulate the implantation and survival of leukemic cells in various tissues (Fig. 2) [50, 51].

CXCR4 (CD184), which is highly expressed on the surface of CLL cells, is regulated by its ligand CXCL12 (SDF1), thereby inducing the chemotaxis and migration of these cells, as well as their resistance to apoptosis-inducing drugs [43]. Also, a pro-survival effect of CXCL12 on CLL cells has been noted [52, 53]. Increased levels of CXCL12 have significantly been associated with the mobilization of HSCs into the peripheral bloodstream [6], and proliferating Ki67-positive CLL cells have been found to express higher CXCR4 and CXCR5 levels than resting cells [54]. CXCR5 (CD185) acts as a receptor of the chemokine CXCL13, thereby regulating the homing of lymphocytes and their orientation in lymphatic follicles, and is continuously secreted by stromal cells in follicular lymphoid B-cell regions [55]. CLL cells express high CXCR5 levels, thereby causing further stimulation of these cells and a long term activation of the MAPK (ERK1/2) pathway [44]. It has been shown that CXCR5 plays an important role in the orientation and interaction between malignant B-cells and CXCL13-secreting stromal cells in lymphoid tissues [44]. Activation of the BCR signaling pathway causes clonal proliferation of normal and malignant B-cells. In response to

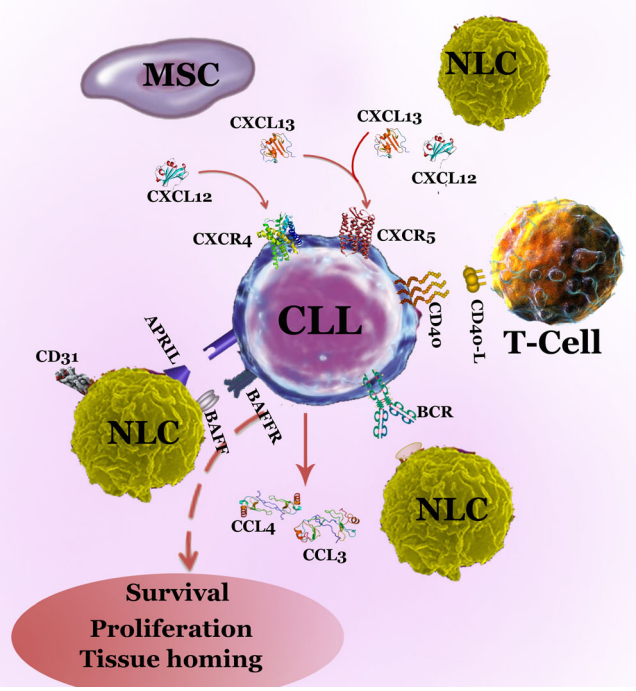

Fig. 2 Increased expression of the CXCR4 and CXCR5 receptors in CLL cells enhances the uptake of the chemokines CXCL12 and CXCL13 produced by MSCs and NLCs, thereby regulating the implantation and survival of leukemic cells in various tissues. Also, hyper-secretion of CCL3/CCL4 by CLL cells as a result of stimulation by CD40-L and BCR can induce the trafficking and implantation of accessory cells in the BM. The expression of BAFF, APRIL and CD31 proteins on NLC cells causes induction of anti-apoptotic pathways and survival of CLL cells. *MSCs* Mesenchymal stem cells, *NLCs* nurse-like cells, *BAFF* B-cell activating factor of the tumor necrosis factor (TNF) family, *APRIL* a proliferation-inducing ligand

BCR activation, CLL cells release the cytokines CCL3 and CCL4 (also called MIP-1α and β), which are likely to be involved in the recruitment of accessory cells like T-reg cells (Fig. 2) [50]. In addition to production of the chemokines CXCL12 and CXCL13, NLC cells express the B-cell activating factor of TNF family (BAFF), proliferation-inducing ligand (APRIL), CD31 and plexin-B1 proteins, which protect CLL cells against apoptosis and activate cell survival pathways (Fig. 2) [56]. CCL3 and CCL4 act as chemical attractants for monocytes and lymphocytes, and their expression is stimulated by BCR and CD40 ligands and suppressed by BCL6 [56, 57]. CLL patients exhibit higher levels of CCL3/CCL4 in their plasma compared to normal individuals, and the CCL3 plasma level has been associated with prognostic factors and treatment duration [24]. Increased secretion of CCL3/CCL4 by CLL cells can induce the trafficking and implantation of accessory cells, especially T-cells and monocytes, in the microenvironment [58]. CCL3 is secreted by CLL cells following activation of the BCR pathway. Inhibition by SYK inhibitors and PI3Kδ inhibits CCL3 secretion. It has been shown that the level of CCL3 in plasma of patients under treatment with these inhibitors reverts to normal after approximately 28 days [24].

ZAP-70 protein expression in CLL patients causes increased responses to the chemokines CCL19, CCL21 and CXCL12 which, in turn, results in an increased migration of CLL cells and an activation of pro-survival signals [24, 59]. The integrin VLA-4 (CD49d) is mainly expressed on the surface of hematopoietic cells, i.e., lymphocytes and monocytes, and plays a role in cell trafficking and implantation. VLA-4 also plays an important role in the adhesion of CLL and other leukemic cells to stromal cells and the extracellular matrix (ECM), and its increased expression in CLL cells has been shown to cause resistance to fludarabine [44, 45]. Thus, changes in expression of various receptors on CLL cells and differences in their affinities can stimulate the activity of relevant signaling pathways, resulting in increases in the survival, implantation and trafficking of these cells. In addition, the altered expression of cell adhesion molecules and the altered proliferation rate of CLL cells enables them to infiltrate into lymph nodes and endows them with the ability to develop lymphadenopathy. Awareness of these alterations may be of relevance for the treatment of patients [51].

3 Cytogenetic abnormalities as diagnostic and prognostic biomarkers

Several numerical and structural chromosomal aberrations in hematologic malignancies can be detected using karyotyping [60]. This cytogenetic technique requires dividing cells, has a relatively low resolution (20–30 Mb) and hampers the identification of small structural changes [60, 61]. A markedly increased sensitivity of this technique was reached by the application of high-resolution banding, through which the chromosomes are evaluated in the prometaphase state (3–5 Mb). This has enabled the identification and demarcation of various structural aberrations such as duplications, deletions and inversions [62]. Nevertheless, karyotyping of CLL cells only allows the detection of 40–69 % of all clinically relevant chromosomal abnormalities present, such as del(11q23), trisomy 12, del(13q14) and del(17p13), mainly due to the low in vitro proliferative capacity of CLL cells, even in the presence of B-cell mitogens [36, 37]. Therefore, high-resolution molecular (cyto)genetic techniques have been developed to more sensitively detect chromosomal aberrations, including multiplex ligation-dependent probe amplification (MLPA) [63] and SNP microarray-based comparative genomic hybridization (array-CGH) [60]. The latter technique allows the detection of small deletions or duplications as well as gross chromosomal imbalances, and has rapidly become a standard method for the evaluation of cytogenetic abnormalities in CLL [64, 65]. A major advantage of this technique over karyotyping is the rapid identification of any chromosomal loss or gain without

requiring dividing cells [66, 67]. Since chromosomal abnormalities are encountered in ~80 % of CLL cases, the use of the above mentioned novel techniques can be applied to the identification of such abnormalities. Below, the most common chromosomal abnormalities and their characteristics are discussed. In Table 2 the most common chromosomal abnormalities are listed.

13q- The 13q14 deletion is the most common chromosomal abnormality and is found in 40–60 % of CLL cases [75, 79]. This deletion can be either heterozygous [mono-allelic (76 %)] or homozygous [bi-allelic (24 %)]. It has been shown that heterozygous deletions mostly occur in the early stages of the disease, and that homozygous deletions occur in the more advanced stages. Detailed analysis of the 13q14 deletion by SNP arrays has revealed the occurrence of two deletion types: type I, which encompasses the miR15a/16 locus but not the *RB1* locus, and type II, which also encompasses the *RB1* locus and is associated with a more aggressive disease type. Large deletions are often homozygous, whereas small deletions are usually heterozygous [12, 80]. CLL patients with del(13q) show a better prognosis and a longer overall survival than CLL patients with del(11q23) or del(17p) (see below;[69]). Also the percentage of CLL cells with del(13q) is associated with survival, i.e., a high percentage (> 80 %) of del(13q) cells results in a shorter survival compared to patients with a lower percentage (<80 %) of these cells [81–83]. In addition, it has been shown that patients with a higher percentage of del(13q) cells have a higher lymphocyte count and tend to exhibit a diffuse pattern of BM infiltration and splenomegaly. In addition, increased expression of genes involved in the MAPK signaling pathway and decreased expression of cell cycle arrest-associated genes (including *CDKN2C, GAS2L1, GAS1, GAS7, ZAK,GAS2L3)* has been observed in such patients, which may lead to increased cell proliferation. In contrast, decreased expression of apoptosis-associated genes (such as *CASP6, CLU, DAPK1* and *E2F1)* has been found to result in a reduced apoptotic activity and an increased survival of CLL cells [81].

11q- The 11q23 deletion is seen in 10–20 % of CLL cases, and most patients with this deletion are relatively young [73]. The deletion is associated with lymphadenopathy, a rapid disease progression, a poor response to treatment and a short

Table 2 Most frequent clinically relevant chromosomal aberrations in CLL

Chromosomal aberration	Prevalence (%)	Gene target	Result of aberration	Prognosis	miRNAs	References
Del 6q	~6 %	….	-Associated with more prominent lymphocytosis -Atypical morphology -Splenomegaly -Higher rates of CD38 positivity	Poor	….	[68, 69]
Del 11q	10–20 %	*ATM*	-Defect in DNA repair -Deregulation of P53 -Deregulation of cell cycle -Extensive lymphadenopathy	Poor	↑miR-29b ↑miR-155 ↓miR-29a ↓miR-34b ↓miR-34c	[70–74]
Del 13q	40–60 %	*miR-15a/16-1*	-Including BCL2 expression -Resistance to apoptosis	….	↑miR-155 ↑miR-7-1 ↑miR-154 ↓miR-15a/16-1 ↓miR-220 ↓miR-221	[70, 71, 75, 76]
Del 17p	3–8 %	*TP53*	-Defect in DNA repair -Deregulation of cell cycle -Associated with an aggressive clinical phenotype	Poor	↑miR-151 ↓miR-29c ↓miR-34a ↓miR-148a ↓miR-181	[25, 77, 78]
Trisomy 12	10–20 %	….	-Atypical morphology -Associated with an aggressive clinical phenotype	Poor	↑miR-148a ↑miR-146a ↑miR-29b ↑miR-155	[25, 31, 71]

Del deletion, *ATM* ataxia telangiectasia mutated, *TP53* tumor suppressor P53

overall survival time [84, 85]. The 11q23 deletion encompasses several genes, including the *ATM* tumor suppressor gene (position 11q23.3-23.1). The various functions of this gene include cell cycle checkpoint control, double-strand DNA break repair, resistance to oxidative stresses, regulation of the P53 protein, as well as protection of telomere regions. The role(s) of other genes located in the 11q23 region remains to be resolved [9, 86, 87].

17p- This chromosomal deletion is usually associated with a high aggressiveness and a lack of response to therapy. Del(17p) is observed in 3–8 % of CLL cases, and is often associated with deletion of the *TP53* tumor suppressor gene [25, 88]. *TP53* plays an essential role in the induction of apoptosis and cell cycle arrest following DNA damage. Patients with 17p deletions or *TP53* gene mutations are resistant to purine analogs. Standard treatment with fludarabine, cyclophosphamide and/or rituximab is, therefore, not very useful for this group of patients. In contrast, however, alemtuzumab is very effective in these patients, especially in combination with methylprednisolone [12, 89, 90]. Nevertheless, the overall survival of these patients is lower than those with less unfavorable genetic aberrations, and allogeneic stem cell transplantation is recommended for this group of patients in case of a good physical condition [12, 91].

Trisomy 12 This anomaly is encountered in 10–20 % of CLL cases, and is associated with a typical morphology and a typical immunophenotype [73, 92]. Small duplications have also been reported encompassing the 12q15 region, which harbors the *MDM2* gene locus. Duplication of 12q22, encompassing the *CLLU1* gene locus, serves as a prognostic risk factor in patients younger than 70 years of age [93, 94]. In addition, impaired Hedgehog (HH) signaling has often been found to be associated with trisomy 12. Increased activity of the HH signaling pathway in CLL patients, which is associated with factors such as SUFU, GLI1 and GLI3, has been found to be the leading cause of CLL disease progression [95, 96]. Treatment of these patients with fludarabine and cyclophosphamide, along with rituximab (anti CD-20), is thought to be effective due to a high level of CD20 expression in CLL cells [12, 26].

6q- Deletion of the long arm of chromosome 6 is a relatively rare aberration occurring in approximately 6 % of CLL cases, and is associated with marked lymphocytosis with an abnormal morphology, splenomegaly, over-expression of CD38 and an unmutated state of the immunoglobulin heavy chain variable region (UM-IGHV) [68, 69].

Other rare chromosomal aberrations in CLL include 14q32 translocations (7 %), trisomy 3q27 (3 %), trisomy 8q24 (5 %), trisomy 18 and trisomy 19 [73].

4 Gene mutations as diagnostic and prognostic biomarkers

Currently, Sanger sequencing is considered as the first generation sequencing technology, whereas more recently developed advanced next generation sequencing (NGS) techniques are considered as second or third generation techniques [97] allowing, among others, comprehensive de novo sequencing, re-sequencing and SNP profiling. NGS has been used for the identification of structural changes, copy number variations, polymorphisms, point mutations and genomic anomalies previously undetectable by Sanger sequencing [40, 98]. Also, mRNA and micro-RNA expression profiling are applications of these latter NGS techniques [98–100]. In the recent past, NGS has also be used for a better delineation of complex DNA mutations in CLL [41, 42, 101].

Mutations in specific genes have been reported to be associated with the etiology, progression and treatment of CLL. Some of these mutations are known to affect the activity of relevant cellular signaling pathways. The most frequent mutations and their roles are listed in Table 3. The B-cell surface receptor (BCR) encompasses the monomeric immunoglobulins IgM and IgD. The variable regions of these receptor molecules recognize foreign antigens, and Igα and Igβ are responsible for their intracellular signaling through phosphorylation of Src family kinases and subsequent activation of downstream effectors. Activation of the BCR signal transduction pathway is involved in the regulation of metabolism, survival, proliferation, differentiation and migration of various cell types, including CLL cells (Fig. 1c) [47, 108]. Therefore, the BCR signaling pathway may serve as a therapeutic target in CLL patients. Ibrutinib (a Bruton tyrosine kinase inhibitor), GS-1101, idelalisib (a PI3K-δ inhibitor) and fostamatinib (a spleen tyrosine kinase inhibitor) are among the inhibitors of this signaling pathway. They prevent its activation via inhibiting Src family kinases and, by doing so, result in apoptosis induction [16, 109]. Approximately 60–65 % of CLL cases show mutations in the immunoglobulin heavy chain variable region *(IGHV)*, which can alter the affinity of BCR for antigens. The remaining 35–40 % of CLL cases lack *IGHV* mutations [25, 102]. *IGHV* gene mutations are markers for a favorable prognosis, i.e., unmutated CLL (U-CLL) is clinically more aggressive than mutated CLL (M-CLL) [110, 111]. The overall survival rate for U-CLL patients is 8–9 years, whereas that for M-CLL patients is more than 24 years. The unmutated cases are associated with the expression of CD38 and ZAP-70, which are markers for a poor prognosis [112, 113].

TP53 mutations DNA damage induces the activation of a variety of protein kinases, such as ATM, ATR, Chk1, Chk2 and DNA-dependent protein kinases. These kinases can form complexes and, subsequently, phosphorylate P53, thereby

Table 3 Most frequent somatic mutations in CLL

Gene mutation	Prevalence (%)	Association with	Functional role	Kind of mutation	Prognosis	References
IGHV	60–65 %	….	M-CLL typically has slower disease progression and longer survival.	….	Good	[102]
TP53	4–12 %	Del (17p13)	An essential role in apoptosis or cell cycle arrest after DNA damage.	inactivation	Poor	[88, 102]
ATM	~12 %	Del (11q23)	Activates cell cycle checkpoints. Induces apoptosis in response to DNA breaks.	inactivation	Poor	[84]
NOTCH1	~10 %	Trisomy 12	Regulates target genes, including *MYC*, *TP53* and molecules of the NFκB pathway.	activation	Poor	[28, 103]
		UM- IGHV	Plays an important role in cell differentiation, proliferation, and apoptosis.			
SF3B1	5–10 %	Del (11q22-q23) ATM mutations, UM- IGHV, ZAP-70 expression	A core component of the spliceosome.	inactivation	Poor	[28, 102]
BIRC3	~4 %	del(11q22-q23)	A negative regulator of the MAP3K14 (activator of NF-κB signaling).	inactivation	Poor	[104, 105]
MYD88	3–5 %	M- IGHV and del (13q14)	Phosphorylated MYD88 resulting in activation the NF-κB pathway.	activation	….	[106, 107]

IGHV immunoglobulin heavy chain variable, *TP53* tumor supresor P53, *ATM* ataxia telangiectasia mutated, *SF3B1* splicing factor 3B subunit 1, *BIRC3* baculoviral IAP repeat-containing protein3, *NOTCH1* neurogenic locus notch homolog protein1

releasing it from MDM2 (a negative regulator of P53) causing cell cycle arrest and activation of the apoptotic pathway [114]. *TP53* gene mutations have been observed in 4–12 % of untreated CLL cases, and in most cases (~70 %) with 17p deletions [88, 102]. The presence of>20 % cells with a *TP53* deletion or mutation is associated with a poor prognosis, whereas the prognosis of patients with<20 % of such cells is similar to the overall prognosis. It has amply been shown that the presence of *TP53* mutations/deletions is associated with disease progression, a poor response to chemotherapy and a short survival of CLL patients [115, 116].

ATM mutations The *ATM* gene is a member of phosphatidylinositol-3 kinase (PI3K) gene family and encodes a nuclear serine/threonine kinase. *ATM* expression increases following double strand DNA damage, causing arrest in the G1/S and G2/M phases of the cell cycle and activation of DNA repair pathways, as well as apoptosis induction in case of lack of suitable DNA repair [117]. *ATM* gene mutations have been reported in 12 % of CLL patients and in about 30 % of CLL patients with a 11q23 deletion [118–120]. Lack of ATM protein expression causes loss of P53 function, chronic severe oxidative stress, rapid telomere shortening, loss of cell cycle checkpoint control, defective DNA repair and, thereby, disease progression [121]. Individuals carrying mutations in this gene have a 70-fold increased risk to develop a lymphoid malignancy compared to individuals without such mutations. The two most common *ATM* mutations include p.R2691C and p.P2699S, occurring in the ATP-binding site of the ATM protein and causing defects in its kinase activity [28, 122]. Bi-allelic mutations or deletions of the *ATM* gene following primary treatment with alkylating agents or treatment with purine analogs have been associated with a poorer prognosis and response to treatment compared to mono-allelic mutations or deletions [123].

NOTCH1 mutations NOTCH1 is a trans-membrane protein encoded by the *NOTCH1* gene and regulates the expression of several downstream target genes, such as *c-MYC* and *TP53,* and genes in the NF-κB pathway such as *SCL, GATA2 and RUNX1*, which are required for normal hematopoiesis [124, 125]. NOTCH1 is involved in determining cell fate, growth, differentiation and self-renewal, as well as the maintenance of stem cells and apoptosis in many tissues during normal embryonic and postnatal development [126]. Activating *NOTCH1* mutations in CLL cells lead to persistent expression of the NOTCH1 protein and its binding to the Jagged-1 (JAG1) ligand, resulting in an increased activity of this pathway. The NOTCH signaling pathway has been shown to play a role in the survival and resistance to apoptosis in CLL cells [127, 128]. *NOTCH1* mutations in aggressive clinical phases of CLL have been associated with a poor survival, resistance to treatment, disease progression and an increased risk of progression to Richter's syndrome [21, 41]. *NOTCH1* activating mutations have been observed in 10 % of B-CLL cases and in 60 % of T-ALL cases [25, 129]. *NOTCH1* mutations are associated with U-CLL and trisomy 12, and are associated with a poor prognosis [130, 131]. *NOTCH1* mutations often represent frame shift mutations causing an increased stability and defective degradation of the NOTCH1 protein, as well as accumulation of an active isoform of NOTCH1 by creating an early stop codon in the C-terminal region of the second

functional PEST domain [132]. C.7544-7545del CT is the most common *NOTCH1* mutation occurring in~80 % of the CLL cases [28, 102].

SF3B1mutations The mRNA splicing process is catalyzed by spliceosomes, which encompass a series of small ribonucleoproteins (snRNPs), including U2. Splicing factor 3B1 (SF3B1) is an important component of U2-snRNP [102, 133]. Inactivating mutations in the *SF3B1* gene, causing defective splicing of various mRNAs followed by defective protein synthesis, have been reported in 5–10 % of the CLL cases. These mutations often represent missense mutations occurring in hot spots in codons 662, 666, 700, 704 encoding the second HEAT domain [101, 133]. These mutations mostly occur in the final stages of CLL development, and are associated with ZAP-70 protein expression as well as 11q23 deletions and unmutated *IGHV* genes, along with adverse clinical symptoms and a poor prognosis [42, 101].

BIRC3 mutations The BIRC3 protein participates in a complex together with the TRAF2 and TRAF3 proteins and acts as a negative regulator of MAP3K14 (an abnormal activator of the NF-κB signaling pathway). Inactivating *BIRC3* mutations cause over-activation of the MAP3K14 pathway and increased NF-κB levels, resulting in enhanced proliferation and resistance to apoptosis [25, 105]. Most *BIRC3* mutations in CLL occur in the C-terminal region of the second RING domain, which is essential for the proteosomal degradation of MAP3K14 by BIRC3 [102]. Overall, *BIRC3* mutations are rare in CLL patients (~4 %), but *BIRC3* inactivation is commonly seen in relapsed and fludarabine-refractory CLLs (~25 % of the cases) [28, 104].

MYD88 mutations The interleukin-1 receptor/Toll like receptor (IL-1R/TLR) signaling pathway is involved in primary immune responses and homeostasis of B-cells, and its activation causes an increased production of pro-inflammatory cytokines [134, 135]. Recently, a correlation has been found between increased expression of TLR1, TLR6, TLR7, TLR9 and an increased expression of downstream effectors, including NF-κB, c-Jun and MAPK, in CLL patients [134, 136]. MYD88 serves as an adapter protein in the IL-1R/TLR signaling pathway [137]. It becomes phosphorylated upon activation of TLR and IL-1R and causes activation of NF-κB after activating kinases and downstream proteins such as TRAF6 (Fig. 1c) [103, 106]. *MYD88* activating mutations have been shown to increase the activity of this pathway in CLL patients and to cause resistance to apoptosis [107]. *MYD88* mutations are found in 3–5 % of CLL cases, as also in other B-cell malignancies such as diffuse large B-cell and marginal zone lymphomas [100, 138].

Other mutations reported in CLL include mutation in the *KRAS*, *SMARCA2*, *NFKBIE* and *PRDK3* genes [12].

5 Epigenetic alterations as diagnostic and prognostic biomarkers

Epigenetic changes do not alter the DNA sequence itself but, instead alter DNA methylation and histone methylation or acetylation patterns. Several studies have highlighted the importance of these processes in CLL and other malignancies [139–141]. Activation of proto-oncogenes through hypomethylation of DNA promoter sequences is a relatively common change observed in CLL. Conversely, promoter hypermethylation has been found to play a role in the silencing of tumor suppressor genes [142], i.e., hyper-methylation of the *P16 INK4A* and *P15INK4B* gene promoters has been reported in a sub-group of CLL patients [143–145]. A high global level of DNA methylation in *IGHV*-unmutated CLL patients has been associated with a poor prognosis and patients with a high methylation index (MI) need to be treated, whereas those with a low MI require a 'wait and see' policy [28]. Hypomethylation of the *BCL-2* gene promoter in CLL patients results in its over-expression [146] and hypo-methylation of *MDR1* and *TCL1* gene promoters results in NF-κB activation, a phenomenon that is frequently seen in CLL patients [78]. Hyper-methylation of the *TWIST-2* gene promoter is an important factor that is associated with *TP53* gene silencing and has been reported to occur in CLL patients [145, 147]. It has also been shown that in patients with *IGHV* mutations, methylation of the *CD38* and *BTG4* gene promoters is associated with a favorable prognosis, whereas methylation of the *HOXA4* gene promoter in these patients is associated with a poor prognosis. The expression level of ZAP-70 is related with methylation of specific CPG islands in the promoter of this latter gene [148–150].

It has also been shown that epigenetic mechanisms are involved in changes in expression of miRNAs (see below) in CLL [151]. It has e.g. been shown that the miR-139 and miR-582 promoters are targets of significant methylation and, thus, silencing in CLL patients [78], whereas hypomethylation of the miR-21and miR-29a promoters is associated with an increased expression of these miRNAs. Conversely, hyper-methylation of the miR-124-2 and miR-129-2 promoters has been associated with a decreased expression of these miRNAs [78, 152]. Hyper-methylation of the miR-9-3 promoter, a miRNA that has a tumor suppressor function, is frequently observed in CLL, causing silencing of its expression and sustained activation of the NF-κB signaling pathway, inducing resistance to apoptosis [153]. Hyper-methylation of the miR-34b/c promoter, leading to silencing, is also commonly seen in CLL [72].

Due to their reversible nature, epigenetic changes serve as prime treatment targets. The application of DNA methyltransferase (DNMT) and histon deacetylase (HDAC) inhibitors, capable of reducing DNA methylation and histone deacetylation, may serve as a novel treatment strategy in

CLL [16, 78]. The efficacy of several of these compounds has already been tested in vitro on CLL cells and are currently subject to clinical trials [154].

6 Micro-RNAs as prognostic biomarkers

Micro-RNAs are small non-coding RNA molecules causing mRNA translation inhibition and/or degradation through binding to its 3′untranslated regions (UTR) [155, 156]. Several studies have reported changes in expression of miRNAs in human malignancies [155, 157]. In CLL, specific miRNAs can be used as etiologic and prognostic (therapy-related) markers, as outlined below.

MiR-15a/16 acts as a tumor suppressor by inhibiting the expression of *BCL-2* [76]. Since the miR-15a/16 locus is located in 13q14, deletion of this region in CLL (see above) is a main cause of its decreased expression. There may, however, also be other causes for this decreased expression, including regulatory *TP53* gene mutations and deletions or mutations in pri-miR transcription factors [158, 159]. Decreased miR15a/16 expression occurs in a considerable proportion of CLL cases (68 %) [76, 160] and leads to increased *BCL-2* expression and resistance to apoptosis, or increased expression of cell cycle-related genes, including *CCND2, CCND3* and *CDK4/6*, which play an important role in the transition of cells from the G0/G1 to S phase [159, 161].

As a tumor suppressor, miR-29b inhibits the expression of several proto-oncogenes, including *TCL1, MCL1, CDK2/6, SP-1*, *DNMT3a* and *DNMT3b* [162, 163]. Reduced miR-29 expression and increased *TCL1* expression in CLL patients has been associated with an aggressive phenotype, an unmutated *IGHV* status, over-expression of ZAP-70 and deletion of 11q23 [71, 146]. *TCL1* proto-oncogene expression has been found to be increased in ~90 % of CLL patients, and is variably expressed during different stages of the disease. The TCL1 protein acts as a co-activator of the PI3K/Akt kinase, which causes apoptosis reduction by phosphorylation and inactivation of BAD (a pro-apoptotic protein). Additionally, activation of NF-κB (a pro-survival protein) by TCL1 has been found to increase the BCL-2 protein level and, as a consequence, to inhibit apoptosis in CLL cells [11, 164]. It has been found that TCL1 expression is regulated by miR-29 and miR-181, and down-regulation of these miRNAs has been shown to cause its increased expression, followed by a decrease in apoptosis [163]. Expression regulation of the MCL1 and BCL-2 family of anti-apoptotic proteins is mediated by miR-29, and reduced expression of miR-29 in CLL increases its resistance to apoptosis [165].

MiR-34a is a direct transcriptional target of the P53-pathway, and its increased expression leads to apoptosis and cell cycle arrest through the regulation of proteins such as BCL-2, cyclin D1 and E2, CDK4 and c-MYC, depending on the cell type [77, 155]. Several studies have shown that deletion or mutation of the *TP53* gene can cause a decreased expression of miR-34a, defective DNA repair and apoptosis responses, and fludarabine resistance of CLL cells [159, 160]. The expression of miR-34a has been found to be decreased in patients with 17p deletions or *TP53* mutations, causing a fludarabine refractory phenotype [18, 166]. Moreover, decreased expression of miR-34a, miR-29c and miR17-5p has been observed in aggressive subtypes of CLL with *TP53* abnormalities, indicating a role of target genes of these miRNAs in CLL pathogenesis [165].

As an oncogene (oncomiR), miR-181 is involved in B-cell CLL development [31], and it has been found that decreased expression of miR-181 increases the levels of the MCL1 and BCL-2 anti-apoptotic proteins [167]. Specifically, miR-181b has been shown to regulate the expression of TCL1, and its reduced expression has been proposed as an unfavorable prognostic factor for CLL and to be related to refractoriness to treatment [19]. In CLL patients with 17p deletions, increased expression of miR-21 has been associated with a poor prognosis. Additionally, in this group of patients decreased expression of miR-223, miR-29b and miR-181 family members has been found to be more prevalent in aggressive than in indolent cases. So, it has been suggested that these miRNAs can be used as markers for the identification of del(17p)-positive CLL patients with an adverse prognosis [168, 169].

Also miR-155 acts as an oncogene and its over-expression has been reported in several solid tumors, including lung, colon and breast carcinomas, as well as in hematological malignancies, including AML and CLL [70, 170]. It has been reported that miR-155, and other miRNAs such as miR-182 and miR-96, regulate several components of the PI3K signaling pathway [171]. Additional studies have shown that miR-155, and also miR-181a and miR-223, are involved in the normal development and differentiation of lymphoid and myeloid cells, and that their altered expression is associated with the development of its respective corresponding leukemias, i.e., increased expression of miR-155 and miR-181a causes enhanced survival and proliferation in both hematopoietic lineages [172].

MiR-221/222 expression has been found to be increased in many solid tumors, including lung, thyroid and hepatocellular carcinomas. The primary target of miR-221/222 is P27, which functions as a tumor suppressor by inhibiting cell cycle progression [173]. In CLL, increased miR-221/222 expression has been found to cause resistance to fludarabine [77]. Additional studies have indicated that miR-223 may serve as a prognostic biomarker that may be used to predict the overall survival (OS) and time to first treatment (TFT) [174, 175].

The miR-146a locus is located on chromosome 5, and its expression is increased in some inflammatory diseases and malignancies, including CLL. Several studies have indicated a relationship between miR-146a expression and

tumorigenesis through inappropriate regulation of inflammatory responses [176]. Increased miR-146a expression has been associated with cell migration, motility and adhesion [168, 177], and increased transcription of the integrin encoding genes *ITGA4* and *ITGB2.* These latter genes are involved in CLL via the inhibition of $\alpha_4\beta_1$ and $\alpha_L\beta_2$ heterodimers in trans-endothelial migration (TEM) during tissue invasion. Therefore, it has been suggested that inhibitors of this miRNA may be useful as therapeutic agents in CLL [176, 177].

Although miRNAs may, as yet, not be very useful as diagnostic indicators, they can be used as biomarkers for predicting response to therapy and survival, and also to better understand the pathogenesis of CLL cells [18, 178]. Since miRNAs can function both as oncogenes and tumor suppressor genes during leukemogenesis, miRNA-based therapies have recently been explored as therapeutic options, and some progress has indeed been made in this field (Table 1). It has e.g. been shown that chemically engineered anti-miRNA oligonucleotides (AMOs or antagomirs) can specifically bind to certain miRNAs and thereby inhibit their function [178]. The use of these oligonucleotides in conjunction with traditional treatment protocols may provide novel treatment strategies for (sub-groups of) CLL patients [29, 30].

7 Conclusions and future directions

CLL is a clinically heterogeneous disease with survival rates ranging from months to decades. Also genetically CLL is heterogeneous, as exemplified by the occurrence of various gross chromosomal alterations (including 11q-, 13q-, 17p- and +12), immunoglobulin heavy chain gene mutations, mutations in several signal transduction pathway genes including *NOTCH1, BIRC3, SF3B1, TP53*, and alterations in miRNA expression patterns. Also serum levels of several proteins, including B2M, TK and LD, and the expression levels of several immunophenotypic markers, such as CD38 and ZAP-70, may be altered. It has been found that there is a strong relationship between specific genetic aberrations and the clinical course of CLL. Specifically, the expression of unmutated immunoglobulin heavy chain variable region genes, chromosomal abnormalities such as del(17p) and del(11q), and the expression of the ZAP-70 and CD38 proteins have been associated with a poor prognosis. Mutations in tumor suppressor genes, such as *TP53* and *ATM,* have been associated with a more aggressive course of the disease and, in addition, to resistance to standard treatment protocols. Since CLL patients usually bear several genetic abnormalities, a combination of various treatments is often used, and its choice should be tailored to the abnormalities present. For example, patients with del(17p) and/or a *TP53* mutation are resistant to fludarabine, cyclophosphamide and rituximab, whereas alemtzumab combined with methylprednisolone has been shown to be highly effective. The identification of new biomarkers will be of help for a proper patient stratification and treatment choice and, in parallel, the efficacy of new drugs should be tested in corollary with the presence of these new biomarkers (i.e., molecular lesions) in CLL patients.

In addition to genetic biomarkers, also epigenetic changes and miRNA expression alterations have been addressed as prognosticators. Although not yet routinely used as diagnostic markers, miRNAs can be applied as biomarkers to predict response to treatment and survival in patients and to better understand disease etiology. Because of the reversible nature of epigenetic changes and the dual role of miRNAs as both oncogenes and tumor suppressor genes in leukemogenesis, they are considered as proper therapeutic targets. Already, a number of them has been tested in vitro with satisfactory results and, based on this, included in clinical trials. Therefore, a further understanding of the (epi)genetic basis in CLL is expected to lead to an improved clinical management through specifically designed targeted therapies.

Acknowledgments We wish to thank all our colleagues in the Shafa Hospital and Allied Health Sciences School, Ahvaz Jundishapur University of Medical Sciences.

Conflict of interest The authors declare no conflict of interest.

References

1. F. Talab, J.C. Allen, V. Thompson, K. Lin, J.R. Slupsky, LCK is an important mediator of B-cell receptor signaling in chronic lymphocytic leukemia cells. Mol. Cancer Res. **11**, 541–554 (2013)
2. T. Robak, Inhibitors of B-Cell receptor signaling for the treatment of chronic lymphocytic leukemia. J. Leuk. **1**, 101 (2013)
3. G. Dighiero, T. Hamblin, Chronic lymphocytic leukaemia. Lancet **371**, 1017–1029 (2008)
4. C. Rozman, E. Montserrat, Chronic lymphocytic leukemia. N. Engl. J. Med. **333**, 1052–1057 (1995)
5. J.A. Zwiebel and B.D. Semin. Oncol. 42–59 (1998).
6. S.E. Herman, A.L. Gordon, A.J. Wagner, N.A. Heerema, W. Zhao, J.M. Flynn, J. Jones, L. Andritsos, K.D. Puri, B.J. Lannutti, Phosphatidylinositol 3-kinase-δ inhibitor CAL-101 shows promising preclinical activity in chronic lymphocytic leukemia by antagonizing intrinsic and extrinsic cellular survival signals. Blood **116**, 2078–2088 (2010)
7. E. Matutes, R. Morilla, K. Owusu-Ankomah, A. Houlihan, D. Catovsky, The immunophenotype of splenic lymphoma with villous lymphocytes and its relevance to the differential diagnosis with other B-cell disorders. Blood **83**, 1558–1562 (1994)
8. E. Matutes, K. Owusu-Ankomah, R. Morilla, M.J. Garcia, A. Houlihan, T. Que, D. Catovsky, The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia **8**, 1640–1645 (1994)
9. R.E. Shackelford, A.R. Bhalodia, J.D. Cotelingam, D.M. Veillon, M. Lowery-Nordberg, Increased transferrin receptor expression following 11q23 deletion as a mechanism of malignant progression in chronic lymphocytic leukemia. Med. Hypotheses **66**, 509–512 (2006)

10. S. Willimott, D. Beck, M.J. Ahearne, V.C. Adams, S.D. Wagner, Cap-translation inhibitor, 4EGI-1, restores sensitivity to ABT-737 apoptosis through cap-dependent and-independent mechanisms in chronic lymphocytic leukemia. Clin. Cancer Res. **19**, 3212–3223 (2013)
11. J. Chen, N.A. McMillan, Molecular basis of pathogenesis, prognosis and therapy in chronic lymphocytic leukaemia. Cancer Biol. Ther. **7**, 174 (2008)
12. A.E. Rodríguez-Vicente, M.G. Díaz, J.M. Hernandez-Rivas, Chronic lymphocytic leukemia: a clinical and molecular heterogenous disease. Cancer Genet. **206**, 49–62 (2013)
13. Y. Pekarsky, N. Zanesi, C.M. Croce, Molecular basis of CLL. Semin. Cancer Biol. **20**, 370–376 (2010)
14. N. Chiorazzi, K.R. Rai, M. Ferrarini, Chronic lymphocytic leukemia. N. Engl. J. Med. **352**, 804–815 (2005)
15. M.L. Palomba, K. Piersanti, C.G. Ziegler, H. Decker, J.W. Cotari, K. Bantilan, I. Rijo, J.R. Gardner, M. Heaney, D. Bemis, Multidimensional single-cell analysis of BCR signaling reveals proximal activation defect as a hallmark of chronic lymphocytic leukemia B cells. PLoS One **9**, e79987 (2014)
16. S. Stilgenbauer, T. Zenz, Genetics in chronic lymphocytic leukemia. Leukemia **16**, 993–1007 (2002)
17. D. Mertens, S. Stilgenbauer, Prognostic and predictive factors in patients with chronic lymphocytic leukemia: relevant in the era of novel treatment approaches? J. Clin. Oncol. **32**, 869–872 (2014)
18. J. Ma, C. Dong, C. Ji, MicroRNA and drug resistance. Cancer Gene Ther. **17**, 523–531 (2010)
19. R. Rosenquist, D. Cortese, S. Bhoi, L. Mansouri, R. Gunnarsson, Prognostic markers and their clinical applicability in chronic lymphocytic leukemia: where do we stand? Leuk. Lymphoma **54**, 2351–2364 (2013)
20. E. San Jose-Eneriz, X. Agirre, P. Rodríguez-Otero, F. Prosper, Epigenetic regulation of cell signaling pathways in acute lymphoblastic leukemia. Epigenomics. **5**, 525–538 (2013)
21. L. Mansouri, N. Cahill, R. Gunnarsson, K.E. Smedby, E. Tjönnfjord, H. Hjalgrim, G. Juliusson, C. Geisler, R. Rosenquist, NOTCH1 and SF3B1 mutations can be added to the hierarchical prognostic classification in chronic lymphocytic leukemia. Leukemia **27**, 512–514 (2012)
22. L. Mansouri, P. Grabowski, S. Degerman, U. Svenson, R. Gunnarsson, N. Cahill, K.E. Smedby, C. Geisler, G. Juliusson, G. Roos, Short telomere length is associated with NOTCH1/SF3B1/TP53 aberrations and poor outcome in newly diagnosed chronic lymphocytic leukemia patients. Am. J. Hematol. **88**, 647–651 (2013)
23. D. Rossi, S. Rasi, V. Spina, A. Bruscaggin, S. Monti, C. Ciardullo, C. Deambrogi, H. Khiabanian, R. Serra, F. Bertoni, Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. Blood **121**, 1403–1412 (2013)
24. J.A. Burger, Inhibiting B-cell receptor signaling pathways in chronic lymphocytic leukemia. Curr. Hematol. Malig. Rep. **7**, 26–33 (2012)
25. G. Gaidano, R. Foà, R. Dalla-Favera, Molecular pathogenesis of chronic lymphocytic leukemia. J. Clin. Invest. **122**, 3432–3438 (2012)
26. M. Hallek, Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. Am. J. Hematol. **88**, 803–816 (2013)
27. E. Tausch, D. Mertens, S. Stilgenbauer, Advances in treating chronic lymphocytic leukemia. F1000Prime Rep. **6**, 65 (2014)
28. R. Foà, I. Del Giudice, A. Guarini, D. Rossi, G. Gaidano, Clinical implications of the molecular genetics of chronic lymphocytic leukemia. Haematologica **98**, 675–685 (2013)
29. P.C. Monroig, G.A. Calin, MicroRNA and epigenetics: diagnostic and therapeutic opportunities. Curr. Pathobiol. Rep. **1**, 43–52 (2013)
30. L.R. Corsini, G. Bronte, M. Terrasi, V. Amodeo, D. Fanale, E. Fiorentino, G. Cicero, V. Bazan, A. Russo, The role of microRNAs in cancer: diagnostic and prognostic biomarkers and targets of therapies. Expert Opin. Ther. Targets **16**, S103–S109 (2012)
31. P. Li, X. Wang, Role of signaling pathways and miRNAs in chronic lymphocytic leukemia. Chin. Med. J. (Engl.) **126**, 4175–4182 (2013)
32. L.P. Frenzel, R. Claus, N. Plume, J. Schwamb, C. Konermann, C.P. Pallasch, J. Claasen, R. Brinker, B. Wollnik, C. Plass, Sustained NF-kappaB activity in chronic lymphocytic leukemia is independent of genetic and epigenetic alterations in the TNFAIP3 (A20) locus. Int. J. Cancer **128**, 2495–2500 (2011)
33. R. Horie, M. Watanabe, T. Okamura, M. Taira, M. Shoda, T. Motoji, A. Utsunomiya, T. Watanabe, M. Higashihara, K. Umezawa, DHMEQ, a new NF-κB inhibitor, induces apoptosis and enhances fludarabine effects on chronic lymphocytic leukemia cells. Leukemia **20**, 800–806 (2006)
34. L. Coll-Mulet, D. Iglesias-Serret, A.F. Santidrián, A.M. Cosialls, M. de Frias, E. Castaño, C. Campàs, M. Barragán, A.F. de Sevilla, A. Domingo, MDM2 antagonists activate p53 and synergize with genotoxic drugs in B-cell chronic lymphocytic leukemia cells. Blood **107**, 4109–4114 (2006)
35. K. Kojima, M. Konopleva, T. McQueen, S. O'Brien, W. Plunkett, M. Andreeff, Mdm2 inhibitor Nutlin-3a induces p53-mediated apoptosis by transcription-dependent and transcription-independent mechanisms and may overcome Atm-mediated resistance to fludarabine in chronic lymphocytic leukemia. Blood **108**, 993–1000 (2006)
36. D.A. Carney, W.G. Wierda, Genetics and molecular biology of chronic lymphocytic leukemia. Curr. Treat Options Oncol. **6**, 215–225 (2005)
37. C. Haferlach, F. Dicker, S. Schnittger, W. Kern, T. Haferlach, Comprehensive genetic characterization of CLL: a study on 506 cases analysed with chromosome banding analysis, interphase FISH. IgVH status and immunophenotyping. Leukemia **21**, 2442–2451 (2007)
38. D. Pfeifer, M. Pantic, I. Skatulla, J. Rawluk, C. Kreutz, U.M. Martens, P. Fisch, J. Timmer, H. Veelken, Genome-wide analysis of DNA copy number changes and LOH in CLL using high-density SNP arrays. Blood **109**, 1202–1210 (2007)
39. J.Y. Hehir-Kwa, M. Egmont-Petersen, I.M. Janssen, D. Smeets, A. Geurts van Kessel, J.A. Veltman, Genome-wide copy number profiling on high-density bacterial artificial chromosomes, single-nucleotide polymorphisms, and oligonucleotide microarrays: a platform comparison based on statistical power analysis. DNA Res. **14**, 1–11 (2007)
40. C. Meldrum, M.A. Doyle, R.W. Tothill, Next-generation sequencing for cancer diagnostics: a practical perspective. Clin. Biochem. Rev. **32**, 177 (2011)
41. G. Fabbri, S. Rasi, D. Rossi, V. Trifonov, H. Khiabanian, J. Ma, A. Grunn, M. Fangazio, D. Capello, S. Monti, Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. J. Exp. Med. **208**, 1389–1401 (2011)
42. L. Wang, M.S. Lawrence, Y. Wan, P. Stojanov, C. Sougnez, K. Stevenson, L. Werner, A. Sivachenko, D.S. DeLuca, L. Zhang, SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. N. Engl. J. Med. **365**, 2497–2506 (2011)
43. S. Azizidoost, S. Babashah, F. Rahim, M. Shahjahani, N. Saki, Bone marrow neoplastic niche in leukemia. Hematology **19**, 232–238 (2014)
44. J.A. Burger, J.G. Gribben, Semin. Cancer Biol. **24**, 71–81 (2014)
45. A.N. Kamdje, G. Bassi, L. Pacelli, G. Malpeli, E. Amati, I. Nichele, G. Pizzolo, M. Krampera, Role of stromal cell-mediated notch signaling in CLL resistance to chemotherapy. Blood Cancer J. **2**, e73 (2012)

46. F. Saba, M. Soleimani, A. Atashi, E. Mortaz, M. Shahjahani, E. Roshandel, K. Jaseb, N. Saki, The role of the nervous system in hematopoietic stem cell mobilization. Lab. Hematol. **19**, 8–16 (2013)
47. A. Wiestner, Emerging role of kinase-targeted strategies in chronic lymphocytic leukemia. Blood **120**, 4684–4691 (2012)
48. N. Saki, S. Abroun, M.F. Hagh, F. Asgharei, Neoplastic bone marrow niche: hematopoietic and mesenchymal stem cells. Cell J. **13**, 131 (2011)
49. M.F. de Rooij, A. Kuil, C.R. Geest, E. Eldering, B.Y. Chang, J.J. Buggy, S.T. Pals, M. Spaargaren, The clinically active BTK inhibitor PCI-32765 targets B-cell receptor–and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. Blood **119**, 2590–2594 (2012)
50. J. Hoellenriegel, S.A. Meadows, M. Sivina, W.G. Wierda, H. Kantarjian, M.J. Keating, N. Giese, S. O'Brien, A. Yu, L.L. Miller, The phosphoinositide 3′-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. Blood **118**, 3603–3612 (2011)
51. S. López-Giral, N.E. Quintana, M. Cabrerizo, M. Alfonso-Pérez, M. Sala-Valdés, V.G.G. de Soria, J.M. Fernández-Rañada, E. Fernández-Ruiz, C. Muñoz, Chemokine receptors that mediate B cell homing to secondary lymphoid tissues are highly expressed in B cell chronic lymphocytic leukemia and non-Hodgkin lymphomas with widespread nodular dissemination. J. Leukoc. Biol. **76**, 462–471 (2004)
52. J.A. Burger, M. Burger, T.J. Kipps, Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. Blood **94**, 3658–3667 (1999)
53. J. Barretina, J. Junca, A. Llano, A. Gutierrez, A. Flores, J. Blanco, B. Clotet, J. Este, CXCR4 and SDF-1 expression in B-cell chronic lymphocytic leukemia and stage of the disease. Ann. Hematol. **82**, 500–505 (2003)
54. F. Bennett, A. Rawstron, M. Plummer, R. Tute, P. Moreton, A. Jack, P. Hillmen, B-cell chronic lymphocytic leukaemia cells show specific changes in membrane protein expression during different stages of cell cycle. Br. J. Haematol. **139**, 600–604 (2007)
55. G. Müller, U.E. Höpken, M. Lipp, The impact of CCR7 and CXCR5 on lymphoid organ development and systemic immunity. Immunol. Rev. **195**, 117–135 (2003)
56. J.A. Burger, M.P. Quiroga, E. Hartmann, A. Bürkle, W.G. Wierda, M.J. Keating, A. Rosenwald, High-level expression of the T-cell chemokines CCL3 and CCL4 by chronic lymphocytic leukemia B cells in nurselike cell cocultures and after BCR stimulation. Blood **113**, 3050–3058 (2009)
57. A. Shaffer, X. Yu, Y. He, J. Boldrick, E.P. Chan, L.M. Staudt, BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity **13**, 199–212 (2000)
58. A. Zucchetto, C. Tripodo, D. Benedetti, S. Deaglio, G. Gaidano, G. Del Poeta, V. Gattei, Monocytes/macrophages but not T lymphocytes are the major targets of the CCL3/CCL4 chemokines produced by CD38+ CD49d + chronic lymphocytic leukaemia cells. Br. J. Haematol. **150**, 111–112 (2010)
59. J.A. Burger, N. Chiorazzi, B cell receptor signaling in chronic lymphocytic leukemia. Trends Immunol. **34**, 592–601 (2013)
60. S. Mehdi Hoseini, F. Montazeri, A.M. Froughmand, M. Dehghani, H.R. Ghadimi, Introduction to genetic testing–applications, advantages and disadvantages. Genet 3rd millennium **12**, 3544–3563 (2014)
61. G. Juliusson, D.G. Oscier, M. Fitchett, F.M. Ross, G. Stockdill, M.J. Mackie, A.C. Parker, G.L. Castoldi, A. Cuneo, S. Knuutila, Prognostic subgroups in B-cell chronic lymphocytic leukemia defined by specific chromosomal abnormalities. N. Engl. J. Med. **323**, 720–724 (1990)
62. J.J. Yunis, High resolution of human chromosomes. Science **191**, 1268–1270 (1976)
63. M. Stevens-Kroef, A. Simons, H. Gorissen, T. Feuth, D.O. Weghuis, A. Buijs, R. Raymakers, A. Geurts van Kessel, Identification of chromosomal abnormalities relevant to prognosis in chronic lymphocytic leukemia using multiplex ligation-dependent probe amplification. Cancer Genet. Cytogenet. **195**, 97–104 (2009)
64. N.P. Carter, Methods and strategies for analyzing copy number variation using DNA microarrays. Nat. Genet. **39**, S16–S21 (2007)
65. M.J. Stevens-Kroef, E. van den Berg, D. Olde Weghuis, A. Geurts van Kessel, R. Pfundt, M. Linssen-Wiersma, M. Benjamins, T. Dijkhuizen, P.J. Groenen, A. Simons, Identification of prognostic relevant chromosomal abnormalities in chronic lymphocytic leukemia using microarray-based genomic profiling. Mol. Cytogenet. **7**, 3 (2014)
66. A.M. Snijders, D. Pinkel, D.G. Albertson, Current status and future prospects of array-based comparative genomic hybridisation. Brief Funct. Genom Proteomic. **2**, 37–45 (2003)
67. L.E. Vissers, J.A. Veltman, A. Geurts van Kessel, H.G. Brunner, Identification of disease genes by whole genome CGH arrays. Hum. Mol. Genet. **14**, R215–R223 (2005)
68. A. Cuneo, G. Rigolin, R. Bigoni, C. De Angeli, A. Veronese, F. Cavazzini, A. Bardi, M. Roberti, E. Tammiso, P. Agostini, Chronic lymphocytic leukemia with 6q − shows distinct hematological features and intermediate prognosis. Leukemia **18**, 476–483 (2003)
69. H. Döhner, S. Stilgenbauer, A. Benner, E. Leupolt, A. Kröber, L. Bullinger, K. Döhner, M. Bentz, P. Lichter, Genomic aberrations and survival in chronic lymphocytic leukemia. N. Engl. J. Med. **343**, 1910–1916 (2000)
70. T.S. Elton, H. Selemon, S.M. Elton, N.L. Parinandi, Regulation of the MIR155 host gene in physiological and pathological processes. Gene **532**, 1–12 (2013)
71. D. Sampath, C. Liu, K. Vasan, M. Sulda, V.K. Puduvalli, W.G. Wierda, M.J. Keating, Histone deacetylases mediate the silencing of miR-15a, miR-16, and miR-29b in chronic lymphocytic leukemia. Blood **119**, 1162–1172 (2012)
72. L.Q. Wang, Y.L. Kwong, K.F. Wong, C.S.B. Kho, D.Y. Jin, E. Tse, A. Rosèn, C.S. Chim, Epigenetic inactivation of mir-34b/c in addition to mir-34a and DAPK1 in chronic lymphocytic leukemia. J. Transl. Med. **12**, 52 (2014)
73. Y.-y. Lai, X.-j. Huang, Cytogenetic characteristics of B cell chronic lymphocytic leukemia in 275 Chinese patients by fluorescence in situ hybridization: a multicenter study. Chin. Med. J. (Engl) **124**, 2417 (2011)
74. U. Rozovski, G.A. Calin, T. Setoyama, L. D'Abundo, D.M. Harris, P. Li, Z. Liu, S. Grgurevic, A. Ferrajoli, S. Faderl, Signal transducer and activator of transcription (STAT)-3 regulates microRNA gene expression in chronic lymphocytic leukemia cells. Mol. Cancer **12**, 50 (2013)
75. T. Herold, V. Jurinovic, M. Mulaw, T. Seiler, A. Dufour, S. Schneider, P.M. Kakadia, M. Feuring-Buske, J. Braess, K. Spiekermann, Expression analysis of genes located in the minimally deleted regions of 13q14 and 11q22-23 in chronic lymphocytic leukemia—unexpected expression pattern of the RHO GTPase activator ARHGAP20. Genes Chromosom. Cancer **50**, 546–558 (2011)
76. G.A. Calin, M. Ferracin, A. Cimmino, G. Di Leva, M. Shimizu, S.E. Wojcik, M.V. Iorio, R. Visone, N.I. Sever, M. Fabbri, A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N. Engl. J. Med. **353**, 1793–1801 (2005)
77. M. Ferracin, B. Zagatti, L. Rizzotto, F. Cavazzini, A. Veronese, M. Ciccone, E. Saccenti, L. Lupini, A. Grilli, C. De Angeli, MicroRNAs involvement in fludarabine refractory chronic lymphocytic leukemia. Mol. Cancer **9**, 123 (2010)

78. N. Cahill, R. Rosenquist, Uncovering the DNA methylome in chronic lymphocytic leukemia. Epigenetics **8**, 138 (2013)
79. S. Kalachikov, A. Migliazza, E. Cayanis, N. Fracchiolla, M. Bonaldo, L. Lawton, P. Jelenc, X. Ye, X. Qu, M. Chien, Cloning and gene mapping of the chromosome 13q14 region deleted in chronic lymphocytic leukemia. Genomics **42**, 369–377 (1997)
80. S. Stilgenbauer, S. Sander, L. Bullinger, A. Benner, E. Leupolt, D. Winkler, A. Kröber, D. Kienle, P. Lichter, H. Döhner, Clonal evolution in chronic lymphocytic leukemia: acquisition of high-risk genomic aberrations associated with unmutated VH, resistance to therapy, and short survival. Haematologica **92**, 1242–1245 (2007)
81. J.Á. Hernández, A.E. Rodríguez, M. González, R. Benito, C. Fontanillo, V. Sandoval, M. Romero, G. Martín-Núñez, A.G. de Coca, R. Fisac, A high number of losses in 13q14 chromosome band is associated with a worse outcome and biological differences in patients with B-cell chronic lymphoid leukemia. Haematologica **94**, 364–371 (2009)
82. D.L. Van Dyke, T.D. Shanafelt, T.G. Call, C.S. Zent, S.A. Smoley, K.G. Rabe, S.M. Schwager, J.C. Sonbert, S.L. Slager, N.E. Kay, A comprehensive evaluation of the prognostic significance of 13q deletions in patients with B-chronic lymphocytic leukaemia. Br. J. Haematol. **148**, 544–550 (2010)
83. M. Dal Bo, F.M. Rossi, D. Rossi, C. Deambrogi, F. Bertoni, I. Del Giudice, G. Palumbo, M. Nanni, A. Rinaldi, I. Kwee, 13q14 deletion size and number of deleted cells both influence prognosis in chronic lymphocytic leukemia. Genes Chromosom. Cancer **50**, 633–643 (2011)
84. J.R. Brown, Insulin receptor activation in deletion 11q chronic lymphocytic leukemia. Clin. Cancer Res. **17**, 2605–2607 (2011)
85. R. Karhu, S. Knuutila, O.P. Kallioniemi, S. Siitonen, R. Aine, L. Vilpo, J. Vilpo, Frequent loss of the 11q14-24 region in chronic lymphocytic leukemia: a study by comparative genomic hybridization. Genes Chromosom. Cancer **19**, 286–290 (1997)
86. S.E. Artandi, L.D. Attardi, Pathways connecting telomeres and p53 in senescence, apoptosis, and cancer. Biochem. Biophys. Res. Commun. **331**, 881–890 (2005)
87. M.F. Lavin, Ataxia-telangiectasia: from a rare disorder to a paradigm for cell signalling and cancer. Nat. Rev. Mol. Cell Biol. **9**, 759–769 (2008)
88. A. Pérez-Perarnau, L. Coll-Mulet, C. Rubio-Patiño, D. Iglesias-Serret, A.M. Cosialls, D.M. González-Gironès, M. de Frias, A.F. de Sevilla, E. de la Banda, G. Pons, Analysis of apoptosis regulatory genes altered by histone deacetylase inhibitors in chronic lymphocytic leukemia cells. Epigenetics **6**, 1228–1235 (2011)
89. D. Rossi, M. Cerri, C. Deambrogi, E. Sozzi, S. Cresta, S. Rasi, L. De Paoli, V. Spina, V. Gattei, D. Capello, The prognostic value of TP53 mutations in chronic lymphocytic leukemia is independent of Del17p13: implications for overall survival and chemorefractoriness. Clin. Cancer Res. **15**, 995–1004 (2009)
90. P. Hillmen, A.B.. Skotnicki, T. Robak, B. Jaksic, A. Dmoszynska, J. Wu, C. Sirard, J. Mayer, Alemtuzumab compared with chlorambucil as first-line therapy for chronic lymphocytic leukemia. J. Clin. Oncol. **25**, 5616–5623 (2007)
91. A.R. Pettitt, R. Jackson, S. Carruthers, J. Dodd, S. Dodd, M. Oates, G.G. Johnson, A. Schuh, E. Matutes, C.E. Dearden, Alemtuzumab in combination with methylprednisolone is a highly effective induction regimen for patients with chronic lymphocytic leukemia and deletion of TP53: final results of the national cancer research institute CLL206 trial. J. Clin. Oncol. **30**, 1647–1655 (2012)
92. M. Bentz, A. Plesch, L. Bullinger, S. Stilgenbauer, G. Ott, H. Konrad Müller-Hermelink, M. Baudis, T.F. Barth, P. Möller, P. Lichter, t (11; 14)-positive mantle cell lymphomas exhibit complex karyotypes and share similarities with B-cell chronic lymphocytic leukemia. Genes Chromosom. Cancer **27**, 285–294 (2000)
93. M. Merup, G. Juliusson, X. Wu, M. Jansson, B. Stellan, O. Rasool, E. Röijer, G. Stenman, G. Gahrton, S. Einhorn, Amplification of multiple regions of chromosome 12, including 12q13–15, in chronic lymphocytic leukaemia. Eur. J. Haematol. **58**, 174–180 (1997)
94. P. Josefsson, C.H. Geisler, H. Leffers, J.H. Petersen, M.K. Andersen, J. Jurlander, A.M. Buhl, CLLU1 expression analysis adds prognostic information to risk prediction in chronic lymphocytic leukemia. Blood **109**, 4973–4979 (2007)
95. C.Y. Ok, R.R. Singh, F. Vega, Aberrant activation of the hedgehog signaling pathway in malignant hematological neoplasms. Am. J. Pathol. **180**, 2–11 (2012)
96. G.V. Hegde, K.J. Peterson, K. Emanuel, A.K. Mittal, A.D. Joshi, J.D. Dickinson, G.J. Kollessery, R.G. Bociek, P. Bierman, J.M. Vose, Hedgehog-induced survival of B-cell chronic lymphocytic leukemia cells in a stromal cell microenvironment: a potential new therapeutic target. Mol. Cancer Res. **6**, 1928–1936 (2008)
97. O. Morozova, M.A. Marra, Applications of next-generation sequencing technologies in functional genomics. Genomics **92**, 255–264 (2008)
98. R. Ekblom, J. Galindo, Applications of next generation sequencing in molecular ecology of non-model organisms. Heredity (Edinb). **107**, 1–15 (2010)
99. D.A. Landau, S.L. Carter, P. Stojanov, A. McKenna, K. Stevenson, M.S. Lawrence, C. Sougnez, C. Stewart, A. Sivachenko, L. Wang, Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell **152**, 714–726 (2013)
100. X.S. Puente, M. Pinyol, V. Quesada, L. Conde, G.R. Ordóñez, N. Villamor, G. Escaramis, P. Jares, S. Beà, M. González-Díaz, Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature **475**, 101–105 (2011)
101. V. Quesada, L. Conde, N. Villamor, G.R. Ordóñez, P. Jares, L. Bassaganyas, A.J. Ramsay, S. Beà, M. Pinyol, A. Martínez-Trillos, Exome sequencing identifies recurrent mutations of the splicing factor SF3B1 gene in chronic lymphocytic leukemia. Nat. Genet. **44**, 47–52 (2012)
102. D. Rossi, C. Ciardullo, V. Spina, G. Gaidano, Molecular bases of chronic lymphocytic leukemia in light of new treatments. Immunol. Lett. **155**, 51–55 (2013)
103. D.A. Landau, C.J. Wu, Chronic lymphocytic leukemia: molecular heterogeneity revealed by high-throughput genomics. Genome Med. **5**, 47 (2013)
104. D. Rossi, M. Fangazio, S. Rasi, T. Vaisitti, S. Monti, S. Cresta, S. Chiaretti, I. Del Giudice, G. Fabbri, A. Bruscaggin, Disruption of BIRC3 associates with fludarabine chemorefractoriness in TP53 wild-type chronic lymphocytic leukemia. Blood **119**, 2854–2862 (2012)
105. S. Vallabhapurapu, M. Karin, Regulation and function of NF-κB transcription factors in the immune system. Annu. Rev. Immunol. **27**, 693–733 (2009)
106. A. Martínez-Trillos, M. Pinyol, A. Navarro, M. Aymerich, P. Jares, M. Juan, M. Rozman, D. Colomer, J. Delgado, E. Giné, Mutations in TLR/MYD88 pathway identify a subset of young chronic lymphocytic leukemia patients with favorable outcome. Blood **123**, 3790–3796 (2014)
107. S.-C. Lin, Y.-C. Lo, H. Wu, Helical assembly in the MyD88–IRAK4–IRAK2 complex in TLR/IL-1R signalling. Nature **465**, 885–890 (2010)
108. D.G. Efremov, A. Wiestner, L. Laurenti, Novel agents and emerging strategies for targeting the B-cell receptor pathway in CLL. Mediterr. J. Hematol. Infect. Dis. **4**, e2012067 (2012)
109. A.K. Ghosh, N.E. Kay, Critical signal transduction pathways in CLL. Adv. Exp. Med. Biol. **792**, 215–239 (2013)

110. A. Rosén, F. Murray, C. Evaldsson, R. Rosenquist, Antigens in chronic lymphocytic leukemia–implications for cell origin and leukemogenesis. Semin. Cancer Biol. **20**, 400–409 (2010)
111. F.K. Stevenson, S. Krysov, A.J. Davies, A.J. Steele, G. Packham, B-cell receptor signaling in chronic lymphocytic leukemia. Blood **118**, 4313–4320 (2011)
112. R.N. Damle, T. Wasil, F. Fais, F. Ghiotto, A. Valetto, S.L. Allen, A. Buchbinder, D. Budman, K. Dittmar, J. Kolitz, Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia presented in part at the 40th annual meeting of The American Society of Hematology, held in Miami Beach, FL, December 4–8, 1998. Blood **94**, 1840–1847 (1999)
113. T.J. Hamblin, Z. Davis, A. Gardiner, D.G. Oscier, F.K. Stevenson, Unmutated Ig VH genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood **94**, 1848–1854 (1999)
114. R. Wickremasinghe, A. Prentice, A. Steele, p53 and Notch signaling in chronic lymphocytic leukemia: clues to identifying novel therapeutic strategies. Leukemia **25**, 1400–1407 (2011)
115. C.S. Tam, T.D. Shanafelt, W.G. Wierda, L.V. Abruzzo, D.L. Van Dyke, S. O'Brien, A. Ferrajoli, S.A. Lerner, A. Lynn, N.E. Kay, De novo deletion 17p13. 1 chronic lymphocytic leukemia shows significant clinical heterogeneity: the MD Anderson and Mayo Clinic experience. Blood **114**, 957–964 (2009)
116. I. Cordone, S. Masi, F.R. Mauro, S. Soddu, O. Morsilli, T. Valentini, M.L. Vegna, C. Guglielmi, F. Mancini, S. Giuliacci, p53 expression in B-cell chronic lymphocytic leukemia: a marker of disease progression and poor prognosis. Blood **91**, 4342–4349 (1998)
117. T. Sperka, J. Wang, K.L. Rudolph, DNA damage checkpoints in stem cells, ageing and cancer. Nat. Rev. Mol. Cell Biol. **13**, 579–590 (2012)
118. P. Ouillette, J. Li, R. Shaknovich, Y. Li, A. Melnick, K. Shedden, S.N. Malek, Incidence and clinical implications of ATM aberrations in chronic lymphocytic leukemia. Genes Chromosom. Cancer **51**, 1125–1132 (2012)
119. B. Austen, J.E. Powell, A. Alvi, I. Edwards, L. Hooper, J. Starczynski, A.M.R. Taylor, C. Fegan, P. Moss, T. Stankovic, Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with B-CLL. Blood **106**, 3175–3182 (2005)
120. B. Austen, A. Skowronska, C. Baker, J.E. Powell, A. Gardiner, D. Oscier, A. Majid, M. Dyer, R. Siebert, A.M. Taylor, Mutation status of the residual ATM allele is an important determinant of the cellular response to chemotherapy and survival in patients with chronic lymphocytic leukemia containing an 11q deletion. J. Clin. Oncol. **25**, 5448–5457 (2007)
121. D. Rossi, G. Gaidano, ATM and chronic lymphocytic leukemia: mutations, and not only deletions, matter. Haematologica **97**, 5–8 (2012)
122. A. Guarini, M. Marinelli, S. Tavolaro, E. Bellacchio, M. Magliozzi, S. Chiaretti, M.S. De Propris, N. Peragine, S. Santangelo and F. Paoloni, ATM gene alterations in chronic lymphocytic leukemia patients induce a distinct gene expression profile and predict disease progression. Haematologica. 2011.049270 (2011)
123. M.J. Rose-Zerilli, J. Forster, H. Parker, A. Parker, A.E. Rodríguez, T. Chaplin, A. Gardiner, A.J. Steele, A. Collins and B.D. Young, ATM mutation rather than BIRC3 deletion and/or mutation predicts reduced survival in 11q-deleted chronic lymphocytic leukemia, data from the UK LRF CLL4 trial. Haematologica. 2013.098574 (2014)
124. C. Lobry, P. Oh, I. Aifantis, Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. J. Exp. Med. **208**, 1931–1935 (2011)
125. J.S. Yuan, P.C. Kousis, S. Suliman, I. Visan, C.J. Guidos, Functions of notch signaling in the immune system: consensus and controversies. Annu. Rev. Immunol. **28**, 343–365 (2009)
126. K.G. Leong, A. Karsan, Recent insights into the role of Notch signaling in tumorigenesis. Blood **107**, 2223–2233 (2006)
127. P. Sportoletti, S. Baldoni, L. Cavalli, B. Del Papa, E. Bonifacio, R. Ciurnelli, A.S. Bell, A. Di Tommaso, E. Rosati, B. Crescenzi, NOTCH1 PEST domain mutation is an adverse prognostic factor in B-CLL. Br. J. Haematol. **151**, 404–406 (2010)
128. D. Rossi, S. Rasi, G. Fabbri, V. Spina, M. Fangazio, F. Forconi, R. Marasca, L. Laurenti, A. Bruscaggin, M. Cerri, Mutations of NOTCH1 are an independent predictor of survival in chronic lymphocytic leukemia. Blood **119**, 521–529 (2012)
129. A. Ahmadzadeh, S. Saedi, K. Jaseb, A.A. Asnafi, A. Alghasi, N. Saki, T-cell acute lymphoblastic leukemia with del (7)(q11. 2q22) and aberrant expression of myeloid markers. Int. J. Hematol. Oncol. Stem Cell Res. **7**, 40 (2013)
130. V. Balatti, A. Bottoni, A. Palamarchuk, H. Alder, L.Z. Rassenti, T.J. Kipps, Y. Pekarsky, C.M. Croce, NOTCH1 mutations in CLL associated with trisomy 12. Blood **119**, 329–331 (2012)
131. I. Del Giudice, D. Rossi, S. Chiaretti, M. Marinelli, S. Tavolaro, S. Gabrielli, L. Laurenti, R. Marasca, S. Rasi and M. Fangazio, NOTCH1 mutations in + 12 chronic lymphocytic leukemia (CLL) confer an unfavorable prognosis, induce a distinctive transcriptional profiling and refine the intermediate prognosis of + 12 CLL. Haematologica. 2011.060129 (2011)
132. S. Weissmann, A. Roller, S. Jeromin, M. Hernández, M. Abáigar, J. Hernández-Rivas, V. Grossmann, C. Haferlach, W. Kern, T. Haferlach, Prognostic impact and landscape of NOTCH1 mutations in chronic lymphocytic leukemia (CLL): a study on 852 patients. Leukemia **27**, 2393–2396 (2013)
133. Y. Wan, C.J. Wu, SF3B1 mutations in chronic lymphocytic leukemia. Blood **121**, 4627–4634 (2013)
134. X. Liang, E.A. Moseman, M.A. Farrar, V. Bachanova, D.J. Weisdorf, B.R. Blazar, W. Chen, Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. Blood **115**, 5041–5052 (2010)
135. E. Bourke, D. Bosisio, J. Golay, N. Polentarutti, A. Mantovani, The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood **102**, 956–963 (2003)
136. E. Arvaniti, S. Ntoufa, N. Papakonstantinou, T. Touloumenidou, N. Laoutaris, A. Anagnostopoulos, K. Lamnissou, F. Caligaris-Cappio, K. Stamatopoulos, P. Ghia, Toll-like receptor signaling pathway in chronic lymphocytic leukemia: distinct gene expression profiles of potential pathogenetic significance in specific subsets of patients. Haematologica **96**, 1644–1652 (2011)
137. D.J. Rawlings, M.A. Schwartz, S.W. Jackson, A. Meyer-Bahlburg, Integration of B cell responses through Toll-like receptors and antigen receptors. Nat. Rev. Immunol. **12**, 282–294 (2012)
138. V.N. Ngo, R.M. Young, R. Schmitz, S. Jhavar, W. Xiao, K.-H. Lim, H. Kohlhammer, W. Xu, Y. Yang, H. Zhao, Oncogenically active MYD88 mutations in human lymphoma. Nature **470**, 115–119 (2011)
139. K. Harikrishnan, S. Bassal, C. Tikellis, A. El-Osta, Expression analysis of the epigenetic methyltransferases and methyl-CpG binding protein families in the normal B-cell and B-cell chronic lymphocytic leukemia (CLL). Cancer Biol. Ther. **3**, 989–994 (2004)
140. J.A. Dubovsky, D. Wang, J.J. Powers, E. Berchmans, M.A. Smith, K.L. Wright, E.M. Sotomayor, J.A. Pinilla-Ibarz, Restoring the functional immunogenicity of chronic lymphocytic leukemia using epigenetic modifiers. Leuk. Res. **35**, 394–404 (2011)
141. E. Yiannakopoulou, Targeting epigenetic mechanisms and microRNAs by aspirin and other non steroidal anti-inflammatory agents–implications for cancer treatment and chemoprevention. Cell. Oncol. **37**, 167–178 (2014)

142. M. Kulis, S. Heath, M. Bibikova, A.C. Queirós, A. Navarro, G. Clot, A. Martínez-Trillos, G. Castellano, I. Brun-Heath, M. Pinyol, Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. Nat. Genet. **44**, 1236–1242 (2012)
143. P. Tsirigotis, V. Pappa, S. Labropoulos, S. Papageorgiou, F. Kontsioti, J. Dervenoulas, E. Papageorgiou, A. Panani, G. Mantzios, T. Economopoulos, Mutational and methylation analysis of the cyclin-dependent kinase 4 inhibitor (p16INK4A) gene in chronic lymphocytic leukemia. Eur. J. Haematol. **76**, 230–236 (2006)
144. S.G. Papageorgiou, S. Lambropoulos, V. Pappa, C. Economopoulou, F. Kontsioti, E. Papageorgiou, P. Tsirigotis, J. Dervenoulas, T. Economopoulos, Hypermethylation of the p15INK4B gene promoter in B-chronic lymphocytic leukemia. Am. J. Hematol. **82**, 824–825 (2007)
145. L. Pei, J.-H. Choi, J. Liu, E.-J. Lee, B. McCarthy, J.M. Wilson, E. Speir, F. Awan, H. Tae, G. Arthur, Genome-wide DNA methylation analysis reveals novel epigenetic changes in chronic lymphocytic leukemia. Epigenetics. **7**, 567 (2012)
146. A.G. Buggins, C.J. Pepper, The role of Bcl-2 family proteins in chronic lymphocytic leukaemia. Leuk. Res. **34**, 837–842 (2010)
147. S.-S. Chen, M.H. Sherman, E. Hertlein, A.J. Johnson, M.A. Teitell, J.C. Byrd, C. Plass, Epigenetic alterations in a murine model for chronic lymphocytic leukemia. Cell Cycle **8**, 3663–3667 (2009)
148. F.B. Rahmatpanah, S. Carstens, S.I. Hooshmand, E.C. Welsh, O. Sjahputera, K.H. Taylor, L.B. Bennett, H. Shi, J.W. Davis, G.L. Arthur, Large-scale analysis of DNA methylation in chronic lymphocytic leukemia. Epigenetics. **1**, 39–61 (2009)
149. M. Corcoran, A. Parker, J. Orchard, Z. Davis, M. Wirtz, O.J. Schmitz, D. Oscier, ZAP-70 methylation status is associated with ZAP-70 expression status in chronic lymphocytic leukemia. Haematologica **90**, 1078–1088 (2005)
150. R. Claus, D.M. Lucas, S. Stilgenbauer, A.S. Ruppert, L. Yu, M. Zucknick, D. Mertens, A. Bühler, C.C. Oakes, R.A. Larson, Quantitative DNA methylation analysis identifies a single CpG dinucleotide important for ZAP-70 expression and predictive of prognosis in chronic lymphocytic leukemia. J. Clin. Oncol. **30**, 2483–2491 (2012)
151. S. Babashah, M. Sadeghizadeh, M.R. Tavirani, S. Farivar, M. Soleimani, Aberrant microRNA expression and its implications in the pathogenesis of leukemias. Cell. Oncol. (Dordr). **35**, 317–334 (2012)
152. K.Y. Wong, C.C. So, F. Loong, L.P. Chung, W.W.L. Lam, R. Liang, G.K.H. Li, D.-Y. Jin, C.S. Chim, Epigenetic inactivation of the miR-124-1 in haematological malignancies. PLoS One **6**, e19027 (2011)
153. L.Q. Wang, Y.L. Kwong, C.S.B. Kho, K.F. Wong, K.Y. Wong, M. Ferracin, G.A. Calin, C.S. Chim, Epigenetic inactivation of miR-9 family microRNAs in chronic lymphocytic leukemia-implications on constitutive activation of NFκB pathway. Mol. Cancer **7**, 11 (2013)
154. C. Mercurio, S. Minucci, P.G. Pelicci, Histone deacetylases and epigenetic therapies of hematological malignancies. Pharmacol. Res. **62**, 18–34 (2010)
155. S.M.A.H. Rad, M.S. Bavarsad, E. Arefian, K. Jaseb, M. Shahjahani, N. Saki, The role of microRNAs in stemness of cancer stem cells. Oncol. Rev. **7**, 8 (2013)
156. L. Rask, E. Balslev, R. Sokilde, E. Hogdall, H. Flyger, J. Eriksen, T. Litman, Differential expression of miR-139, miR-486 and miR-21 in breast cancer patients sub-classified according to lymph node status. Cell. Oncol. **37**, 215–227 (2014)
157. Y. Wang, M. Li, W. Zang, Y. Ma, N. Wang, P. Li, T. Wang, G. Zhao, MiR-429 up-regulation induces apoptosis and suppresses invasion by targeting Bcl-2 and SP-1 in esophageal carcinoma. Cell. Oncol. **36**, 385–394 (2013)
158. A. Veronese, F. Pepe, J. Chiacchia, S. Pagotto, P. Lanuti, S. Veschi, M. Di Marco, A. D'Argenio, I. Innocenti, B. Vannata, Allele-specific loss and transcription of the miR-15a/16-1 cluster in chronic lymphocytic leukemia. Leukemia (2014). doi:10.1038/leu.2014.139
159. S. Srivastava, G.J. Tsongalis, P. Kaur, Recent advances in microRNA-mediated gene regulation in chronic lymphocytic leukemia. Clin. Biochem. **46**, 901–908 (2013)
160. S.P. Nana-Sinkam, C.M. Croce, MicroRNA in chronic lymphocytic leukemia: transitioning from laboratory-based investigation to clinical application. Cancer Genet. Cytogenet. **203**, 127–133 (2010)
161. U. Klein, R. Dalla-Favera, New insights into the pathogenesis of chronic lymphocytic leukemia. Semin. Cancer Biol. **20**, 377–383 (2010)
162. J.L. Mott, S. Kobayashi, S.F. Bronk, G.J. Gores, mir-29 regulates Mcl-1 protein expression and apoptosis. Oncogene **26**, 6133–6140 (2007)
163. Y. Pekarsky, U. Santanam, A. Cimmino, A. Palamarchuk, A. Efanov, V. Maximov, S. Volinia, H. Alder, C.-G. Liu, L. Rassenti, Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. Cancer Res. **66**, 11590–11593 (2006)
164. M. Herling, K. Patel, J. Khalili, E. Schlette, R. Kobayashi, L. Medeiros, D. Jones, TCL1 shows a regulated expression pattern in chronic lymphocytic leukemia that correlates with molecular subtypes and proliferative state. Leukemia **20**, 280–285 (2005)
165. M. Mraz, M. Mraz, S. Pospisilova, K. Malinova, I. Slapak, J. Mayer, MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes. Leuk. Lymphoma **50**, 506–509 (2009)
166. T. Zenz, S. Häbe, T. Denzel, J. Mohr, D. Winkler, A. Bühler, A. Sarno, S. Groner, D. Mertens, R. Busch, Detailed analysis of p53 pathway defects in fludarabine-refractory chronic lymphocytic leukemia (CLL): dissecting the contribution of 17p deletion, TP53 mutation, p53-p21 dysfunction, and miR34a in a prospective clinical trial. Blood **114**, 2589–2597 (2009)
167. F. Caligaris-Cappio, Chronic lymphocytic leukemia (CLL): a model for understanding and treating chronic B-cell malignancies. Rinsho Ketsueki. **54**, 1838 (2013)
168. R. Visone, L.Z. Rassenti, A. Veronese, C. Taccioli, S. Costinean, B.D. Aguda, S. Volinia, M. Ferracin, J. Palatini, V. Balatti, Karyotype-specific microRNA signature in chronic lymphocytic leukemia. Blood **114**, 3872–3879 (2009)
169. S. Rossi, M. Shimizu, E. Barbarotto, M.S. Nicoloso, F. Dimitri, D. Sampath, M. Fabbri, S. Lerner, L.L. Barron, L.Z. Rassenti, microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. Blood **116**, 945–952 (2010)
170. C. Baer, R. Claus, L.P. Frenzel, M. Zucknick, Y.J. Park, L. Gu, D. Weichenhan, M. Fischer, C.P. Pallasch, E. Herpel, Extensive promoter DNA hypermethylation and hypomethylation is associated with aberrant microRNA expression in chronic lymphocytic leukemia. Cancer Res. **72**, 3775–3785 (2012)
171. M. Xu, Y.-Y. Mo, The AKT-associated microRNAs. Cell. Mol. Life Sci. **69**, 3601–3612 (2012)
172. M.A. Lindsay, microRNAs and the immune response. Trends Immunol. **29**, 343–351 (2008)
173. M. Frenquelli, M. Muzio, C. Scielzo, C. Fazi, L. Scarfò, C. Rossi, G. Ferrari, P. Ghia, F. Caligaris-Cappio, MicroRNA and proliferation control in chronic lymphocytic leukemia: functional relationship between miR-221/222 cluster and p27. Blood **115**, 3949–3959 (2010)
174. B. Stamatopoulos, N. Meuleman, B. Haibe-Kains, P. Saussoy, E. Van Den Neste, L. Michaux, P. Heimann, P. Martiat, D. Bron, L. Lagneaux, microRNA-29c and microRNA-223 down-regulation has in vivo significance in chronic lymphocytic leukemia and improves disease risk stratification. Blood **113**, 5237–5245 (2009)
175. K. Zhou, S. Yi, Z. Yu, Z. Li, Y. Wang, D. Zou, J. Qi, Y. Zhao, L. Qiu, MicroRNA-223 expression is uniformly down-regulated in B cell lymphoproliferative disorders and is associated with poor survival

in patients with chronic lymphocytic leukemia. Leuk. Lymphoma **53**, 1155–1161 (2012)

176. Z. Hua, W. Chun, C. Fang-yuan, MicroRNA-146a and hemopoietic disorders. Int. J. Hematol. **94**, 224–229 (2011)
177. J. Redondo-Muñoz, E. Escobar-Díaz, R. Samaniego, M.J. Terol, J.A. García-Marco, Á. García-Pardo, MMP-9 in B-cell chronic lymphocytic leukemia is up-regulated by α4β1 integrin or CXCR4 engagement via distinct signaling pathways, localizes to podosomes, and is involved in cell invasion and migration. Blood **108**, 3143–3151 (2006)
178. J. Hayes, P.P. Peruzzi, S. Lawler, MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol. Med. **8**, 460–469 (2014)

Authors' contributions

N.S. and M.Sh conceived the manuscript and revised it. M.S, J.M., M.Sh. and F.N. wrote the manuscript. F.S and F.N. prepared the figures.