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Sami Malek *Editor*

Advances in Chronic Lymphocytic Leukemia

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Sami Malek
Editor

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Editor
Sami Malek
Department of Internal Medicine
University of Michigan
Ann Arbor, MI, USA

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Preface

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world but is rare in Asia. CLL has a highly varied clinical course. While advances in CLL therapy are noted, many patients still succumb to this illness. To date, most CLL patients are diagnosed in early stages and are not in need of immediate therapeutic interventions. These patients are, however, in need of careful counseling and guidance. Once therapy is medically indicated, CLL patients begin a phase of their illness marked by periods of remission, interrupted by relapses and medical complications. Most patients with CLL that are young and in need of therapy will, ultimately, die of their illness. While improvements in CLL therapy over the last 2 decades have resulted in many patients with CLL living active lives, many of these patients are, nevertheless, in need of ongoing psychological and medical attention and intervention: a situation that is likely to intensify with the advent of multiple novel therapies that appear to be of substantial clinical activity in CLL.

Like most progress in medicine, solid advances in the diagnosis, prognosis, and treatment of CLL are rooted in an in-depth understanding of the basic and translational biology of CLL. Major biological principles that underlie and contribute to CLL pathogenesis relate to (1) the invariant but infrequent (1 %/year) descendence of CLL from CD5+ monoclonal B-cell lymphocytosis (MBL); (2) molecular aberrations that contribute to the substantial inter-patient variability in clinical progressiveness and aggressiveness of CLL; (3) characteristic recurrent CLL cell-intrinsic abnormalities, including acquired genomic copy number aberrations, gene mutations, transcriptome deregulations and epigenetic deregulations; (4) the important role of anti-apoptotic proteins in CLL biology; (5) the central role of the B-cell antigen receptor in CLL pathogenesis; (6) the important contribution of CLL cell-to-cytokine and CLL cell-to-cell interactions collectively referred to as the microenvironment; (7) the acquisition of CLL therapy resistance and the ultimate progression to drug resistant CLL; and (8) CLL-associated immune system deregulations resulting in substantial morbidity including infection proneness and autoimmune cytopenias.

In this book, CLL experts have contributed state-of-the-art summaries of various important aspects of CLL biology and have discussed the translational implication of such findings. This book, which is directed at physicians and researchers alike, aims to educate broadly and deeply. Intentionally, the many aspects and nuances of CLL clinical care that can only really be appreciated through direct patient care are not covered here, but instead, basic aspects of CLL are presented that underlie many of the contemporary decisions that are made in CLL research and clinical settings.

Individual and comprehensive chapters in this book will collectively describe (1) inherited susceptibility of CLL; (2) the B-cell antigen receptor and the role of antigens in CLL; (3) the CLL microenvironment; (4) the anatomy and clinical relevance of recurrent acquired copy number aberrations and gene mutations, including mutations in *TP53*, in CLL; (5) the current understanding of epigenetic deregulation in CLL and the important role of microRNAs in CLL pathogenesis; (6) apoptosis deregulation in CLL; (7) our improving knowledge of Richter's transformation of CLL; (8) critical signal transduction pathways in CLL that may offer novel therapeutic opportunities; (9) molecular CLL biomarkers and information regarding their judicious use; and (10) selected, very exciting novel, but still experimental, therapeutic approaches to CLL.

In summary, we hope that this book will critically inform the community and stimulate interest in CLL, which will ultimately translate into better CLL research, prognostication, and therapy, with the end goal of providing a better outlook for patients afflicted with this common leukemia.

Ann Arbor, MI Sami Malek

Contents

Contributors

Andreas Agathangelidis B Cell Neoplasia Unit, Istituto Scientifico San Raffaele, Milan, Italy

Veronica Balatti Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center and the Wexner Medical Center, The Ohio State University, Columbus, OH, USA

Renier J. Brentjens Department of Medicine, Leukemia Service, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Jan A. Burger Unit 428, Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Elías Campo Hematopathology Unit, Hospital Clinic, University of Barcelona, Barcelona, Spain

Carlo M. Croce Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center and the Wexner Medical Center, The Ohio State University, Columbus, OH, USA

Chris Fegan CLL Research Group, Institute of Cancer and Genetics, Cardiff University, Heath Park, Cardiff, UK

Gianluca Gaidano Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy

Asish K. Ghosh Division of Hematology, Mayo Clinic, Rochester, MN, USA

Richard S. Houlston Division of Genetics and Epidemiology, Institute of Cancer Research, Sutton, Surrey, UK

Jade Jones Hematology Branch, National Heart, Lung, Blood Institute, National Institutes of Health, Bethesda, MD, USA

Neil E. Kay Division of Hematology, Mayo Clinic, Rochester, MN, USA

Carlos López-Otín Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología-IUOPA, Universidad de Oviedo, Oviedo, Spain

Jitka Malcikova Department of Molecular Medicine, Central European Institute of Technology, Masaryk University (CEITEC MU), Brno, Czech Republic

Sami Malek Department of Internal Medicine, Division of Hematology and Oncology, University of Michigan, Ann Arbor, MI, USA

Alejandra Martínez-Trillos Unidad de Hematopatologia, Departamento de Anatomía Patológica, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain

Carsten U. Niemann Hematology Branch, National Heart, Lung, Blood Institute, National Institutes of Health, Bethesda, MD, USA

Stavroula Ntoufa Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece

Institute of Applied Biosciences, Center for Research and Technology Hellas, Thessaloniki, Greece

Peter Ouillette Department of Internal Medicine, Division of Hematology and Oncology, University of Michigan, Ann Arbor, MI, USA

Jae H. Park Department of Medicine, Leukemia Service, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Yuri Pekarky Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center and the Wexner Medical Center, The Ohio State University, Columbus, OH, USA

Chris Pepper CLL Research Group, Institute of Cancer and Genetics, Cardiff University, Heath Park, Cardiff, UK

Xose S. Puente Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología-IUOPA, Universidad de Oviedo, Oviedo, Spain

Víctor Quesada Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología-IUOPA, Universidad de Oviedo, Oviedo, Spain

Lara Rizzotto Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center and the Wexner Medical Center, The Ohio State University, Columbus, OH, USA

Davide Rossi Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy

Georgina Sava Division of Genetics and Epidemiology, Institute of Cancer Research, Sutton, Surrey, UK

Rita Shaknovich Department of Medicine, Weill Cornell Medical College, New York, NY, USA

Department of Pathology, Weill Cornell Medical College, New York, NY, USA

Helen E. Speedy Division of Genetics and Epidemiology, Institute of Cancer Research, Sutton, Surrey, UK

Kostas Stamatopoulos Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece

Institute of Applied Biosciences, Center for Research and Technology Hellas, Thessaloniki, Greece

Martin Trbusek Department of Molecular Medicine, Central European Institute of Technology, Masaryk University (CEITEC MU), Brno, Czech Republic

Neus Villamor Unidad de Hematopatologia, Departamento de Anatomía Patológica, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Barcelona, Spain

Adrian Wiestner Hematology Branch, National Heart, Lung, Blood Institute, National Institutes of Health, Bethesda, MD, USA

Chapter 1
B Cell Receptor and Antigens in CLL

Andreas Agathangelidis, Stavroula Ntoufa, and Kostas Stamatopoulos

Abstract Nowadays, chronic lymphocytic leukemia (CLL) is considered as a prototypic antigen-driven lymphoma, with antigenic stimuli from the microenvironment promoting tumor outgrowth. Antigen recognition is a function of both the clonotypic B cell receptor immunoglobulin (BcR IG) and various other immune sensors, e.g., the Toll-like receptors. The critical role of BcR IG-mediated signaling in CLL development and evolution is underscored by the following: the disease-biased IG gene repertoire; the subdivision of CLL based on the somatic hypermutation load of the BcR IG into two broad categories with vastly different prognosis and eventual outcome; the existence of subsets of cases with distinct, quasi-identical (stereotyped) BcR IGs; and the clinical efficacy of novel therapeutics inhibiting BcR signaling. Here, we trace the immunogenetic evidence for antigen selection in CLL and also consider the types of implicated antigens as well as the immune signaling pathways relevant for CLL ontogeny and clonal progression.

Keywords B cell receptor • Immunoglobulin • Antigen • Signaling • VH CDR3 • Somatic hypermutation

A. Agathangelidis
B Cell Neoplasia Unit, Istituto Scientifico San Raffaele, Milan, Italy

S. Ntoufa • K. Stamatopoulos, M.D. (✉)
Hematology Department and HCT Unit, G. Papanicolaou Hospital,
Thessaloniki 57010, Greece

Institute of Applied Biosciences, Center for Research and Technology Hellas,
Thessaloniki, Greece
e-mail: kostas.stamatopoulos@gmail.com

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Introduction

Chronic lymphocytic leukemia (CLL) is a disease of aged populations and the most common adult leukemia in the Western world. Clinically, it is very heterogeneous, with some patients never requiring treatment and sharply contrasting others who experience aggressive disease [1]. This clinical heterogeneity is linked to and likely reflects the underlying molecular and cellular heterogeneity [2].

CLL can be subdivided into subgroups with distinct biological features, extending from genomic abnormalities [3] to immune cell signaling [4] in the context of interactions of the CLL clones with their microenvironment. Here we will attempt an overview of the types of stimuli that are relevant for CLL ontogeny and evolution as well as the implicated sensor molecules, with a great emphasis on the specific antigen receptors expressed by the neoplastic cells.

Antigen Selection in CLL: Early Immunogenetic Evidence

The importance of immunogenetics, i.e., the analysis of antigen receptor genes, in unraveling the pathogenesis of CLL was first noticed in the mid-1990s when it was reported that the immunoglobulin (IG) gene repertoire of the malignant clones was restricted [5–10]. However, due to limited number of patients in these studies, no definitive conclusions could be drawn. Solid evidence was obtained in 1998 by the first comprehensive analysis of IG genes demonstrating that the immunoglobulin heavy variable (*IGHV*) gene repertoire in CLL is not only remarkably restricted but also distinct from that of IgM+ CD5+ normal blood B cells [11]. CLL cells clearly favor the expression of specific *IGHV* genes, such as *IGHV1-69*, *IGHV4-34*, and *IGHV3-7*. Restrictions are not confined to the *IGHV* gene repertoire but also concern the usage of immunoglobulin heavy diversity (*IGHD*) and immunoglobulin heavy joining (*IGHJ*) genes [12–17]. Taking a step further, the analysis of rearrangements of the most frequent *IGHV* genes revealed biased IG gene combinations. As an example, rearrangements utilizing the *IGHV1-69* gene showed a preference for the *IGHD3-3* and the *IGHJ6* genes. Other frequent *IGHV* genes, particularly *IGHV4-34*, *IGHV3-7*, and *IGHV3-23*, exhibited a different behavior with weaker or no gene combination biases [11, 18]. Constraints were also evident at the level of somatic hypermutation (SHM): rearrangements of the *IGHV1-69* gene carry minimal or no somatic mutations, whereas most *IGHV3-7*, *IGHV3-23*, and *IGHV4-34* rearrangements are significantly affected by the SHM machinery [11, 15, 16].

Altogether, these findings argued against serendipity and were interpreted as evidence for antigen selection in CLL ontogeny, at least for a fraction of cases.

Somatic Hypermutation of the Clonotypic IG Genes: A Highly Accurate Molecular Prognosticator for CLL

Perhaps the most important milestone in the timeline of modern CLL research came in 1999, when the Hamblin, Stevenson, and Chiorazzi groups independently reported that the mutational status of the rearranged *IGHV* genes can directly predict patient survival. The load of SHMs was the first highly accurate molecular marker for disease prognostication and remains one of the strongest independent markers in CLL [19, 20].

In particular, the impact of SHM classifies CLL patients into two distinct disease subtypes, namely unmutated or mutated, associated with a different clinical course. Assignment of a case to the mutated or unmutated subtype depends on the identity of the clonotypic *IGHV* genes to the closest germline gene (GI): in particular, cases displaying *IGHV* genes with GI equal to or greater than 98 % are classified as unmutated, whereas those with GI below 98 % belong to the mutated subtype [19, 20]. The application of this cut-off value was deemed necessary since at that time the sequence of the human genome was not completely determined. In this way, all possible polymorphisms could be excluded without the need to know the respective germline *IGHV* gene sequence of each individual CLL patient [21].

IG Genes and Prognostication in CLL: Beyond Somatic Hypermutation?

The 2 % GI cut-off value, though practical and clinically useful, underplays the biological significance of SHM given ample evidence from normal, autoreactive or malignant B cells that a low level of SHM or even single mutations can be functionally significant [16, 22, 23], though still translating to "unmutated" status following the CLL prognostication cut-off. Furthermore the clinical course of cases of "borderline" SHM status (GI close to 98 %) is difficult to predict accurately, since these cases form a heterogeneous group of benign and malignant cases rather than a homogeneous group with intermediate clinical aggressiveness [24].

Rosenquist's group provided the first evidence that other molecular characteristics of the clonotypic B cell receptor (BcR) IGs beyond SHM status were important for prognostication. In particular, CLL patients utilizing the *IGHV3-21* gene were proposed to comprise a distinct clinical group with adverse prognosis, even in cases with a high impact of SHM [25]. This finding led to the notion that the usage of specific *IGHV* genes may be used for prognostication, perhaps independently of their SHM status [26]. In the ensuing years, independent studies reached the same conclusion, reporting different outcomes for CLL clones with BcR IGs encoded by the *IGHV3-23* (adverse) [27], *IGHV3-30* [28], and *IGHV3-72* [29] (both favorable) genes, as well as others [30]. However, with the exception of the *IGHV3-21* gene, where the evidence is undeniable, these studies were performed on small series of patients and, hence, should be viewed with caution.

Amazing Restrictions: Tracing the Footprints of Highly Homologous BcR IGs in CLL

Initial hints for the existence of highly similar antigen-binding sites among different patients with CLL can be traced back to 1995 [31], at a time when CLL was presumed to be a disease of naive, antigen-inexperienced B cells that mostly express somatically unmutated *IGHV* genes [32]. However, the turning point in the study of sequence restrictions in CLL BcR IGs came in 2003, when Rosenquist's group demonstrated that a major fraction of patients carrying clonotypic IGs encoded by the *IGHV3-21* gene carried short and highly conserved heavy complementarity-determining region 3 (VH CDR3) and exhibited biased association with *IGLV3-21* light chains [33]. These findings were justifiably viewed as evidence for interaction with a common antigenic epitope, perhaps of pathogenetic significance. At roughly the same time, independent studies from both Europe and the United States reached the same conclusion: at variance with the logistics of IG synthesis, different patients with CLL carried BcR IGs with quasi-identical antigen-binding sites—in other words, these BcR IGs complied with a specific or general prototype and, for this reason, were termed "stereotyped" [12–17, 34, 35]. The timeline of BcR IG stereotypy studies is depicted in Fig. 1.1.

One-Third of Patients with CLL Express Stereotyped BcRs

Until quite recently, the actual frequency of BcR IG stereotypy and, therefore, the true impact of this phenomenon remained elusive. Published work indicated that as the size of the cohort increases the frequency of BcR IG stereotypy also rises [12, 14–17, 35]. However, the mathematical relation, if any, connecting these two values remained underestimated. In our recent study of more than 7,500 *IGHV-IGHD-IGHJ* gene rearrangements from patients with CLL [18] it became evident that although the proportion of stereotyped BcRs in CLL may indeed continuously increase when larger series of patients are analyzed, the rate of this increase is rather limited: indeed, the upward trend in BcR IG frequency begins to decline after a critical cohort size, eventually reaching a plateau at about 30 % of all cases, irrespective of the cohort size. These findings, along with our random set simulations of the actual IG sequence data, led us to argue that not all CLL cases will end up in stereotyped subsets and that CLL essentially comprises two different groups of patients: one with stereotyped and the other with heterogeneous (non-stereotyped) BcR IGs at an approximate ratio of 1:2 [18].

Furthermore, comparison of the stereotyped versus the heterogeneous IG rearrangements clearly showed that the *IGHV* gene expression biases reported as typical for CLL are almost exclusive to the stereotyped subentity, providing evidence for a clear immunogenetic distinction [35]. On these grounds, we proposed a

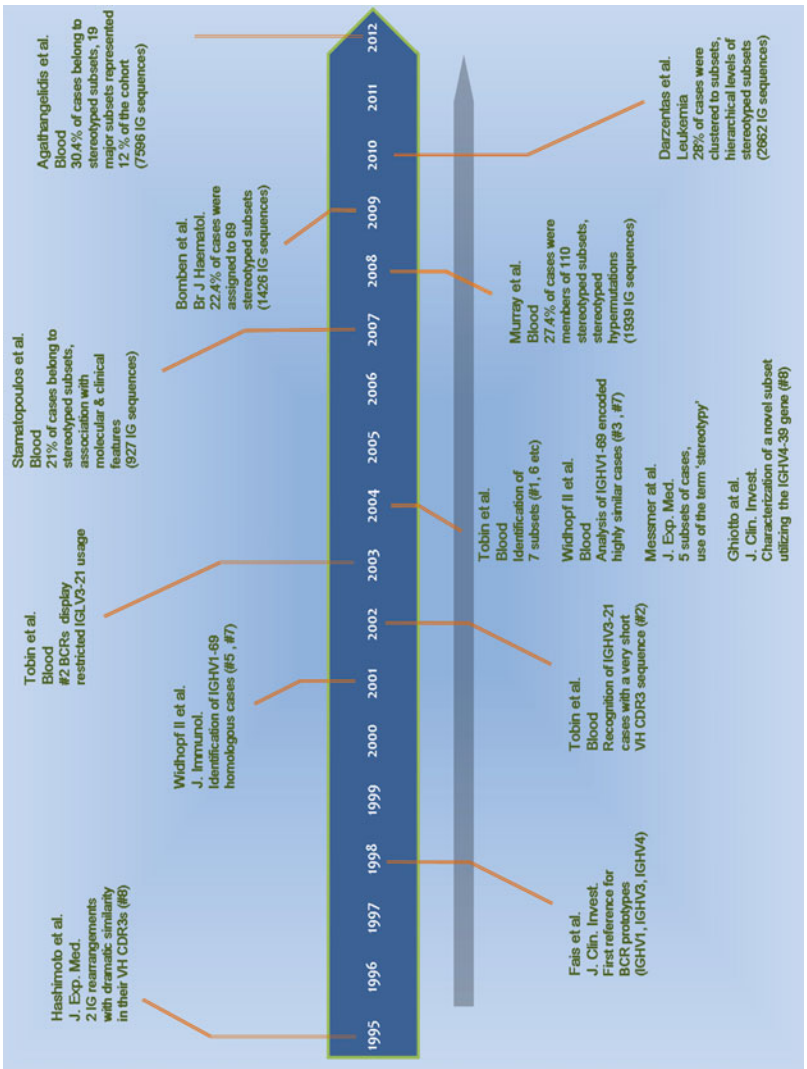

Fig. 1.1 Timeline of studies on B cell receptor immunoglobulin (BcR IG) stereotypy in chronic lymphocytic leukemia (CLL). For each study, a short summary of its most important contributions in deciphering the BcR stereotypy phenomenon in CLL is given

different ontogeny for the two broad CLL categories, namely stereotyped and heterogeneous, whereby different antigens and immune mechanisms are implicated in shaping the respective IG repertoires [35].

Types of Stereotyped BcRs: From Conserved Landmark Positions to Absolute Identity

Detailed analysis of stereotypy in CLL identified a series of distinctive molecular features regarding the composition and exact positioning of the shared amino acid patterns that render certain BcR IGs stereotyped. Through this analysis, two main types of patterns were identified [18]: (1) "mainly combinatorial," which were essentially encoded by the unmutated germline sequences of specific *IGHD* and *IGHJ* genes and (2) "combinatorial + junctional," which were to a large extent defined by junctional residues located between the *IGHV-IGHD* and *IGHD-IGHJ* genes. These findings indicate that in some cases VH CDR3 stereotypy stems from biased *IGHV-IGHD-IGHJ* gene combinations, whereas in other instances, several IG diversity mechanisms are at play [18].

Two well-characterized CLL subsets exemplify each pattern type, namely subsets #8 and #4. Subset #8 consists of *IGHV4-39*/*IGHD6-13*/*IGHJ5* gene rearrangements [18, 34] with the *IGHD6-13* gene always in reading frame 1. The VH CDR3 is 19 amino acids long, with the common pattern limited to the *IGHD*- and *IGHJ*-encoded regions, whereas the composition of the junctional residues is variable. One might argue that "mainly combinatorial" patterns, like that of subset #8, could not be highly specific due to the fact that they "merely" represent IG gene combinations. Yet, this particular VH CDR3 composition proved to be unique for this CLL subset, as revealed by comparisons against thousands of IG gene rearrangements from public databases [18].

The great differences between the two pattern types become immediately apparent when studying subset #4 rearrangements, which are encoded by the *IGHV4-34* and *IGHJ6* genes and carry VH CDR3s of 20 amino acids. The part of the VH CDR3 that lies between what is irrefutably *IGHV4-34*-derived or *IGHJ6*-derived, encompassing the *IGHV-IGHD* junctions along with the *IGHD*-encoded sequences and the *IGHD-IGHJ* junctions, is characterized by heterogeneity of such magnitude that conclusive *IGHD* gene assignment was in many cases very difficult. The classification of an *IGHV4-34*/*IGHJ6* rearrangement with a 20-amino acid long VH CDR3 to subset #4 is based on the identification of the R(K)RYYYY pattern that is encoded by both N2-junctional and *IGHJ6* germline amino acids. Again, public database enquiries identified this pattern as highly specific for subset #4 [18].

A remarkable feature of certain BcR IG stereotypes concerns the extreme restriction observed in certain positions of the VH domain which can be considered as truly subset-defining molecular landmarks. This is amply exemplified by subset #2 rearrangements encoded by the *IGHV3-21* gene and carrying short VH CDR3s

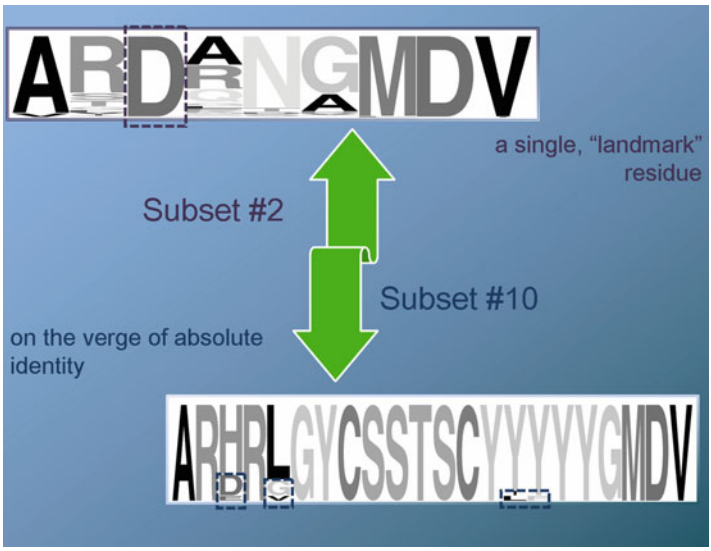

Fig. 1.2 The defining sequence features of stereotyped VH CDR3 patterns range from a single amino acid residue to almost the entire VH CDR3. Prime examples for the most extreme cases are subsets #2 and #10 (see text)

of only nine amino acids, despite utilizing the *IGHJ6* gene, which is the longest human *IGHJ* gene [15, 18, 25, 35]. Again, likely due to short VH CDR3 length, reliable *IGHD* gene assignment was impossible, yet almost all cases bore an aspartic acid (D) residue at VH CDR3 position 3, which lies between the *IGHV3-21*- and *IGHJ6*-encoded parts. In the rare outliers identified thus far, position 3 in VH CDR3 was occupied by a glutamic acid (E) residue, which belongs to the same physicochemical class as D (both D and E are acidic). In sharp contrast, subset #10 stands on the verge of absolute sequence identity. This subset included unmutated *IGHV4-39*/*IGHD2-2*/*IGHJ6* gene rearrangements with a 22-amino acid long VH CDR3. Only ten amino acid differences were identified in a total of 396 VH CDR3 amino acid residues (2.5 %) of subset #10 rearrangements, rendering it the most homogeneous subset described to date [18]. Differences between subsets #2 and #10 VH CDR3 sequences are illustrated in Fig. 1.2.

From Stereotyped VH CDR3s to Stereotyped BcR IGs: Recurrent Mutations and Sequence Convergence Induced by Somatic Hypermutation

In 2008 we published the first evidence that stereotypy in CLL may extend from shared VH CDR3 patterns to shared somatic mutations [16]. Specifically, the impact of SHM on subset #2 rearrangements was distinctive and very different from that of

non-subset #2 rearrangements utilizing the same *IGHV* gene, namely *IGHV3-21*. In particular, a string of recurrent, nonconservative mutations were identified, mostly centered at the VH CDR2, including a single amino acid deletion present at a very high frequency among subset #2 cases [36], which is notable given that deletions and/or insertions are very infrequently introduced by the SHM process in both normal and malignant B cells [37–39]. Altogether, these results were interpreted as further supporting the idea that antigenic selection plays a pivotal role in CLL ontogeny.

More recently, we presented additional evidence for the role of antigen-driven affinity maturation in shaping the IG repertoire of CLL by analyzing subset #77 [18]. This subset included mutated IG rearrangements with highly similar VH CDR3s that nonetheless express different yet phylogenetically connected *IGHV* genes, namely *IGHV4-4* or *IGHV4-59*. Notably, these rearrangements exhibited sequence convergence induced by SHM: in particular, at certain positions where the germline sequences of the *IGHV4-4* and *IGHV4-59* genes differed, a tendency for SHM to even out differences was documented.

In summary, antigen selection in CLL can lead not only to restricted VH CDR3 composition but also to restricted SHM patterns throughout the variable domain.

A Sizeable Fraction of CLL Can Be Characterized by Just a Few BcR Stereotypes, a.k.a. Major Stereotyped Subsets

An essential step towards obtaining a better insight into BcR IG stereotypy was the development of computational tools powered enough to handle ever-increasing IG gene sequence datasets. In 2010, our group introduced a purpose-built, sophisticated bioinformatics algorithm specifically developed for sequence pattern discovery within VH CDR3 amino acid sequences [35]. Aside from the undeniable efficacy of this algorithm in identifying stereotyped VH CDR3 sequences within large IG sequence datasets, another very important feature was its unique ability to provide an integrated view of the VH CDR3 stereotypy through the formation of a network highlighting more distant sequence relationships with more widely shared sequence patterns. Specifically, the algorithm takes advantage of the existence of common amino acid patterns between VH CDR3 sequences belonging to different subsets and assigns them to subsets at progressively higher levels, offering a comprehensive overview of the VH CDR3 “landscape” in CLL with the creation of a tree-like hierarchy [35].

In our latest study of more than 7,500 IG gene rearrangements from patients with CLL [18], we identified, at the highest level, 19 different subsets comprising at least 20 sequences each, with the two largest subsets numbering 213 and 184 cases, respectively. These 19 subsets, due to their size, were defined as major. En masse, major subsets accounted for 12 % of the cohort and a remarkable 41 % of all stereotyped cases, respectively. The relative size of each major subset is given in

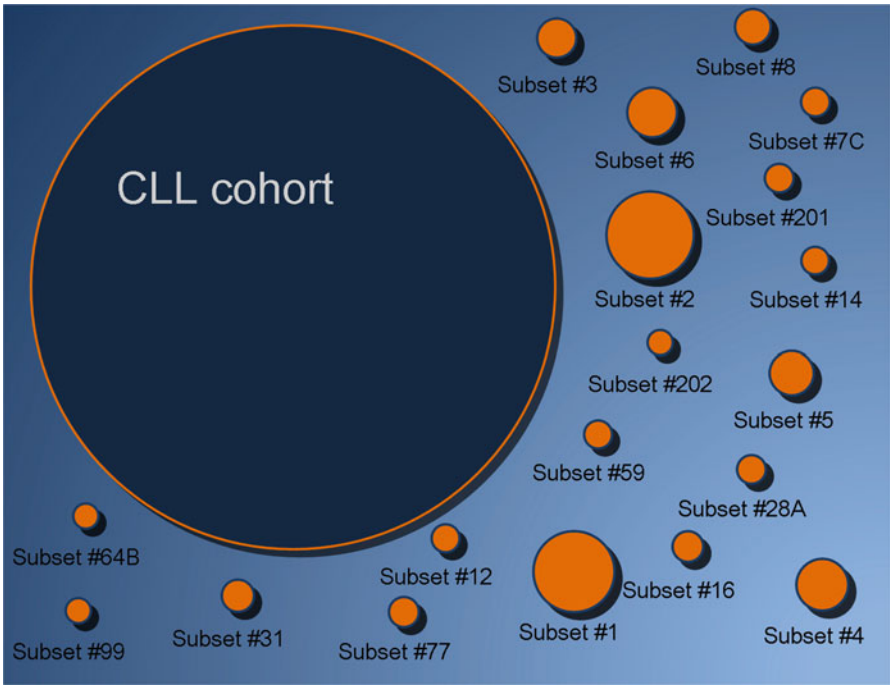

Fig. 1.3 Schematic illustration of the relative size of major subsets from our recent large-scale analysis of the IG gene repertoire in CLL [18]. Collectively, major subsets account for 12 % of the cohort and 41 % of the stereotyped fraction, indicating that a sizable proportion of CLL can be represented by only a bunch of VH CDR3 stereotypes

Fig. 1.3. Major subsets were heterogeneous in terms of IG gene usage, VH CDR3 length and composition, *IGHV* mutational status, as well as stereotyped type.

BcR IG Stereotypy Is Not Confined to CLL; However, CLL Stereotypes Exhibit "CLL-Specific" Features

Recently, immunogenetic evidence for antigen interactions has also been obtained for other B cell malignancies, namely mantle cell lymphoma (MCL) [40–45] and splenic marginal-zone lymphoma (SMZL) [46–49], both exhibiting biased *IGHV* gene repertoires and distinctive SHM patterns with disease-biased features. Notably, clonotypic IG rearrangements with stereotyped VH CDR3s were identified in both entities, albeit at much lower frequencies than CLL: ~10 % in MCL [50], even lower (~7 %) in SMZL [51, 52]. Thus, BcR IG stereotypy is not exclusive to CLL, though much more prevalent than in other B cell malignancies.

With the realization that stereotyped BcR IGs exist in various entities, the obvious next question concerns if they are "disease-biased" or common.

To investigate this issue, we performed cross-entity comparisons of the VH CDR3-stereotyped patterns in rearrangements utilizing the same *IGHV* gene [18]. Thus, in the case of MCL, since most stereotyped rearrangements (67 %) utilized either the *IGHV4-34* or the *IGH3-21* gene, accordingly, these stereotypes were compared to major CLL subsets utilizing the same *IGHV* genes, namely subsets #4, #16 (both *IGHV4-34*), and #2 (*IGHV3-21*), respectively. Comparisons revealed fundamental differences in terms of VH CDR3 sequence length and amino acid composition. Likewise, for the comparison between CLL and SMZL stereotypes, the attempt to identify similarities between stereotyped rearrangements of the *IGHV1-2* gene, which predominates by far in the repertoire of SMZL, failed completely.

In conclusion, the IG molecular features of stereotyped BcR IGs in CLL were clearly distinct from those in MCL or SMZL and can therefore be considered as "CLL-specific" [18]. By logical extension, the immune interactions with the antigen(s) leading to CLL development and evolution are also expected to be distinct from the corresponding ones for MCL or SMZL.

Stereotypy Extends from Primary IG Sequence Features to Shared Biological and Clinical Characteristics

The identification of stereotyped VH CDR3s in different, unrelated patients with CLL was widely viewed as strong evidence for the recognition of discrete antigens or classes of structurally similar epitopes. This gave rise to the idea that BcR stereotypy may have important clinical implications in addition to but also perhaps independently of the mutational status of the clonotypic *IGHV* genes. Early findings supported this idea, as subset #2 cases, even if they displayed a high *IGHV* mutational load, were shown to have a very poor prognosis [33]. Soon thereafter, similar results were reported for subset #1, whereas, in contrast, patients with BcR IGs assigned to subset #4 followed a very indolent disease course [15].

Since these initial observations, an increasing body of evidence from independent research groups clearly suggests that stereotypy in CLL may indeed extend from shared amino acid patterns to shared biological and clinical characteristics and, perhaps, outcome [15, 17, 34]. In the following paragraphs, some paradigmatic examples will highlight the case.

Subsets #1 and #2 are the largest subsets in CLL, collectively accounting for almost 5.5 % of the entire cohort [18]. Subset #1 patients are often diagnosed with advanced stage disease; express high levels of CD38, CD49d, and ZAP70; exhibit various adverse features [15, 17], including a high propensity to develop immune thrombocytopenia (ITP) [53]; and have an overall poor prognosis [15]. The bad prognosis puzzle of subset #1 is completed by the high frequency of unfavorable genomic aberrations, with del17p being the most prevalent [54]. Patients belonging to subset #2 have a similar adverse prognosis [15]. At the genomic level, a high frequency of aberrations is indicative of general genomic instability, with deletions

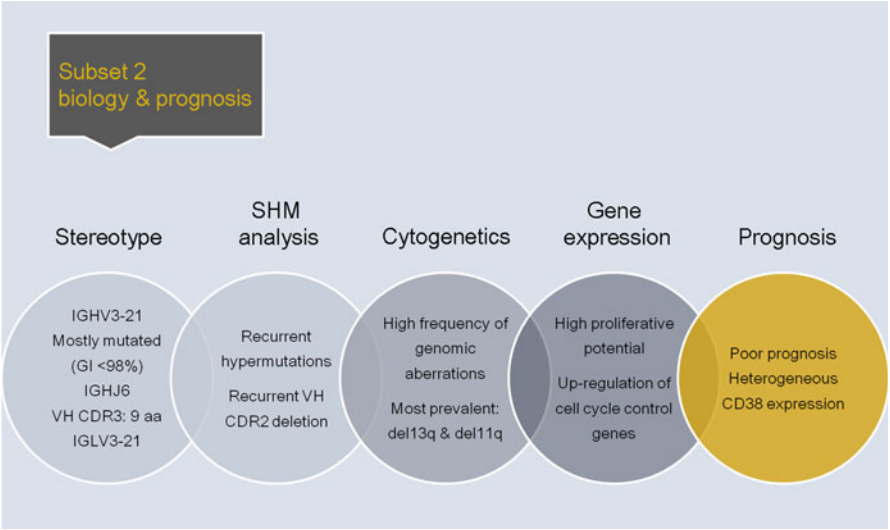

Fig. 1.4 A synopsis of the reported biological and clinical characteristics of CLL subset #2

involving the chromosomal regions 13q and 11q being particularly frequent [55]. Furthermore, gene expression profiling (GEP) of subset #2 cases revealed high proliferative potential, with up-regulation of genes involved in cell cycle control [56]. A schematic account of the biological and clinical features of subset #2 is given in Fig. 1.4.

Subset #8 is much less frequent, yet it has the distinction of being the first identified stereotyped subset—though not named as such at the time of the original publication back in 1995 [31]. Patients assigned to this subset display a particularly aggressive clinical course [34] and have the highest risk of transformation to Richter's syndrome (RS) of all CLL [57]. A high frequency of trisomy 12 has been reported for this subset [57], which is also distinctive among all other CLL for an association with a recurrent chromosomal translocation, namely t(14;19)(q32;q13), leading to deregulation of the *BCL3* gene locus [58].

In sharp contrast to the aforementioned bad prognosis subsets, subset #4, defined by the expression of mutated *IGHV4-34/IGKV2-30* BcR IGs, exhibits a very indolent clinical behavior [15], even when compared to other mutated CLL. Subset #4 cases do not express CD38 or ZAP70 [54] and have low genomic complexity [55]. GEP is suggestive of low proliferation [59]. Furthermore, another distinguishing feature of this subset concerns a much younger age at diagnosis (below 50) compared to CLL in general [15].

Altogether, this evidence links BcR IG molecular structure and functional attributes with the biological behavior of the clone underlying clinical behavior and, perhaps, the eventual outcome. Hence, molecular classification of CLL based on BcR IG molecular features is clinically relevant and may pave the way towards a better, compartmentalized understanding of the disease with obvious implications for patient-specific and hence, improved, patient care.

CLL Cells Are Antigen-Experienced: Evidence from Immunophenotype and Gene Expression Profiling Studies

Leukemic B cells from all CLL patients, regardless of their *IGHV* gene mutational status, exhibit an immunophenotype of activated B cells, characterized by high expression of the activation markers CD23, CD25, CD69, and CD71, and low expression of molecules usually down-regulated after cell activation (CD22, Fcγ receptor IIb, CD79b, and IgD). Furthermore, all CLL cells express CD27, an identifier of memory B cells [60].

Pioneering GEP studies using DNA chip microarrays supported the notion that all CLL cells, irrespective of the mutational status of their IG receptor, have a rather uniform GEP signature, being more related to memory B cells [61, 62]. Nevertheless, these same studies have identified a restricted number of genes whose differential expression can distinguish *IGHV*-mutated (M-CLL) from *IGHV*-unmutated (U-CLL) cases. Subsequent studies investigating GEP in relation to BcR IG features have reported distinctive profiles in subgroups of CLL cases defined by the molecular characteristics of their IG receptors. For instance, among CLL cases expressing mutated IGHV4-34 receptors, distinctly different GEPs were identified for two subsets (namely, subsets #4 and #16) whose major significant difference concerned different antigen-binding sites (i.e., different VH CDR3s). Genes with differential expression between these subsets were mostly involved in cell cycling, proliferation, and immune response and were down-regulated in subset #4, in accordance with the low-proliferative disease of subset #4 CLL patients [59]. Along the same lines, we recently reported differential GEPs for TLR pathway-associated genes between cases belonging to distinct stereotyped subsets even beyond *IGHV* gene mutational status [63].

Antigens in CLL: The Truth Exposed?

The precise nature of the antigens (Ags) recognized by CLL cells has been a matter of investigation for many years. Early studies from the late 1980s, mainly using hybridoma technologies, have offered the first evidence that CLL monoclonal antibodies (mAbs) are polyreactive, recognizing the Fc of IgG, ssDNA, dsDNA, histones, cardiolipin, or cytoskeletal components [64, 65]. Significant progress, however, was achieved much later through the application of recombinant DNA techniques to obtain CLL mAbs. With these technologies, it was convincingly demonstrated that CLL cells exhibit properties of auto/polyreactive cells that either retain (U-CLL) or lose (M-CLL) polyreactivity as a result of SHM. Indeed, the spectrum of epitopes reacting with CLL mAbs is generally broader in U-CLL clones; that notwithstanding, most recombinant mAbs from M-CLL clones that were not polyreactive per se acquired polyreactivity when their sequence was reverted to their germline equivalent [66].

In 2008, Rosen's group utilized cell lines derived from the neoplastic CLL clone by Epstein–Barr virus (EBV) transformation as well as primary ex vivo CLL cultures and showed that several CLL mAbs react with molecular motifs present on apoptotic cells and common bacteria, like vimentin, filamin B, cofilin-1, PRAP-1, phosphorylcholine, cardiolipin, oxLDL, and *S. pneumoniae* polysaccharides. U-CLL mAbs were again found to be polyreactive, contrasting those from M-CLL. These findings led to speculations that bacterial infections acting in synergy with neo–self-antigen/apoptotic cells may drive CLL evolution by continuously triggering BcR IGs with distinctive molecular features [67].

The reactivity profiles of CLL mAbs are strongly reminiscent of those described for natural antibodies that serve housekeeping functions such as clearance of material produced during apoptosis and normal metabolism [68]. Moreover, the antibacterial and anti-oxLDL reactivities point to a link between CLL and autoimmunity, since apoptotic cells are often targets of autoantibodies in systemic autoimmunity [69, 70]. Notably, microbial associations have been reported in several B cell lymphomas including CLL, e.g., with epidemiological studies showing that respiratory tract infections increase the risk of developing CLL [71].

Except for bacteria, viruses have also been implicated in CLL development. A recent study found higher CMV seropositivity in CLL cases compared with the general population [72]. The same group later reported that six different CLL mAbs encoded by the *IGHV1-69* or the *IGHV3-21* genes reacted specifically with the large structural phosphoprotein pUL32 of human CMV, whereas they failed to bind other bacterial, viral, or cellular structures tested [73].

Our group studied the possibility that common herpes viruses might be relevant to the pathogenesis of CLL, focusing particularly on clones expressing antigen receptors utilizing the *IGHV4-34* gene. This gene encodes antibodies that are intrinsically autoreactive by virtue of recognizing the N-acetyllactosamine (NAL) antigenic epitope present on various self-antigens (I/i blood group antigen, CD45 B cell isoform) but also on pathogens (EBV, CMV, *Mycoplasma pneumoniae*) [74]. Although the *IGHV4-34* gene is frequent among the normal peripheral B cell repertoire, such antibodies are rarely detectable in sera from healthy individuals, alluding to an anergic status for IGHV4-34 B cells. Nevertheless, IGHV4-34 antibodies have been found in high titers in the sera of patients with systemic lupus erythematosus (SLE), as well as healthy individuals after acute infections with EBV, CMV, and *M. pneumoniae* [75]. Along these lines, our overtime molecular monitoring of a large cohort of CLL patients provided evidence for CMV and EBV persistence specifically in cases carrying stereotyped IGHV4-34 receptors typical of subset #4 [76]. These findings imply that persistence of common herpes viruses could perhaps activate CLL progenitors expressing distinctive IGHV4-34 BcR IGs, promoting clonal expansion and, eventually, CLL outgrowth and, perhaps, evolution. Along these lines, it is relevant to mention that subset #4 is outstanding among all CLL examined thus far for a pronounced level of intraclonal diversification of the IG receptors through ongoing SHM activity, implying functional interactions with (auto)antigen(s) in the premalignant phase and after leukemic transformation as well [77, 78].

Very recently, we reported results from functional and molecular studies implicating for the first time the hepatitis C virus (HCV) in the development of a minor CLL subset with stereotyped IGHV4-59/IGKV3-20 receptors (subset #13). In particular: (1) the BcR IGs of subset #13 IG bore striking similarities to rheumatoid factors (RF) developing in various disease states some of which are associated with HCV infection; (2) several patients of this subset had positive anti-HCV serology; and (3) the mAb secreted from heterohybridomas of a typical CLL case in subset #13 was not directly binding to HCV, while it was exhibiting strong RF activity. On these grounds, we proposed that the stereotyped IGHV4-59/IGKV3-20 mIG with RF activity would not be binding directly to HCV but receiving indirect stimulation, as RF may bind immune complexes involving polyclonal IgG directed against HCV antigens, similar to mixed cryoglobulinemia type II [79]. The complete reactivity profiles of certain stereotyped subsets of mutated and unmutated status are given in Fig. 1.5a, b.

Antigen Reactivity Can Be Linked to Prognosis

In 2008 Chiorazzi's group demonstrated that stereotyped IGHV1-69/IGKV3-20 recombinant CLL mAbs typical of subset #6 bound nonmuscle myosin heavy chain IIA (MYHIIA) [80]. MYHIIA, normally an intracellular protein, is cleaved during apoptosis and translocated to apoptotic blebs, defining a subset of apoptotic cells called myosin-exposed apoptotic cells or MEACs.

Subset #6 CLL mAbs recognize MEACs but not apoptotic cells without exposed MYHIIA or live cells. However, MEAC binding is not confined to subset #6 as MEACs were targeted by several CLL mAbs, especially of the unmutated subtype. Perhaps more importantly, high binding to MEACs significantly correlated with poor patient survival, even beyond *IGHV* gene mutational status [81]. Equally noteworthy, CLL mAbs from the same stereotyped subset showed similar MEAC binding profiles strengthening the notion that stereotyped CLL clones might recognize and be selected by similar epitopes and that the clustering of CLL cases into distinct subsets based on BcR IG stereotypy is functionally and clinically relevant [15, 16].

BcR Signaling in CLL

BcR-proximal Lyn and Syk kinases induce PLCγ2 phosphorylation and Ras activation. Ras binds to and activates Raf kinase that subsequently activates MEK1 and MEK2 that lay immediately upstream of extracellular signal-related kinase (ERK)1/2. In a parallel signaling cascade, PI3K phosphorylates and activates AKT. BcR stimulation induces gene transcription also through activation of nuclear factor (NF)-kB and nuclear factor of activated T cells (NF-AT) transcription factors

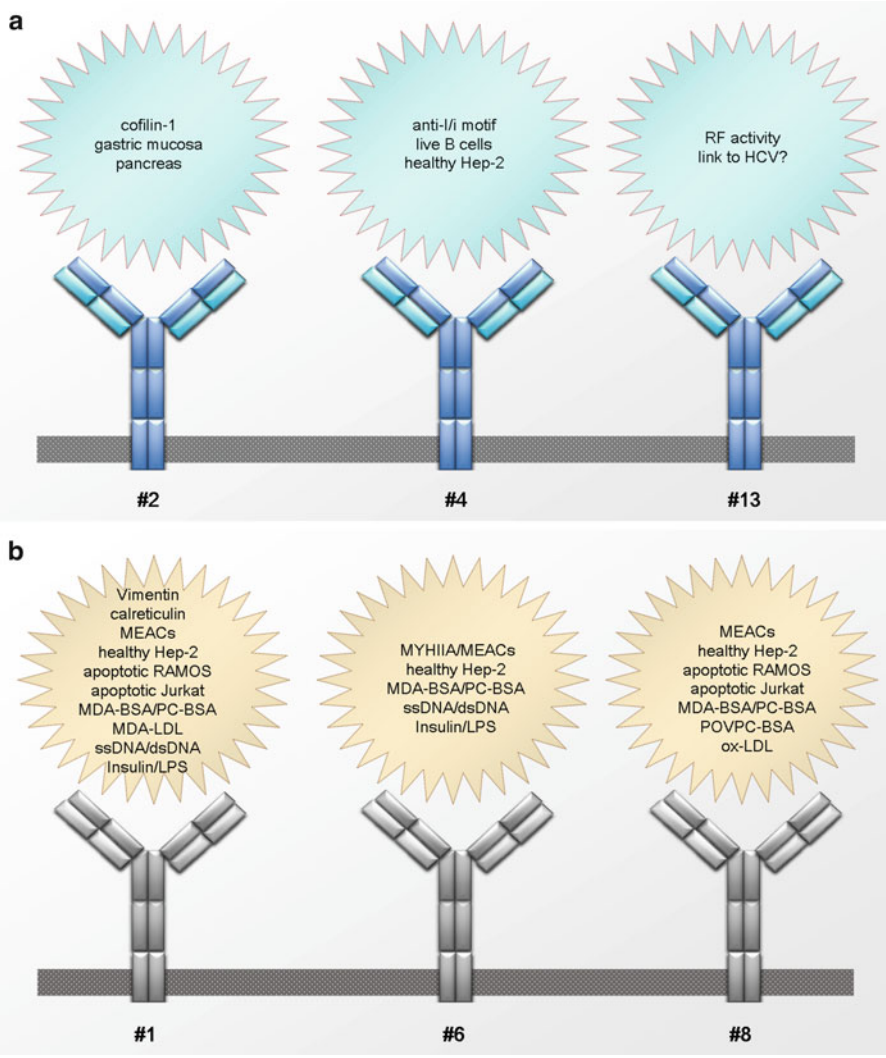

Fig. 1.5 Antigen reactivities of selected CLL stereotyped monoclonal antibodies (mAbs). Unmutated CLL mAbs react with autoantigens in a polyreactive manner in contrast to mutated Abs that are not polyreactive per se. (**a**) Stereotyped subsets #2, #4, and #13 carry mutated BcRs and show distinct and relatively restricted reactivity profiles. (**b**) Stereotyped subsets #1, #6, and #8 carry unmutated BcRs and are polyreactive yet with subset-specific reactivity profiles. For a comprehensive account of these profiles, see relevant sections of the chapter

[82]. CLL B cells are characterized by low surface IG expression; however, this does not necessarily mean that BcR signaling is defective. In fact, ~50 % of CLL cases were able to respond to in vitro cross-linking of their surface immunoglobulin (sIg) with effective activation, as measured by membrane-proximal events such as Syk

phosphorylation, PLCγ2 activation, and intracellular calcium release [83–85]. In contrast, the remaining cases were unresponsive to BcR cross-linking, thereby resembling B cells anergized after antigen stimulation. Nevertheless, the BcR signaling pathway in such cases appeared to be intact, since BcR unresponsiveness was reversible in vitro and dependent on the levels of sIgM [86].

Anergy is one of the physiological mechanisms employed to silence autoreactive B cells [87–89]. Interestingly, CLL cases unresponsive to BcR cross-linking were found to exhibit constitutive phosphorylation of MEK1/2 and ERK1/2 together with NF-AT activation, while at the same time lack AKT phosphorylation. This molecular profile resembles the biochemical signature of anergy in mice, leading to the hypothesis that these CLL cases could correspond to previously anergized autoreactive B cells aberrantly expanded as a result of the malignant process [83].

The molecular explanation of this functional difference among CLL cases is not yet clear. Although the aforementioned study did not find a correlation between the anergic profile and *IGHV* gene mutational status, in general, cases responsive to BcR ligation carry unmutated *IGHV* genes and exhibit high expression of ZAP70 and/or CD38 [90]. Given the adverse outcome of such cases, it would not be unreasonable to claim that increased signaling through the BcR underlies clinical aggressiveness in CLL. Nevertheless, even in these cases, BcR ligation did not elicit a full immune response, since it did not induce proliferation [86]. However, more sophisticated studies show that immobilized anti-IgM antibodies promote CLL cell survival probably by engaging the BcR for longer time-periods, implying that BcR cross-linking with soluble anti-IgM antibodies might not accurately recapitulate the in vivo antigenic stimulation [91, 92].

In CLL, the BcR Collaborates with Innate Immunity Receptors

BcR is the specific antigen receptor for normal and CLL B cells with abundant evidence pointing to its critical importance in CLL development as both a sensor and a mediator of interactions with the microenvironment. However, the BcR is not the only means of sensing the microenvironment: indeed, other sensors play important roles, e.g., CD40, receptors for complement, cytokines and chemokines, as well as the Toll-like receptors (TLRs), the major class of innate immunity receptors.

TLRs are pattern recognition receptors (PRRs) due to their ability to recognize widely conserved pathogen-associated molecular patterns (PAMPs) [93]. In humans, the TLR family consists of 11 members and each member recognizes different pathogen structures [94]. TLR stimulation acts as a direct third signal, that synergizes with BcR triggering (signal one) and T cell help (signal two), amplifying human B cell responses to antigen [95, 96]. Furthermore, functional interactions between the BcR and TLR signaling pathways are implicated in the control of B

cell anergy and/or TLR tolerance. For instance, dual engagement of the BcR and TLR9 in B cells has been proposed as a mechanism leading to autoimmunity, since it has been shown that triggering the BcR and TLR9 of RF^+ autoreactive B cells in a sequential manner is required for the production of autoantibodies [97]. Some years later, another study showed that combined engagement of BcR/TLR7 by RNA-associated autoantigens effectively activates autoreactive B cells [98]. Other evidence that TLR7 and TLR9 are implicated in autoimmunity is the fact that deficiency of TLR7 or TLR9 in lupus abrogates the production of autoantibodies against RNP and dsDNA, respectively [99]. Interestingly, chronic activation of ERK appears to act as a mechanism of resistance to CpG DNA-induced autoimmunity in anergic B cells [100].

The first studies of TLR signaling in CLL focused on TLR7 and TLR9, which are located within endosomal compartments and recognize RNA and DNA, respectively [94]. TLR9 stimulation induces the expression of co-stimulatory molecules, thereby augmenting the immunogenicity of CLL cells, and also has variable effects on their proliferation and/or apoptosis [101, 102]. TLR7 stimulation has been shown to regulate CLL cell immunogenicity [103, 104] and is possibly involved in apoptosis resistance [105].

We very recently reported that subgroups of CLL cases with distinct BcR IG molecular features show differential TLR pathway-associated gene expression [63] and distinct functional profiles after TLR stimulation. For instance, subset #4 cases were unresponsive to TLR7 stimulation with imiquimod despite expressing high TLR7 mRNA levels, indicating a TLR7-tolerant state [106]. In contrast, they responded avidly to TLR1/2 and TLR2/6 stimulation, up-regulating the expression of co-stimulatory molecules and showing reduced apoptosis (Fig. 1.6). Distinct gene expression and functionality profiles were also observed for other CLL subsets (the case of subset #1 is also depicted in Fig. 1.6) with stereotyped BcR IGs, both mutated and unmutated, indicating particular modes of collaboration of the clonotypic BcRs with innate immunity receptors.

Concluding Remarks

Microenvironmental stimulation is critical for CLL development and evolution. This is supported not only by molecular and functional studies showing that patient outcome can be linked to antigenic stimulation through the BcR IG, very likely in the context of other co-signals, but also by the clinical efficacy of drugs targeting the BcR [107, 108]. Therefore, it is only reasonable to argue that elucidation of the implicated immune mechanisms will assist towards the development of individualized treatment approaches in CLL: studying antigens and antigen receptors has a major part to play in this endeavor.

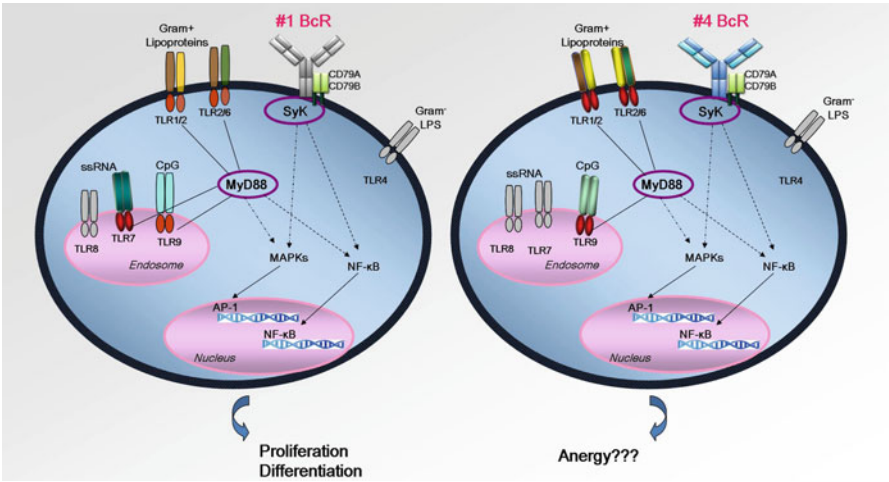

Fig. 1.6 In CLL, stereotyped BcR IGs collaborate with certain Toll-like receptors (TLRs), regulating CLL B cell responses. TLR7 stimulation in CLL subset #1 CLL (*left*) induces co-stimulatory molecule expression in contrast to subset #4. In contrast, TLR1/2, TLR2/6, and TLR9 stimulation induce strongest responses in subset #4 compared to subset #1. TLR4 and TLR8 (in *grey*) are expressed in low levels but are not functional in CLL B cells. The Syk kinase (kinase of the SRC family) and the MyD88 adaptor are key molecules for BcR and TLR signaling receptors which conduct the events near the membrane. Finally, through the action of multiple signaling molecules, the AP-1 and NF-κB transcription factors enter the nucleus and regulate the expression of target molecules

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Chapter 2
The CLL Cell Microenvironment

Jan A. Burger

Abstract Cross talk between CLL cells and accessory stromal cells in specialized tissue microenvironments, such as the secondary lymphoid organs, favors CLL progression by promoting malignant B cell growth and drug resistance. Disrupting the cross talk between CLL cells and their milieu is an attractive, novel strategy for treating CLL patients. This chapter summarizes current knowledge about cellular and molecular interactions between CLL cells and their supportive tissue microenvironment and the therapeutic targets that are emerging, focusing on the CXCR4–CXCL12 axis and small molecule inhibitors that are targeting the B cell receptor-associated kinases SYK, BTK, and PI3Kδ. Clinically relevant aspects of these new therapeutics will be discussed, along with an outlook into future biologically oriented therapeutic strategies. The rapid progress in dissecting the CLL microenvironment and the promising early results of these new targeted treatments in CLL indicate that CLL has become a role model for microenvironment-dependent cancers.

Keywords Chronic lymphocytic leukemia • CLL • Microenvironment • Nurselike cells • Stromal cells • CXCR4 • CXCL12 • B cell receptor • BCR • SYK • BTK • PI3Kδ • Chemokines • Chemokine receptors

Introduction to the CLL Microenvironment

CLL cells expand in specialized tissue microenvironments, such as the bone marrow (BM) and secondary lymphoid organs, where CLL cells interact with different populations of accessory cells, such as mesenchymal stromal cells [1]

J.A. Burger, M.D., Ph.D. (✉)
Unit 428, Department of Leukemia, The University of Texas MD Anderson Cancer Center, PO Box 301402, Houston, TX 77230-1402, USA
e-mail: jaburger@mdanderson.org

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and monocyte-derived nurselike cells (NLC) [2–4], as well as T cells [5, 6]. While the vast majority of circulating CLL cells in the blood are resting, nondividing cells, tissue CLL cells proliferate at a relatively high rate, accounting for a daily birth rate of approximately 0.1–1 % of the entire clone, as demonstrated by deuterated water labeling in CLL patients [7]. CLL cell proliferation occurs in microanatomical sites called proliferation centers, also called pseudofollicles, a hallmark histopathology finding in CLL [8, 9]. Conceptually, CLL and other mature B cell malignancies, such as follicular lymphoma (FL), mucosa-associated lymphoid tissue (MALT) lymphomas, and multiple myeloma (MM), are characterized by the expansion of the neoplastic B cells in a friendly, regulated coexistence with the microenvironment [9]. These interactions between CLL cells and the microenvironment resemble the pattern that normal counterpart B cells engage in. Particularly in the secondary lymphatic tissues, CLL cell B cell receptors (BCRs) become activated [10] either by currently still ill-defined microbial or autoantigens [11] or in an antigen-independent fashion [12, 13]. BCR activation then sets in motion a signaling cascade that results in the expansion of the CLL clone, in concert with other signals, such as CD40 ligand (CD154), BAFF and APRIL, and plexin B1 (reviewed in [14, 15]).

Consequently, the proliferative drive for the malignant cells is, at least initially, largely dependent upon external signals from the microenvironment, and the CLL cells undergo apoptosis unless their survival is reinforced by these external stimuli. Early evidence of microenvironment dependency came from the notion that CLL cells normally undergo spontaneous apoptosis in suspension culture unless they are cocultured with accessory stromal cells such as mesenchymal bone marrow stromal cells (BMSC) [2, 16, 17] or monocyte-derived NLC [2, 18]. Microenvironment dependence is also reflected by the difficulty of establishing CLL cell lines in the absence of EBV [19]. CLL–stroma interactions are not targeted by current "conventional" treatments, which may explain why, despite major therapeutic advances, CLL still remains an incurable disease. Based on this concept, CLL and other related mature B cell malignancies are expected to be particularly responsive to microenvironment-directed treatment approaches, and the clinical success of small molecule inhibitors of BCR-associated kinases in CLL patients in early stage clinical trials [20, 21] suggests that the microenvironment dependency of CLL cells may indeed be the "Achilles' heel" of CLL.

Tissue Microenvironments in the Bone Marrow and Secondary Lymphoid Organs: Cellular Players and Model Systems

The BM and secondary lymphoid organs have entirely different, distinct microenvironments, supporting lymphocyte maturation and differentiation. The BM harbors hematopoietic stem cells (HSC) and fosters the development of mature B cells from committed progenitors. B cell lymphopoiesis in the marrow results in

the generation of B cells with functional antigen (Ag) receptors (BCRs). Mature B cells then migrate to secondary lymphoid organs where they are exposed to Ag within germinal centers (GC) of secondary lymphoid follicles. The microenvironment of GC allows maturing B cells to interact with $CD4^+$ T cells for the necessary help upon Ag recognition and with specialized stromal cells (follicular dendritic cells/FDC) for the required quality control following affinity maturation [22, 23]. Each of these finely regulated steps ultimately results in the proliferation, maturation, and differentiation into Ag-specific effector plasma cells and memory B cells [24].

In the BM, stromal "feeder" cells maintain HSC in specialized "niches" which are close to the marrow vasculature (vascular niche) or to the endosteum (osteoblast niche) [25]. The importance of stromal cells for hematopoiesis was initially demonstrated in long-term BM cultures [26] and was utilized by Whitlock and Witte to develop a culture system to study the early stages of B cell maturation [27]. In vitro [28], CLL cells are attracted to BMSC, and the protective effects of BMSC require the close proximity between CLL and the stromal counterparts [2, 17, 28, 29]. The high affinity of CLL cells for stromal cells is exemplified by a striking in vitro phenomenon termed pseudoemperipolesis [28]. Pseudoemperipolesis describes the spontaneous migration of a fraction of CLL cells beneath BMSC, which occurs within a few hours of coculture. In phase contrast microscopy, pseudoemperipolesis is characterized by the dark appearance of lymphocytes that migrated into the same focal plane as the stromal cells. Pseudoemperipolesis describes symbiotic complexes of leukemia cells with their stromal cell component [30, 31]. During this cell interaction, leukemia cells migrate beneath the adherent BMSC or are trapped by membrane projections, but do not become internalized by the stromal cells. Coculture systems of CLL cells with BMSC, typically BMSC cell lines, have been standardized [29] and represent a reliable tool for studying CLL cell activation by BMSC, as well as stroma-mediated drug resistance. Intrinsic qualitative and quantitative abnormalities of CLL patient-derived primary BMSC have recently been characterized [32], as well as the effects of more physiologic hypoxia present in the marrow microenvironment on BMSC function [33]. NLC, on the other hand, activate CLL cells in a different fashion than BMSC, as demonstrated by gene expression profiling (GEP) in vitro [34, 35] and in vivo [10]. Specifically, BMSC induced a GEP pattern with prominent upregulation of the lymphoid proto-oncogene TCL1, paralleled by decreases of TCL1-interacting FOS/JUN [35]. In contrast, NLC induced a GEP response in CLL cells with characteristic induction of genes in the BCR and NFκB pathways [34] that is strikingly similar to the GEP of CLL cells isolated from lymph nodes of CLL patients [10]. Several other genes of potential importance were also differentially upregulated by BMSC (e.g., TNFRSF17, VPREB3, TNFSF10) and NLC (i.e., TNFRSF17, EGR2 and 3, MYCN), but their precise functions in the CLL microenvironment remain to be explored.

NLC owe their name to the similarities with thymic nurse cells that nurture developing thymocytes [2]. In vitro, NLC differentiate from blood monocytes cocultured with CLL cells in high-density culture conditions after 7–14 days [2].

In vivo, NLC can be found in the spleen and lymphoid tissues of CLL patients [3, 36], and the importance of NLC for CLL disease progression was highlighted in recent CLL animal models [37, 38]. NLC attract CLL cells by secreting CXCL12 [2] and CXCL13 [3] and protect CLL cells from spontaneous or drug-induced apoptosis through CXCL12 [2, 39], BAFF, APRIL [39], CD31, plexin-B1 [40], and activation of the BCR-signaling cascade [34]. Collectively, these data indicate that CLL cell cocultures with BMSC and NLC represent validated model systems for studying the impact of different microenvironments on CLL cell biology and for drug testing.

The Importance of BCR Activation in the CLL Microenvironment

BCR activation and signaling have emerged as a key mechanism for CLL cell expansion, even though the precise mechanism of BCR stimulation and the nature of the antigen(s) that activate the BCR remain largely unknown [11, 14, 41]. Direct evidence for the importance of BCR signaling in CLL comes from recent comparative GEP data that revealed BCR signaling as the most prominent pathway activated in CLL cells isolated from lymphatic tissues [10]. BCR activation can be induced either by antigen or be ligand independent ("tonic" BCR signaling) [12], and it activates a cascade of signaling events which normally cause B cell selection, proliferation, differentiation, and antibody production. Thereby, BCR signaling allows for the expansion of selected, specific normal B cells, and hence the deletion of unwanted, self-reactive B cells [42]. Engagement of BCRs by antigen induces phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmatic tails of Ig-α and Ig-β [43] by Lyn and other Src family kinases (Fyn, Blk), which also activate BTK, CD19, and PI3K. These events are associated with BCR oligomerization and BCR microcluster growth, leading to the recruitment and activation of SYK via ITAMs [44]. Upon phosphorylation, SYK, BTK, and PI3K activate downstream signaling pathways, including calcium mobilization and activation of AKT kinase, extracellular signal-related kinase 1/2 (ERK, also called p44/42 mitogen-activated protein kinase/MAPK), and myeloid cell leukemia-1 (Mcl-1) [45, 46]. In CLL cells, BCR activation also causes phosphorylation of SYK, activation of phospholipase Cγ2, and intracellular calcium mobilization and facilitates DNA binding of the calcium–calcineurin-dependent transcription factor NFAT2 [47], as well as MEK1/2-dependent expression of the proto-oncoprotein MYC [48]. The clinically most relevant targets within the BCR-signaling cascade in CLL are BTK, PI3Kδ, and SYK.

BCR-Associated Kinases BTK, PI3Kδ, and SYK

Bruton's Tyrosine Kinase, BTK

BTK is a non-receptor tyrosine kinase of the Tec kinase family and plays a central role in BCR signaling. BTK is primarily expressed in hematopoietic cells, particularly in B cells, but not in T cells or plasma cells [49]. BTK deficiency due to mutations in BTK is the genetic basis for X-linked agammaglobulinemia (XLA) [50, 51], a primary immunodeficiency characterized by low serum immunoglobulin levels and lack of peripheral B cells. Upon BCR activation, BTK becomes activated by other tyrosine kinases, such as Lyn and SYK, resulting in activation of transcription factors necessary for B cell proliferation and differentiation [52]. In addition to its role in BCR signaling, BTK also is involved in signaling of other receptors related to B cell migration and adhesion, such as the CXCR4 and CXCR5 chemokine receptors and adhesion molecules (integrins) [53–55].

Ibrutinib, previously called PCI-32765, is the first in-human BTK inhibitor which binds specifically and irreversibly to a cysteine residue in the BTK kinase domain and inhibits BTK phosphorylation and its enzymatic activity [56]. Ibrutinib shows encouraging clinical activity in patients with B cell malignancies, particularly in CLL patients [21]. Herman et al. reported about ibrutinib-induced CLL cell apoptosis in the presence of CLL pro-survival factors (CD40L, BAFF, IL-6, IL-4, TNF-α, fibronectin, stromal cell contact) [57]. We recently reported that ibrutinib inhibits CLL cell survival and proliferation, as well as leukemia cell migration towards tissue-homing chemokines (CXCL12, CXCL13) [58]. Ibrutinib also downregulated secretion of BCR-dependent chemokines (CCL3, CCL4) by the CLL cells, both in vitro and in CLL patients receiving therapy with ibrutinib. These data demonstrate that ibrutinib effectively inhibits CLL cell migration and survival, possibly explaining some of the characteristic clinical activity (CLL cell redistribution) of ibrutinib. Along the same lines, de Rooij and colleagues recently reported about ibrutinib's interference with CLL cell chemotaxis and integrin-mediated CLL cell adhesion [59], suggesting that these BCR-independent actions of ibrutinib explain the redistribution of CLL cells from the tissues into the peripheral blood, characteristically seen during the first months of treatment with ibrutinib and other BCR-signaling inhibitors [20, 21, 60].

Phosphoinositide 3′-Kinase Delta, PI3Kδ

PI3Ks integrate and transmit signals from different surface molecules, such as the BCR [61], chemokine receptors, and adhesion molecules, thereby regulating cellular functions, such as cell growth, survival, and migration [62]. PI3Ks are divided into three classes (I through III). Class I kinases contain four isoforms designated PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ. While the PI3Kα and PI3Kβ isoforms are

ubiquitously expressed and the PI3Kγ isoform has a particular role in T cell activation, PI3Kδ expression is largely restricted to hematopoietic cells, where it plays a critical role in B cell homeostasis and function [63]. Mice with inactivating PI3Kδ mutations have reduced numbers of B1 and marginal zone B cells, low levels of immunoglobulins, poor responses to immunization, defective BCR and CD40 signaling, and can develop inflammatory bowel disease [63, 64]. In CLL cells, PI3K are constitutively activated [65], and unmutated high-risk CLL patients show overexpression of PI3K by quantitative polymerase chain reaction [66]. Furthermore, growth and survival signals from the microenvironment, such as adhesion to stromal cells [67], CXCR4 activation [28], and BCR activation [46], cause PI3K activation in CLL cells.

GS-1101, previously called CAL-101, is a potent and highly selective PI3Kδ inhibitor and represents the first and currently the only PI3Kδ inhibitor in clinical use [68]. GS-1101 induces apoptosis in B cell lines and primary cells from patients with different B cell malignancies, including CLL [69], mantle cell lymphoma, and multiple myeloma [68, 70]. GS-1101 also inhibits constitutive and CD40-, TNF-alpha-, fibronectin-, and BCR-induced PI3K activation [68–70]. In patients receiving GS1101 therapy there is an initial redistribution of CLL cells from the tissues into the blood, along with a rapid lymph node size reduction and a transient lymphocytosis during the first weeks of treatment [71], which is not explained by inhibition of pro-survival signaling. GS-1101 inhibits CLL cell chemotaxis towards CXCL12 and CXCL13 and migration beneath stromal cells (pseudoemperipolesis) [72]. These in vitro results are corroborated by clinical data showing marked reductions in circulating CCL3, CCL4, and CXCL13 levels, paralleled by a surge in lymphocytosis during GS-1101 treatment [72]. Therefore, it appears that GS-1101 has several mechanisms of action, directly decreasing cell survival while reducing interactions that retain CLL cells in the tissue microenvironments.

Spleen Tyrosine Kinase, SYK

SYK belongs to the SYK/ZAP-70 family of non-receptor kinases and activates signaling pathways downstream of the BCR. SYK-deficient mice have severely defective B lymphopoiesis [73, 74], with a block at the pro-B to pre-B transition, consistent with a key role for SYK in pre-B cell receptor signaling. Moreover, in vivo studies recently demonstrated that SYK is critical for survival and maintenance of mature normal and malignant B cells [73, 75]. Besides their role in immune responses, SYK activation also modulates cell adhesion and chemotaxis of normal cells, such as B cells [76, 77], suggesting that SYK participates in tissue homing and retention of activated B cells.

R788 (fostamatinib disodium, FosD) is the only SYK inhibitor in clinical use to date. Fostamatinib, the clinically used oral formulation, is a prodrug that rapidly converts in vivo into the bioactive form called R406 [78, 79]. Previous studies established that R406 is a relatively selective SYK inhibitor, although R406 also

displayed activity against other kinases including Flt3, Jak, and Lck [79]. After encouraging results in a phase I/II study in patients with relapsed B cell lymphomas, particularly in patients with CLL, where the objective response rate was 55 % [20], further development of this drug is focused on rheumatoid arthritis (RA) [80]. As such, there is at this time only one ongoing clinical trial of fostamatinib in patients with diffuse large cell B cell lymphoma (DLBCL, NCT01499303). Alternative SYK-specific inhibitors are under development and have demonstrated promising preclinical activity in CLL models [81]. Importantly, similarities in clinical response pattern of CLL patients to treatment with SYK, BTK, or PI3Kδ inhibitors (transient lymphocytosis due to redistribution, rapid lymph node shrinkage) suggest overlapping functions of these kinases in BCR signaling, CLL cell migration, and homing [60]. The transient lymphocytosis caused by these new agents has complicated response assessment in these patients, given that progressive lymphocytosis could be interpreted as progressive disease (PD). However, given that CLL patients on these drugs typically show clear signs of response (reduced lymph node sizes, normalization of hemoglobin and platelet counts, resolution of constitutional symptoms) even before stabilization and then resolution of lymphocytosis, this lymphocytosis in the absence of other signs of PD should not be confused with true PD. This interpretation was supported by a group of CLL experts [82], and CLL response criteria may need to be formally revised when these new agents become more widely used.

Mechanism of CLL Cell Migration and Adhesion

Normal B cell trafficking and function largely depend upon interactions between B cells and accessory stromal cells [83, 84]. For example, stromal cells in secondary lymphatic tissues constitutively express chemokines such as CXCL12 and CXCL13 that provide guidance for B cell positioning within distinct lymph node compartments [83, 85–87]. According to Springer's multistep paradigm [88], lymphocyte trafficking and homing require the cooperation between chemokine receptors and adhesion molecules, such as integrins, CD44, and L-selectins, which are expressed on normal and malignant lymphocytes. Lymphocytes actively enter and home within tissue microenvironments, such as the secondary lymphatic tissues, where stromal cell networks provide guidance cues by secreting chemokines, establishing chemokine gradients, and expressing ligands for lymphocyte adhesion molecules. Coordinated lymphocyte entry, migration, and territoriality are essential during immune surveillance and induction of specific immune responses [85, 89–91]. In B cell lymphomas/leukemias, the neoplastic B cells largely retain the capacity of their normal counterparts for trafficking and homing, as demonstrated in CLL and B cell acute lymphoblastic leukemia (ALL), both in vitro [3, 28] and in vivo [92].

The term chemokines initially was coined in 1992 as a short form of "chemotactic cytokines." Currently, the human chemokine system includes more than

40 chemokines and 18 chemokine receptors [93]. Chemokines are small secreted proteins that are released either constitutively or in response to stimulation and cause migration of cells towards a gradient of the chemokine (chemotaxis). The two main subfamilies of chemokines, CXC and CC chemokines, are distinguished based upon two conserved cysteine residues, which are separated by either an intervening or adjacent amino acid, accounting for CXC or CC chemokines, respectively [93]. Chemokines bind to chemokine receptors, which belong to the large family of seven transmembrane domain G-protein-coupled cell surface receptors (GPCRs). Following activation, the intracellular domains cause dissociation of G-proteins, which are composed of three distinct subunits (α-, β-, γ-heterotrimers). This leads to formation of the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG), resulting in cytoplasmatic calcium mobilization and activation of multiple downstream signaling cascades, such as the phosphatidylinositol 3-kinase (PI3K)/Akt and the Ras/MAPK (also called ERK 1/2) signaling pathways.

T and B lymphocytes express receptors for various chemokines, and their expression and function is modulated during lymphocyte differentiation and activation [94]. Circulating lymphocytes interact transiently and reversibly with vascular endothelium through adhesion molecules (selectins, integrins) in a process called rolling. Chemokines displayed on the luminal endothelial surface activate chemokine receptors on the rolling cells, which triggers integrin activation [88]. This results in the arrest, firm adhesion, and transendothelial migration into tissues where chemokine gradients guide localization and retention of the cells [95]. These steps are collectively referred to as "homing" and are essential for normal development of the organism, organization and function of the immune system, and tissue replacement.

Chemokine Receptors on CLL Cells

CXCR4 (CD184)

CXCR4 is expressed at high levels on the surface of peripheral blood CLL cells [28, 96–98] and mediates CLL cell chemotaxis, migration across vascular endothelium, actin polymerization, and migration beneath and underneath CXCL12-secreting BMSC [28, 96, 99, 100]. CXCL12 also has a pro-survival effect on CLL cells [2, 39, 101], which is not surprising, given that CXCL12 initially was characterized as pre-B cell growth-stimulating factor (PBSF) [102]. CXCR4 surface expression is regulated by its ligand CXCL12 (previously called stromal cell-derived factor-1/SDF-1) via receptor endocytosis [28], with downregulation of surface CXCR4 on tissue CLL cells by CXCL12 present at high levels in the tissues. This characteristic can be used to distinguish tissue (lymphatic tissue- and marrow-derived) from blood CLL cells, which express low or high CXCR4 levels,

respectively [10, 28]. Proliferating, Ki-67$^+$ CLL cells from marrow and lymphatic tissue displayed significantly lower levels of CXCR4 and CXCR5 than nonproliferating CLL cells [103]. In vivo deuterium (^{2}H) labeling of CLL cells revealed that patients with higher CXCR4 expression on their CLL cells had delayed appearance of newly produced CD38$^+$ cells in the blood and increased risk for lymphoid organ infiltration and poor outcome [104]. These ^{2}H studies also revealed intraclonal heterogeneity of CXCR4 expression, with an enrichment of CLL cells expressing lower CXCR4 surface levels in the CD38$^+$/CD5^{bright} fraction, along with increased ^{2}H incorporation [104]. These in vivo data indicate that lower blood CXCR4 surface levels label a fraction of CLL cells that has recently exited the tissues into the blood.

B cell antigen receptor (BCR) signaling results in down-modulation of CXCR4 [105, 106], along with enhanced chemotaxis towards CXCL12 and CXCL13, at least in our hands [105]. This may explain why ZAP-70$^+$ CLL cells display increased chemotaxis and survival in response to CXCL12 when compared to ZAP-70-negative CLL cells [101], given that ZAP-70 expression is associated with a higher responsiveness to BCR stimulation [107]. CD38$^+$ CLL cells also display higher levels of chemotaxis [108], and CD38 activation enhanced chemotaxis towards CXCL12, whereas a blocking anti-CD38 mAb inhibited chemotaxis [109]. CXCR4 signaling in CLL cells is pertussis toxin sensitive and induces calcium mobilization, activation of PI3 kinases [28] and p44/42 MAP kinases [2], and serine phosphorylation of signal transducer and activator of transcription 3 (STAT3) [110]. CXCR4 signaling can be inhibited by isoform-selective PI3 kinase inhibitors [111], including CAL-101 [112], and inhibitors of SYK [105] and BTK [58], leading to impaired CLL cell migration.

Other Chemokine Receptors in CLL (CXCR3, CXCR5, CCR7)

CXCR3 (CD182) is the receptor for the CXC chemokines CXCL9, CXCL10, and CXCL11. These interferon-gamma (IFNγ)-induced chemokines are secreted at sites of inflammation and function in a paracrine or autocrine fashion [113]. CXCR3 is expressed on subsets of normal B and T cells [114]. CXCR3 is consistently expressed on CLL and splenic marginal zone lymphoma B cells, but not on normal CD5$^+$ B cells, and more inconsistently on neoplastic B cells from patients with other B cell lymphomas [115, 116]. CXCR3 expression levels on CLL cells are variable, and low CXCR3 expression was associated with advanced stages (Rai III/IV), diffuse marrow infiltration, other risk factors, and poor survival in one study [117], although the functional role of CXCR3 expression in CLL remains unclear.

CXCR5 (CD 185) is the receptor for CXCL13, a chemokine that regulates lymphocyte homing and positioning within lymph follicles [118]. CXCR5 is expressed by mature B cells, a small subset of T cells, and skin-derived dendritic cells (reviewed in [119]). CXCR5 gene-deleted mice display defective formation of

primary follicles and germinal centers in the spleen and Peyer's patches and lack inguinal lymph nodes [120]. Subsequently, the ligand for CXCR5 was identified and termed B cell-attracting chemokine 1 (BCA-1) [121] and now is designated CXCL13. CXCL13 is constitutively secreted by stromal cells in B cell areas of secondary lymphoid tissues (follicles), where B cells encounter antigen and differentiate [118, 122]. CXCR5 induces the recruitment of circulating naïve B cells to follicles [118, 122] and is also responsible for the microanatomic positioning within the germinal center (GC) [84, 86, 91, 123]. In addition, it has been suggested that the primordial function of CXCL13 may be the recruitment of primitive B cells to body cavities for T-independent responses, prior to its involvement in the complex lymphocyte positioning during T-dependent antibody responses [124]. CLL cells express high levels of CXCR5 [3, 97, 100, 116, 125]. CXCR5 expression levels are similar on CLL B cells and normal, $CD5^+$ B cells and higher when compared to normal, CD5-negative B cells, T_{FH} cells, or neoplastic B cells from other B cell neoplasias [3]. Stimulation of CLL cells with CXCL13 induces actin polymerization, CXCR5 endocytosis, chemotaxis [100], and prolonged activation of MAPK (ERK 1/2). In CLL, CXCR5 signals through Gi proteins, PI3 kinases, and the p44/42 MAPK pathway [3]. CXCL13 mRNA and protein is expressed by NLC in vitro and in vivo [3]. These data suggest that CXCR5 plays a role in CLL cell positioning and cognate interactions between CLL- and CXCL13-secreting stromal cells, such as NLC in lymphoid tissues.

The CCR7 (CD197) receptor has two ligands, CCL19 and CCL21. CCL19 and CCL21 are constitutively expressed by reticular cells, high endothelial venules (HEVs), and dendritic cells (DC) and play a role in lymph node homing of naïve and regulatory T cells and DC [126]. Moreover, the CCR7–CCL19/CCL21 axis is involved in organizing the architecture and function of the thymus. CCR7 is expressed by DCs, thymocytes during defined stages of their development, and B and T cell subpopulations. CCR7 is also expressed by various neoplastic cells, and CCR7 expression correlates with lymph node metastasis in solid tumors [127], including malignant melanoma and colorectal and prostate cancer. In sharp contrast to CXCR5-deficient mice, which show reduced peritoneal B-1 and B-2 B cells, CCR7 deficiency results in a massive accumulation of T cells and B-2 B cells in the peritoneal and pleural cavities, caused by an impaired egress of CCR7-deficient lymphocytes from body cavities [128]. CLL cells express CCR7 and migrate across vascular endothelium in response to CCL19 and CCL21 [99, 125]. Moreover, expression levels of CCR7 correlated with lymphadenopathy [99, 125] and expression of ZAP-70 and CD38 [101]. CCL19- and CCL21-induced migration and actin polymerization of $ZAP\text{-}70^+/CD38^+$ CLL cells were higher when compared to CLL cells lacking ZAP-70 and CD38 [101]. Moreover, CCL21 significantly increased B-CLL metalloproteinase-9 (MMP-9) production in MAP kinase (ERK1/2-)-dependent fashion [129], suggesting cross talk between these pathways during trafficking and tissue homing. CCR7 signaling for chemotaxis in response to CCL19 and CCL21 involves PI3 kinases and the Rho kinase [130]. Anti-CCR7 mAbs recently were shown to cause complement-dependent cytotoxicity against CLL cells and therefore were proposed as a potential therapeutic [131]. Overall,

these data support the concept that CCR7 plays an important role in trafficking and homing of CLL cells to the lymphatic tissues.

Chemokines Secreted by CLL Cells: CCL3, CCL4, and CCL22

CCL3 and CCL4 are chemoattractants for monocytes and lymphocytes [132]. CCL3 expression in normal B cells is induced by BCR triggering and CD40 ligand [133–135] and repressed by Bcl-6 [136]. Activated CLL cells express and secrete CCL3/4 [34, 137, 138] in response to BCR stimulation and in coculture with NLC [34]. This BCR- and NLC-dependent induction of CCL3/4 is sensitive to inhibition of BCR signaling, using, for example, a SYK inhibitor [34, 105]. CLL patients display elevated CCL3/4 plasma levels [34], and plasma levels of CCL3 were strongly associated with established prognostic markers and time to treatment. A multivariable analysis revealed that CCL3, advanced clinical stage, poor risk cytogenetics, and CD38 expression were independent prognostic markers in a cohort of 351 CLL patients [139]. The function of CCL3/4 in CLL remains poorly defined, but based upon the function of B cell-derived CCL3/4 in normal immune responses, increased CCL3/4 secretion by CLL cells may induce trafficking and homing of accessory cells, particularly of T cells and monocytes, to CLL cells in the tissue microenvironments [34, 140].

Regulatory T cells (T_{reg}), identified by expression of the transcription factor FoxP3, typically express the chemokine receptor CCR4 and migrate towards the ligands for CCR4, called CCL22 and CCL17. It was proposed that CCL17 and/or CCL22 secretion could be responsible for an accumulation of $FoxP3^+$ T_{reg} cells in the tumor microenvironment, which might suppress local immune responses and favor tumor progression in diseases such as breast cancer or Hodgkin's disease [141, 142]. CLL cells obtained from the tissues, but not from the blood, express CCL22 and variable levels of CCL17 mRNA. After CD40 ligation, CCL22 and CCL17 mRNA became induced in blood CLL cells, and CCL22 protein was released into CLL cell supernatants, which in turn attracted $CCR4^+$ T cells. Conceivably, by attracting T cells and other immune cells, CLL cell-derived chemokines foster the coevolution of CLL cells and their supportive microenvironment, actively creating a favorable microenvironment in which CLL cells interact with T cells and other accessory cells that deliver survival and proliferation signals.

VLA-4 (CD49d) Adhesion Molecules in CLL

Integrins are a superfamily of heterodimeric glycoproteins, consisting of various α- (1 through 11) and β- (1 through 6) subunits, whose function is to mediate cell–cell and cell–matrix adhesion in various cell types. The term "integrin" was first proposed in 1986 to describe membrane complexes involved in the transmembrane

association between fibronectin as part of the extracellular matrix (ECM) and the actin cytoskeleton [143]. Integrins are categorized into subfamilies, with members sharing a common β-subunit pairing with a unique α-subunit. β_1-integrins are very late activation (VLA) antigens that have the same β_1-subunit but various α-chains (α 1 through 6) [144]. The $\alpha_4\beta_1$-integrin VLA-4 (CD49d) is a receptor for fibronectin (FN) and vascular cell adhesion molecule-1 (VCAM-1/CD106). VLA-4 is expressed on lymphocytes, monocytes, and most other hematopoietic cells (except for neutrophils); VLA-4 is involved in both cell–cell and cell–ECM adhesions and plays a role in lymphocyte trafficking and homing as part of immune surveillance [94], the trafficking and homing of other hematopoietic cells, and inflammation. Integrins are highly versatile adhesion molecules; their adhesiveness can rapidly be regulated by the cells on which they are expressed: for example, by chemokine receptor activation [88]. VLA-4 mediates lymphocyte adhesion to the VCAM1, also known as CD106, which is expressed on cytokine-activated endothelium. VCAM1 mediates leukocyte–endothelial cell adhesion and may play a role in the development of artherosclerosis and rheumatoid arthritis. VLA-4 also binds fibronectin, an ECM component expressed on MSC [145], by interacting with at least three fibronectin sites: CS-1 and REDV in the IIICS region and H1 in the HepII region [146]. VLA-4 plays a particularly important role for interactions between normal and malignant hematopoietic cells and the marrow microenvironment. VLA-4 integrins cooperate with chemokine receptors in CLL cell adhesion to stromal cells [28, 147], they cooperate with CD38 [148], and their function can be inhibited by the BTK inhibitor ibrutinib [59]. Moreover, VLA-4 expression on CLL cells has prognostic impact [149, 150], indicating the relevance of these interactions in vivo. Collectively, these studies indicate that VLA-4 integrins play a key role for the adhesion of CLL and other leukemia cells to stromal cells and ECM, and they provide a rationale to further explore and target this molecule in CLL.

Therapeutic Targeting of Chemokines and Their Receptors in CLL

The CXCR4–CXCL12 Axis

CXCR4 antagonists initially were developed as new drugs for the treatment of HIV-1 infection (reviewed in [151]), where CXCR4 functions as a co-receptor for HIV-1 entry into T cells. However, their use in HIV-1 was abandoned because of lack of oral bioavailability and low efficacy. CXCR4 antagonists inhibit CLL cell activation by CXCL12 on functional and signaling levels, and they reverse, at least in parts, stromal cell-mediated drug resistance [110]. Several classes of CXCR4 antagonists are in clinical development, such as small modified peptide CXCR4

antagonists (BKT140), small molecule CXCR4 antagonists (AMD3100, now called plerixafor), and antibodies to CXCR4 (MDX-1338/BMS 93656). Plerixafor, a bicyclam, is a specific small molecule antagonist of CXCL12, inhibiting CXCL12-mediated calcium mobilization, chemotaxis, and GTP binding, and it does not cross-react with other chemokine receptors [152]. Plerixafor causes the mobilization of various hematopoietic cells, including CD34-positive HSC, to the blood [153, 154] and was approved by the FDA for administration together with G-CSF for mobilization of HSC in lymphoma and multiple myeloma patients. BKT140 is a high-affinity inverse CXCR4 agonist, which finished phase I/II testing in multiple myeloma patients that undergo stem cell mobilization.

The preclinical data of plerixafor and BTK140 were the basis for a recent clinical trial in relapsed CLL patients, in which patients were treated with rituximab in combination with plerixafor. The goal of this proof-of-principle trial was to determine whether leukemia cells can be mobilized from the tissues, using a CXCR4 antagonist, and then targeted outside of the protective tissues. Data from this trial demonstrated a plerixafor dose-dependent mobilization of CLL cells from the tissues into the blood [155].

Conclusions and Perspective

During the coming years increasing emphasis will be placed towards targeting the microenvironment in CLL and other cancers. Clinically, inhibitors of BCR-associated kinases (SYK, BTK, and PI3Kδ) represent the most advanced therapeutics for targeting the microenvironment in CLL, and when compared to other B cell malignancies, these agents display the highest clinical activity in CLL [20, 21]. The current enthusiasm for these novel agents is justified based on the clinical activity and the lack of major side effects, such as myelosuppression, but the precise mechanism of action, the potential benefit of combinations with conventional agents, usefulness of biomarkers such as CCL3 [58, 72, 139], durability of responses, and potential resistance mechanism remain to be explored. Despite these open questions, we can expect a paradigm shift towards kinase inhibitor-based treatment in CLL, which hopefully will benefit large numbers of CLL patients in the near future and which will set a new standard for other diseases to follow.

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Chapter 3
Acquired Genomic Copy Number Aberrations in CLL

Peter Ouillette and Sami Malek

Abstract Approximately 80 % of chronic lymphocytic leukemia (CLL) carries somatically acquired genomic copy number aberrations (aCNAs). These include gains of entire chromosomes (trisomy 12) and recurrent genomic losses, including interstitial deletions of various lengths at 13q14, 11q, and of more uniform length at 17p. In addition, approximately 10–15 second-tier aCNAs, with frequencies of 1–5 %, have been identified. In this chapter, we will discuss the biology and clinical significance of these CLL-associated aCNAs in detail and also discuss generic aspects of aCNAs relevant to all cancer cells. The hypothesis is advanced that most if not all aCNAs in CLL deregulate multiple target genes as a consequence of aCNA-associated gene mutations and through stable deregulation of gene expression. The concept of elevated genomic complexity (multiple aCNAs per CLL case) is reinforced as one of the strongest biological traits associated with aggressive CLL with short survival. Further, all inherited polymorphic copy number variations as detected through SNP 6.0 array profiling of T-cell-derived DNA of 255 CLL patients are listed to allow the reader a more critical appraisal of the somatic status of CLL-associated aCNAs as reported in the literature. Finally, given that aCNAs and gene mutations coexist in many CLL cells, we stress the importance of understanding in detail the relative biological and clinical roles each mutation type serves in individual CLL patients; this is a research area in need of more in-depth investigation.

Keywords CLL • Genome • aCNA • LOH

P. Ouillette • S. Malek, M.D. (✉)
Department of Internal Medicine, Division of Hematology and Oncology,
University of Michigan, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0936, USA
e-mail: pouillet@med.umich.edu; smalek@med.umich.edu

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Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western World and remains incurable with conventional chemotherapy treatment approaches. CLL is a heterogeneous disease and comprises multiple related illnesses [1, 2]. The heterogeneity in CLL biology is reflected in substantial patient-to-patient variations in CLL clinical presentations, the speed of disease progression, the response to therapies, and ultimately, survival times from diagnosis [3, 4].

Acquired genomic copy number aberrations (aCNAs), as in most cancers, also serve a central role in CLL pathogenesis [5–7]. Over the last 2 decades, the anatomy and incidence of aCNAs in CLL have been described in great detail and at a spatial resolution that now has reached the sub-megabase range. Using latest SNP array-based technologies, somatically acquired aCNAs in the 50–100 kilobase range can be easily identified with high sensitivity and specificity, provided paired normal DNA is analyzed in parallel to tumor samples to identify polymorphic copy number variants (CNVs). From an aggregate review of data from CLL karyotyping, CLL fluorescent in situ hybridization (FISH) testing and SNP array-based profiling, it is clear that overall, CLL is characterized by a relatively stable genome. The majority of CLL cases at diagnosis (80 %) harbor 0–2 aCNAs and 20 % of all CLL carry $\geq$3 aCNAs; this is in contrast to other hematological malignancies like DLBCL, FL, MCL, or AML, which carry substantially higher mean aCNA loads per genome.

CLL-associated aCNAs primarily consist of chromosomal material loss on one affected chromosome; recurrent homozygous losses are only detected in the epicenter of a subset of del13q14 at ~50 Mb physical position. Gains (3 N states) of chromosomal material are less frequent and mostly reflect trisomy 12—trisomies of other chromosomes are rare, and recurrent high-level amplifications (greater than 3 N) are not observed in the CLL genome. Besides aCNAs, CLL occasionally demonstrates structural changes such as balanced chromosomal translocations and isochromosomes. These are detectable through stimulated karyotyping, but unless associated with the loss of chromosomal material, they may escape detection via SNP arrays.

The recurrent aCNAs in CLL are listed here in order of descending frequency: del13q14 (~50 %), trisomy 12 (15–18 %), del11q (10–15 %), and del17p (7–10 %). Largely as a result of high-resolution SNP array profiling, approximately 10–15 second-tier recurrent aCNAs, which occur at frequencies of 1–5 %, depending on the characteristics of the CLL cohort analyzed, have been identified as well.

In the following paragraphs, we briefly outline generic pathomechanisms as they relate to aCNAs in cancer in general and specifically to CLL. The biological and clinical characteristics of selected recurrent aCNAs in CLL and associated recurrent gene mutations, as applicable, are then discussed in detail.

Generic Aspects of the Biology of aCNAs as Applied to CLL

Individual aCNAs in CLL may represent random genomic damage that lacks immediate biological consequences for the afflicted CLL cell (passenger aCNAs), or they may be drivers of CLL biology (driver aCNAs). As a general notion, recurrence of an aCNA in an otherwise relatively stable genome likely indicates a selection for the associated biological effects of that aCNA and therefore a contributing role to CLL biology. Clearly, for the most frequent aCNAs in CLL, del13q14, trisomy 12, del11q, and del17p, the identification of deregulated and mutated genes and pathways, as detailed below, supports an important biological role. However, for the infrequent second-tier recurrent aCNAs such a driver role or other disease-modifying characteristic has not yet been established or identified, and for sporadic aCNAs a driver role may very well not exist at all.

Regarding the possible effects any individual aCNA can have on an afflicted CLL cell, a variety of issues deserve consideration. First, the aCNA removes one allele of a critical gene or genes and the retained allele of the gene(s) is mutated. This is the classic scenario for tumor suppressor genes, and this exists in CLL at 17p for *TP53* and at 11q for a small subset of cases with *ATM* mutations. For all other recurrent aCNAs in CLL, including del13q14, this is not the prevailing pathological scenario, and conversely, most recurrently mutated genes in CLL as detected through large-scale sequencing studies are not located in recurrent aCNAs. Secondly, an aCNA removes one allele of a critical gene or genes and the retained allele is insufficient to maintain full gene function; this scenario is referred to as haplo-insufficiency. Although haplo-insufficiency (or compound haplo-insufficiency involving more than one gene) likely contributes substantially to the biology of aCNAs in CLL cells, this mechanism has not been definitively proven to be operational for individual genes. In some cases, such as an 11q deletion with one residual intact *ATM* allele, there is in fact no evidence for ATM haplo-insufficiency. In the third scenario, an aCNA removes one allele of a gene or genes and the retained allele is epigenetically downregulated. This is an evolving research topic and is not yet proven to be critically involved in the pathogenesis of aCNAs in CLL. Fourthly, specific aCNAs and other events in the CLL cell, such as aberrantly regulated pathways like those of the B cell receptor (BCR) or pathways involving mutated genes like *NOTCH1*, cooperate to effect specific phenotypic changes. For example, a statistical association of co-occurrence between *NOTCH1* mutations and trisomy 12 or between *MYD88* mutations and del13q14 has been detected. Finally, an aCNA may critically affect multiple independent genes or pathways simultaneously and therefore cannot be reduced to a single pathobiological principle. In particular, the concept of a minimally deleted or altered region (MDR or MAR), although useful in narrowing down regions of interest for single pathogenetic gene discoveries, defies the full biological and clinical impact of all major aCNAs in CLL and in fact has stymied research progress in this area for years.

Acquired Genomic Copy Number Aberrations and Loss-of-Heterozygosity in CLL

Acquired CLL Genomic Complexity

aCNAs, loss-of-heterozygosity (LOH), and copy-neutral LOH (cnLOH) have been studied in paired highly purified CD19+ and CD3+ cell-derived DNA samples from 255 CLL patients utilizing the SNP 6.0 array platform(5), as well as other SNP array-based reports which incorporated from 70 to 369 patients and employed DNA of varying purity and/or DNA from tumor only [8–14]. Array CGH platforms have been used in other published CLL studies [15, 16]. A rather complete view of genomic aCNAs in CLL has emerged from this data: generally, the CLL genome is relatively stable, with most cases demonstrating between 0 and 2 aCNAs. Observed LOH is usually associated with copy loss in CLL. cnLOH, also referred to as acquired uniparental disomy (aUPD), is infrequent. When it does occur, however, it is clinically relevant, such as in the case of cnLOH at 17p that is associated with homozygous *TP53* mutations [17].

The subset of CLL with elevated aCNA counts is clinically important. Two or more aCNA are detected in ~35 % of all cases; three or more aCNA in ~20 %. As such, an elevated aCNA count, referred to as elevated genomic complexity, delineates a CLL subset with progressive and aggressive disease and short survival [5, 6, 18]. Furthermore, the estimates for survival shorten for each additional aCNA, indicating that hypercomplex CLL genomes are associated with the shortest survival times. Elevated genomic complexity was found to be the dominant predictor of short overall survival (OS) in CLL in comprehensive bivariate and multivariate analysis and superseded knowledge of all other frequently tested molecular predictors. High-risk CLL cases were identified in all CLL cohorts that were previously stratified by other markers, like del17p/del11q, *TP53* mutations, IgV_H status unmutated, and ZAP70 positivity. It is thus clear that there is a strong association between an inability to maintain genomic stability and an aggressive CLL phenotype.

Genomic complexity has also been identified and described in large-scale CLL karyotyping studies (see below). Three or more genomic aberrations were detected in approximately 20 % of cases, and the presence of complex aberrant karyotypes predicted for short OS [19]. Although formally unproven, it is likely that both SNP array-based and karyotype-based genomic analyses identify subsets of CLL patients that largely overlap, with SNP arrays additionally detecting microdeletions or cnLOH and karyotype analyses detecting chromosomal changes that do not result in genomic copy loss, such as balanced translocations, chromosomal additions, or isochromosomes.

Known and measurable CLL properties, when correlated with genomic complexity as detected by SNP arrays, independently associate with elevated genomic complexity in the following descending order of strength: del17p/*TP53* mutations, del11q, and del13q14 type II (large enough to include *RB*) [20]. Elevated CLL

genomic complexity was weakly associated with diminished ATM activation as measured through auto-phosphorylation after ex vivo CLL cell irradiation, but the effect could not be separated from the presence of del11q [20].

What is the likely mechanism behind the strongly negative prognostic effects of elevated genomic complexity in CLL? The association with *TP53* mutations is one possibility, as most cases of *TP53*-mutated CLL are also genomically complex. However, two-thirds of all CLL with two or more aCNAs are *TP53* wild type. It remains unclear, despite strident research efforts, what molecular aberrations cause or are associated with genomic complexity in this CLL subset. Possible hypotheses that are currently being experimentally tested are (1) stable changes in the expression of genes relevant to DNA repair and maintenance that predispose to genomic instability (judged to be unlikely, based on unpublished observations), (2) genomic complexity is an invariable byproduct of CLL cell accumulation/proliferation as seen in progressive CLL (also unlikely, as many ZAP70+ or IgV_H unmutated CLL are not genomically complex), (3) telomere dysfunction-associated genomic instability (it is unclear if this mechanism can create interstitial deletions), and (4) mutations in multiple genes that are individually engaged in pathways that affect DNA repair and maintenance. The latter hypothesis is likely at least in part correct and is currently being tested using next-generation sequencing-based approaches in genomically complex CLL.

Three complementary hypotheses are put forth to explain the clinical aggressiveness of genomically complex CLL: the first is a frequent association with del17p, *TP53* mutations, and other defects which impair the cell's apoptotic response to genotoxic therapies [6, 20]. The second is ongoing clonal evolution, which has been documented for CLL cases that received therapy in the setting of preexisting *TP53* mutations; third, a relationship to telomere attrition and associated genomic instability. This last possibility would put genomic instability in the position of being a consequence of telomere dysfunction. This remains an attractive concept, but it is as yet unproven [21–25]. Moreover, it remains uncertain whether telomere dysfunction can give rise to the most frequent aCNA type in CLL: interstitial deletions.

In the ensuing subsections, established, high-frequency CLL aCNAs and associated gene mutations are examined individually, followed by a brief synopsis of novel, low-frequency (but recurrent) aCNAs for which little detailed information is presently available.

Deletion 13q14

The most frequent chromosomal abnormalities in CLL, present in 50 % of cases [7, 26–32], are the interstitial deletions in chromosome 13, which are frequently referred to as del13q14. These deletions are centered near chromosomal physical position 50 Mb, may range in length from less than one megabase to over forty Mb, and can extend in either the centromeric or telomeric direction. In about 15–20 % of del13q14-afflicted CLL cases, a lesion is present on both chromosomes. It may be

the same deletion or a mosaic of two different lesions of different lengths. This knowledge is possible largely as a result of high-resolution SNP array profiling [33]. Figure 3.1 summarizes these findings through a previously published copy number heatmap of 255 CLL cases and a schema of various observed presentations of del13q14 [5, 34]. The conclusions that can be drawn from these studies are several. The striking length heterogeneity of del13q14 has already been mentioned—it is likely that these various lesions deregulate differing but overlapping sets of genes. Previous work had identified very short lesions which were used to define a minimally deleted region (MDR) that included the microRNA *mir15a/16-1* locus. This microdeletion or a related deletion was not found in large CLL genomic profiling studies [33]. Rather, approximately 60 % of all CLL with del13q14 were found to have a relatively uniform lesion type, about 0.8–1.0 Mb in length that we have termed type I. It should be noted that some type I lesions are biallelic and occur flanked by tens of megabases of cnLOH, which is otherwise rare in CLL. Considering the centromeric breakpoint patterns observed, multiple discrete anatomic del13q14 lesion clusters can be identified. This suggests divergent but largely unknown biological or clinical consequences. Finally, 98 % of all del13q14 do share in common a loss of the *miR15a/16-1* locus, *DLEU7*, and all intervening genes [14, 34, 35].

The frequent occurrence of del13q14 in CLL suggests that it is a driver of CLL pathogenesis. However, del13q14 lesions, indistinguishable from those found in CLL, have been detected in other diverse cancer types, including multiple myeloma, diffuse large B cell lymphoma, acute myeloid leukemia, prostate cancer, and others. Therefore, del13q14 is unlikely to confer CLL-specific biological properties upon the CLL cell. In addition, there is a 50 % frequency of del13q14, detectable by FISH, present in CD5+ CLL-like monoclonal B cell lymphocytosis (MBL), but only 1 % of these MBL cases convert to CLL yearly—clearly indicating that del13q14 by itself is not sufficient to cause CLL in humans [36, 37]. Finally, it should be noted that gene deregulations resulting from del13q14 remain incompletely understood despite many years of research efforts, and of the genes located within Type I deletions, none are recurrently somatically mutated.

The *miR15a/16-1* locus is a contributing genetic element to del13q14 biology [38]. This locus is included in nearly all del13q14 lesions [33], and it is occasionally biallelically deleted, suggesting a tumor suppressor gene function. However, in humans, at approximately 160.122 Mb on chromosome 3, a second locus encodes for the highly related *miR15b* and *16.2* (http://www.mirbase.org/index.shtml), which may potentially compensate for loss of *miR15a/16-1* in del13q14. With this caveat in mind, other lines of evidence support a role for *miR15a/16-1*. Observed effects of low *miR15a/16-1* levels on anti-apoptotic and cell cycle-regulating molecules are consistent with a role in both CLL pathobiology and cancer in general. In mice, a CLL-like illness develops upon ablation or alteration of murine homologues of hsa-*miR15a/16-1* in a spontaneously occurring or artificially engineered context [39–41]. Finally, epigenetic downregulation of *miR15a/16-1* occasionally occurs in CLL lacking del13q14 [42].

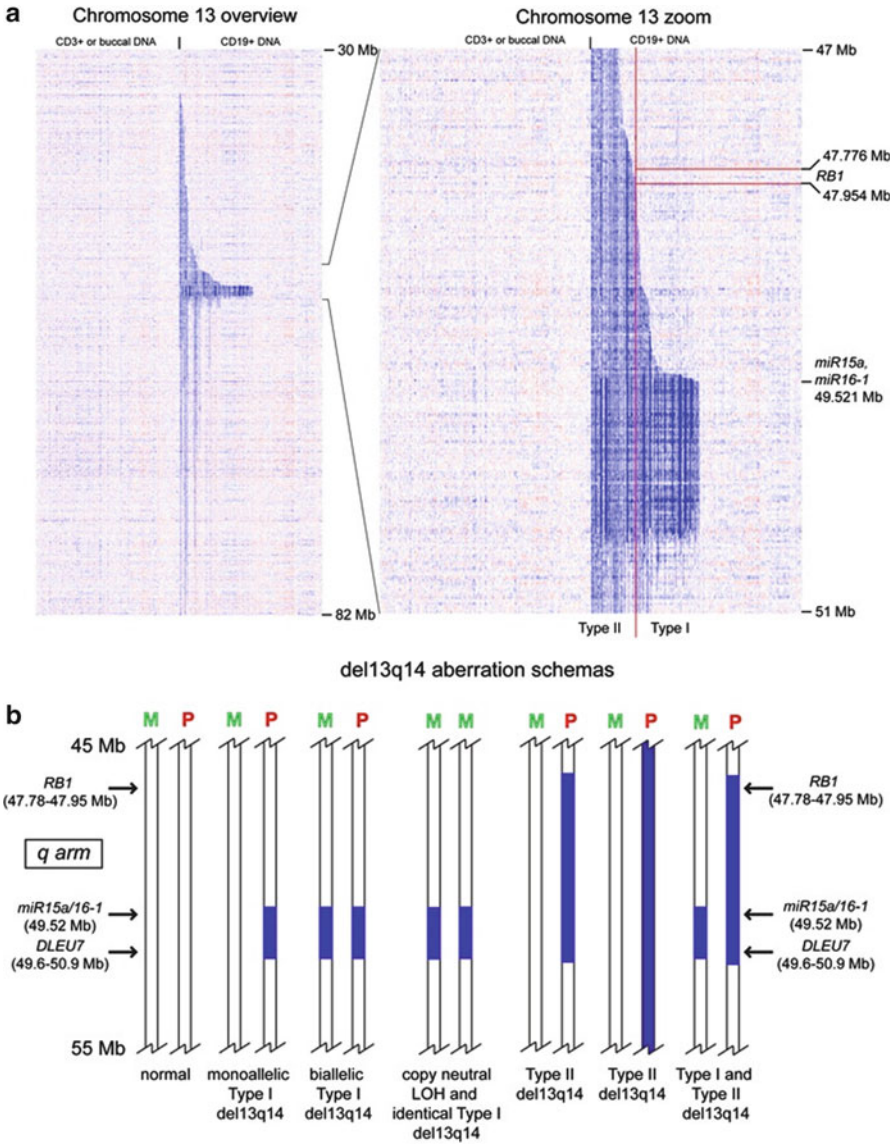

Fig. 3.1 Genomic copy number heatmap display of chromosome 13q of 255 CLL cases ranked by the position of centromeric 13q14 deletion break points: (**a**) Copy number heatmap displays for paired DNA samples based on SNP 6.0 array profiling were generated using dChipSNP. *Left half of each heatmap*: CD3+ or buccal DNA; *Right half of each heatmap*: CLL CD19+ DNA. *Blue* indicates copy loss, *red* indicates copy gain. Each column represents one patient. (**b**) Schemas of various del13q14 in CLL. *M* maternal chromosome, *P* paternal chromosome. The approximate location of selected genes (*miR15.a/16.1*, *DLEU7*, and *RB*) is indicated. Deletions are indicated by *blue bars*

However, experimental evidence is published which suggests a less fundamental and possibly more contributory role of miR15a/16-1 to CLL pathogenesis. Mice have been engineered to have either a deletion of only the *miR15a/16-1* region or a larger deletion, similar to the deletion size which actually occurs in humans. CLL-like illnesses occur less frequently in the former mice and more frequently in the latter. In expression studies, miR levels (particularly miR16-1) are not substantially affected by monoallelic del13q14 [34]. Lastly, there is a lack of recurrent *miR15a/16-1* mutations in CLL.

DLEU7, as stated previously, is deleted in nearly all del13q14 lesions, and has been the subject of recent attention, as it is frequently underexpressed in CLL [43]. In heterologous cell systems, DLEU7 demonstrates an effect on pro-survival cell signaling pathways. In addition, a family with multiple CLL cases has shown inherited CNVs which span the *DLEU7* gene [44, 45]. Therefore, it is conceivable that the mutual ablation of *miR15a/16-1* and *DLEU7* (additional genes may also be involved) codetermines the biology of del13q14. This research area constitutes a work in progress.

It bears repeating that no recurrent, somatically mutated gene has been found within any of the various CLL-associated 13q14 deletions, even including an ad-hoc summary analysis of recent large-scale sequencing studies. The yet-to-be-identified critical genes located within del13q14 are most likely therefore subject to haplo-insufficiency, or inactivated through epigenetic regulation.

In the clinical setting, del13q14 in the CLL patient is commonly detected through FISH, and it is important to be cognizant of several considerations. First, there is no independent prognostic significance than can be ascribed to (1) a monoallelic vs. a biallelic del13q14 finding when the length of 13q14 deletions is accounted for, or (2) the expression levels of *miR15a/16-1*, which reside on 13q14. Second, del13q14 long enough to include the *RB1* gene near 48 Mb (termed type II lesions) indicate a CLL subset with del13q14 that is clinically more aggressive and demonstrates shortened survival, *especially after therapy*, and the clinical FISH assay cannot discriminate between type I and type II lesions—the location of the probe will detect all del13q14. It is important to note that an association has been detected between del13q14 type II lesions and an elevated genomic lesion load, i.e., elevated genomic complexity [14, 34, 35, 46–48]. It follows that future inclusion of a second del13q14 FISH probe centered on the *RB1* gene will further refine CLL risk stratification. The last consideration is that the detection of a del13q14 of any type plus any additional FISH abnormalities or additional aCNAs indicates a more aggressive disease course than a del13q14 found in isolation.

Deletion 17p

Approximately 7 % of newly diagnosed CLL are afflicted with a deletion of the short arm of chromosome 17 (del17p) [7, 49] as a consequence of various structural abnormalities, such as deletions, unbalanced translocations, and isochromosomes.

In CLL, del17p is quite uniform in length, extending from the p-arm telomere to 18–22 Mb. Small 17p deletions that are centered on *TP53*, which is located at the 7.5 Mb physical position on 17p, have also been described, but are rare. When present, del17p tends to occupy a large percentage of the CLL clone as quantified by FISH, indicating a strong growth or survival advantage. The del17p always results in the deletion of the critical *TP53* locus on one allele, and the remaining *TP53* copy is reported mutated in from 64 to 100 % of cases, according to various studies [6, 17, 50–52]. It should be pointed out, however, that these studies differ in technical aspects of *TP53* mutation analysis, and they differ substantially in the frequency of mutations detected in *TP53* exons 4, 9, and 10. Large, unselected CLL cohorts with 193–400 CLL patients as described in [6, 50, 59], respectively, found that in CLL cases already carrying del17p, from 94 to 100 % carried a *TP53* mutation. In the author's opinion, nearly all CLL cases with del17p have concomitant *TP53* mutations on the remaining allele. Figure 3.2 shows a published chromosome 17 copy number heatmap for a large cohort of CLL cases, along with a schema to visualize the relationships between chromosomal aberrations, LOH, and *TP53* mutations.

The following facts describe the important clinical facets of del17p and *TP53* mutations. The clinical CLL FISH assay utilizes a probe for del17p centered on the *TP53* locus [53]; therefore all del17p are detectable. Some cases with del17p duplicate the remaining allele, however, resulting in cnLOH, which is undetectable by FISH. Yet such cases always carry homozygous *TP53* mutations: a falsely reassuring clinical test result in the context of an aggressive CLL [17]. Unbiased frequencies of cnLOH at 17p in untreated or relapsed/refractory CLL patients are not available, although in [17], three of 255 cases (1 %) were positive for cnLOH at 17p.

TP53 mutations do occur which are not associated with del17p (commonly referred to as monoallelic *TP53* mutations), but these are infrequent, and some of the reported *TP53* mutations that exist independently of del17p have an unclear relation to cnLOH at 17p. Large CLL cohorts report occurrences from 1 to 5 %; in highly selected, fludarabine-refractory CLL, this frequency can be as high as 18 %. In the nonacademic or non-referral setting, the frequency may well be 1 %, although accurate estimates are unavailable. These monoallelic *TP53* mutations are also associated with aggressive disease [50–52, 54, 55].

Somatically acquired *TP53* mutations can be detected in approximately 10 % of newly diagnosed CLL using direct sequencing of PCR products templated on genomic DNA or functional assays [50, 56, 57], although in community-based assessments this frequency may be substantially lower [58]. Clearly, most published reports suffer from referral biases that result in overestimates of the *TP53* mutation frequencies in CLL. Functional assays for the detection of *TP53* mutations have been described as well [17, 59]. In CLL, *TP53* mutations tend to occur in the DNA binding domain and are largely restricted to exons 5–9. *TP53* exon 4 harbors mutations occasionally, and they are rare in exon 10 [17, 52, 54, 55]. Missense mutations predominate, with the remainder being indels, nonsense, or located at splice acceptor or donor sites. These latter mutations result in truncated or altered TP53 proteins and are assumed to be inactivating mutations.

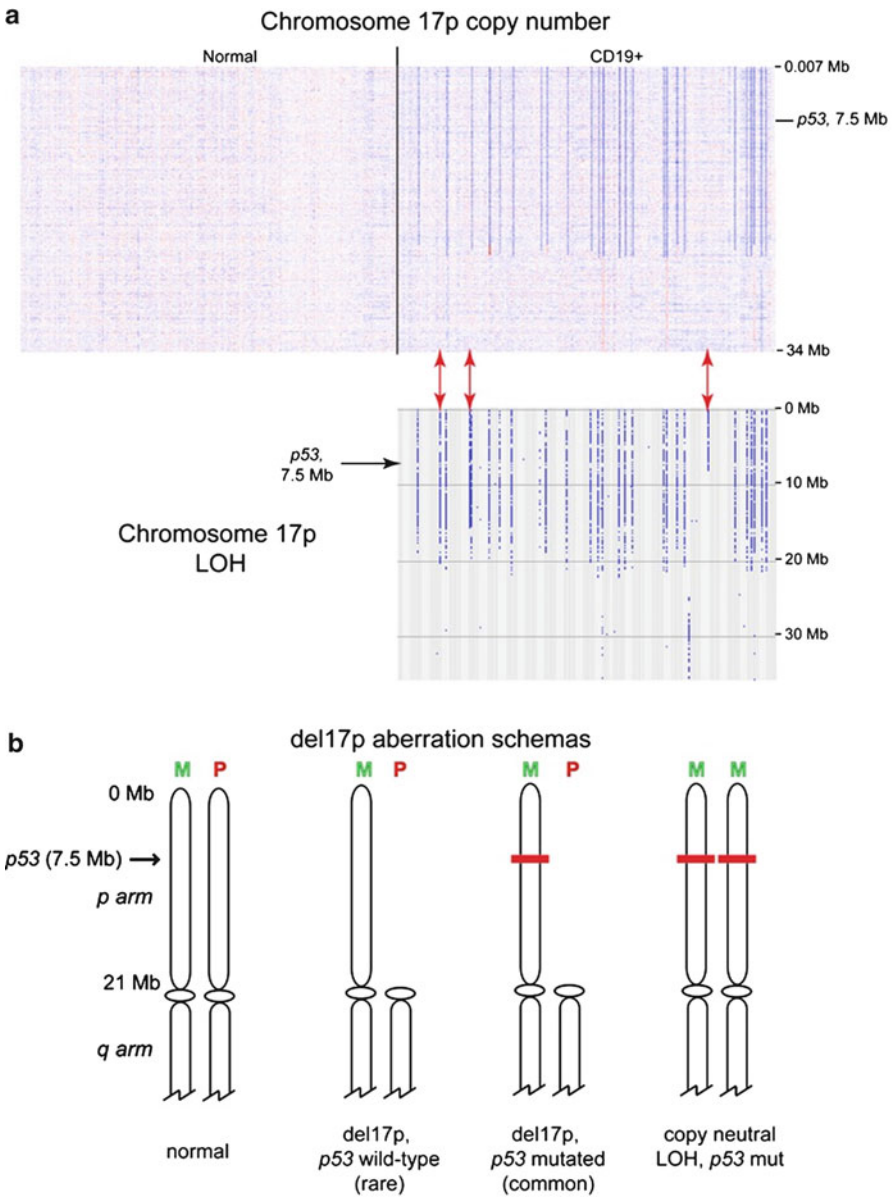

Fig. 3.2 (**a**) Genomic copy number heatmap display of chromosome 17p of 255 CLL cases: Copy number heatmap displays for paired DNA samples based on SNP 6.0 array profiling were generated using dChipSNP. *Left heatmap half*: CD3+ or buccal DNA; *Right heatmap half*: CLL CD19+ DNA. *Blue* indicates copy loss, *red* indicates copy gain. Each column represents one patient. *Lower panel*: 17p-LOH (loss-of-heterozygosity); *red arrows* indicate cases with cnLOH-17p. (**b**) Schemas of del17p in relation to *TP53* mutations and LOH at 17p in CLL. *M* maternal chromosome, *P* paternal chromosome. The approximate location of *TP53* is indicated. *Red bars* indicate mutated *TP53*

Within the CLL cell, there exists a central, absolute requirement for functional TP53 protein to permit a proper apoptotic response to double-stranded breaks in DNA, as evidenced by the complete resistance of *TP53*-mutated CLL cells to death by irradiation [20, 60]. Other observations and hypotheses lack clarity at present: the pathophysiological relevance of high-level intracellular accumulation of missense-mutated TP53 protein; the degree to which other TP53 functions contribute to the profoundly negative clinical phenotype of *TP53*-mutated CLL; and whether some mutants have additional gain-of-function phenotypes and their precise nature.

TP53-mutated CLL is clinically aggressive [61, 62], and the *TP53* mutation frequency increases slightly with both disease duration and at relapse following genotoxic therapy. The clinical response of *TP53*-mutated CLL can range from a complete response (CR), which is rare, to progressive disease, but for CLL cohorts, the remission duration is invariably substantially shorter than for comparator cohorts free of *TP53* mutations. *TP53*-mutated CLL is currently treated with a variety of risk-adapted therapies, and it provides the best-studied indication for the use of reduced-intensity allogeneic stem cell transplantation (RIC-allo-TX) as consolidation therapy [4, 63] after frontline therapy.

Patients carrying *TP53* mutations who receive conventional therapies have a survival measured in years when assessed from the time point of *TP53* mutation detection, but substantial variability exists [64, 65]. Incomplete responses and short response durations appear to account for much of the negative survival impact conferred by *TP53* mutations, but a subset of patients display frank therapy resistance, which is clinically catastrophic and not well understood in molecular terms.

Regarding del17p and therapy, (1) in the context of genotoxic therapy, i.e., purine analogues and alkylators, del17p is particularly deleterious, as such therapies rely on apoptosis induction following DNA damage triggered by functional TP53 protein [66]; (2) the presence of del17p serves as a basis for risk-adapted therapies in CLL, which increasingly involve non-genotoxic therapy approaches and RIC-allo-TX as consolidation [67–69]; and (3) the incidence of del17p increases with the amount of time from diagnosis, and with prior therapies for CLL.

CLL with del17p is nonetheless clinically heterogeneous. If del17p is associated with mutated IgV_H loci or an early Rai stage, the disease may be clinically stable for years [70, 71]. Specifically, in a survival model employing risk factors of Rai stage 1 or higher, unmutated IgV_H genes, and 17p loss in >25 % of nuclei, having $\leq$1, 2, or 3 of these risk factors, their 3-year survival was 95 %, 74 %, and 22 %, respectively [70]. Finally, the occurrence of del17p alone should not be used as an indication for the initiation of therapy, as no data suggest benefit, and patients should only be treated if indicated and standard treatment criteria of symptomatic disease are fulfilled.

The exact mechanism conferring the observed clinical aggressiveness of CLL with del17p is somewhat unclear. While del17p effects are likely due to apoptosis resistance and the resulting lack of complete CLL cell eradication, del17p and *TP53* inactivation also create a permissive environment for new genomic changes to persist—some of these changes are likely to result in more aggressive CLL subclones [72–75].

As stated previously, del17p in CLL, with rare exceptions, is an 18- to 22-megabase long lesion. Therefore, its full biological impact on CLL cells likely involves additional deregulated genes besides *TP53*. This, too, is an area of future research interest, as little information is currently available. Recent genome-wide studies of CLL gene mutations have not identified a frequently mutated gene within del17p besides *TP53*. Aberrant expression of microRNAs in del17p-afflicted CLL has been reported; high expression of miR-21 has been proposed to further increase the odds of short survival in CLL [76]. However, hundreds of statistically deregulated genes have been identified in unpublished data, which complicates further in-depth analysis.

Deletion 11q

Approximately 10 % of CLL patients have interstitial material loss on the q arm of chromosome 11 (del11q) at diagnosis. Most del11q lesions in CLL are large, ranging in length from several megabases to tens of Mb, and substantial length heterogeneity exists [77–79]. All del11q which include the *ATM* locus are considered "classical" lesions; these all involve one chromosome only—homozygous del11q do not exist. Atypical del11q, distinct from "classical" del11q by virtue of being smaller, located closer to the centromere, and having a different MDR, have also been observed, but nothing is known about their biology or clinical relevance [6, 11].

The *ATM* gene has received most of the attention of the CLL del11q research, despite the considerable diversity in the 11q lesion's size. In contrast to del17p and *TP53*, mutated *ATM* is only detected in a minority of 11q-deleted CLL and at varying frequencies. The published literature often does not clearly distinguish between somatically acquired *ATM* mutations and germline mutations, or even SNPs. In addition, mutations have been detected in BIRC3—located approximately 6 Mb distant from *ATM* at 101.7 Mb—albeit not in de novo CLL but rather in a small subset of cases which had received fludarabine-based therapy and then quickly relapsed [80]. Other frequently mutated genes on 11q have not been detected in large-scale gene sequencing studies in CLL at the present time.

As stated previously, in contrast to the nearly universal association in CLL of del17p with mutated *TP53*, the *ATM* gene is estimated to be mutated in only 8–30 % of cases with del11q [81, 82], and among CLL cases as an unselected whole, the somatically acquired *ATM* mutation frequency is unknown [83–85]. If extrapolations from large-scale CLL sequencing studies are used, and assuming that all exons in this large gene were adequately covered, it is substantially less than 10 %. Nonsense, missense, and frameshift mutations have all been found in *ATM*; the latter mutation type suggests gene product inactivation and is classic for tumor suppressor genes. Little information on the function of ATM proteins carrying single amino acid substitutions is available (either somatically acquired or due to non-synonymous polymorphisms) and it is unclear what fraction of these constitute complete or partial loss of function mutations.

Studies of an unselected CLL cohort of 250 cases which focused upon actual ATM protein levels and the ability of ATM to autophosphorylate in response to radiation-induced DNA damage demonstrated that ATM aberrations are in fact infrequent, and only of modest effect on clinical CLL outcome [20, 82].

An unpublished observation, based on quantitative analysis of radiation-induced CLL apoptosis in 210 cases with wild type *TP53*, showed that CLL cells with aberrant ATM fall neither into a radiation-resistant nor a particularly radiosensitive group, which contrasts with data based on clonogenic plating assays in various cell types demonstrating a radiosensitive phenotype with concurrent *ATM* mutations. This is clearly very distinct from the findings in *TP53*-mutated CLL. Even though ATM functions upstream of TP53 in the DNA double-strand break repair and response pathway, a defect in ATM, either phenotypically or clinically, does not equal a defect in TP53, suggesting redundant TP53 activation pathways within the CLL cell.

The existing literature is conflicted regarding the clinical importance of *ATM* mutations in CLL [81, 86–88]. Most importantly, effects of *ATM* mutations and effects of del11q are not separated in published work, and when mutations are specifically discussed, germline mutations in *ATM* are not distinguished from somatically acquired mutations. Further, prospective assessments which employ multivariate analyses to evaluate the effect of *ATM* mutations on CLL survival in well-defined CLL cohorts are not available.

With an absence of frequently mutated genes located within del11q, what drives the clonal selection of these CLL cells? A second question, which may or may not be related to the first, involves the fact that nearly all CLL with del11q associate with unmutated IgV_H status (defined as $\geq$98 % homology to germline). This observation remains unexplained.

Recently, 60–70 % of del11q-afflicted CLL were found to have aberrantly elevated levels of the insulin receptor (INSR) [79]. Treating such CLL cells with insulin ex vivo demonstrated anti-apoptotic and pro-growth stimuli. Elevated INSR expression was also associated with early disease progression. It is presently assumed that the observed aberrant INSR upregulation stems from the monoallelic deletion of one or a few genes residing within del11q, plus other, unknown defects. Normally these genes would function in the negative regulation of INSR expression. Experiments are underway to identify these genes, which will provide additional insight into the mechanistic picture of INSR expression regulation in CLL.

In summary, the best available data on the pathophysiology of del11q in CLL point, in general, to defects in the DNA double-strand (ds) break and repair responses, to pro-survival and pro-growth pathway activation, and to a permissive state for the accumulation of genomic lesions. The DNA ds break defects are due to several factors: occasional *ATM* mutations, ATM dysfunction (hypoactivation) due to unknown mechanisms, additional gene deletions located within 11q, and unidentified 11q-associated mechanisms [59]. The additional, compound gene deletions inflicted through del11q may include *MRE11A* and *H2AX*, in addition to *ATM* [20]; 50 % of all del11q co-delete ATM and *MRE11A* or *H2AX*. Overexpression of the INSR or TCL1 provides credible mechanisms for a selective proliferative growth

advantage on clones that would enrich for the presence of del11q [89]. And lastly, in CLL there exists a strong association between del11q and elevated genomic complexity, which may underlie clonal evolution/diversification and the resultant emergence of more aggressive clones. Future del11q research will likely reveal additional molecular aberrations relevant to various del11q subsets.

From current evidence, the presence of del11q in CLL implies clinically progressive disease in essentially all cases; stable disease, not requiring initial therapy, does not exist at appreciable frequencies. Furthermore, CLL cases with del11q experience shorter remission durations following frontline therapy than non-del11q (and non-del17p) cases, although recent improvements have been noted following the inclusion of cyclophosphamide into treatment regimens [90]. Disproportional lymph node sizes can be associated with del11q-positive CLL, and this may be related to aberrantly elevated INSR expression [91]. Overall, CLL cohorts with del11q experience somewhat shorter survival than CLL without del11q (and without del17p) [7, 49].

Trisomy 12

Trisomy 12, the least studied of all CLL subsets as defined by FISH, is found in approximately 15–18 % of CLL at diagnosis. Partial gains, as opposed to gains of the whole chromosome, are found in sporadic CLL cases, but few, if any, of these lesions are recurrent. Eighteen percent is a substantial fraction of all CLL, yet scant concrete information is available on the molecular mechanisms which underlie this aberration and its effect on CLL cells.

However, two interesting observations have recently been reported. Mutations in exon 34 of *NOTCH1* are found in 4–6 % of unselected, de novo CLL, but CLL with *NOTCH1* mutations also carries a trisomy 12 at a frequency of 50 % [92–95]. Secondly, in a study of hedgehog (HH) pathway inhibitors, CLL cells with trisomy 12 were found to have elevated levels of desert hedgehog (DHH) and GLI1; this led to the preliminary finding of a heightened dependence of these cells on HH pathway activation [96].

There is no adverse prognostic information for an isolated trisomy 12 in the clinical context. Notably, CLL patients with an isolated trisomy 12 nearly always respond to modern chemoimmunotherapy approaches and usually enjoy remission durations above median [6, 97]. A strong negative association of trisomy 12 with *TP53* mutations or elevated genomic complexity may possibly underlie this phenomenon.

Recurrent aCNAs in CLL with Frequencies of 1–5 %

Certain relatively rare aCNAs are detected recurrently in CLL at frequencies of approximately 1–5 %, largely as a result of high-resolution SNP array experiments. It should be noted that a firm understanding of the biological consequences and

target genes and incidences of these "second-tier" lesions is unavailable at present. Methodological differences among published studies and, often, a lack of analysis of paired normal DNA complicate the picture and prevent estimation of unbiased frequency estimates of these second-tier aCNAs in CLL. To assist the reader with interpretation of published data we have identified all inherited polymorphic CNVs in our published cohort of 255 CLL patients and have listed the data in the Appendix. As can be seen, such inherited CNVs occur in positions that overlap reports of somatically acquired aCNA in CLL, justifying caution in data interpretation. Of note, second-tier aCNA often co-occur with other aCNAs or *TP53* mutations, further muddying reductionistic research efforts and the conclusions derived therefrom, especially with regards to prognosis.

With these caveats in mind, Table 1 in [5] lists aCNAs found by [6], a SNP array study based on direct comparisons of paired normal and tumor DNA samples from 255 CLL patients, first purified by flow cytometry sorting. Other recent publications [8, 12–14] also describe recurrent, low-frequency aCNA, such as gains at 3q26 and 8q24 and losses of varying lengths at 8p. These lesions tend to be observed in association with del11q, del17p, or elevated aCNA counts: hence it is not yet possible to discriminate any driver function these lesions may impart onto the CLL clone. However, one study [11] determined that infrequent gains on 3q26 are associated with increased expression of PIK3CA, a member of the PI3K pathway, one of the pathways though which BCR signals are relayed. BCR signaling is currently being investigated as an attractive therapeutic target of interest in CLL.

Clinically, none of the existing data are based upon CLL cohorts of sizes sufficient to allow for multivariate analyses and independent effect assessments on survival prognosis or other clinical outcome parameters for rare but recurrent second-tier aCNA. Individual lesions plausibly affect the clinical progression or chemotherapy resistance of CLL, but no direct evidence is available to confirm such a hypothesis.

The Landscape of aCNA and Chromosomal Translocations in CLL as Defined Through CLL Cell Karyotyping

CLL karyotyping using basic cell culture conditions has resulted in valuable information on gross structural genomic aberrations in CLL cells [98–101]. The major limitations of this tool are the inability to generate karyotypes for the majority of patients, as well as the unreliable detection of genomic lesions in the sub-megabase to 5 Mb range. Recently, novel CLL cell culture conditions have been established that now allow for the generation of so-called "stimulated karyotypes" in the vast majority of patients [102], and results from karyotyping efforts of large CLL patient collections have been reported [103–106]. The largest

study of CLL genomics, using stimulated karyotypes of 506 CLL patients, has been reported by C. Haferlach et al. [19].

Some of the caveats regarding the routine use of stimulated karyotypes in CLL management are the following: (1) the prognostic value of stimulated karyotyping results in CLL cohorts has not been assessed prospectively and in relation to other strong prognostic factors and using multivariate analysis; (2) stimulated karyotyping misses smaller genomic lesions: for instance, short del13q14 is often not detected through CLL FISH; (3) interlaboratory differences in karyotyping results exist; and (4) the results of a stimulated karyotype are somewhat dependent on what specific culture conditions are used.

The following is a brief summary of pertinent findings from CLL stimulated karyotypes as described by Haferlach et al. [19], which is based on >500 CLL karyotyping results:

1. Eighty-three % of CLL cases carried at least one aCNA; the median was 1.7 aCNA per case; two or more or three or more aCNAs were detected in ~44 % and 21 % of cases, respectively.
2. Reciprocal translocations in CLL, the biological consequences of which are largely unknown, were detected in ~20 % of cases. Of these, translocations involving the Ig loci (heavy and light chain loci) as well as cytoband 13q14 were the most frequent. Additional studies on translocations involving cytoband 14q32 identified BCL2 t(14;18)(q32q21) and BCL3 t(14;19)(q32;q13) as recurrent translocation partners; t(14;19)(q32;q13)/(Ig-BCL3) may be associated with more aggressive CLL as well as atypical CLL presentations [107–110].
3. aCNAs as detected by karyotyping in CLL with normal FISH are prognostically adverse [111, 112].
4. A complex aberrant karyotype identifies a high-risk subgroup of CLL with short survival. Not all CLL cases with a complex aberrant karyotype carried either del17p or del11q,
5. The mean number of aCNAs in *TP53*-mutated CLL was substantially higher than in the *TP53* wild-type group (5 vs. 1.5).

Appendix

Listing of all polymorphic copy number variants (pCNVs) present in the constitutional DNA of 255 CLL patients as identified through visual inspection of dChipSNP-based heatmap displays of CD3 cell- or buccal cell-derived DNA analyzed on SNP 6.0 arrays as published by Ouillette et al. [6].

List of normal copy number variants found in CD3 or buccal DNA of 255 CLL cases

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
1	2268170	9.238	478103	9.325	0.087	Gain
1	12036106	21.871	7526162	21.94	0.069	Gain
1	550252	23.952	7355010	24.324	0.372	Gain
1	10914933	34.612	10753308	34.68	0.068	Loss
1	4332387	65.632	17127673	65.728	0.096	Gain
1	11162075	75.086	11162286	75.2	0.114	Gain
1	810915	84.289	12746741	84.312	0.023	Loss
1	12402272	113.086	12145713	113.541	0.455	Gain
1	1752370	116.403	10802163	116.495	0.092	Loss
1	4083420	157.461	11265165	157.55	0.089	Loss
1	1016815	173.696	487948	173.994	0.298	Gain
1	3908615	180.125	1281294	180.165	0.04	Loss
1	653791	184.465	1795011	184.473	0.008	Loss
1	814969	188.773	12084012	188.876	0.103	Loss
1	515299	194.973	3748556	195.194	0.221	Loss
1	449847	194.998	6674522	195.183	0.185	Loss
1	384006	195.038	6428369	195.111	0.073	Loss
1	384006	195.038	1853883	195.148	0.11	Loss
1	384006	195.038	1853883	195.148	0.11	Loss
1	800249	195.077	7538501	195.168	0.091	Loss
1	7542235	195.09	7538501	195.168	0.078	Loss
1	7542235	195.09	6674522	195.183	0.093	Loss
1	6656059	195.102	7538501	195.168	0.066	Gain
1	1202543	229.033	731824	229.09	0.057	Loss
1	1151659	245.905	11801427	246.295	0.39	Gain
1	7555595	245.924	1934553	246.643	0.719	Gain
1	12028988	246.311	6587444	246.505	0.194	Gain
2	2293085	0.686	4854295	0.913	0.227	Gain
2	1900853	14.244	16861513	14.299	0.055	Gain
2	16861426	14.266	1947205	14.282	0.016	Gain
2	1118202	14.271	12479278	14.286	0.015	Gain
2	1118202	14.271	1947206	14.282	0.011	Gain
2	1118202	14.271	7608366	14.297	0.026	Gain
2	11892304	15.021	10195777	15.263	0.242	Gain
2	10172510	32.474	218222	33.185	0.711	Gain
2	2710629	32.619	11896237	32.883	0.264	Gain
2	6707049	35.727	6716654	35.787	0.06	Loss
2	4952732	45.114	11691323	45.166	0.052	Loss
2	12995518	50.734	17502019	50.815	0.081	Loss
2	6727517	51.224	7425298	51.364	0.14	Gain
2	1446439	53.073	2357016	53.131	0.058	Loss
2	4995220	53.104	17044121	53.196	0.092	Loss
2	17044623	53.489	1874057	53.514	0.025	Loss
2	7582028	56.492	10779943	57.202	0.71	Loss
2	10189319	56.56	1978530	56.597	0.037	Loss
2	12713334	57.248	6419630	57.323	0.075	Gain

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
2	1424626	57.248	4395280	57.295	0.047	Gain
2	6707919	57.257	12622457	57.336	0.079	Loss
2	17617459	63.514	11895173	65.429	1.915	Gain
2	6761711	64.982	6740379	64.996	0.014	Loss
2	2861798	68.203	6729652	68.391	0.188	Gain
2	17838437	88.911	13394748	90.922	2.011	Loss
2	3849324	110.207	10170120	110.335	0.128	Gain
2	2165055	110.218	11900983	111.143	0.925	Loss
2	17009474	123.638	1515158	123.645	0.007	Loss
2	780032	124.745	11902093	125.172	0.427	Gain
2	524398	137.432	6750400	137.484	0.052	Loss
2	1345959	163.536	12468570	163.652	0.116	Loss
2	6706308	180.638	2056786	180.681	0.043	Loss
2	2368335	183.67	16824100	183.754	0.084	Gain
2	7423787	232.914	790027	233.015	0.101	Loss
2	7581318	242.311	12478296	242.697	0.386	Loss
2	4675886	242.328	12478296	242.697	0.369	Loss
2	6733823	242.345	12478296	242.697	0.352	Loss
2	6733823	242.345	12478296	242.697	0.352	Loss
2	7423193	242.367	12478296	242.697	0.33	Loss
2	7423193	242.367	12478296	242.697	0.33	Loss
2	7423193	242.367	12478296	242.697	0.33	Loss
2	7423193	242.367	12478296	242.697	0.33	Loss
2	7423193	242.367	12478296	242.697	0.33	Loss
2	7423193	242.367	12478296	242.697	0.33	Loss
2	7423193	242.367	12478296	242.697	0.33	Loss
2	9973663	242.374	12478296	242.697	0.323	Loss
2	9973663	242.374	12478296	242.697	0.323	Loss
2	9973663	242.374	12478296	242.697	0.323	Loss
2	9973663	242.374	12478296	242.697	0.323	Loss
2	12987376	242.566	12478296	242.697	0.131	Loss
2	12987376	242.566	12478296	242.697	0.131	Loss
2	12987376	242.566	12478296	242.697	0.131	Loss
2	12987998	242.566	12478296	242.697	0.131	Loss
2	12987376	242.566	12478296	242.697	0.131	Loss
2	12987376	242.566	12478296	242.697	0.131	Loss
2	12987376	242.566	12478296	242.697	0.131	Loss
2	12987376	242.566	12478296	242.697	0.131	Loss
2	13427735	242.603	12478296	242.697	0.094	Loss
2	13427735	242.603	12478296	242.697	0.094	Loss
3	—	0.06	17047384	0.103	0.043	Gain
3	1949341	0.061	6778252	0.101	0.04	Gain
3	6442152	1.055	17493901	1.217	0.162	Loss
3	6792367	1.146	1579005	1.686	0.54	Gain
3	9839671	1.307	17786361	1.803	0.496	Loss
3	7614111	1.577	9815216	1.696	0.119	Gain
3	364432	1.644	2731522	1.666	0.022	Loss

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
3	7634053	1.726	17194161	2.181	0.455	Gain
3	2174019	2.448	17023680	2.978	0.53	Loss
3	9811218	4.01	17039952	4.131	0.121	Loss
3	317618	4.041	17040046	4.159	0.118	Loss
3	17039991	4.141	310694	4.288	0.147	Loss
3	588845	4.149	314418	4.292	0.143	Loss
3	2442807	12.605	17037220	12.793	0.188	Gain
3	1898300	21.217	13080495	21.327	0.11	Loss
3	779166	22.078	1491878	22.244	0.166	Loss
3	1426766	25.944	7638008	26.148	0.204	Loss
3	17075303	43.223	9311335	43.25	0.027	Loss
3	7635362	60.048	732380	60.252	0.204	Loss
3	7635362	60.048	12636197	60.238	0.19	Loss
3	4677001	75.48	7613806	75.723	0.243	Loss
3	4677001	75.48	544020	75.822	0.342	Loss
3	1810375	75.487	1703205	75.669	0.182	Loss
3	1810375	75.487	7613806	75.723	0.236	Loss
3	9809797	75.499	7613806	75.723	0.224	Loss
3	9809707	75.499	1711586	75.66	0.161	Loss
3	9809707	75.499	12639451	75.628	0.129	Loss
3	9809707	75.499	4677549	75.691	0.192	Loss
3	4054258	75.522	1703205	75.669	0.147	Loss
3	4054258	75.522	12639451	75.628	0.106	Loss
3	4054258	75.522	1703205	75.669	0.147	Loss
3	4054258	75.522	12639451	75.628	0.106	Loss
3	4054258	75.522	7624950	75.731	0.209	Loss
3	4054258	75.522	3009005	75.741	0.219	Loss
3	4054258	75.522	2944644	75.759	0.237	Loss
3	4054258	75.522	1703205	75.669	0.147	Loss
3	4054258	75.522	7613806	75.723	0.201	Loss
3	4054258	75.522	7624950	75.731	0.209	Loss
3	4054258	75.522	2944644	75.759	0.237	Loss
3	4054258	75.522	7613806	75.723	0.201	Loss
3	6793001	75.536	11926504	75.843	0.307	Loss
3	6793001	75.536	7624950	75.731	0.195	Loss
3	6793001	75.536	2944628	75.78	0.244	Loss
3	6793001	75.536	4677549	75.691	0.155	Loss
3	6793001	75.536	7624950	75.731	0.195	Loss
3	6793001	75.536	7613806	75.723	0.187	Loss
3	6793001	75.536	2944628	75.78	0.244	Loss
3	9875783	82.56	2132599	82.911	0.351	Gain
3	7627378	101.731	1144106	101.925	0.194	Gain
3	1620897	101.823	3773921	101.938	0.115	Gain
3	1144126	101.833	1144106	101.925	0.092	Gain
3	1144126	101.833	1143777	101.91	0.077	Gain
3	7632036	109.378	7622810	109.454	0.076	Gain
3	6437804	110.089	1356076	110.432	0.343	Gain

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
3	4687835	120.202	11917432	120.301	0.099	Gain
3	4687836	120.214	11916676	120.325	0.111	Gain
3	35860	120.235	13082731	120.362	0.127	Gain
3	11926466	120.357	2922290	120.464	0.107	Gain
3	1401968	152.944	10935878	153.046	0.102	Loss
3	12152284	152.976	1546686	153.026	0.05	Loss
3	16863667	152.991	1520216	153.073	0.082	Loss
3	16863667	152.991	1546687	153.026	0.035	Loss
3	1401968	152.994	1546687	153.026	0.032	Loss
3	1401968	152.994	1546687	153.026	0.032	Loss
3	7643257	152.995	1546686	153.026	0.031	Loss
3	16824307	156.003	4680160	156.517	0.514	Loss
3	6801320	162.88	1157705	163.034	0.154	Loss
3	16835297	163.346	12053991	163.436	0.09	Gain
3	13096521	163.349	7653283	163.436	0.087	Gain
3	4856674	163.354	12633693	163.444	0.09	Gain
3	4538407	197.652	1619646	197.95	0.298	Gain
4	2291157	5.772	3856955	5.826	0.054	Gain
4	3857184	7.136	3852121	7.443	0.307	Gain
4	10032245	8.89	2061995	9.171	0.281	Loss
4	9291627	8.986	12501535	9.066	0.08	Gain
4	4235342	10.338	13110946	11.336	0.998	Loss
4	11937874	26.207	9998368	26.498	0.291	Gain
4	3857024	29.692	12641117	29.933	0.241	Loss
4	1908806	44.66	4469117	44.701	0.041	Gain
4	10866443	57.744	10027042	57.793	0.049	Gain
4	6857279	57.749	17087713	57.795	0.046	Gain
4	6857528	57.749	9999844	57.797	0.048	Gain
4	6857528	57.749	10027042	57.793	0.044	Gain
4	6857279	57.749	17425986	57.795	0.046	Gain
4	282729	59.176	6853837	59.369	0.193	Loss
4	7670819	70.516	7656541	70.551	0.035	Loss
4	17713792	71.189	1994625	71.347	0.158	Loss
4	4412018	80.795	9790680	80.905	0.11	Loss
4	6813942	81.576	10025495	81.759	0.183	Gain
4	13136977	92.16	11732866	92.253	0.093	Loss
4	5017329	97.409	7682919	97.503	0.094	Loss
4	11098478	120.225	10034453	120.307	0.082	Gain
4	973877	138.328	7664552	138.432	0.104	Loss
4	13130345	145.062	2875026	145.14	0.078	Loss
4	4499663	162.233	17040737	162.321	0.088	Loss
4	2319239	168.538	7689336	168.713	0.175	Gain
4	6831239	172.631	1919495	172.741	0.11	Loss
4	330502	176.26	7676096	176.377	0.117	Loss
4	7668312	188.695	12509381	188.843	0.148	Gain
5	659757	3.989	465560	4.321	0.332	Gain
5	16878631	7.469	—	7.532	0.063	Gain

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
5	8180420	9.955	10866597	9.977	0.022	Loss
5	8180420	9.955	10866597	9.977	0.022	Loss
5	8180420	9.955	10866597	9.977	0.022	Loss
5	11959975	9.956	11741133	9.976	0.02	Loss
5	902502	23.84	10942029	24.235	0.395	Loss
5	13174324	28.933	1692334	29.083	0.15	Loss
5	10050837	33.117	7707459	33.177	0.06	Loss
5	17358298	36.482	6868930	36.514	0.032	Loss
5	216399	37.335	—	37.59	0.255	Gain
5	6879102	66.556	281455	67.324	0.768	Gain
5	4869387	97.054	17087964	97.359	0.305	Gain
5	10866785	97.061	4573015	97.125	0.064	Loss
5	10866785	97.061	4573015	97.125	0.064	Loss
5	10866785	97.061	6865508	97.142	0.081	Loss
5	10866785	97.061	4573015	97.125	0.064	Loss
5	4869388	97.071	11958522	97.152	0.081	Loss
5	4869388	97.071	4573015	97.125	0.054	Loss
5	4869388	97.071	4573015	97.125	0.054	Loss
5	4869388	97.071	6862260	97.171	0.1	Loss
5	2914928	97.074	4573015	97.125	0.051	Loss
5	2914928	97.074	6865508	97.142	0.068	Loss
5	2914928	97.074	6890685	97.142	0.068	Loss
5	2914928	97.074	6865508	97.142	0.068	Loss
5	2914928	97.074	4573015	97.125	0.051	Loss
5	2163157	97.076	12655673	97.178	0.102	Loss
5	2115767	97.076	4573015	97.125	0.049	Loss
5	2682158	98.951	11960460	99.454	0.503	Loss
5	294152	104.487	652575	104.685	0.198	Loss
5	4308513	115.628	37181	115.658	0.03	Loss
5	12522824	120.224	6861434	120.284	0.06	Loss
5	7408	141.361	6869301	141.434	0.073	Gain
5	300228	162.674	10746556	162.739	0.065	Loss
5	7720865	170.918	10053587	170.965	0.047	Loss
5	10475626	175.504	4428433	175.698	0.194	Loss
6	4959515	0.11	3800250	0.28	0.17	Gain
6	719065	0.111	12210593	0.175	0.064	Loss
6	6922303	0.199	2797303	0.33	0.131	Loss
6	6921918	0.199	3866814	0.324	0.125	Loss
6	6921918	0.199	3866814	0.324	0.125	Loss
6	6921918	0.199	7773324	0.328	0.129	Loss
6	2181107	0.215	3866814	0.324	0.109	Loss
6	2181107	0.215	7753579	0.321	0.106	Loss
6	2181107	0.215	7753579	0.321	0.106	Loss
6	6925262	0.223	7773324	0.328	0.105	Loss
6	6925262	0.223	3866814	0.324	0.101	Loss
6	9328228	4.198	1265264	4.419	0.221	Loss
6	11963128	25.184	6910965	25.28	0.096	Loss

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Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
6	7746418	40.889	870032	40.947	0.058	Loss
6	11964138	54.803	6902422	54.919	0.116	Gain
6	17683329	55.346	9475321	55.479	0.133	Gain
6	11964721	65.66	4134138	65.714	0.054	Loss
6	9453668	67.043	1708557	67.106	0.063	Loss
6	7757213	67.053	1708557	67.106	0.053	Loss
6	9453679	67.059	1708561	67.105	0.046	Loss
6	17644076	67.062	851860	67.112	0.05	Loss
6	17644076	67.062	1708558	67.106	0.044	Loss
6	17644076	67.062	1708561	67.105	0.043	Loss
6	17644076	67.062	1708561	67.105	0.043	Loss
6	17644076	67.062	1708558	67.106	0.044	Loss
6	17644076	67.062	1708561	67.105	0.043	Loss
6	17644076	67.062	1708558	67.106	0.044	Loss
6	17644076	67.062	3846814	67.117	0.055	Loss
6	17644076	67.062	1708561	67.105	0.043	Loss
6	2106553	67.065	1708558	67.106	0.041	Loss
6	2106553	67.065	1708561	67.105	0.04	Loss
6	9345808	67.065	1708558	67.106	0.041	Loss
6	9345808	67.065	1708558	67.106	0.041	Loss
6	9345808	67.065	1708562	67.105	0.04	Loss
6	9345808	67.065	851861	67.111	0.046	Loss
6	9345808	67.065	851861	67.111	0.046	Loss
6	9345808	67.065	1708562	67.105	0.04	Loss
6	9345808	67.065	1708561	67.105	0.04	Loss
6	2106553	67.065	1708561	67.105	0.04	Loss
6	9345808	67.065	1708561	67.105	0.04	Loss
6	9345808	67.065	1708561	67.105	0.04	Loss
6	9345808	67.065	1708558	67.106	0.041	Loss
6	2106553	67.065	1708561	67.105	0.04	Loss
6	2106553	67.065	851861	67.111	0.046	Loss
6	2106553	67.065	1708561	67.105	0.04	Loss
6	2106553	67.065	1708562	67.105	0.04	Loss
6	10440810	67.066	1708558	67.106	0.04	Loss
6	10440810	67.066	1708562	67.105	0.039	Loss
6	10440810	67.066	1708561	67.105	0.039	Loss
6	10440810	67.066	1708558	67.106	0.04	Loss
6	10440810	67.066	1708557	67.106	0.04	Loss
6	1418689	71.936	12208481	72.003	0.067	Gain
6	11966981	94.391	6454980	94.579	0.188	Gain
6	2184906	114.814	4946040	115.279	0.465	Loss
6	1933470	115.412	1856336	115.475	0.063	Loss
6	9375230	123.534	1510295	124.371	0.837	Gain
6	1930951	127.273	9401937	127.43	0.157	Loss
6	4263608	158.319	828004	158.697	0.378	Gain
6	7739202	164.659	11969444	164.775	0.116	Loss
6	206990	167.619	2981987	167.79	0.171	Gain

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Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
6	7772997	168.081	2880102	168.326	0.245	Gain
6	7772997	168.081	9455595	168.34	0.259	Gain
6	683465	168.091	9455595	168.34	0.249	Gain
6	2843010	168.144	9455973	168.326	0.182	Gain
7	12666071	0.138	11970804	0.161	0.023	Gain
7	6463626	7.007	7793374	7.055	0.048	Gain
7	9655529	8.795	12154877	8.832	0.037	Loss
7	7791718	9.094	17160303	9.196	0.102	Loss
7	2057819	34.911	7792475	35.136	0.225	Gain
7	17563764	54.725	4947488	55.084	0.359	Gain
7	10224201	55.432	815961	55.512	0.08	Loss
7	1717942	56.944	10238392	57.625	0.681	Gain
7	2167871	61.716	6965334	62.283	0.567	Gain
7	2123597	61.718	2871906	62.344	0.626	Gain
7	4718180	64.1	11971971	64.706	0.606	Loss
7	6954820	69.954	7777301	70.004	0.05	Gain
7	2868366	75.888	17842722	76.331	0.443	Loss
7	2868366	75.888	3909200	76.404	0.516	Loss
7	2908182	76.269	3909200	76.404	0.135	Gain
7	10261840	89.324	1079527	90.206	0.882	Gain
7	17619605	89.396	11980089	89.55	0.154	Loss
7	799578	96.762	11979492	96.822	0.06	Loss
7	3807513	100.752	17469756	100.924	0.172	Gain
7	7799285	100.755	13232646	100.914	0.159	Gain
7	10953865	117.712	6959273	117.829	0.117	Loss
7	2402465	118.612	170099	118.787	0.175	Loss
7	1860681	145.687	17170238	146.074	0.387	Loss
8	2581568	2.117	10107849	2.166	0.049	Loss
8	2618819	2.118	10107849	2.166	0.048	Loss
8	17759031	2.119	4876197	2.165	0.046	Loss
8	17063040	2.309	7827228	2.577	0.268	Gain
8	4875968	2.335	13439436	2.536	0.201	Gain
8	17692852	2.36	7005280	2.57	0.21	Gain
8	1481821	3.602	2046199	3.643	0.041	Loss
8	2912292	3.796	1991279	3.808	0.012	Loss
8	4875356	4.371	11136750	4.4	0.029	Loss
8	2724962	4.507	2617055	4.549	0.042	Loss
8	9329219	7.825	11994648	8.176	0.351	Gain
8	3989610	8.125	7836064	8.185	0.06	Gain
8	2980437	8.132	17602876	8.183	0.051	Gain
8	572366	8.616	481061	8.667	0.051	Loss
8	17732433	9.372	6601327	9.433	0.061	Loss
8	1534862	11.681	9325708	12.447	0.766	Gain
8	7817551	13.483	12677921	13.569	0.086	Loss
8	17116929	13.54	11203513	13.617	0.077	Loss
8	352766	15.689	10106193	16.315	0.626	Gain
8	12543352	15.994	351572	16.066	0.072	Loss

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
8	12543352	15.994	351572	16.066	0.072	Loss
8	11987039	16.504	4922125	16.58	0.076	Loss
8	17384769	18.891	2898484	18.903	0.012	Loss
8	13248405	18.894	2898484	18.903	0.009	Loss
8	13248405	18.894	7010324	18.911	0.017	Loss
8	13248405	18.894	2898484	18.903	0.009	Loss
8	13248405	18.894	2898484	18.903	0.009	Loss
8	2950324	24.93	11989437	25.003	0.073	Gain
8	17054092	25.558	17054228	25.644	0.086	Loss
8	2565122	40.405	2565085	40.493	0.088	Gain
8	10095687	53.832	17340869	53.977	0.145	Gain
8	7821091	71.894	3103860	71.997	0.103	Loss
8	6470733	87.288	12681287	87.339	0.051	Gain
8	13256627	92.192	7823844	92.253	0.061	Loss
8	16875098	107.886	1433176	108.462	0.576	Gain
8	16877399	109.275	7824725	109.363	0.088	Gain
8	10108049	130.339	16904024	130.372	0.033	Loss
8	894089	137.747	3861778	137.926	0.179	Loss
8	894089	137.747	17597927	137.932	0.185	Loss
8	894089	137.747	17597927	137.932	0.185	Loss
8	894089	137.747	4084897	137.933	0.186	Loss
8	894089	137.747	17597927	137.932	0.185	Loss
8	2919682	137.751	4084896	137.933	0.182	Loss
8	2919682	137.751	17597927	137.932	0.181	Loss
8	2919682	137.751	17597927	137.932	0.181	Loss
8	2919682	137.751	4084896	137.933	0.182	Loss
8	2919682	137.751	3861778	137.926	0.175	Loss
8	2919682	137.751	17597927	137.932	0.181	Loss
8	2919682	137.751	3861778	137.926	0.175	Loss
8	2919682	137.751	17597927	137.932	0.181	Loss
8	2919682	137.751	4084896	137.933	0.182	Loss
8	90190	137.757	4124848	137.924	0.167	Loss
8	17646599	137.757	4124848	137.924	0.167	Loss
8	17646599	137.757	4124848	137.924	0.167	Loss
8	90190	137.757	3861778	137.926	0.169	Loss
8	17646599	137.757	17597927	137.932	0.175	Loss
8	17646599	137.757	17597927	137.932	0.175	Loss
8	12548622	140.19	10101305	140.221	0.031	Loss
9	9407292	0.517	7039523	0.804	0.287	Gain
9	7047146	1.491	2050757	1.504	0.013	Loss
9	13285858	2.22	10965905	2.354	0.134	Loss
9	17546584	7.721	7047293	7.763	0.042	Loss
9	10977616	9.241	9644896	9.324	0.083	Loss
9	10491919	9.792	16930649	9.932	0.14	Loss
9	1805197	10.47	6474717	12.579	2.109	Gain
9	10809254	10.943	1104513	11.018	0.075	Gain
9	3861712	11.703	1446262	12.061	0.358	Loss

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
9	7045470	11.746	7036007	11.94	0.194	Loss
9	10960243	11.786	10809655	11.948	0.162	Loss
9	10960309	11.832	4449884	12.068	0.236	Loss
9	10960309	11.832	1446256	12.01	0.178	Loss
9	10511574	11.941	2166422	12.137	0.196	Loss
9	1929409	11.943	16928450	12.106	0.163	Loss
9	2121246	12.028	10960465	12.082	0.054	Loss
9	7863023	12.058	10121735	12.196	0.138	Loss
9	10960462	12.072	10121483	12.171	0.099	Loss
9	2145661	17.5	1755272	17.97	0.47	Gain
9	556958	22.471	9644863	22.555	0.084	Loss
9	13440446	28.242	2860817	28.312	0.07	Loss
9	10968561	28.389	914537	28.433	0.044	Loss
9	2891316	28.578	321728	28.752	0.174	Loss
9	10813247	30.41	10481581	30.557	0.147	Loss
9	7021184	71.29	7869376	71.308	0.018	Gain
9	11793607	134.515	10115625	134.632	0.117	Gain
9	7861295	137.618	7037014	137.784	0.166	Gain
10	17347211	17.119	2356829	17.264	0.145	Gain
10	7093985	19.796	1537346	19.816	0.02	Loss
10	16927268	27.653	11015752	27.742	0.089	Loss
10	11239623	41.956	2796577	42.694	0.738	Gain
10	7076300	44.539	17157129	44.678	0.139	Gain
10	17159090	45.528	4414152	47.166	1.638	Gain
10	17696117	45.531	3013811	47.103	1.572	Gain
10	17696112	45.531	4635023	47.163	1.632	Gain
10	17696112	45.531	2805173	47.43	1.899	Gain
10	2442927	45.535	6602880	47.016	1.481	Gain
10	2812916	46.111	12247533	46.569	0.458	Loss
10	2812916	46.111	4414152	47.166	1.055	Gain
10	2812916	46.111	11259802	47.212	1.101	Loss
10	2812916	46.111	1539630	46.557	0.446	Loss
10	2812916	46.111	11259802	47.212	1.101	Gain
10	2812916	46.111	11259802	47.212	1.101	Gain
10	2812916	46.111	1539630	46.557	0.446	Loss
10	1855862	46.363	1539630	46.557	0.194	Loss
10	1855862	46.363	6602880	47.016	0.653	Gain
10	1855862	46.363	4128664	47.174	0.811	Gain
10	1855862	46.363	12247533	46.569	0.206	Loss
10	1475815	46.398	6602880	47.016	0.618	Gain
10	1475815	46.398	2338327	47.431	1.033	Gain
10	1336003	46.414	4503470	46.559	0.145	Gain
10	1105347	46.415	7899396	47.165	0.75	Gain
10	1977887	46.495	4128664	47.174	0.679	Gain
10	1539630	46.557	2338327	47.431	0.874	Gain
10	4503470	46.559	11259802	47.212	0.653	Gain
10	12247533	46.569	2805173	47.43	0.861	Gain

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
10	4013008	46.574	4073429	47.147	0.573	Gain
10	4013008	46.574	11259802	47.212	0.638	Loss
10	12246443	47.038	11259785	47.148	0.11	Gain
10	12246443	47.038	3013795	47.155	0.117	Gain
10	12246443	47.038	4073429	47.147	0.109	Gain
10	12250640	47.057	4385821	47.121	0.064	Gain
10	17157069	47.057	3013819	47.099	0.042	Gain
10	12250640	47.057	3013795	47.155	0.098	Gain
10	12250640	47.057	3013795	47.155	0.098	Gain
10	7072255	47.063	11259785	47.148	0.085	Gain
10	2338050	51.475	10825837	51.815	0.34	Gain
10	10761884	66.387	10761899	66.457	0.07	Loss
10	10997049	67.735	4459177	67.778	0.043	Loss
10	4370822	67.742	4459177	67.778	0.036	Loss
10	4434898	67.743	4459177	67.778	0.035	Loss
10	10997057	67.748	4459177	67.778	0.03	Loss
10	6480159	67.749	7897679	67.796	0.047	Loss
10	7087155	67.88	10997205	67.935	0.055	Loss
10	1911303	67.896	2260006	67.97	0.074	Loss
10	1911355	67.948	4615912	68.213	0.265	Loss
10	1034289	73.845	7921361	73.991	0.146	Loss
10	2935713	123.422	1219505	123.451	0.029	Loss
10	9971131	123.422	1219508	123.452	0.03	Loss
10	2935713	123.422	1219505	123.451	0.029	Loss
10	10745295	135.045	2492664	135.237	0.192	Gain
10	11101727	135.045	2492664	135.237	0.192	Gain
10	2297031	135.083	2070676	135.201	0.118	Gain
10	2245170	135.093	8181436	135.221	0.128	Gain
10	2245170	135.093	10857760	135.225	0.132	Gain
10	12241015	135.102	2492664	135.237	0.135	Gain
10	12241015	135.102	915908	135.917	0.815	Gain
10	2251083	135.125	2492664	135.237	0.112	Gain
10	743535	135.199	7903897	135.283	0.084	Gain
11	2735691	4.074	471698	4.292	0.218	Gain
11	4716198	4.292	1426378	4.371	0.079	Gain
11	12789492	4.751	10500633	4.864	0.113	Gain
11	11029757	26.939	2245826	27.197	0.258	Gain
11	628029	30.715	10488798	30.782	0.067	Gain
11	4756645	41.407	12271374	41.453	0.046	Gain
11	4639920	42.222	7105041	42.278	0.056	Loss
11	10839307	49.566	11245714	50.437	0.871	Gain
11	1807793	50.023	10902283	51.078	1.055	Gain
11	11822622	50.072	10902283	51.078	1.006	Gain
11	11230518	55.17	12098903	55.423	0.253	Loss
11	295635	55.201	12808932	55.36	0.159	Loss
11	2512730	55.441	2449127	55.584	0.143	Loss
11	2512730	55.441	11826500	55.586	0.145	Loss

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
11	4930497	67.232	308346	67.419	0.187	Gain
11	10791923	67.258	308350	67.489	0.231	Gain
11	1399622	83.688	7127845	83.793	0.105	Loss
11	3018579	98.028	11217332	98.261	0.233	Loss
11	2656173	98.827	2466903	98.921	0.094	Loss
11	648646	99.018	666825	99.08	0.062	Loss
11	587925	99.026	17134227	99.081	0.055	Loss
11	17103112	104.2	644121	104.247	0.047	Loss
11	7931348	109.573	1870962	109.721	0.148	Gain
11	1729408	116.18	2071521	116.203	0.023	Loss
11	10791352	133.657	10894798	133.709	0.052	Loss
11	10791352	133.657	11223765	133.722	0.065	Loss
11	4592433	133.853	11224035	134.224	0.371	Gain
11	10894831	133.855	2661977	134.222	0.367	Gain
12	7969162	7.855	10772877	8.008	0.153	Gain
12	10845990	7.862	4883463	8.008	0.146	Loss
12	12369506	7.899	4628755	8.035	0.136	Gain
12	12369506	7.899	7964540	8.022	0.123	Gain
12	2445420	15.059	12300553	15.272	0.213	Loss
12	7297169	19.358	12230328	19.468	0.11	Gain
12	7296810	25.405	11048022	25.468	0.063	Loss
12	11048888	27.185	17801352	27.689	0.504	Gain
12	16934110	29.328	12814180	29.352	0.024	Loss
12	11051217	31.092	12423595	31.302	0.21	Gain
12	12303225	31.094	12423595	31.302	0.208	Gain
12	6487966	31.116	7954014	31.298	0.182	Gain
12	10843879	31.128	12824643	31.276	0.148	Gain
12	2005900	31.153	1025623	31.298	0.145	Gain
12	2005900	31.153	1025624	31.298	0.145	Gain
12	2005900	31.153	7954014	31.298	0.145	Gain
12	2005900	31.153	625227	31.301	0.148	Gain
12	4931443	31.169	1025623	31.298	0.129	Gain
12	2536827	31.172	7954014	31.298	0.126	Gain
12	2536827	31.172	12423595	31.302	0.13	Gain
12	2536827	31.172	10771819	31.287	0.115	Gain
12	2536827	31.172	10771819	31.287	0.115	Gain
12	2536827	31.172	10771819	31.287	0.115	Gain
12	2536827	31.172	7954014	31.298	0.126	Gain
12	2536827	31.172	10771819	31.287	0.115	Gain
12	2536827	31.172	1025624	31.298	0.126	Gain
12	2536827	31.172	1025623	31.298	0.126	Gain
12	2536827	31.172	625227	31.301	0.129	Gain
12	2536827	31.172	625227	31.301	0.129	Gain
12	2536827	31.172	1025623	31.298	0.126	Gain
12	2536827	31.172	7133993	31.286	0.114	Gain
12	10843909	31.24	4931472	31.277	0.037	Gain
12	7136266	31.244	1025624	31.298	0.054	Gain

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
12	11051321	31.249	1025623	31.298	0.049	Gain
12	2128614	31.89	1150990	31.954	0.064	Gain
12	2128614	31.89	1150990	31.954	0.064	Gain
12	10844027	31.891	1150990	31.954	0.063	Gain
12	16919008	31.904	1150996	31.96	0.056	Gain
12	16919008	31.904	1150996	31.96	0.056	Gain
12	10844032	31.911	1150990	31.954	0.043	Gain
12	12307593	36.604	4073360	37.161	0.557	Gain
12	10880404	41.68	17652045	43.345	1.665	Gain
12	2214526	46.228	7977613	46.366	0.138	Gain
12	10783205	46.233	7977613	46.366	0.133	Gain
12	1945299	50.952	2271084	51.062	0.11	Gain
12	10876266	50.963	1506566	51.048	0.085	Gain
12	10876272	50.965	3759194	51.048	0.083	Gain
12	7295468	50.975	1732261	51.072	0.097	Gain
12	11173462	59.117	11173505	59.2	0.083	Loss
12	4763094	62.261	7312734	62.438	0.177	Gain
12	12311315	71.807	1915291	72.007	0.2	Loss
12	2571342	92.842	10459183	92.878	0.036	Gain
12	11110078	98.792	934159	98.821	0.029	Loss
12	12316047	106.918	11113739	107.014	0.096	Loss
12	2888963	106.934	—	107.008	0.074	Loss
12	12299767	117.45	1356950	117.508	0.058	Loss
12	10744299	126.27	7305741	126.443	0.173	Loss
12	11059862	127.792	11059908	127.836	0.044	Gain
12	6486642	130.293	4237834	130.392	0.099	Loss
12	6486642	130.293	10848359	130.402	0.109	Loss
12	7952982	130.298	4759568	130.4	0.102	Loss
13	2104352	42.332	1575518	42.411	0.079	Gain
13	9567121	42.632	9533429	42.681	0.049	Gain
13	9563409	55.974	806418	56.154	0.18	Loss
13	6562181	60.999	4430624	61.042	0.043	Loss
13	——	63.29	9540058	63.832	0.542	Loss
13	7330473	66.757	1937502	66.914	0.157	Loss
13	12867665	83.006	9602228	83.07	0.064	Loss
13	12867665	83.006	7318828	83.048	0.042	Loss
13	9586847	104.861	12864685	104.942	0.081	Loss
13	7327673	105.237	1927571	105.263	0.026	Gain
13	9555715	109.951	8000390	110.032	0.081	Gain
13	9577555	113.162	11619206	113.243	0.081	Loss
14	1713459	20.004	1652045	20.089	0.085	Gain
14	10136680	20.286	7142320	20.344	0.058	Gain
14	10140911	23.985	2007316	24.054	0.069	Gain
14	12590795	44.231	229709	44.302	0.071	Loss
14	6572276	44.252	229709	44.302	0.05	Loss
14	6572276	44.252	229709	44.302	0.05	Loss
14	1999620	45.93	10131343	46.066	0.136	Loss

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Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
14	2790512	48.289	10498415	48.404	0.115	Loss
14	6573476	62.57	10133798	62.879	0.309	Gain
14	10131727	65.093	17103113	65.479	0.386	Gain
14	7154841	71.143	4899409	71.508	0.365	Gain
15	12440521	20.073	11263693	20.774	0.701	Gain
15	8027379	20.318	7170324	20.646	0.328	Gain
15	6576666	21.908	8040759	22.15	0.242	Loss
15	11853911	22.008	8031733	22.134	0.126	Loss
15	17118728	22.055	7496441	22.278	0.223	Loss
15	17118727	22.055	7496441	22.278	0.223	Loss
15	10519467	22.066	7496441	22.278	0.212	Gain
15	8031733	22.134	6576317	22.301	0.167	Gain
15	2883495	22.14	6576317	22.301	0.161	Gain
15	11636778	52.827	12592633	52.927	0.1	Loss
15	8025686	54.078	1983193	54.139	0.061	Loss
15	11855269	64.048	471751	64.123	0.075	Loss
15	2464605	85.609	2009305	85.678	0.069	Loss
15	9920857	85.632	8033636	85.671	0.039	Loss
16	1978318	6.594	17140825	6.625	0.031	Gain
16	574661	21.447	8050407	21.648	0.201	Loss
16	226005	21.528	8050407	21.648	0.12	Gain
16	10871268	34.282	238005	34.615	0.333	Gain
16	748250	34.295	237990	34.54	0.245	Gain
16	12716730	34.299	11860567	34.648	0.349	Gain
16	12716730	34.299	171125	34.618	0.319	Gain
16	1684568	34.307	7198487	34.56	0.253	Gain
16	1684568	34.307	4887603	34.722	0.415	Gain
16	1684568	34.307	8054993	34.68	0.373	Gain
16	1684568	34.307	238005	34.615	0.308	Gain
16	1684568	34.307	17724893	34.597	0.29	Gain
16	7500312	34.328	—	34.591	0.263	Gain
16	7500312	34.328	171125	34.618	0.29	Gain
16	7500312	34.328	17724893	34.597	0.269	Gain
16	7500312	34.328	11647180	34.672	0.344	Gain
16	7500312	34.328	—	34.591	0.263	Gain
16	7500312	34.328	8054993	34.68	0.352	Gain
16	7500312	34.328	11647180	34.672	0.344	Gain
16	7500312	34.328	11860567	34.648	0.32	Gain
16	7500312	34.328	17724893	34.597	0.269	Gain
16	7500312	34.328	—	34.591	0.263	Gain
16	11860956	34.329	17841568	34.587	0.258	Gain
16	11860956	34.329	7198892	34.671	0.342	Gain
16	11860956	34.329	17841568	34.587	0.258	Gain
16	11860956	34.329	17724893	34.597	0.268	Gain
16	11860956	34.329	11860567	34.648	0.319	Gain
16	12149499	34.337	—	34.591	0.254	Gain
16	12149499	34.337	17841568	34.587	0.25	Gain

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Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
16	17841516	34.344	8057388	34.708	0.364	Gain
16	9927120	34.403	1123289	34.586	0.183	Gain
16	8055603	34.426	17841568	34.587	0.161	Gain
16	8055603	34.426	—	34.591	0.165	Gain
16	11859395	75.501	284000	75.58	0.079	Loss
16	12598771	81.747	4404057	81.767	0.02	Loss
16	6565143	81.751	8049648	81.775	0.024	Loss
16	9933360	81.753	4404057	81.767	0.014	Loss
16	9933360	81.753	9931994	81.775	0.022	Loss
16	9933360	81.753	9937714	81.766	0.013	Loss
16	9933360	81.753	4404057	81.767	0.014	Loss
16	12446219	83.006	4782994	83.11	0.104	Loss
16	1693947	84.819	7205575	84.862	0.043	Loss
17	2304958	0.906	902967	1.18	0.274	Loss
17	11654477	1.687	12453691	2.378	0.691	Gain
17	4791889	7.724	4792239	7.957	0.233	Gain
17	180750	12.303	237347	12.368	0.065	Gain
17	7221571	22.388	11870888	22.508	0.12	Loss
17	957979	31.461	9909180	31.899	0.438	Gain
17	1357365	31.461	916841	31.502	0.041	Gain
17	1357365	31.461	916841	31.502	0.041	Gain
17	957979	31.461	916841	31.502	0.041	Gain
17	1357365	31.461	1015673	31.502	0.041	Gain
17	1357365	31.461	916841	31.502	0.041	Gain
17	1357365	31.461	916841	31.502	0.041	Gain
17	957979	31.461	916841	31.502	0.041	Gain
17	4796153	31.468	916841	31.502	0.034	Gain
17	4796153	31.468	916841	31.502	0.034	Gain
17	16971990	31.468	916841	31.502	0.034	Gain
17	16971990	31.468	916841	31.502	0.034	Gain
17	4796153	31.468	916841	31.502	0.034	Gain
17	4796153	31.468	916841	31.502	0.034	Gain
17	4796153	31.468	916841	31.502	0.034	Gain
17	4796153	31.468	916841	31.502	0.034	Gain
17	12945586	31.471	916841	31.502	0.031	Gain
17	12945586	31.471	916841	31.502	0.031	Gain
17	12939695	31.472	8072238	31.89	0.418	Gain
17	1465472	47.927	7222644	48.213	0.286	Loss
18	6505953	1.704	12709578	1.825	0.121	Loss
18	754905	1.815	12955130	2.254	0.439	Gain
18	754905	1.815	16942140	1.843	0.028	Loss
18	7229858	1.88	1940693	1.986	0.106	Loss
18	312995	1.89	12604106	1.971	0.081	Loss
18	312995	1.89	12604106	1.971	0.081	Loss
18	312995	1.89	12604106	1.971	0.081	Loss
18	7239989	1.901	12970542	1.97	0.069	Loss
18	9949006	1.907	12970542	1.97	0.063	Loss

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
18	6505984	1.961	1940678	2.049	0.088	Gain
18	4121620	7.679	1374105	7.751	0.072	Loss
18	1470471	7.723	1374105	7.751	0.028	Loss
18	1298527	13.82	9951717	13.948	0.128	Gain
18	9948548	25.242	11083374	25.791	0.549	Gain
18	8111681	48.346	12609638	48.538	0.192	Loss
18	7236127	56.174	11152266	56.672	0.498	Loss
18	17075671	61.857	2715282	61.908	0.051	Loss
18	7234351	63.838	9319770	64.687	0.849	Gain
18	584173	63.997	664358	64.047	0.05	Loss
18	584173	63.997	664358	64.047	0.05	Loss
18	12957718	64	512684	64.047	0.047	Loss
18	1482539	64	—	64.034	0.034	Loss
18	12957718	64	664358	64.047	0.047	Loss
18	12957718	64	664358	64.047	0.047	Loss
18	12454536	68.216	1897375	68.287	0.071	Loss
18	6565969	73.273	12957544	73.372	0.099	Gain
19	16993897	6.833	2967661	7.058	0.225	Gain
19	16994004	6.909	768489	7.036	0.127	Loss
19	10409326	23.413	1821290	23.873	0.46	Loss
19	1035478	38.008	10410304	38.086	0.078	Loss
19	4099161	46.126	7250745	46.195	0.069	Gain
19	10419516	47.974	4803565	48.231	0.257	Loss
19	10419516	47.974	4803598	48.504	0.53	Gain
19	10419516	47.974	4803565	48.231	0.257	Loss
19	7246390	47.979	10424212	48.283	0.304	Loss
19	7246390	47.979	12426	48.389	0.41	Loss
19	4341855	47.983	4803565	48.231	0.248	Loss
19	7249066	48.015	4803565	48.231	0.216	Loss
19	7249066	48.015	4335867	48.249	0.234	Loss
19	7254439	48.016	3922338	48.229	0.213	Loss
19	4558510	48.018	10410062	48.339	0.321	Gain
19	4558510	48.018	4803565	48.231	0.213	Loss
19	10419095	48.157	4803565	48.231	0.074	Loss
19	2078350	48.335	4802160	48.386	0.051	Loss
19	10410062	48.339	13382034	48.479	0.14	Loss
19	10410062	48.339	4803591	48.462	0.123	Loss
19	10410062	48.339	8109311	48.459	0.12	Loss
19	2354278	48.34	10415726	48.405	0.065	Loss
19	11667152	48.344	7257560	48.559	0.215	Loss
19	8111681	48.346	12609638	48.538	0.192	Loss
19	2190835	48.364	12609638	48.538	0.174	Loss
19	1058259	48.372	12609638	48.538	0.166	Loss
19	2617751	58.634	1284521	58.714	0.08	Gain
19	660575	59.992	11672983	60.075	0.083	Loss
20	6042859	14.624	6110431	14.71	0.086	Loss
20	6074799	14.719	8118172	14.778	0.059	Loss

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
20	6074799	14.719	432501	14.78	0.061	Loss
20	6135263	14.73	8118172	14.778	0.048	Loss
20	6135263	14.73	407097	14.771	0.041	Loss
20	6135263	14.73	407097	14.771	0.041	Loss
20	6079575	14.756	407600	14.792	0.036	Loss
20	2423849	14.852	175269	15.146	0.294	Loss
20	6034104	15.011	7267540	15.051	0.04	Loss
20	11699601	17.713	6105818	17.749	0.036	Loss
20	6014601	36.353	8120088	36.41	0.057	Loss
20	1015387	40.651	17748052	40.686	0.035	Loss
20	232278	43.776	462145	43.812	0.036	Loss
20	232262	43.784	462145	43.812	0.028	Loss
20	522962	47.577	235038	47.672	0.095	Loss
21	2821803	13.61	17207824	14.07	0.46	Loss
21	2824994	18.976	2825011	19	0.024	Gain
21	2824994	18.976	2825014	19.003	0.027	Gain
21	2824995	18.978	2825014	19.003	0.025	Gain
21	2824995	18.978	2825014	19.003	0.025	Gain
21	9680185	18.98	2825018	19.004	0.024	Gain
21	9680185	18.98	2825017	19.004	0.024	Gain
21	2826250	20.722	1735837	20.755	0.033	Loss
21	12482742	36.386	12627688	36.539	0.153	Gain
22	140378	15.257	5748657	15.668	0.411	Gain
22	2252257	17.02	16982953	17.387	0.367	Gain
22	3180408	17.031	6518517	17.41	0.379	Loss
22	2543958	17.258	6518517	17.41	0.152	Gain
22	2543958	17.258	3747041	17.386	0.128	Gain
22	17742907	17.271	6518517	17.41	0.139	Gain
22	7288481	17.342	3747041	17.386	0.044	Gain
22	2016805	23.973	680355	24.249	0.276	Gain
22	4822592	23.98	680355	24.249	0.269	Gain
22	17608968	23.982	680355	24.249	0.267	Loss
22	9620506	23.989	599794	24.26	0.271	Loss
22	9620506	23.989	4820650	24.255	0.266	Loss
22	12158362	23.991	4822639	24.255	0.264	Gain
22	6004508	23.992	686025	24.241	0.249	Gain
22	5996883	24	560065	24.246	0.246	Loss
22	5996883	24	4822638	24.255	0.255	Gain
22	5996883	24	680355	24.249	0.249	Gain
22	5996884	24.002	2742651	24.239	0.237	Gain
22	4083400	24.031	489157	24.241	0.21	Gain
22	4083400	24.031	489157	24.241	0.21	Gain
22	6004567	24.042	489157	24.241	0.199	Gain
22	2331038	24.129	489157	24.241	0.112	Gain
22	1811828	24.137	484400	24.241	0.104	Gain
22	16992342	32.17	9621706	32.197	0.027	Loss
X	311161	2.704	11796830	2.821	0.117	Gain

(continued)

Chrom	Start rs	Start phys pos	End rs	End phys pos	Size (megabases)	Loss or gain
X	5962032	6.411	4830389	8.095	1.684	Gain
X	5989582	6.756	6639703	6.838	0.082	Loss
X	6654876	8.541	16985123	8.673	0.132	Gain
X	5970631	22.58	4828949	22.91	0.33	Loss
X	5970976	22.943	5970985	22.965	0.022	Loss
X	12392817	37.662	1884694	37.901	0.239	Gain
X	570429	47.704	5905651	47.889	0.185	Loss
X	857884	47.709	17148218	47.866	0.157	Loss
X	478846	47.756	5905651	47.889	0.133	Loss
X	537861	47.768	17148218	47.866	0.098	Loss
X	2379044	63.689	7891562	63.736	0.047	Gain
X	1503645	75.454	1112337	75.509	0.055	Loss
X	5921140	97.731	2001096	97.788	0.057	Loss
X	1180799	103.054	178124	103.235	0.181	Gain
X	527454	103.13	178100	103.216	0.086	Gain
X	510819	103.133	178101	103.216	0.083	Gain
X	510819	103.133	178100	103.216	0.083	Gain
X	510819	103.133	178100	103.216	0.083	Gain
X	1180790	103.137	178101	103.216	0.079	Gain
X	1180790	103.137	6523769	103.192	0.055	Gain
X	531800	103.145	6523769	103.192	0.047	Gain
X	531800	103.145	178100	103.216	0.071	Gain
X	17332029	103.146	178101	103.216	0.07	Gain
X	17332029	103.146	3213519	103.182	0.036	Gain
X	16997612	116.505	5911947	116.511	0.006	Loss
X	5976553	126.368	4609321	126.567	0.199	Loss
X	956823	126.497	226617	126.747	0.25	Loss
X	17330766	134.573	3123483	134.741	0.168	Gain
X	6654427	140.383	205595	140.623	0.24	Gain
X	205595	140.623	6636474	140.694	0.071	Gain
X	12843534	141.672	1569855	142.143	0.471	Loss
X	7877383	148.091	2222954	148.267	0.176	Gain
X	11797711	148.693	7064596	148.775	0.082	Gain
X	5925319	151.965	5925379	152.227	0.262	Gain

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Chapter 4
Recurrent Gene Mutations in CLL

Alejandra Martínez-Trillos, Víctor Quesada, Neus Villamor, Xose S. Puente, Carlos López-Otín, and Elías Campo

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

A. Martínez-Trillos • N. Villamor
Unidad de Hematopatologia, Departamento de Anatomía Patológica,
Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS),
Universitat de Barcelona, Barcelona, Spain

V. Quesada • X.S. Puente • C. López-Otín
Departamento de Bioquímica y Biología Molecular, Instituto Universitario de
Oncología-IUOPA, Universidad de Oviedo, Oviedo, Spain

E. Campo (✉)
Hematopathology Unit, Hospital Clinic, University of Barcelona, Villarroel 170, Barcelona
08015, Spain
e-mail: ecampo@clinic.ub.es

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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].

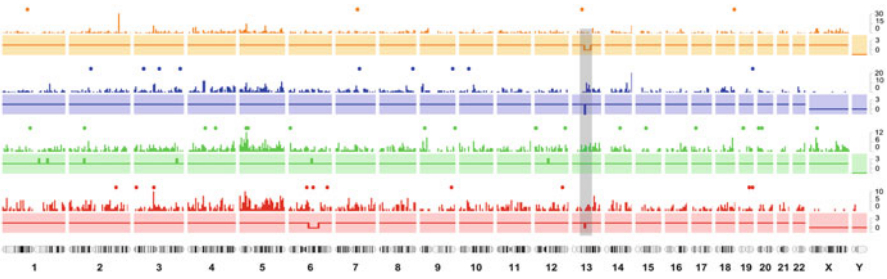

Fig. 4.1 Somatic mutations in the whole genome of four patients with chronic lymphocytic leukemia (CLL). For each case the density of mutations per 5-Mb window is represented with *bars* and protein-altering mutations by *dots*. The copy number alterations are represented by the *solid bar* and the *shaded 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 ± 0.2 % vs. 6.2 ± 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

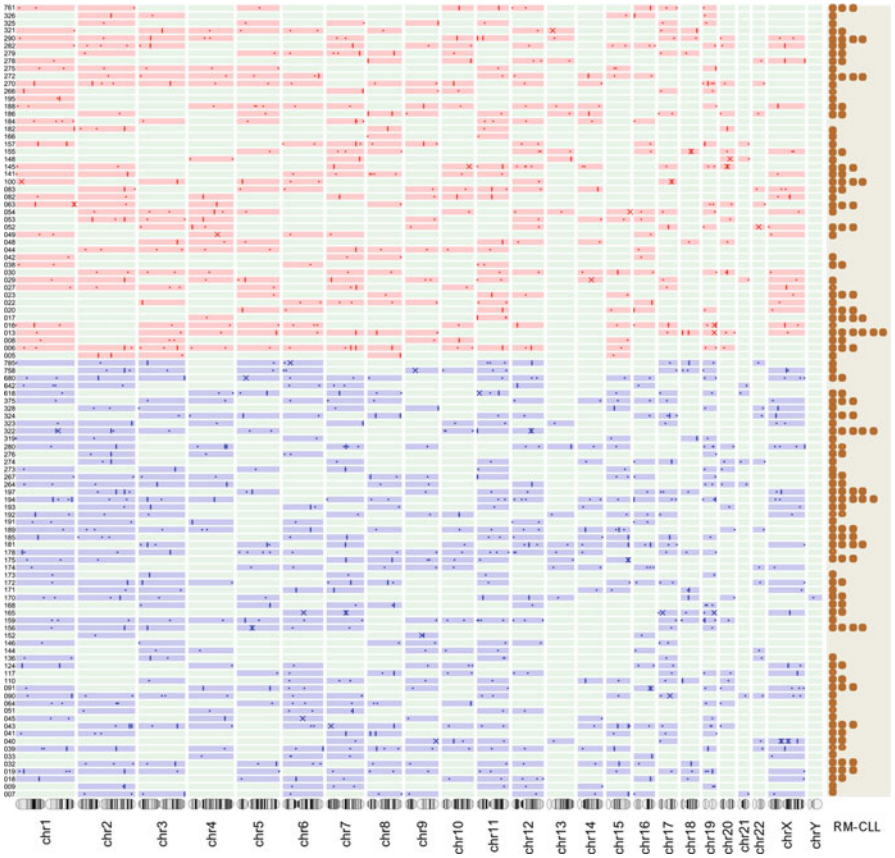

Fig. 4.2 Somatic mutations in the exome of CLL. Distribution and location of protein-coding mutations (*dots*), insertions, and deletions (*X*) in 60 CLL cases with mutated (*blue*) and 45 CLL cases with unmutated (*red*) *IGHV*. Recurrent mutated genes are highlighted with *vertical bars* and summarized 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, Waldenstrom 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

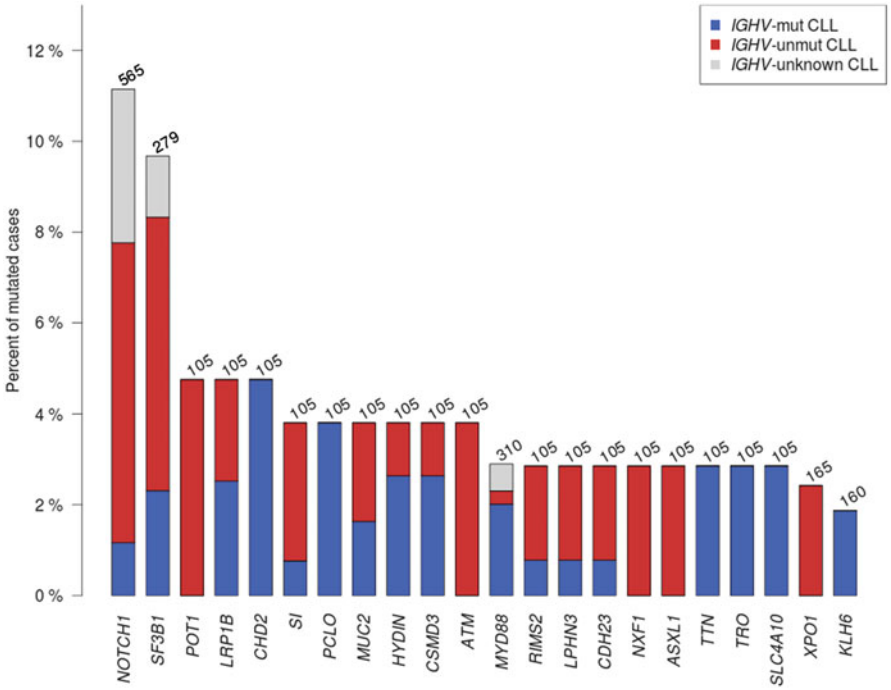

Fig. 4.3 Repertoire of mutations in CLL. Frequency of the most common somatic mutations in CLL according to the IGHV mutational status (data are taken from Quesada et al. [9])

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

[a]Mutation percentage between 61 chemotherapy-naïve and 30 chemo-treated CLL samples
[b]Includes 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

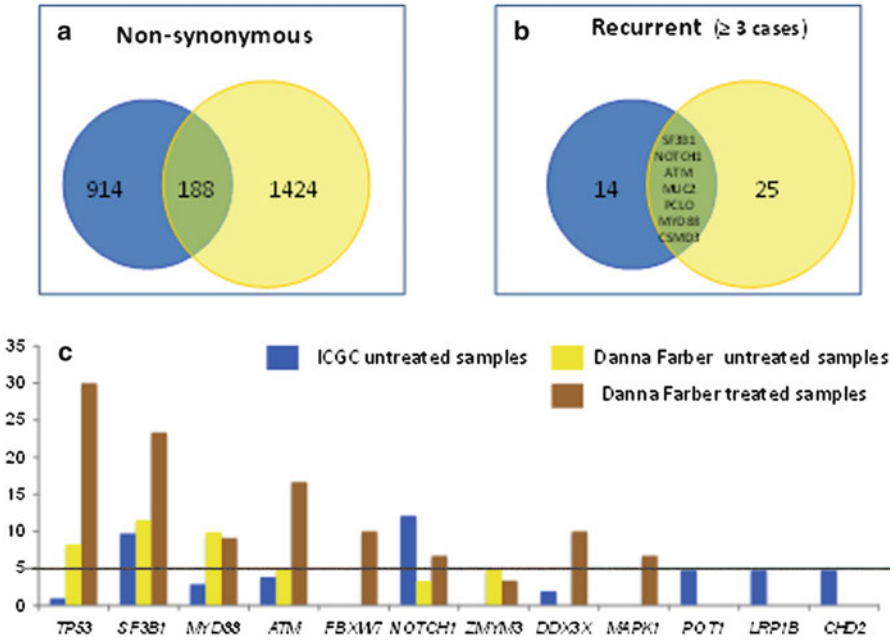

Fig. 4.4 Comparison of mutations in two independent whole exome studies [9, 10]. *Blue* identifies mutations in the ICGC exome study [9], *yellow* and *brown* identify mutations in the Dana Farber exome study [10]. (**a**) Nonsynonymous mutations with commonly mutated genes found in both series. (**b**) 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	*n*	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 NFkB 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 β2-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

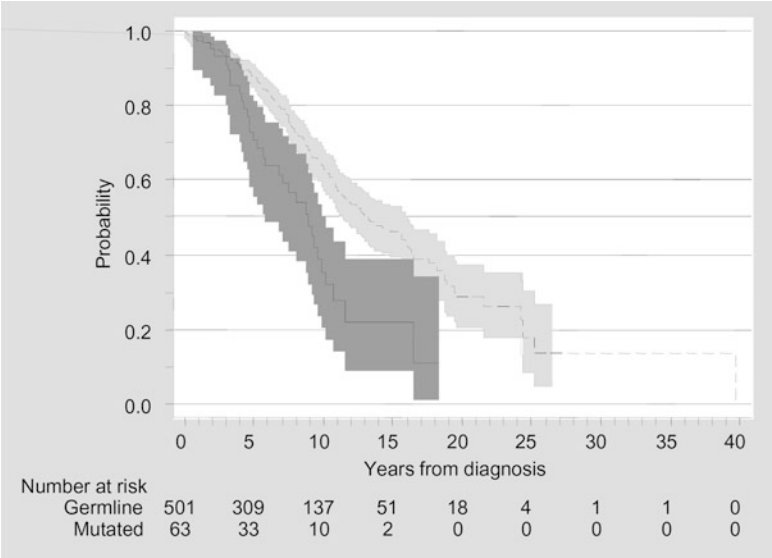

Fig. 4.5 Overall survival of CLL patients according to NOTCH1 mutations. Overall survival in *NOTCH1*-mutated (*solid line*) and *NOTCH1*-unmutated (*dashed line*) CLL patients ($p < 0.001$). The 95 % confidence interval for each group of patients is depicted (with permission from Villamor et al. [37])

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	*n*	%
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

[a]Other: 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 *FOXP1*, 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 β2-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 NFkB. 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 NFkB 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 NFkB 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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Chapter 5
TP53 Aberrations in Chronic Lymphocytic Leukemia

Martin Trbusek and Jitka Malcikova

Abstract CLL patients harboring *TP53* defects remain the most challenging group in terms of designing rational and effective therapy. Irrespective of the treatment employed—chemotherapy, chemoimmunotherapy, or pure biological drugs—median survival of these patients does not exceed 3–4 years. This adverse outcome is caused by a less effective response to therapeutics acting through DNA damage induction and relying on the subsequent initiation of apoptosis as well as by virtually inevitable aggressive relapse. Patient proportions with *TP53* defects at diagnosis or before first therapy were reported within the range 5–15 %, but they increase dramatically in pretreated cohorts (reported up to 44 %), and also in patients with Richter transformation (50 % harbor *TP53* defects). Currently, most laboratories monitor *TP53* defect as presence of 17p deletion using I-FISH, but 23–45 % of *TP53*-affected patients were shown to harbor only mutation(s). In other patients with intact *TP53*, the p53 pathway may be impaired by mutations in *ATM* gene coding for the p53-regulatory kinase; however, prognosis of *ATM*-defective patients is not as poor as those with *TP53* abnormalities. Though many novel agents are under development, the monoclonal antibody alemtuzumab and allogeneic stem cell transplantation remain the basic treatment options for *TP53*-affected CLL patients.

Keywords *TP53*/p53 mutation • Deletion 17p • Apoptosis • Prognosis • Relapse • Chemo-refractoriness • Alemtuzumab • ATM

M. Trbusek, Ph.D. (✉) • J. Malcikova, Ph.D.
Department of Molecular Medicine, Central European Institute of Technology, Masaryk University (CEITEC MU), Kamenice 5, 62500 Brno, Czech Republic
e-mail: mtrbusek@fnbrno.cz; jmalcikova@fnbrno.cz

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Tumor Suppressor p53

p53: A Tumor Suppressor with Unique Properties

In 1979, two independent research groups [1, 2] published their reports noting a physical interaction between a large T-antigen of the SV40 virus and a cellular protein of approximately 53–54 kDa. This is when the fascinating story of p53 research commenced. Although this yet unknown protein attracted a lot of attention from the very beginning, determination of its principle role in tumor cells has not always been straightforward. Still, in 1984, p53 had been erroneously assigned among oncoproteins (for review see [3]). This incorrect classification was influenced by two basic factors: (a) protein accumulation was frequently observed in tumor cells, by contrast to normal cells, which resembled an oncogenic behavior, and (b) complementary DNA (cDNA) clones used for transfection experiments into human cells harbored missense mutation, and—as disclosed later—some missense mutations exert the ability to switch the p53 from a tumor suppressor to a powerful oncoprotein. Only later studies from the end of 1980s confirmed the tumor-suppressive behavior of wild-type p53 [4, 5] and, finally, 13 years from its discovery, the p53 protein was officially proclaimed as the "Guardian of the Genome" [6].

Although p53 activity was recently shown to impact the pathogenesis of several nonmalignant diseases, the p53 role in cancer prevention is substantially more elaborated. Currently, both experimental data and clinical observations recognize the p53 as the most important tumor-suppressor protein: (a) *TP53*$^{-/-}$ mice invariably develop tumors [7], (b) heterozygous inherited mutations predispose to the Li-Fraumeni cancer-prone syndrome in humans [8], and (c) somatic mutations are frequent in many different types of human tumors [9].

The p53 plays a critical role in an anti-cancer barrier preventing an organism from malignant cell proliferation [10–12]. During early cancerogenesis, tumor cells experience genotoxic stress, which elicits a DNA damage response (DDR) pathway—the hierarchically ordered machinery detecting DNA lesions and signaling their presence to protein complexes that either promptly repair the damaged DNA or arrest the cell cycle if DNA repair requires additional time. Alternatively—in case when DNA damage is too extensive and repair is not possible—the DDR pathway induces apoptosis or replicative senescence. For effective induction of the above-mentioned processes, p53 activity is crucial.

Considering these facts, it is not surprising that the central axis of the DDR pathway, involving both the p53 and its positive regulator, the ATM kinase (*A*taxia *T*elangiectasia *M*utated), is under enormous pressure to be impaired during malignant conversion [11, 12]. Indeed, *TP53* mutations in particular are frequent in many different tumors and most often observed in ovarian, colorectal, and esophageal cancer [13]. Even a low *TP53* mutation frequency, which is typical for some types of tumors, may not mean that p53 is irrelevant in prevention of their development. In cervical carcinoma, for instance, a typically non-mutated p53

protein is inactivated by direct physical interaction with an E6 oncoprotein encoded by the high-risk human papillomavirus (type 16 or 18) [14]. In other tumors with an intact *TP53* gene, the p53 pathway may also be abolished by enhanced activity of p53 inhibitors (e.g., MDM2) or defects in upstream p53 activators (such as ATM in CLL) or downstream target genes (e.g., inactivation of NOXA and others in different B-cell lymphomas [15]). Frequency of p53 inactivation in hematological malignancies is lower in comparison with solid tumors, usually reaching 10–15 % in unselected patient cohorts. However, in contrast with the solid tumor situation, virtually all relevant studies agree with a severe prognosis for patients with hematological malignancies and p53 inactivation [16].

p53 Protein Structure

The *TP53* gene is located at the short arm of chromosome 17 (17p13.1) and contains 11 exons, 10 of which (2–11) are coding. The full-length protein consists of 393 amino acids and harbors several structural domains: (a) the N-terminal domain, which ensures target gene transactivation; (b) the central DNA-binding domain, which directly interacts with consensus DNA sequence in the target promoters; (c) the oligomerization domain, through which the four monomeric polypeptide chains join together to form a final tetramer molecule; and (d) the C-terminal domain, which harbors important regulatory sites for the DNA-specific and also nonspecific p53 binding. Besides the basic full-length protein, analysis of *TP53* gene sequence also revealed 12 putative p53 isoforms (for review see [17]). Expression of individual isoforms is tissue-specific, and they differ in subcellular localization. In quiescent lymphocytes, isoform p53β is typically expressed [18].

The *TP53* gene contains many polymorphisms, with 11 of them being non-synonymous. The most frequent polymorphism P72R (changing the ancestral proline allele to arginine, which is notably more frequent in some populations) was described to have functional impact, and its role in cancer susceptibility, prognosis, and treatment response was studied in several malignancies with inconsistent results (for review see [19]). Similarly, in early CLL studies no relation between this polymorphism and clinical outcome was evident [20, 21]; in a more recent study, the proline allele in the homozygous state was associated with a shorter time to first treatment among the group of patients with mutated *IGHV* locus [22].

Regulation of p53 Protein

The p53 protein level in a cell is low under normal conditions—undetectable by western blot or immunohistochemistry. This is because the p53 induces—among other targets—expression of *MDM2* gene coding for a protein which serves as a negative regulator, targeting p53 protein for ubiquitin-mediated degradation

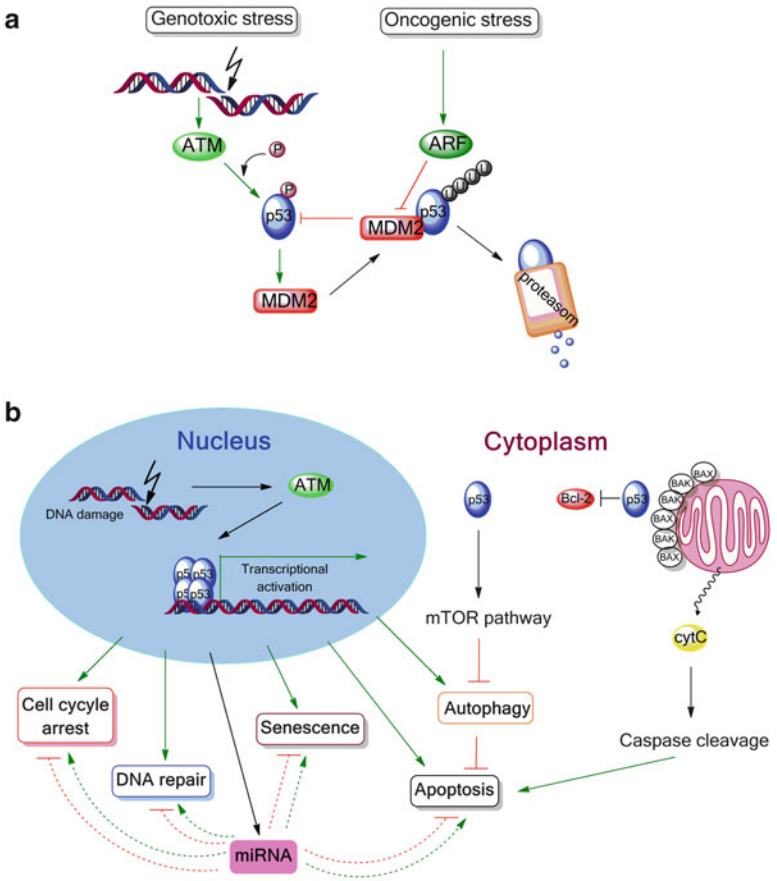

Fig. 5.1 Overview of p53 stabilization (**a**) and activity (**b**). In case of a genotoxic stress the p53 protein is phosphorylated by ATM kinase and thus prevented from MDM2-mediated degradation. Under oncogenic stress p53 is protected from degradation by $p14^{ARF}$ protein. After stabilization the p53 protein regulates through transcriptional activation of target genes several divergent but interconnected processes, which are decisive for a cell fate. The p53 also localizes on mitochondria, where it interacts with Bcl2 protein family members. This translocation contributes to permeabilization of the mitochondrial membrane, cytochrome c release, and subsequent apoptosis

(Fig. 5.1a). p53 protein stabilization following genotoxic stress is then ensured through delicate posttranslational modifications namely involving phosphorylations, but also acetylations or sumoylations [23]. The p53 Ser-15 phosphorylation elicited by ATM kinase is critical for p53 protein stabilization after induction of DNA double-strand breaks (DSBs) [24, 25]; this phosphorylation prevents MDM2 binding to p53. Another situation arises in case of oncogenic stress (activation of oncogenes, e.g., BCR-ABL1), when p53 is stabilized by the product of a *CDKN2A* gene, i.e., $p14^{ARF}$ protein that directly inhibits p53-MDM2 binding [26] (Fig. 5.1a).

Cellular Functions of p53

The p53 is a transcription factor with a consensus binding site consisting of two copies of the 10 base pair motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0–13 base pairs [27]. It is estimated that p53 regulates more than 125 structural genes [28]. The expression of many p53-regulated proteins is critical for decisions made within the DDR (Fig. 5.1b). Regarding cell cycle arrest, the cyclin-dependent kinase inhibitor *CDKN1A* (coding for p21 protein) seems to be the most prominent target upregulated by p53, while *PUMA* (p53-upregulated modulator of apoptosis) is a key mediator of p53's apoptotic activity [28]. Transcriptional activity of p53 is tissue-specific [29] and is distinct in lymphocytes compared to other cells [30]. Among p53 target genes there are also numerous microRNAs, including miR-34a, with a proposed role in CLL pathogenesis [31, 32]. Recently, p53's role in posttranscriptional maturation of several microRNAs with growth-suppressive properties (including, e.g., miR-16) has been reported [33].

How p53 can discriminate between cell cycle arrest, senescence, or apoptosis still remains a matter of intense debate [34]. The p53 molecule conformation represents perhaps the most critical factor, and this conformation depends on two basic events: (a) posttranslational modifications, such as phosphorylations, methylations, or acetylations; and (b) DNA-binding itself that determines which cofactors will participate in target gene transactivation and thus influence transcription extent.

The p53 is also able to trigger apoptosis independently on transcription initiation. This mechanism facilitates a fast response to genotoxic stress, when p53 localizes on mitochondria, it interacts with Bcl2 protein family members, enabling oligomerization of bak and bax proteins and subsequent permeabilization of the mitochondrial membrane and cytochrome c release, which leads to caspase cleavage (Fig. 5.1b, for review see [35]). It was even suggested by Steele et al. [36] that transcriptional-independent apoptosis is a major route to cell death induction by p53 in CLL cells, as they showed that blocking of p53-mediated transcription paradoxically augmented apoptosis induction by chlorambucil and fludarabine through accelerating the proapoptotic conformation change of the Bax protein.

Another important p53 cytoplasmic function is inhibition of autophagy through mTOR pathway promotion. Interestingly, p53 plays an ambiguous role in autophagy control, as nuclear p53 can induce autophagy through transcriptional activation of positive autophagy regulators (including mTOR inhibitors) (for review see [37]). Autophagy is tightly connected with apoptosis, although their exact interplay remains a matter of debate. Autophagy inhibition facilitates cell death, yet on the other hand, its activation promotes the cell's attempt to cope with stress and to survive. However, with excessive autophagy, an autophagocytic death via necrosis may occur. Therefore, the p53 decision between autophagy induction and inhibition is crucial for cancer treatment, and autophagy was identified as an important mechanism of drug resistance. Early studies also suggested its relevance in CLL resistance to treatment by flavopiridol [38] or dasatinib [39].

p53 Mutagenesis: Dominant-Negative Effect and Gain-of-Function

In contrast with certain other tumor-suppressors, the *TP53* gene doesn't need to be inactivated on both alleles to eliminate p53 function. The p53 pathway is highly sensitive to p53 protein level changes, and the protein produced by only one allele may not be sufficient to ensure proper function. This effect, termed "haploinsufficiency," was documented on mouse models [40] and is assumed in patients with sole 17p deletion (del(17p)) or sole truncating mutation. However, simple loss of function (LOF) may not be the most grievous p53 defect contributing to tumor progression. This could have been predicted from the highly predominant occurrence of p53 missense substitutions (forming approximately 75 % of all mutations) leading to expression of aberrant protein in cancer patients. Indeed, besides LOF, another two effects clearly attributable to mutated p53 have been evidenced: (a) - dominant-negative effect (DNE) of monoallelic mutation towards the second intact (wild-type) allele, and (b) gain-of-function (GOF) effect probably acting independently on the allele status.

DNE is most likely caused by final p53 molecule inhibition through hetero-oligomerization of mutated and non-mutated p53 polypeptide chains. The p53 functions as a tetramer composed of a dimer of dimers with co-translational forming of individual dimers. Once one p53 allele is mutated in the DNA-binding region, half of the dimers would be active, the other half would be inactive, and final posttranslational tetramerisation would render inactive 75 % of p53 tetramers [41]. Accompanying del(17p), frequently observed in mutated patients, then probably eliminates the rest of p53 activity. By contrast, a sole del(17p) eliminates only 50 % of p53 molecules, and this may potentially explain why monoallelic missense mutation, but not sole del(17p), is frequently selected in CLL patients [42].

In addition to the DNE, it has also been well evidenced through numerous studies and several experimental systems that some missense mutations exert a strong GOF effect. This effect was initially demonstrated in cell lines lacking endogenous p53, when the mutated *TP53* gene had been expressed and the phenotype did not copy a simple loss of p53 function [43]. A pivotal mechanism of the mutated p53 GOF seems to be an interference with the p53-related proteins, i.e., p63 and p73, which prevents their tumor-suppressive functions [44]. In addition, some p53 mutants have been shown to upregulate genes and miRNAs, supporting cancer progression or precluding effective therapy. For instance, mutated p53 was shown to enhance the expression of multidrug-resistance gene 1 (*MDR1*) [45]. A number of other GOF mechanisms were described (for review see [46]) including direct protein–protein interactions, e.g., interaction with NF-κB leading to prominent enhancement of a cancer progression [47], or interaction with the nuclease Mre11, which suppresses the binding of the Mre11-Rad50-NBS1 (MRN) complex to DNA DSBs, leading to impaired ATM activation [48]. Mutated p53 has also been implicated in abrogation of the mitotic spindle checkpoint [49]. Both DNE and

GOF are mutant and cell-type-specific, and preliminary data suggests that a strong mutated p53 GOF effect may also be present in CLL patients with particular p53 mutations [50].

p53 Activity: More than Cancer Protection

Despite its indisputable role in protecting an organism from developing a tumor, p53 protein activity may not always be desirable. One example might be the adverse effect of anti-cancer therapy (namely chemotherapy and radiotherapy) on normal tissues; in this respect, a transient p53 inhibition during therapy could be a reasonable way how to protect healthy cells from unwanted apoptosis [51]. Recently, p53 protein contribution to the pathological elimination of nonmalignant cells is gradually being recognized. Specifically, p53 protein activation has been proven to result in cardiomyocyte necrosis during ischemic heart disease, and p53-dependent apoptosis then appears to lead to the pathological neurodegeneration in Alzheimer's, Parkinson's, and Huntington's diseases [28].

p53 Mutation Functional Impact Assessment

While some *TP53* mutations manifest obvious effects on p53 function (e.g., nonsense or frame-shift mutations which abolish the DNA-binding domain), identified missense substitutions should always be checked for severity and predicted clinical impact. Comprehensive analysis of individual *TP53* missense mutations is available via the web pages of the International Agency for Research on Cancer (IARC; http://www-p53.iarc.fr). The following statistics are available in this database: (a) mutated protein activity assessed towards eight selected target promoters (percentage in comparison with wild-type protein); this activity was analyzed using a yeast functional assay and is available for all potential 2 314 p53 missense mutations [52]; for some mutants, information about functionality in human cells is also available; (b) structural impact of mutations; (c) available data about DNE and GOF; (d) frequency of particular mutations in cancer patients and Li-Fraumeni families; (e) list of described p53 polymorphisms.

TP53 Gene Abnormalities in CLL

The frequency of *TP53* defects in hematological malignancies varies between 5 and 20 %, which is low in comparison with solid tumors, where it can reach up to 80 % (http://www-p53.iarc.fr); however, their impact on disease course is unequivocal, with p53 abnormalities having a well-documented role in chronic lymphocytic

leukemia. Within 1–2 years, patients with *TP53* defects almost uniformly require treatment, are often chemo-refractory, and their expected survival is distinctively reduced, with almost exclusive disease-related death. CLL patients with *TP53* gene defects were therefore assigned to a small but challenging subgroup of patients that was defined as "ultra high-risk CLL" [53].

History of TP53 Gene Defects Examination

Initial *TP53* gene defects in relation to CLL were reported as early as in 1991, when Gaidano et al. [54] identified *TP53* mutations in different human lymphoid malignancies and described their presence in late CLL stages. Subsequent studies showed the presence of *TP53* mutations in 10–15 % of patients, confirmed association with advanced stages [55], and delineated the association of mutations with chemo-refractoriness and poor clinical outcome [56]. Loss of the *TP53* locus (del(17p)) was not considered an important recurring event in CLL during early studies using conventional karyotyping, which most distinctively identified trisomy of chromosome 12 [57, 58]. Significance of del(17p) was proven only when interphase fluorescence in situ hybridization (I-FISH) was introduced [59]. Adverse prognostic impact of del(17p) was definitely confirmed in the year 2000 by Dohner et al. [60]. In this study, a comprehensive set of FISH probes was employed with del (17p) being the strongest predictor of poor survival and reduced time to treatment, followed by 11q deletion and trisomy 12; deletion 13q as the sole abnormality exhibited the best prognosis. Based on this observation, a hierarchical prognostic stratification model was suggested that is still referenced today, and assessment of del(17p) using I-FISH was introduced into general practice. Recommendation for a del(17p) examination, at least in clinical trials, was also included in a report from the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) updating guidelines for the diagnosis and treatment of CLL [61].

The clinically relevant cut-off value for del(17p) presence has long been sought after. In the LRF CLL4 trial comparing Chlorambucil (Chl), Fludarabine (F) or Fludarabine, and Cyclophosphamide (FC), no difference in progression-free survival (PFS) or response duration was observed between the patients having 5–20 % of cells with del(17p) and those without this deletion. It was therefore suggested to use 20 % positivity as a clinically relevant cut-off for del(17p) [62]. However, there is still insufficient evidence to use the uniform clone size cut-off in diagnostic procedure.

While the assessment of the del(17p) presence is relatively easy, sensitive, and provides quantitative information on the proportion of affected cells, examination of *TP53* gene mutations is more complicated, and no standardized approach for CLL patients has been established. Initially, it was assumed that correlation between deletion and mutation of the second allele is high in cancer cells [63], although some reports noted a common presence of sole mutation in later stages of tumor development [64]. Currently, most laboratories still investigate the *TP53*

defect in CLL patients only as del(17p) by I-FISH both in routine clinical practice and in clinical trials according to official worldwide recommendations [61]. *TP53* mutations only became a renewed subject of interest after several studies showed that quite a large proportion of patients carry *TP53* mutations in the absence of del(17p) and that such mutations have an independent prognostic impact [42, 65–69]. Based on these observations, the European Research Initiative on CLL released the recommendations on *TP53* mutation analysis in CLL in 2012 [70]. Thus, examination of both mutations and deletions is currently recommended before any treatment initiation.

Prognostic Impact of 17p Deletions and TP53 Mutations

Despite rare cases with del(17p) and indolent disease course (usually manifesting a mutated *IGHV* status) [71, 72], a strong adverse impact of del(17p) was observed in numerous studies and clinical trials. With these patients both a short PFS and overall survival (OS) were documented [73, 74]. The p53-affected patients exhibit markedly poor responses to various chemotherapy-based regimens involving alkylating agents or purine analogues [75–78] as well as their combination with the anti-CD20 monoclonal antibody rituximab [79, 80]. Even chemoimmunotherapy involving rituximab in combination with fludarabine and cyclophosphamide (FCR), which is currently considered the first treatment option for physically fit CLL patients, did not abrogate the negative del(17p) prognostic effect. Although patients with this genetic abnormality enrolled in a CLL8 study comparing FC and FCR manifested a prolonged PFS in the FCR arm (FCR: 11.3 months vs. FC: 6.5 months; HR 0.47), only 5 % of patients achieved complete remission after FCR therapy, and OS was significantly shorter in comparison with all other cytogenetic subgroups, reaching a median of approximately 3 years [81].

Deletion 17p is usually accompanied by *TP53* mutation in CLL cells, but both sole del(17p) and sole *TP53* mutations occur. Their frequencies and mutual proportion vary among different studies (Fig. 5.2). In addition, a mutation in the absence of del(17p) may be accompanied by uniparental disomy (UPD) resulting in duplication of the mutant allele [82]. The first study which examined the impact of mutations in the absence of del(17p) was performed on patients enrolled in US Intergoup E2997 trial (F vs. FC), and in contrast to del(17p) presence, no independent impact on PFS was observed for *TP53* mutations. However, this output could be influenced by including polymorphisms and intronic as well as unconfirmed mutations in this study [76]. By contrast, later studies recorded a reduced time to first treatment [83], reduced PFS [68], and also adverse OS [42, 65, 68] in patients with sole *TP53* mutations. Independent negative prognostic impact of *TP53* mutations was also subsequently confirmed in prospective clinical trials [66, 69, 84].

A majority of mutations identified in CLL are missense substitutions localized in the DNA-binding domain with a mutation profile similar to other cancers, though several specific features were described (Fig. 5.3). A high incidence of an unusual

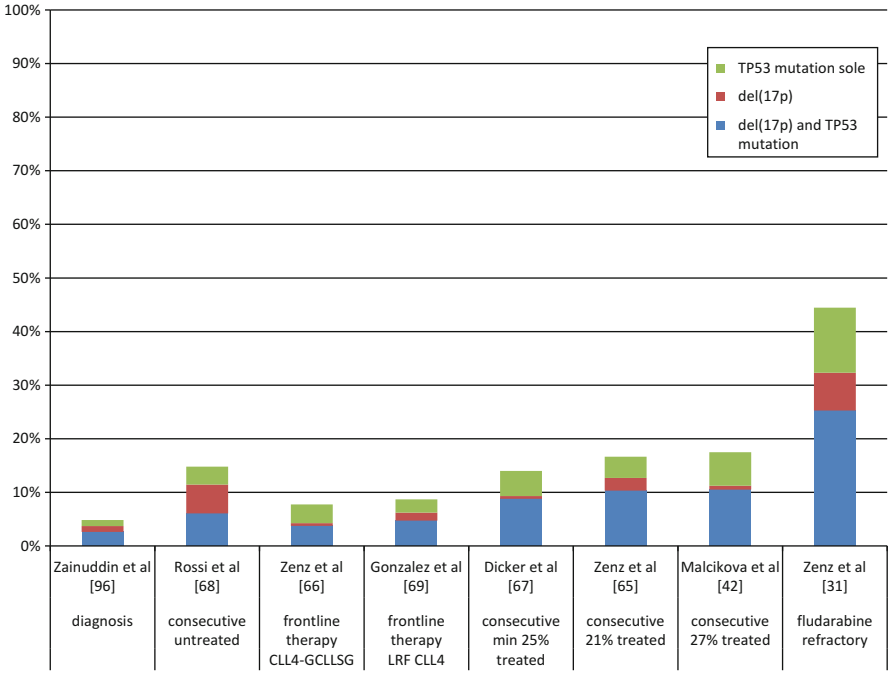

Fig. 5.2 Frequency of *TP53* defects and their composition in selected relevant CLL studies

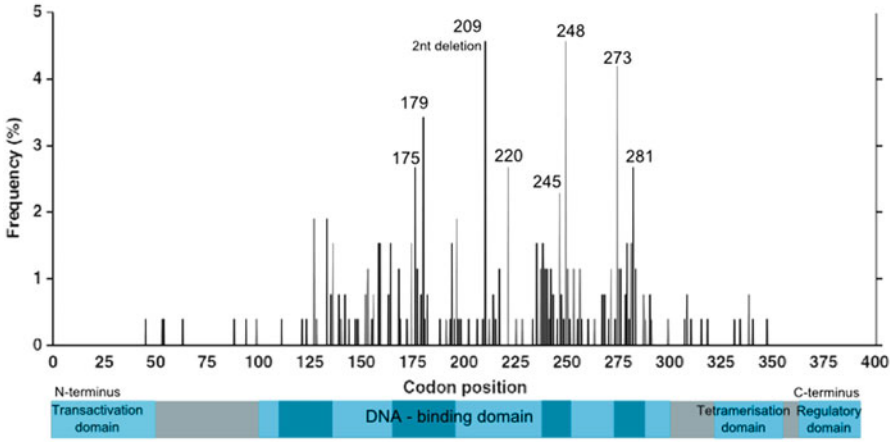

Fig. 5.3 Distribution of mutated codons in CLL patients based on the most comprehensive study by international collaborative group ($n = 268$ *TP53* mutations) [86]. Structure of p53 protein with highlighted DNA-binding motifs inside the DNA-binding domain

two-nucleotide deletion in codon 209 was documented [85] and later confirmed by an international collaborative study analyzing 268 mutations from four independent cohorts [86]. The same study also revealed a decreased percentage of transitions at CpG sites with bias favoring G-A exchange when compared to C-T exchange. Interestingly, G-A transitions were shown to be preferentially selected in quiescent, nondividing cells as a mirror reflection of cytosine deamination (C-T mutation) in the coding, non-transcribed DNA strand [87].

Data from in vitro studies suggest that not only the presence of a *TP53* mutation, but also the type of mutation and its position matter. Indeed, it was shown in other malignancies that specific *TP53* mutations are associated with either a poorer prognosis or a worse response to treatment than other *TP53* mutations; however, the results are often contradictory due to the complexity of p53 pathway defects, unpredictable other genetic context, and lack of prospective studies [88–92]. Preliminary data in CLL [50] showed that patients with mutations in p53 DNA binding motifs (DBMs) (codons directly involved in DNA binding localized in loops L2 and L3 and in the loop-sheet-helix motif) have clearly reduced survival rates compared with patients carrying other p53 mutations. All mutations included in this study led to a basic loss of p53 transactivation activity. Substantially worse survival of patients with DBMs mutations may, therefore, most likely be attributed to a strong mutated-p53 GOF. It is important to study this potential phenomenon in CLL cells since there are innovative studies focusing on activation of p53 homologs in patients with a *TP53* defect [93–95]. There might be a critical difference in utility of this approach between patients with absent p53 and those harboring p53 missense mutation connected to the GOF effect, which could potentially interfere with the activity of homologs.

Clonal Evolution of TP53 Defects

Frequency of mutations and deletions strongly varies depending on the disease stage and the cohort analyzed. At diagnosis, only 4.9 % of patients were reported to carry 17p deletion and/or *TP53* mutation [96]. In untreated cohorts and in patients analyzed before first therapy the frequency varies between 8.5 and 14.8 % [66, 68, 69], and occurrence sharply increases after treatment, where it can reach up to 44 % in fludarabine refractory disease [31] and 50 % after CLL transformation to Richter syndrome [97] or to prolymphocytic leukemia [98]. The increasing proportion of *TP53* abnormalities suggests that clonal selection of adverse genetic defects plays an important role during CLL progression. The Mayo Clinic report [99] illustrated the del(17p) selection during the CLL course and associated this selection with the high expression of zeta-associated protein (ZAP-70) and the presence of treatment. Another study [100] associated clonal evolution of del(17p) specifically with foregoing therapy presence. Several research groups analyzing p53 mutations then similarly documented their selection under therapy pressure [42, 65, 68, 101].

The higher frequency of *TP53* mutations after therapy administration and also the higher frequency of transversions as opposed to transitions led to the notion that these mutations might directly be induced by chemotherapy [85, 102], similarly to what was evidenced with the chemical compound aflatoxin or tobacco smoke in other types of cancer [103, 104]. However, the most extensive collaborative study involving 268 p53 mutations didn't record any differences in the mutation profiles of CLL patients with or without previous therapy [86], which indicated that treatment probably did not contribute to mutation origination. This view is currently supported by direct analyses of *TP53* mutation presence in samples taken before therapy administration from patients who were later (after therapy) shown to acquire clonal *TP53* mutation [65, 105].

Association of TP53 Defects with Other Genetic Variables

Abnormalities in the *TP53* gene mostly occur in patients with unfavorable unmutated *IGHV* locus [73, 86] and constitute an independent adverse prognostic factor within this subgroup [50]. In line with the role of p53 as the guardian of the genome (see above), *TP53* abnormalities were also associated with elevated genomic complexity in CLL patients [106, 107].

In 2001, Pettitt et al. [108] reported that CLL samples, which manifested p53 dysfunction (assessed through defective response to ionizing radiation) but did not harbor any *TP53* defect, have ATM kinase impaired by a mutation. Since ATM is a positive regulator of p53 protein, it has been suggested that ATM inactivation may represent an alternative to p53 dysfunction. This notion has subsequently been confirmed in other study [109] and is usually observed in routine clinical practice when investigating del(17p) and del(11p) presence, since these two deletions are rarely observed in the same patient. Importantly, with respect to p53 dysfunction, these two defects should not be considered as equivalent, as the prognosis of *ATM*-defective patients is not as poor as those with *TP53* abnormalities.

A polymorphism within the *p21* gene has also been linked to p53 pathway dysfunction [110]. In addition, impaired p21 up-regulation despite an intact p53 response, which is not connected to any known gene defect, has been associated with early relapse [111]. Recently, mutations in the *BIRC3* gene, a negative regulator of NF-κB signaling, have been shown as the cause of fludarabine chemo-refractoriness in CLL patients having an intact *TP53* gene, with patient prognosis being as poor as with *TP53*-defective group [112].

Current and Future Therapeutic Options for Patients with TP53 Defects

Chemotherapeutic regimens based on alkylation agents and/or nucleoside analogues act through DNA damage induction and therefore require functional

p53 for efficient triggering of apoptosis. Consequently, pure chemotherapy and also its combination with rituximab proved to be ineffective in p53-affected CLL patients (see above; reviewed in [113]).

Currently these patients are treated most often with the anti-CD52 monoclonal antibody alemtuzumab, which functions independently of p53. Initially, effectiveness of alemtuzumab was noted in relapsed/refractory CLL, with the patients with del(17p) responding similarly to other patients [114–117]. A CAM307 study comparing alemtuzumab and chlorambucil as frontline therapy also yielded promising results in relation to treatment response, although response duration was very short in all patients, i.e., also in the alemtuzumab arm [118].

Subsequent trials combined alemtuzumab with chemotherapy and also non-chemotherapy agents. Alemtuzumab with fludarabine alone (FluCam) [119, 120] or in combination with other agents (cyclophosphamide—FCCam [121, 122], or cyclophosphamide and rituximab—CFAR [123]) were reported to produce high response rates in del(17p) patients, but such regimens show extended risk of toxicity. On the other hand, the combination of alemtuzumab with glucocorticoids appears to be meaningful, as glucocorticoids act independently of the p53 pathway, and high dose methylprednisolone (HDMP) on its own was shown to induce remissions in patients with *TP53* abnormalities [124]. Glucocorticoids, by contrast to alemtuzumab, are effective in reducing lymphadenopathy and are able to wash out CLL cells from tissues into the bloodstream, where the cells can be more susceptible to alemtuzumab elimination. Accordingly, alemtuzumab in combination with methylprednisolone (CamPred) [125] or dexamethasone (CamDex) [126] was proven to be highly effective for del(17p) CLL patients. Glucocorticoids were combined not only with alemtuzumab but also with rituximab: R-HDMP [127, 128], *R*-dexamethasone [129], or humanized anti-CD20 antibody ofatumumab (*O*-dexamethasone); a phase II trial is ongoing and will be completed soon.

However, none of the currently approved therapeutic strategies noted above are able to attain long-term remissions in p53-defective patients. Therefore, ASCT still remains a viable option for young and physically fit p53-defective patients, with the potential to induce long-term disease-free survival [130, 131]. ASCT, however, is only available for a small subset of patients and is connected to nonrelapse mortality associated with development of graft-vs.-host disease (GVHD); both early and late relapses frequently occur.

A number of novel compounds are in various phases of clinical or preclinical testing. Biological targeted treatment functions via p53-independent triggering of apoptosis, which includes diverse mechanisms of action: (a) targeting of CLL cell surface molecules (CD20, CD23, CD37) by monoclonal antibodies or small modular immunopharmaceuticals (SMIP) [132]; (b) use of immunomodulatory and microenvironment modulating agents (IMiDs) [133]; (c) promoting the apoptotic pathway using, e.g., Bcl-2 antisense oligonucleotide, BH3 mimetics, or Bcl-2 inhibitors (for review see [134]); (d) altering histone modification by inhibition of histone deacetylases [135]; (e) targeting cell signaling through inhibition of cyclin-dependent kinases; [136] or by inhibition of the BCR signaling pathway—inhibition of NFκB and kinases PI3K-delta, SYK, AKT, Lyn, and mainly Bruton's

tyrosine kinase—this approach belongs among the most promising, as it directly impacts B-cell proliferation and survival (for review, see [137]). Another approach is activation of p53 homologs in patients with a *TP53* defect [93, 94] or direct targeting of mutant p53 protein and thus sensitizing the CLL cells to chemo/chemoimmunotherapy treatment using: (a) suppression of mutated p53 by antisense oligonucleotide [138]; (b) reactivation of p53 by specific compounds directed to wt or mutated p53 molecules [139]; (c) inhibition of heat-shock protein 90 that leads to destabilization of many tumor-promoting proteins, including mutant p53 molecules [140, 141] .

Notwithstanding progressive scientific achievements, there is currently no optimal treatment available for CLL patients with 17p deletions and/or *TP53* mutations. The patients still have dismal expectations and should be scheduled to participate in applicable trials whenever possible [61].

Techniques for *TP53* Mutation Analysis

No standardized methodology is currently used for *TP53* mutational analysis in CLL samples and individual centers utilize different approaches. A list of available methods with more detailed description is provided in ERIC recommendations on *TP53* mutation analysis, published in 2012 [70].

Direct Sequencing

Direct (Sanger) sequencing is still one of the most often used methods. Genomic DNA (gDNA) rather than cDNA is preferred, as RNA-based analysis may omit some mutations which lead to nonsense-mediated mRNA decay (i.e., some nonsense or frame-shift mutations) [142]. The detection limit of Sanger sequencing may not be sufficient to detect small subclones with *TP53* mutation, especially in cases without deletion of the second allele. Primers and reaction conditions can be found at the IARC p53 website (http://www-p53.iarc.fr/p53sequencing.html). Sequencing may be restricted to exons 4–9 or alternatively 4–10, as mutations in exons 2, 3, and 11 are very rare and exon 10 contains only about 4 % of all mutations [86].

Prescreening Methods

The usage of prescreening methods such as denaturing high performance liquid chromatography (DHPLC) or high resolution melting (HRM) makes the mutational screening faster, cheaper, and more sensitive. However, identification of the particular mutation by Sanger sequencing is always essential. Primers and conditions for

DHPLC can be also found at the IARC p53 website. DHPLC can reach a sensitivity of up to 5–10 % of mutated alleles (depending on the particular sequence); however, mutation confirmation by the less-sensitive Sanger sequencing method is not always possible.

Another screening option represents the yeast functional assay (FASAY—Functional Analysis of Separated Alleles in Yeast) that directly identifies inactivating mutations, thus distinguishing them from silent alterations, polymorphisms, and partially or fully functional mutations. In this assay, the *TP53* gene from patient cells is expressed in yeasts that function as reporter cells. FASAY is fast, cheap, and has a sufficient detection limit (10 %) [143, 144]. Underlying mutations should always be determined by sequencing. The mutation identification is based on DNA sequencing from yeast clones, which is more sensitive than direct sequencing of gDNA. However, since FASAY is a RNA-based method, it may not detect mutations leading to RNA degradation due to nonsense-mediated mRNA decay.

Microarrays

Microarray resequencing provides high sensitivity, with detection limits reaching up to 3 % of the mutated clone; however, this limit varies depending on the particular sequence. The Roche Amplichip p53 test, based on Affymetrix platform, is currently under development. The procedure is fast and user-friendly and is intended as in vitro diagnostic tool without need for confirmatory sequencing. This microarray was already tested in CLL studies [84, 145], but is not commercially available yet. The main shortcoming of microarray resequencing is the ability to detect only mutations for which the probes are printed on the array (for Amplichip, e.g., all single base pair substitutions and single nucleotide deletions in exons 2–11 and splicing sites).

Functional Tests of the p53 Pathway

Several partially modified tests have been suggested, based on DNA DSBs induction followed by the monitoring of p53 accumulation and subsequent p21 induction [108–111, 146, 147] or the induction of p21 together with other p53-downstream genes [146, 148]. An alternative approach utilized etoposide and nutlin-3a for efficient distinguishing of *TP53* and *ATM* defects [149]. Another alternative represents the measurement of miR34a base level. In this assay, no cell treatment is required since the miR34a basal level is decreased in patients with p53 defects in comparison with patients carrying functional p53 [31, 32]. Although a functional assessment of the p53 pathway seems to be an elegant means of identifying potential *TP53* and *ATM* defects, this testing is tricky and partially provides inconsistent results in relation to cell abnormalities [146] which still precludes its application in routine diagnostics.

Next-Generation Sequencing

Current rapid development of next-generation sequencing (NGS) technologies has allowed their utilization within a wider scientific community. Initial upfront cost of instrumentation is still very high, but with high throughput of samples or more screened target genes the methodology becomes cost-effective. Usage of highly sensitive technologies such as ultra-deep sequencing allows a detection of very small clones carrying *TP53* mutation, but the clinical impact and the relevance of these "minor" mutations for their subsequent selection is currently uncertain and under investigation.

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Chapter 6
Gene Expression and Epigenetic Deregulation

Rita Shaknovich

Abstract The last decade resulted in many scientific discoveries illuminating epigenetic mechanisms of gene regulation and genome organization. DNA methylation emerged as playing a pivotal role in development and cancer. Genome-wide changes in DNA methylation, including hypermethylation of tumor suppressor genes and genome-wide loss of methylation, are two dominant mechanisms that deregulate gene expression and contribute to chromosomal instability. In this chapter we give an overview of how methylation patterns are established during B-cell development and what machinery is necessary to maintain those patterns. We summarize the current state of knowledge of aberrant changes taking place during and contributing to lymphoid transformation in general and to the development of CLL in particular. We discuss key deregulated biomarkers extensively studied using single-gene approaches and give an overview of a wealth of data that became available from genome-wide approaches, focusing on pathways that are critical for lymphomagenesis. We also highlight epigenetic differences between known prognostic groups of CLL.

Keywords Lymphomagenesis • Chronic lymphocytic leukemia • smRNA • DNA methylation • Histone modifications • DNA methyltransferases (DNMT) • MBD proteins • Cell of origin • CpG

R. Shaknovich, M.D., Ph.D. (✉)
Department of Medicine, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10021, USA

Department of Pathology, Weill Cornell Medical College, 1300 York Avenue, New York, NY, USA
e-mail: ris9004@med.cornell.edu

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Biological Function and Mechanism of DNA Methylation

DNA methylation at CpG dinucleotides plays a fundamental role in imprinting [1–3], X-chromosome inactivation [4], tissue differentiation, and cancer [5, 6]. Sequences rich in CpG dinucleotides are found in the promoters of various genes [7]. These promoter-associated CpG islands have been maintained across evolution and are highly conserved [7]. These elements are believed to play an important regulatory role in gene expression. Regional hypermethylation of cytosines is associated with heterochromatin formation, which is also associated with chromatin conformation including specific histone tail modifications [8]. Methylation of CpG dinucleotides is mediated by DNA methyltransferases (DNMTs). These include DNMT1, which is proposed to maintain existing CpG methylation patterns after DNA replication, and DNMT3A and 3B, which are believed to introduce de novo methylation into previously unmethylated sites [8]. Although this model is generally accepted, it appears that the different DNMTs do not strictly adhere to these differential and specialized functions. Recruitment of DNMTs is both sequence independent, possibly through protein complexes that associate with specific histone code configurations, and sequence dependent through interactions with transcription factors, such as the case of p21 repression, where c-myc recruits DNMT3A [9]. CpG methylation can alter gene regulation by blocking access of transcription factors such as MYC to their target genes, since they do not recognize their binding sites when methylated [10]. This passive effect could block both activators and repressors of transcription. In contrast, active silencing of genes by DNA methylation is dependent on recruitment of methyl-CpG-binding proteins that repress transcription by recruiting histone deacetylases (HDACs) and other chromatin-modifying proteins [8].

Expression of methyltransferases is highly compartmentalized within the germinal center, with DNMT1 and DNMT3b being the most highly expressed within GC B cells, but not naïve B cells. Formation of the GC is dependent on the amount of DNMT1, with significant decreases in the size of GC in DNMT1 hypomorphic mice and complete absence in DAC-treated animals [11] (DAC is a known DNMT1 inhibitor). In addition to the key role in DNA methylation, DNMT1 was recently implicated in repair of double-stranded breaks in DNA, thus preserving DNA integrity by interacting with 9-1-1 PCNA-like sliding clamp and CHK1 [12].

Two families of methyl-CpG-binding proteins have been identified that mediate DNA methylation-dependent transcriptional repression: the methyl-binding domain (MBD) family (among which MBD1, MeCP2, and MBD2 have intrinsic transcriptional repressor activity) and the Kaiso subfamily of C2H2 *Krüppel*-like zinc finger proteins which include KAISO, ZBTB4, and ZBTB38 (also called ZENON) [13, 14]. MBD proteins and KAISO mediate repression at least in part through recruitment of HDAC complexes [15, 16]. All three of the repressor MBDs have been implicated in cancer. For example, MBD2 deficiency in tumor-susceptible APC mice significantly reduces adenoma formation [17], and MBD2 siRNA knockdown reduces tumorigenesis of cancer cell lines [18]. In the C2H2

subfamily, only KAISO has been studied to date in cancer. KAISO knockdown caused methylated silenced genes to be reexpressed, reactivated tumor suppressor pathways, and killed cancer cells [19].

In the human genome 5-methylcytosine is found in the context of CpG, while methylation in other sequence contexts (e.g., CT or CA) is common in plants and fungi. Non-CpG methylation has been only recently reported in mammals and remains controversial [20]. Data from multiple groups suggests that unmethylated CpGs are found in clusters around CpG islands, nearly half of which are associated with transcriptional start sites. Genome-wide studies revealed that more than half of the genes contain CpG islands. Thus, it appears that CpG-rich sequences that are devoid of transcription factor binding sites are susceptible to DNA methylation. Approximately 40 % of orphan CpG islands are associated with transcriptional initiation and represent TSS for ncRNAs and are marked by active histone marks. Many orphan CpG island-associated promoters are active in a tissue-specific manner [21]. The function of DNA methylation is context-dependent and does not have a simple inverse linear relationship with gene expression. For example, methylation of CpG islands and around TSS blocks initiation of transcription, while gene body methylation may stimulate elongation, thus positively affecting gene expression [6]. In cancer, genes that are already silenced by Polycomb complexes are more likely to be methylated, thus implicating methylation in stabilizing inhibitory mark in cancer [22–24].

Do Experimental Methods Matter?

The answer is definitely yes. Several popular methods that allow the study of DNA methylation exist with differential genomic coverage and underlying principles, all of which have different strengths and weaknesses. Critical evaluation of reported results is required in the context of the utilized method in order to understand the limitation of any particular study, some lack of reproducibility between different studies and the potential for future revision of the findings with the advent of newer techniques. The coverage and comparison of existing technology can be found in several recent papers [25–27]. In brief, most popular methods of DNA preparation for the study of methylation include digestion by methylation-sensitive restriction enzymes, followed by enrichment using either MBD proteins, like MeCP2 or MBD2; immunoprecipitation using anti-5-methylcytosine antibody (MeDIP); or size selection with enrichment for shorter fragments that tend to contain a greater density of 5mC. Another popular method of DNA preparation is based on bisulfite conversion, which converts only unmethylated Cs to Us, which are subsequently converted to Ts during PCR. Bisulfite conversion is also commonly followed by size selection and sequencing.

The resultant DNA fragments can be studied either by hybridization to arrays or by sequencing. A large selection of arrays exist, many with custom designs that allow study of variable numbers and variable subsets of genomic loci. Due to these differences, results of some studies can not be compared to one another.

Role of DNA Methylation in Normal B-Cell Development

DNA methylation is part of epigenetic programming that is required for normal B-cell development and is disrupted during lymphomagenesis [28]. Understanding normal patterns of DNA methylation thus is of paramount importance in an effort to find the cell of origin and to understand the pathobiology of lymphomas. In the field of hematologic malignancies and particularly lymphomas, the cell of origin has been linked to specific developmental pre- or post-germinal center stages of B-cell development: to naïve pre-germinal center stage for U-CLL [29] and U-MCL [30], to the germinal center B cell for FLs and GCB-like DLBCLs [31, 32], to plasma cell stage for multiple myeloma [33, 34], and possibly to memory cell stage for mutated CLLs (M-CLLs) [29]. The dynamic nature of the methylome during hematopoietic development and lineage commitment was studied by Hong Ji et al. in mouse model using comprehensive high-throughput array-based relative methylation analysis (CHARM), which examined 4.6 million CpGs in the genome [35]. This study demonstrated differentially methylated regions between consecutive stages of lymphoid development and revealed that lymphoid commitment requires more DNA methylation than myeloid lineage, with myeloid skewing of lineages in DNMT1 hypomorphic animals. Loss of methylation predominated during progression of multipotent progenitors (MPPs) to common lymphoid progenitors (CLPs), while progression to the thymocyte progenitor DN1 stage was characterized by predominant hypermethylation. Deaton et al. [36, 37] showed that the methylation landscape has specificity not only within differentially methylated regions around TSS but also in the intergenic areas [36]. Shaknovich et al. addressed changes in the epigenome during germinal center transit and revealed that the transition from naïve B cells to centroblasts is associated with predominant loss of methylation in 235 differentially methylated genes that affect the NF-kB and MAP kinase pathways [11]. This loss of methylation was specific for DMRs and was not accompanied by an overall loss of methylcytosine content in the genome, as measured using LC-MS.

Role of DNA Methylation in B-Cell Lymphomas

Aberrant DNA methylation is believed to contribute to the pathobiology of hematopoietic neoplasms. DNMTs are constitutively expressed in lymphocytes, monocytes, neutrophils, and normal bone marrow cells [38], and changes in methyltransferases and MBD protein expression correlate with silencing of tumor suppressor genes in lymphomas. For example, in CLL MBD2 and MeCP2 are upregulated by more than tenfold [39]. Methylation states of specific genes may serve as biomarkers and contribute to disease pathogenesis. Esteller et al. [40] reported that hypermethylation of DNA methyltransferase (MGMT) correlates with favorable prognosis in DLBCLs and serves as a better prognostic predictor than any other International Prognostic Index (IPI) criteria. The 9p21 region contains the

cyclin-dependent kinase inhibitors p14, p15, and p16, which have tumor suppressor activity and block cell cycle progression [41, 42] and is frequently silenced by promoter methylation in several B-cell neoplasms [43–45]. Promoter methylation and tumor suppressor expression vary between types of lymphomas [44]. For example, follicular lymphomas usually express p14, while 39 % of DLBCL have lost its expression [46]. Several reports have now shown that the INK4/ARF locus and specifically p15 and p16 genes are hypermethylated in DLBCL [43]. In DLBCL, p16 was methylated in nearly 30 % of cases at the onset of the disease and at relapse, suggesting that methylation is an early event in lymphomagenesis. On the other hand, in Hodgkin lymphomas, 25 % of cases contained methylated p16 at diagnosis and 83 % at relapse [47]. These examples highlight the fact that epigenetic changes play an important role in lymphomagenesis, but are disease-specific. The rest of this chapter will focus on changes that are specific to CLL.

DNA Methylation and the Cell of Origin in CLL

Better understanding of CLL biology would be aided by knowing what cell it originates from. The answer to the question of the cell of origin for CLL has been elusive for many years, and the debate between one-cell and two-cell origin theories continues. Sequence analysis of IGVH genes revealed that 35–45 % of CLL patients have unmutated genes, while the rest of the patients have evidence of somatic hypermutations that renders them a more favorable prognosis [48–50]. This observation suggested that unmutated CLLs (UM-CLLs) arise from naïve B cells and M-CLL arises from the germinal center or post-germinal center B cell. Klein et al. [29, 51] attempted to answer the question of the cell of origin by doing GE profiling on a panel of 34 CLLs characterized by the presence or absence of IGVH mutations. The group used U95A Affymetrix GeneChips with close to 12,000 genes represented and profiled naïve B cells, centroblasts, centrocytes, and memory B cells from tonsillar tissue and CD5+ B cells from cord blood in addition to CLL cases. The unsupervised analysis demonstrated that both UM-CLL and M-CLL can be differentiated from other lymphomas and from normal B cells, but appear very similar to each other, with only 23 genes being differentially expressed. Supervised analysis comparing CLLs to normal B-cell subsets revealed that CLLs were more related to memory B cells independently of their mutational status. This GE study delineated connection of both M-CLLs and UM-CLLs to memory B cells, with the limitation that memory B cells were isolated as a total of CD27+ B cells representing a mixture of functional normal subpopulations. These limitations, in combination with the evidence from Chiorazzi et al. [52] that CLL has functional similarities to splenic marginal zone B cells (sMGZ), prompted more extensive GE studies. Siefert et al. [53] showed that based on the transcriptome, UM-CLL derives from mature CD5+ B cells and M-CLL derives from a small subset of mutated CD5+ CD27+ post-germinal center B cells.

Deaton et al. [36] showed that the epigenome may be more stable and reliable than gene expression when addressing the questions of cell of origin in tumors. A recent paper by Kulis et al. [54] followed, trying to use the methylome of CLL to address the question of the cell of origin. The group profiled the methylome of 1 M-CLL and 1 UM-CLL along with 3 control B-cell subpopulations (naïve B cells (NB)), class-switched memory B cells (csMBC), and non-class-switched memory B cells (ncsMBC) using whole-genome bisulfite sequencing (WGBS) and 139 CLLs using high-density microarrays. Correlation of GE and methylation revealed poor correlation, with only methylation data allowing reliable differentiation between M-CLL and UM-CLL. Using microarray data, the group defined 3,265 differentially methylated CpGs. Hierarchical clustering of CLL cases based on the 3,265-CpG signature revealed that UM-CLLs were closer to NBCs and M-CLL was closer to MBCs. This result highlights the promising nature of stable methylation mark in allowing delineation of the cell of origin in CLLs. However, the limited number of CLL samples in the study and the limited number of normal controls leave uncertainty about the true origin of CLL. It appears that additional studies profiling larger numbers of cases and a more extensive representation of potential precursor B-cell subsets using high-resolution techniques addressing the genome, epigenome, and possibly the proteome will be necessary to find an elusive clonogenic precursor for CLL.

An interesting insight into the temporal relationship of methylation changes and development of CLL comes from a TCL1 mouse model, which leads to CLL leukemogenesis as a result of *Eμ-TCL1* transgene expression [55, 56]. It is still not clear if methylation changes are the consequence or the cause of neoplastic transformation: there is evidence to support both models in various cancers. In lung and colon cancer, epigenetic changes have been demonstrated to be an early event and to be detectable in early preneoplastic lesions [57]. It is less clear in lymphomas, but the existing CLL mouse model sheds some light on that question. Some CLL cases are characterized by overexpression of TCL1 and a poor response to therapy [58]. The *Eμ-TCL1* mouse model recapitulates human CLL, with development of polyclonal B lymphocytosis at 3 months and development of clonal leukemia at 9–11 months. Using restriction landmark genomic scanning (RLGS), Chen et al. [56] determined that at the onset of disease 2.5–5.5 % of RLGS fragments had already increased methylation, which is comparable to reported frequencies in human disease [59]. Several validated genes like *PCDH10*, *FOXD3*, *FIGN*, *AXIN1*, *PKP4*, *DLX1*, and *EPHA7* were also methylated and silenced in CLL patients. The study of methylation events preceding the onset of CLL in CD19+ B cells from *Eμ-TCL1* mice revealed that methylation increased progressively during the preneoplastic stage when mice had polyclonal lymphocytosis from 0.4 % of methylated fragments at 1 month to 1.0 % at 5 months to 1.8 % at 7 months and to 4.1 % in mice with advanced CLL. Thus, an increase in methylation took place in subclinical disease in non-clonal expanding B lymphocytes. Interestingly, there was an increase in expression of DNMT3A and DNMT3B in expanding B lymphocytes in *Eμ-TCL1* model. In addition, centromeric repeat sequences revealed an early loss of methylation at 7–9 months in *Eμ-TCL1* model. In conclusion, mouse model

highlights an early onset of epigenetic changes in non-clonal expanding B lymphocytes that recapitulates changes observed in human disease. This observation is different from the stable methylation in paired samples of CLL at diagnosis and relapse reported by Cahill et al. [60]. It is possible that the methylome is more dynamic during the process of neoplastic transformation and undergoes fewer changes after the clonal disease evolves. It is known that CLL is relatively stable in reference to its genome, and possibly CLL is also relatively stable epigenetically. This underscores the importance of detecting the early changes in epigenome, which are more likely to represent "driver" epimutations, as opposed to "passenger" epimutations accumulating during disease progression.

Controversy about the cell of origin in CLL persists pending further confirmation by additional studies that will use large numbers of samples, control subsets representing most stages of B-cell development, and genome-wide methods allowing single-nucleotide resolution along with unbiased statistical approaches.

Genome-Wide Changes in DNA Methylation in CLL and Hypomethylation of the Genome

The first observation that the CLL genome undergoes global loss of methylation came from the work of Wahlfors et al. [61] using digestion of genomic DNA with isoschizomer enzymes HpaII and MspI, followed by validation using HPLC. Yu et al. demonstrated loss of methylation as measured by ratio of 5-mCyt/Cyt with age. Advent of higher-resolution techniques confirmed the above findings and revealed that aberrant hypomethylation is centered in repetitive sequences, like ALU and LINES, and is particularly pronounced in CLL with TP53 mutations [62]. Hypomethylation of SATα is greater in CLLs with TP53 mutations.

Sequencing technology allowed genome-wide coverage and single-nucleotide resolution, resulting in the study of aberrant epigenomic changes at unprecedented detail. Kulis et al. confirmed using WGBS marked loss of DNA methylation in CLLs: out of 1,838,346 differentially methylated regions between U-CLL and NBCs, 1,779,168 were hypomethylated and 59,178 were hypermethylated, while out of 1,254,527 differentially methylated regions between M-CLL and MC, 982,683 were hypomethylated and 271,844 were hypermethylated [54]. The same study revealed that hypermethylated CpGs were enriched in 5′ regulatory regions, CpG islands, and 5′ regions of introns, while hypomethylated CpGs were located in the gene bodies outside of CpG islands. These data confirmed what has been observed in other lymphomas, but importantly for CLL pathogenesis, hypomethylation affected genes enriched in B-cell receptor signaling, NF-kB signaling, and calcium-activated pathways, along with cytokine–cytokine receptor interaction. This sheds some light on the mechanism of BCR pathway activation that has been reported previously in CLL [63]. Inhibitors of several kinases in the pathway, like spleen tyrosine kinase (SYK) and Bruton's tyrosine kinase (BTK),

decrease CLL cell viability and are active in de novo and relapsed CLL. NF-kB is also dysregulated in CLL, having higher levels of nuclear NF-kB and increased responses to CD40L stimulation [64, 65]. Activation of NF-kB also leads to the overexpression of its target genes that regulate proliferation and survival, like *BCL2*, *c-MYC*, *c-MYB*, and *cyclin D1*.

BCL2 serves as a good example of a key pro-survival gene that is dysregulated via multiple epigenetic pathways in CLL, and we would like to use it as an example illustrating the complexity of epigenetic regulation. Besides being upregulated via NF-kB activation, BCL2 is also upregulated via promoter hypomethylation and decreased inhibition by *mir29a* and *mir29b*. Hanada et al. reported that 19 out of 20 studied CLL cases had BCL2 overexpression, and all of the cases revealed hypomethylation of the *BCL2* gene [66]. Hypomethylation was only present in cases that lacked t(14;18) and did not correlate directly with the level of BCL2 expression. Dysregulation and downregulation of miRNA expression by epigenetic HDAC treatment in CLL leads to upregulation of BCL2 expression and higher proliferative capacity of CLL [67–69]. Expression of many miRNAs seems to be deregulated by changes in promoter methylation, thus representing a complex network of epigenetic regulation. Baer et al. reported 128 miRNAs that display changes in promoter methylation. Most of those had loss of methylation and resulted in increased expression of *miR-21*, *miR-29a*, *miR-34a*, among others [70]. Hypermethylation of *miR124-2*, *miR-9-2*, and *miR-551* resulted in their decreased expression. Other genes hypomethylated in CLL are *MDR1* and *TCL1*, which contribute to the pathogenesis of disease [71, 72].

The role of hypomethylation in CLL can be multifold. On one hand, the state of gene body methylation had the greatest correlation with gene expression in CLL [54], thus providing a direct link between methylation and gene expression. On the other hand, hypomethylation of intergenic areas was implicated in causing genomic instability [73–75], thus linking epigenetic changes to genomic events.

Single Gene Silencing in CLL by Hypermethylation

Numerous studies identified aberrantly methylated genes in CLL with proposed biological and clinical significance. The list of aberrantly methylated genes is extensive and includes *DAPK1*, *ID4*, *WIF1*, *ZAP70*, cell cycle regulators like *CDKN2A* and *CDKN2B*, and *TWIST2* regulator of *p53*, among many others.

A known biomarker of CLL, *ZAP70*, has been reported to be differentially methylated between M-CLL and UM-CLL, with hypomethylation and overexpression associated with UM-CLL cases [76]. Interestingly, Claus et al. [77] identified that the loss of methylation in a single nucleotide within the *ZAP70* promoter not only influenced its expression but also could be used as a biomarker predictive of prognosis. Tong et al. used methylated CpG island amplification (MCA) coupled with a promoter microarray, but did not identify any

correlation between ZAP70 expression and methylation status of the gene [75]. The function of methylation in regulating ZAP70 expression remains unclear.

Another gene helix-loop-helix transcription factor ID4 was found to be hypermethylated in CLL, with consistent downregulation of protein expression [78]. Increased promoter methylation of *ID4* correlated with shorter overall survival in univariable analysis. Interestingly, without prior understanding of the biological role of ID4 in the pathogenesis of CLL, its role has been delineated using animal CLL modeling. Crossing of *ID4*+/− mice with *Eμ-TCL1* mice resulted in more aggressive murine CLL disease with slightly worse survival probability.

In up to 10 % of CLL cases, there is familial predisposition with more than one family member affected [79]. A significant breakthrough was made by Raval et al. identifying aberrant promoter methylation of the *DAPK1* gene in nearly all sporadic CLL and in familial cases [80]. In familial cases hypermethylation of the *DAPK1* promoter cooperated with increased binding by HOXB7 to further reduce protein expression. The functional relevance of DAPK1 downregulation has been proven in in vitro experiments using siRNAs in Jurkat cells and revealing increased resistance to apoptosis. Other studies confirmed aberrant hypermethylation of *DAPK1* [81, 82]. When universally present in a disease subtype, methylation of the gene may serve as a biomarker that may allow correct classification of the disease or prediction of its clinical behavior. The clinical utility of DAPK1 as a biomarker is still to be proven.

M-CLL has a heterogeneous clinical course, so Irving et al. discovered methylation biomarkers that collectively contributed to the estimation of methylation score which was composed of *CD38*, *HOXA4*, and *BTG4* [83]. Multivariate Cox regression analysis showed that methylation score was most predictive of TTFT and can be used as a biomarker for the disease.

Next-generation sequencing allowed identification of new recurrent mutations in CLL, which included *NOTCH1* in nearly 12 % of cases. *NOTCH1* mutation appears to be activating, with a preference for UM-CLL [84, 85]. It is thought that activating *NOTCH1* mutations are involved in survival and apoptosis resistance in CLL [86]. *NOTCH1* is a good example of a gene that is important for the pathobiology of CLL and is deregulated by multiple mechanisms, including both genomic and epigenomic. In addition to being commonly mutated, *NOTCH1* has been also identified to be targeted by aberrant methylation. Using 450 K high-resolution array, Cahill et al. were able to identify that NOTCH1 is differentially methylated between M-CLL and UM-CLL [60]. Additional complexity in multilayered epigenetic regulation comes from the discovery that *NOTCH1* is a target of *miR34a* in many tumor types and that *miR34a* is hypermethylated in 4 % of CLL cases [87], thus making it likely for NOTCH1 to be overexpressed at least in part due to *miR34a* downregulation in CLL [88–90]. The role of NOTCH1 in the pathogenesis of CLL and the mechanisms of its epigenetic deregulation is subject to further investigation.

As technology continued to develop and interrogation of whole pathways and networks became possible using microarrays, several breakthrough discoveries have been made in the early 2000s, including discovery of aberrant upregulation and constitutive activation of the WNT signaling pathway [91]. WNT family

members belong to a large family of secreted glycoproteins that are involved in normal B-cell development and in control of proliferation and differentiation. WNT signaling inhibits the activity of the enzyme glycogen synthase kinase-3b and leads to β-catenin activation and translocation to the nucleus where it affects its downstream targets. Lu et al. determined that many WNT family members and its receptor Frizzled (Fzd) were overexpressed in CLL and led to downstream upregulation of cyclin D1 and increased CLL cell survival. The insight into the mechanism of WNT activation came from the later epigenetic studies that determined that many inhibitors of the WNT pathway are epigenetically silenced in CLL [92, 93]. *CDH1*, *DKK1*, *DKK2*, *DKK3*, *SFRP1*, *SFRP2*, *SFRP3*, *SFRP4*, *SFRP5*, and *WIF1* genes were shown repeatedly to be hypermethylated in CLL as compared to normal B cells [92, 93]. The advent of next-generation sequencing allowed a more comprehensive study of epigenetic changes in the WNT pathway, and Pei et al. used RRBS to show aberrant hypermethylation in all *HOX* gene clusters and in the WNT signaling pathway [94]. Gandhirajan et al. demonstrated that small molecular inhibitors of Wnt/beta-catenin/lef-1 signaling (CGP049090 and PKF115-584) efficiently kill CLL cells in vitro [95]. Epigenetic treatment with 5-aza-2′-deoxycytidine in CLL in order to reverse aberrant methylation of WNT inhibitors and FZD proteins represents a rational epigenetic therapy. Liu et al. tested such therapy in vitro and achieved reversal of aberrant inhibition [96].

Peu et al. used RRBS approach to study CLL samples and CD19+ B cells from normal B cells and identified 1,764 promoters to be differentially methylated. Distribution of aberrant hypermethylation confirmed prior findings of selective aberrant hypermethylation on HOX gene clusters and WNT signaling pathway genes [94].

We are currently in the discovery stage about epigenetic lesions and their function in CLL. Further studies are necessary to fully take advantage of all the novel discoveries.

Clinical Relevance of Methylome in CLL

Even further depths of understanding of the clinical significance that the methylome plays in CLL came from the whole-genome bisulfite sequencing by Kulis et al. that revealed three prognostic CLL groups based on DNA methylation [54]. Using the signature genes that defined the similarity of M-CLL to MBC and of U-CLL to NBs, which together comprised 1,649 CpGs, and performing consensus clustering analysis, they identified three subgroups of CLLs with different TTFT. Twenty percent of MBC-like CLLs required treatment at 10 years, 43 % of intermediate CLLs, and 100 % of NBC-like CLLs. The multivariate Cox model showed that methylation signature, CD38 expression, and LDH were the only independent prognostic factors. The genome-wide patterns of change in CLL showed that hypomethylated CpGs were enriched for B-cell enhancers in both M-CLL and UM-CLL. Gene body CpGs that inversely correlated to gene expression were

also enriched for enhancers. On the other hand, hypermethylated chromatin marks were enriched for Polycomb gene-related chromatin marks. The emerging data points to the possibility of using methylation to define the biological and clinical subgroups of disease, but more evidence is necessary before we can fully rely on these findings in our clinical practice.

Emerging Role of Chromatin Remodelers in the Pathogenesis of CLL

Recent discoveries implicated chromatin remodelers in the pathogenesis of CLL. Chromatin remodelers can be subdivided into several families based on the core ATPase subunit: SWI/SNF (SWItch/Sucrose NonFermentable), ISWI (Imitation of SWItch), CHD (Chromodomain Helicase DNA binding), and INO (INOsitol). Several mutations involving chromatin remodelers have been identified in hematologic malignancies, including in CLL, that affect ARID1A and CHD2 genes [97].

ARID1A is a tumor suppressor that promotes formation of SWI/SNF chromatin-remodeling complexes that have been shown to be mutated in many tumors, including uterine, ovarian, breast, and few hematologic malignancies like CLL and BL [98, 99]. In gynecologic cancers, ARID1A was shown to directly interact with p53 and to regulate downstream genes like *CDKN1A* and *SMAD3* that control growth and proliferation [100]. Quesada et al. performed whole-exome sequencing of 105 CLL samples and determined some known and many novel recurrent mutations, among which two patients contained mutation in *ARID1A*. Both cases with mutated *ARID1A* had also mutated IGVH [99] and lacked *p53* mutations. The specific mechanisms by which mutated ARID1A causes lymphoid neoplasia are still not known, but in gynecologic cancers mutations in ARID1A and in p53 were mutually exclusive, pointing to the fact that the likely mechanism of ARID1A action is in its cooperativity with p53. Hypothetically, ARID1A mutation may explain the existence of genomically complex but wild-type p53 cases.

The functional role of CHD2 has been recently elucidated by Harada et al., who demonstrated CHD2-dependent deposition of histone H3.3 to mark lineage-determining genes during muscle differentiation [101]. Histone H3.3 marks actively transcribed genes or genes that are poised to be expressed and also may contribute to epigenetic memory of actively transcribed chromatin [102]. Expression of mutant CHD2 lacking DNA-binding domain resulted in delayed development, perinatal lethality, and gross developmental abnormalities in multiple organs [103]. Quesada et al. detected CHD2 mutation in 5 out of 105 CLL cases and all of them had mutated IGHVs. The functional consequence of the mutations may be the inability to activate the differentiation program in B lymphocytes, resulting in tumorigenesis.

The role of chromatin remodelers in the pathogenesis of CLL is intriguing but needs to be tested in the future experimentally.

Future Directions

Understanding the role epigenetic regulatory mechanisms play in the pathogenesis of CLL is of paramount importance for making further strides in designing targeted rational therapies for the disease and also for early preventive interventions. We are currently assembling a vast body of knowledge regarding various levels of epigenetic regulation, including DNA methylation, histone modifications, chromatin folding, and small noncoding RNAs. Integration of the data and understanding how aberrant combinatorial epigenetic program effects the genome, transcriptome, and proteome in CLL are big challenges for the next several years. Better understanding of the driving epigenetic mutations will aid in delineating the cell of origin and the pathogenic role of the epigenome.

Great strides have been made in other subtypes of non-Hodgkin lymphoma, where several driving epimutations have been identified, and the pivotal role of the balance between EZH2 and MLL2 written marks has been revealed in the pathogenesis. Study of larger numbers of well-characterized CLL cases is needed using next-generation sequencing techniques in order to better understand the epigenome of CLL. Functional mouse models will more unequivocally prove the role of various components of epigenetic machinery and their contribution to the evolution of CLL.

Development of effective therapies targeting the epigenome is the final challenge that will make the biggest impact on the well-being of patients suffering from the disease. Epigenetic therapies are not currently effective in the treatment of CLL, and their potential is not even fully understood.

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Chapter 7
Apoptosis Deregulation in CLL

Chris Fegan and Chris Pepper

Abstract The description of apoptosis and the identification of the genes that regulate it have proved pivotal to our understanding of how cancer cells accumulate and ultimately cause morbidity and mortality. It has become increasingly clear that in CLL the balance between the pro- and anti-apoptotic members of the BCL2 family of apoptotic regulatory proteins is critical in the development and clinical progression of CLL. Furthermore, the apoptotic potential of the CLL cell determines chemotherapy sensitivity and ultimately progression-free and overall survival. The unravelling of the BCL2 story in CLL has led to the development of a whole new class of therapeutic agents—the BH3 mimetics—which are significantly more targeted than conventional chemo-immunotherapy and therefore promise potent clinical activity coupled with reduced toxicity.

Keywords Apoptosis • BCL2 family • BH3 mimetics

Introduction

Haematopoietic stem cells are capable of both self-renewal and differentiation. The fate of a differentiated cell, once it is unable to perform its prescribed function, is senescence or cell death. This latter process was originally described by Kerr and colleagues in 1972, who observed cell membrane blebbing, cytoplasmic shrinkage, chromatin condensation, and later DNA fragmentation or "laddering," implying a controlled, active, cell death, a process they termed "apoptosis" or programmed cell death [1, 2].

C. Fegan, M.D. (✉) • C. Pepper, Ph.D.
CLL Research Group, Institute of Cancer and Genetics, Cardiff University,
Heath Park, Cardiff, Wales CF14 4XN, UK
e-mail: Christopher.Fegan@wales.nhs.uk

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Cells that are dysfunctional but retain the ability to survive and/or divide are by their very nature neoplastic. We now understand that neoplasms grow as a result of an imbalance between cell division/proliferation and cell apoptosis/death. Cancer cells can gain a proliferative advantage through dysregulation in the machinery that controls cell division or they can gain a survival advantage through a failure in apoptosis. Cells that fail to undergo perfect mitosis or that acquire DNA damage during cell division should stall the cell cycle through the triggering of a DNA checkpoint pathway. If the DNA damage be irreparable, the cell should then undergo apoptosis as the major effector mechanism for removing these potentially neoplastic cells. Research has shown that for almost all neoplasms the failure of apoptosis is a primary neoplastic event, with enhanced proliferation normally a secondary event in the transformation process [3, 4]. Considering the number of cell divisions that are performed each day, and the enormous potential for cells to be exposed to DNA damage, it is a testament to the efficiency of the apoptotic system that cancer is not more common than it is. Understanding the normal mechanism of cellular apoptosis and hence how cancer cells avoid it is a pivotal aim on the path towards curing or at least containing cancer. Chronic lymphocytic leukaemia is an archetypal example of a human cancer with both intrinsic and extrinsic defects in apoptotic signalling and represents an unrivalled model for examining how these deficiencies impact upon clonal evolution and drug resistance.

Cell Apoptosis

The process of apoptosis ultimately leads to the activation of caspase-activated DNase (CAD), which cleaves DNA in areas not covered by histones called inter-nucleosomal linker regions. These fragments of ~180 bp form multimers which give rise to the characteristic "laddering of DNA" as the final morphologically recognisable feature of apoptosis [5]. The caspase family consists of many zymogenic proteases broadly splitting into effector and activator caspases (caspases 3, 6 and 7 and caspases 8, 9 and 10, respectively) with two distinct but not completely independent activation pathways [6]. Normally CAD is complexed with an inhibitor (ICAD), but effector caspases cleave this complex allowing CAD to fragment DNA. Caspases normally exist as homodimers (the so-called pro-caspases) but when activated become oligo-dimers [7].

In the so-called "death receptor" or "extrinsic" apoptotic pathway, the best-studied membrane-bound death receptor, Fas (CD95), has a cytoplasmic death domain which when activated by its ligand interacts with FADD (Fas-associated death domain), leading to a death-inducing signalling complex (DISC) which results in the activation of caspase-8 and ultimately the downstream activation of the effector caspases 3 and 7 [8, 9]. Caspase-8 activation also activates BID, leading to BAX and BAK activation and ultimately cytochrome c release from mitochondria—see below. Other death receptors include TRAIL R1 and TRAIL R2 [10].

In the so-called "mitochondrial" or "intrinsic" apoptotic pathway, activation is principally regulated by the evolutionarily conserved BCL2 family of proteins

which can be subdivided according to the number of BCL2 homology domains they contain or by their pro- or anti-apoptotic functions [11, 12]:

BCL2, MCL1, BCL-X_L, BCL-W, BFL-1, BCL-B and A1—contain BH1–4 domains—pro-survival
BAX and BAK—contain BH1–3 domains—pro-apoptotic
BAD, BFM, BID, BIM, BIK, HRK, NOXA and PUMA—BH3-only domains—pro-apoptotic

Activation of the mitochondrial pathway can be triggered by oxidative stress, free radicals, growth factor deprivation, endoplasmic reticulum stress and DNA damage. The BH3-only domain members are structurally distinct from the other members and are able to directly activate BAX and BAK and subsequently lead to cytochrome c release [13]. However, BH3-only proteins are unable to activate apoptosis in the absence of BAX and BAK. The BH1–4 and BH1–3 members can both homodimerise and heterodimerise via BH3 groove interactions such that the balance between pro- and anti-apoptotic signals can be altered by excess of a pro-survival protein, e.g. BCL2, or deficiency of a pro-apoptotic member, e.g. BAX, and vice versa [14, 15]. In effect, BCL2, MCL1 and other pro-survival proteins, by heterodimerising with BAX, inhibit apoptosis, which can be reversed by excess BAX production or by displacement of the heterodimeric complex by BH3-only proteins. All pro-survival proteins can sequester BAX, but only BCL-X_L binds to BAK. Likewise, BAD only interacts with BCL2, BCL-W and BCL-X_L, whilst NOXA binds only MCL1 and A1 [16]. Most of the BCL2 proteins reside in the cytoplasm, but BCL2 has been shown to bind to many organelle membranes including endoplasmic reticulum, mitochondria and the nucleus [17]. When apoptosis is triggered, the mitochondrial outer membrane permeability (MOMP) changes, leading to the release of cytochrome c, which binds with the apoptosis protein activator-1 (APAF-1) and pro-caspase-9 to form the so-called apoptosome [18]. Pro-caspase-9 is then cleaved, leading to the activation of effector caspases 3, 6 and 7 and CAD activation. The BH3-only domain proteins when activated lead to a conformational change in BAX and BAK, homodimerisation and the induction of MOMP [19, 20].

An alternative mechanism of cell death, independent of caspases, has been described whereby cytochrome c activates apoptosis-inducing factor (AIF) and endonuclease G, leading to cell DNA fragmentation and chromatin condensation [21].

Regulation of Apoptosis

Given the complexity of apoptotic signalling and the diverse range of apoptosis-inducing stimuli, a full understanding of the regulatory mechanisms is still the subject of ongoing controversy and research. However, it is incontrovertible that the BCL2 family of pro- and anti-apoptotic proteins plays a critical role. In some tumours the methylation status and miRNA interactions with various BCL2 genes have been identified, but their physiological role has yet to be established.

Inhibitors of apoptosis protein (AIPs) have a BIR (Baculovirus IAP Repeat) domain which binds to and in some cases inactivates caspases [22].

AIPs are split into three groups:

- Group 1—contain Ring finger motifs (XIAP, cIAP1, cIAP2) as well as BIR domains
- Group 2—have three BIR domains
- Group 3—contain a single BIR domain (survivin)

Exactly how all the IAPs interact with the caspases is still unclear as some appear to bind but not inactivate caspases, whereas others such as XIAP can bind and inactivate caspases 3 and 7 via their BIR2 domain [23, 24]. XIAP has many other functions, including binding caspase-9 and keeping it in its inactive monomeric form via its BIR3 domain and activation of NF-κB, which regulates many apoptotic and survival genes, including MCL1 [25]. The cIAPs are also able to activate the classical NF-κB pathway but in addition prevent caspase-8 activation following TNFR1 binding. A major regulator of the extrinsic apoptotic pathway is the DISC-recruited and NF-κB-regulated protein c-FLIP, which has three splice variant isoforms—c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_s$ [26]. At low levels c-FLIP$_L$ acts as an activator of caspase-8 promoting apoptosis but has the opposite effect at high concentrations.

Several inhibitors of IAPs have been described, including Smac and the serine protease OMI, which compete with IAPs for caspase binding [27, 28]. Smac interacts with several IAPs, including XIAP, cIAP1, cIAP2 and survivin, promoting apoptosis by preventing IAP binding to caspases 3, 7 and 9 [29]. When MOMP occurs the serine protease OMI, which normally resides in the mitochondrial intermembrane space, is released into the cytoplasm. This inhibits XIAP binding to caspases, which degrades not only IAPs but also cytoskeletal proteins [30]. The Ring domain has E3 ubiquitin ligase activity and promotes ubiquitination and subsequent degradation of caspases 3 and 9 [31]. Other key regulators of the ubiquitin system are heat shock proteins (HSPs) which are a family of chaperone proteins whose primary role is to assist cells undergoing stressful stimuli, e.g. heat, irradiation and cytotoxic drugs [32]. Stresses typically result in protein changes, including denaturation and degradation, and HSPs prevent the accumulation of non-functional proteins via the ubiquitin pathway. HSPs have various roles in regulating apoptosis, including HSP70 and HSP90 preventing apoptosis by binding to APAF-1, preventing its oligomerisation [33]. BAG-1 was originally identified by its ability to heterodimerise with BCL2, but more recently it has been shown to bind to the ATPase domain of HSP70 and assist in ubiquitin-dependent protein degradation [34].

The Role of the Bcl-2 Family in CLL

The exact aetiology of CLL is yet to be determined, although both hereditary and acquired factors are required, as evidenced by highly variable frequencies of CLL in differing countries, the identification that ~5 % of CLL cases are familial and the

fact that the incidence increases with age [35, 36]. Several groups around the world have performed genome-wide associated studies (GWAS) and identified a polygenic inheritance of low-risk alleles. One of these is a BCL2 family member; a single nucleotide polymorphism of intron 1 of BAK was identified in meta-analysis of GWAS studies [37]. It would be expected that this polymorphism would result in reduced BAK expression and hence a more anti-apoptotic phenotype. Furthermore, the prevalence of this particular BAK polymorphism is significantly higher in parts of the world with a high frequency of CLL, suggesting that it may have a pathogenic role. Historically, CLL was described as a lymphoaccumulative disease caused by failed apoptosis. This assumption was derived from the fact that CLL cells from the peripheral blood of patients are largely arrested in G0/G1 of the cell cycle but still accumulate in vivo despite a propensity to undergo spontaneous apoptosis once removed from the patient. However, in recent times this model has been challenged by the discovery that CLL cells often have high rates of proliferation, with up to 1 % of the tumour turning over per day [38–44]. This indicates that there are clearly proliferation centres in the lymphoid tissues, but there are also pro-survival, anti-apoptotic signals emanating from the microenvironment. In keeping with this notion, Herishanu et al. showed that CLL cells derived from the lymph nodes manifested a pro-survival gene expression signature that was dominated by upregulation of NF-κB-regulated genes when compared to the CLL cells derived from bone marrow and peripheral blood [45]. These findings support a previous study which showed that lymph node-derived CLL cells exhibited reduced NOXA but increased expression of another NF-κB-regulated gene, MCL1, resulting in a reduced NOXA/MCL1 ratio in lymph nodes compared to peripheral blood [45, 46]. Davids et al. reported that peripheral blood CLL cells are BCL2- and in some cases MCL1- and BCL-X_L-dependent [47]. Furthermore, PB-derived CLL cells had a higher apoptotic threshold than bone marrow, which was most likely due to pro-survival signalling through CD40L and CXCR4 signalling from stromal cells within the bone marrow. Clearly the biological mechanisms that regulate apoptosis in CLL are complex, but overexpression of the anti-apoptotic proteins BCL2 and MCL1 is the hallmark of CLL [48–53]. In a study of BCL2 family expression in 185 CLL patients, a number of clinically relevant differences were found in the expression of BCL2, BAX and MCL1 [54]. In terms of *IGHV* gene mutation status and ZAP70 expression, only MCL1 was differentially expressed between unmutated and mutated and $ZAP70^+$ and $ZAP70^-$ subsets. In contrast, all three BCL2 family proteins were differentially expressed in $CD38^+$ patients, with higher BCL2 and MCL1 and reduced BAX expression. Furthermore, BCL2 and MCL1 were significantly higher and BAX was significantly lower in patients with advanced Binet stage. In addition, high MCL1/BAX ratio was associated with shorter LDT, with low BAX expression also associated with adverse cytogenetics ($11q^-/17p^-$), consistent with data showing that BAX expression is transcriptionally regulated by the p53/ATM pathways. The correlations with CD38 and BCL2 family expression were in keeping with an earlier study of CLL patients with bimodal expression of CD38. In that study, CD38-positive cells had a significantly higher expression of MCL1 than the CD38-negative cells derived from the same patient [55].

Longitudinal analysis of BCL2 and BAX indicates that the expression of these apoptosis-regulating proteins may vary over the clinical course, particularly after chemotherapy [52]. A 48-month longitudinal analysis showed an upward trend in BCL2 expression that was particularly marked in patients who received chemotherapy during the period of study, whilst BAX expression showed a downward trend, again especially in the treated patient group [54]. In contrast, MCL1 expression remained relatively stable. However, MCL1 expression and MCL1/BAX ratios were both associated with a shorter time to first treatment and overall survival in the total cohort and in those patients with early stage disease.

Although as outlined above there is a wealth of corroborative data identifying a role for the BCL2 family in the progression and/or response to therapy in CLL, it remains unclear as to whether these are the result of clonal selection or transcriptional induction/suppression. Also, clarity of their exact prognostic role has not been helped by failure to standardise the measurement of their expression, with some studies using flow cytometric methods—% of cells (using differing thresholds) and/or mean fluorescent intensity (MFI)—some using Western blot whilst others used RNA analysis. This is coupled with many of the studies either not undertaking multivariate analysis or being underpowered to show a difference compared to other potential pathogenic factors or other better established prognostic markers. For example, in the largest study to date of 185 patients, median MFI for BCL2, MCL1 and BAX was used, which was probably not the most sensitive use of the assay, as recursive partitioning showed a continuum of impact with rising (BCL2 and MCL1) and falling (BAX) protein levels (Fegan, Pepper, unpublished data). Even in this study a full multivariate analysis was not undertaken. Although the MCL1/BAX ratio did split the CD38, ZAP70 and *IGHV* gene mutation status curves, it did not show independent prognostication using the median MFI. In contract in one of the only other studies to attempt to define an independent role for BCL2, intracellular levels of BCL2, Cyclin D1, PCNA, ATM, Fas (CD95), BAX, RARα, RXRβ, Flt1, VEGF and cellular β_2-microglobulin were determined by Western blot analysis [50]. Perhaps unsurprisingly, increasing levels of BCL2 correlated with most of the poor prognostic factors such as high white blood cell (WBC) count, peripheral blood lymphocytosis, advanced Rai or Binet stage, decreased haemoglobin, low platelet counts and high levels of serum β_2-microglobulin, and although it showed a role for BCL2, Cyclin D1, PCNA and ATM in survival in CLL, the model did not include CD38 and ZAP70 expression or *IGHV* gene mutation status. Thus, to date no study has convincingly shown an independent role for any of the BCL2 family members in the progression or outcome in CLL.

Phosphorylation of two isoforms of BIM, BIM_{EL} and BIM_L, is triggered via B-cell receptor signalling and correlated with progressive disease in patients with both unmutated and mutated *IGHV* genes. BIM phosphorylation was also shown to be dependent on MEK1/2 kinase activity, suggesting a possible role in apoptosis regulation in CLL cells, by coordinating antigen and microenvironment-derived survival signals [56].

There have been conflicting reports as to the importance of AIP expression. In one study, expression of anti-apoptotic cIAP1 and cIAP2 was significantly higher in CLL cells compared to normal lymphocytes, whereas the IAP-antagonist, Smac,

was decreased. Higher expression of AIPs was associated with advanced stage disease, higher lymphocyte counts and high β2M expression but not with age, LDH, CD38 and ZAP70 expression [57].

The almost ubiquitous overexpression of BCL2 in CLL was originally attributed to hypomethylation of the promoter region of the BCL2 gene [58]. However, the discovery of small non-coding microRNAs (miRs) that interact with RNA transcripts to induce silencing complexes raises the possibility that BCL2 expression is regulated by multiple mechanisms [59]. It has been shown that miR-15 and miR-16, which are located on chromosome 13q14, are deleted or downregulated in the majority of CLL cells and especially those with the commonest genetic abnormality in CLL, deletion of 13q [60, 61]. Both of these miRs negatively regulate BCL2 at the post-transcriptional level and are therefore effectively acting as tumour suppressor genes. Further, they were shown to induce a CLL-like phenotype in mice [62]. Overexpression of BCL2, MCL1 and XIAP has been associated with miR-181b and an inferior clinical outcome with reduced progression-free and overall survival [63–65]. In the study of Visone et al. miR-181b expression decreased with disease progression and with an increase in both BCL2 and MCL1 expression [64]. A putative binding site for miR-181b was proposed in the 3′ UTR of MCL1 based on genome sequencing and such a site being identified in HeLa cells (cervical carcinoma). In an analysis of 114 patients, miR-181b expression was as significant if not more significant than *IGHV* gene mutation status and ZAP70 expression with regard to progressive disease, but MCL1 expression was not included in this analysis, and no formal multivariate analysis was undertaken. In another study using primary CLL cells, miR-181a/miR-181b was shown to directly bind to the 3′ UTR of *BCL2*, *MCL1* and *XIAP,* leading to their inhibition. miR-181a/b expression was shown to be significantly lower in patients with unmutated IGHV genes, a dysfunctional p53 pathway and 11q deletions. Unsurprisingly, a reduced treatment-free and overall survival was shown, but alas again no multivariate analysis was undertaken [65]. Two of the four commonest cytogenetic abnormalities are 13q and 11q (ATM) deletions, and in proximity to these two chromosome arms are miR-15/miR-16 and miR-34b/*c*, respectively. Fabbri et al. proposed a feedback loop between 17p and 13q/11q via miRs [66]. Patients with 13q deletions not only had lower expression of miR-15/miR-16, they had higher expression of BCL2 and p53. Similarly they showed that patients with 11q deletions had reduced levels of miR-34b/miR-34c and higher expression of ZAP70. Using chromatin precipitation and various cell lines (MEG-01—megakaryocytic leukaemia cell, K562-CML, H1299 lung cancer and HeLa-cervical carcinoma), they identified an miR-34 family binding site within the *ZAP70* open reading frame and a p53 binding site in the pre-*miR-34b*/*miR-34c* binding site on 11q. In this model, deletion of the *miR-15*/*miR-16* locus promotes an anti-apoptotic phenotype and augments p53 expression, *miR-34b*/*miR-34c* cluster activation, and ultimately ZAP70 suppression. Conversely, the poor outcomes associated with an 11q deletion are likely a manifestation of dampening the p53-mediated *miR-34b*/*miR-34c* inhibition of ZAP70. A mutation or chromosomal aberration inactivating *p53* would impair both loops of this circuit, as may be evidenced in patients with a 17p deletion

who experience the worst CLL outcomes. However, although the findings are very novel, they need to be confirmed in primary CLL cells. Also, these data do not explain the role of the two *IGHV* gene mutation groups and their differing expression of the BCL2 family proteins—or for that matter ZAP70—or indeed take into account that both hyper- and hypomethylation of DNA promoter regions of microRNAs and that a reproducible disease-specific pattern of methylation has been identified in CLL [67]. Clearly the picture is very complex and far from complete, with at least a further 11 microRNAs having been identified and implicated in the pathogenesis of CLL [65]. To date, no large-scale studies showing an independent role for miRs in the outcome of CLL have been published.

Perhaps surprisingly given the pivotal role of the BCL2 family in CLL, very few constitutive genetic factors underlying the differential expression of BCL2, MCL1 and BAX have been described. A study identified a common polymorphism G(−248)A in the promoter region of the *BAX* gene which resulted in lower BAX protein expression, an increased BCL2/BAX ratio and a shorter overall survival once treatment was initiated [68]. Another study reported nucleotide insertions in the promoter region of the MCL1 gene correlated with increased RNA and protein levels in CLL cells and influenced clinical outcome [69]. Although this presented an attractive rationale for the relative overexpression of MCL1 found in some CLL patients, the findings of this study were refuted by many, often much larger, studies, including one of 173 CLL patients [70]. This study found no association between MCL1 promoter insertion sequences and *IGHV* gene status, Binet stage or overall survival and concluded that they have no prognostic value in CLL.

CLL cells rapidly die in vitro, indicating that in vivo they are probably receiving constant tonic pro-survival signals via interactions with their microenvironment. Interaction of CLL cells with follicular dendritic cells is able to upregulate MCL1 through CD44. Numerous other signalling pathways have been implicated in the upregulation of MCL1, including the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway, B-cell receptor signalling and CD40 ligand pathways [71–75]. BCR signalling leads to a reduction in apoptotic threshold which is PI-3 kinase-dependent [47]. Inhibition of NF-κB results in the reduction of both BCL2 and MCL1, suggesting that NF-κB contributes to the maintenance of these proteins [76]. The same study revealed a strong correlation between *IGHV* mutational status and constitutive MCL1 expression, suggesting that CLL cells with unmutated *IGHV* genes may be more sensitive to, or have a higher capacity for, processing intrinsic survival signals, resulting in elevated basal expression of MCL1. Surprisingly in the light of Herishanu's data showing NF-κB-dependent signalling and the fact CD40L is an NF-κB downstream pathway, Davids showed no difference in apoptotic threshold between peripheral blood and lymph node-derived CLL cells [47].

The short half-life of MCL1 (1–2 h) indicates that circulating CLL cells with high MCL1 expression most probably receive regular stimulation from circulating chemokines, cytokines or cell-cell contact in the peripheral vasculature, as it seems implausible that CLL cells recirculate to lymph nodes with sufficient frequency to maintain a pro-survival MCL1 signal. In this regard, previous work has shown that $CD38^{+}$ CLL cells have higher VEGF and MCL1 expression. This suggests a

positive autocrine loop where phosphorylation of the VEGF receptor leads to increased MCL1 [55, 77]. Furthermore, co-culture of endothelial cells with primary CLL cells leads to upregulation of both CD38 and CD49d on the CLL cells [78]. In addition, NF-κB is activated within the CLL cells, leading to increased expression of BCL2, MCL1 and BCL-X_L [78]. This data strongly supports at least a two-compartment model of CLL cell survival, with the lymph node providing proliferative and anti-apoptotic signals and the peripheral vasculature also providing additional anti-apoptotic signals. The bone marrow may also be exerting an independent role, as suggested by Davids et al. [47]. Indeed, given the data to date, it is highly likely that differing BCL2 family members are exerting their effects in different tissues as the CLL cells migrate from one compartment to another.

Therapeutic Targeting of the BCL2 Family

We have known for some time that chemotherapy and radiotherapy induce DNA damage, and hence their main mechanism of action is actually through the intrinsic apoptotic pathway. Indeed, the propensity of many chemotherapy agents to kill malignant haematopoietic cells is determined by the apoptotic threshold of the mitochondria as determined by the BCL2 family profile within each tumour cell type [47, 79].

The apoptotic threshold is one of many determinants of clinical response and several studies have assessed the role of the BCL2 family in response to therapy/drug resistance [47]. High levels of BCL2 have been associated with shorter overall survival in previously treated CLL patients and increased chemoresistance to treatment with fludarabine [39, 40]. However, the relative expression of BCL2 to BAX appears to be a more important determinant of CLL cell apoptosis; BCL2/BAX ratios correlate with progressive disease and resistance to chlorambucil and fludarabine in vitro [51, 54, 80, 81]. Further, we have previously shown that chemoresistance to chlorambucil is mediated, at least in part, by in vitro selection of subclones with high BCL2 expression and low BAX expression [81]. Likewise, MCL1 has been associated with chemoresistance to chlorambucil, fludarabine and rituximab both in vitro and a failure to achieve a complete response in vivo [54, 82–84]. In addition, targeted downregulation of MCL1 has been shown to enhance rituximab-mediated apoptosis and complement-dependent cytotoxicity [84]. Fludarabine and glucocorticosteroids are also able to upregulate the BH3-only pro-apoptotic proteins, BIM, NOXA and PUMA, as well as BAX [85, 86]. The pan cyclin-dependent kinase (CDK) inhibitor flavopiridol prevents phosphorylation of RNA polymerase, leading to a reduction in MCL1 and XIAP expression and apoptosis via a p53-independent mechanism [87, 88]. Similar results were obtained with another pan-CDK inhibitor, roscovitine [89]. In a study of 100 patients, higher expression of cIAP1, cIAP2, XIAP and survivin and lower levels of Smac were all associated with disease progression. Simultaneous expression of both cIAP1 and survivin was associated with a poor response to

chemotherapy, an inferior outcome and indeed was an independent prognostic factor in a model containing clinical stage, LDH, β2M and CD38 and ZAP70 expression, but importantly not *IGHV* gene mutation status [57].

The p53 tumour suppressor gene is deleted or mutated in approximately 50 % of all solid tumours and 10–20 % of CLL cases; in both cases it is associated with a very poor outlook. The p53 protein plays a key role in modulating the apoptotic response of CLL cells to genotoxic agents [90]. Although transcriptional induction of pro-apoptotic proteins, including BAX, PUMA and NOXA, has been shown to mediate p53-dependent apoptosis in response to DNA damage, recent studies have identified a novel non-transcriptional mechanism, involving direct binding of p53 to anti-apoptotic proteins including BCL2 at the mitochondrial surface. One study showed in vitro that in CLL cells, in response to chlorambucil and fludarabine damage, p53 can bind directly to mitochondrial bound BCL2 [91]. PUMA, which is constitutively expressed in a p53-independent manner, was modestly upregulated following this p53 induction, and inhibition of p53 blocked upregulation of PUMA and p21 and accelerated the conformational change of BAX [91]. The authors propose that direct interaction of p53 with mitochondrial anti-apoptotic proteins, including BCL2, is the "major route" for apoptosis induction in CLL cells and that p53's transcriptional targets include proteins that impede this non-transcriptional pathway. This work awaits confirmation by other groups. In the study by Zhu et al., transfection of primary CLL cells with miR-15a, miR-16-1, miR-34 and especially miR-181a/b sensitised the CLL cells to fludarabine-induced killing, although this required an intact p53 pathway as this was not evident in samples with p53 abnormalities [65]. The expression of BCL2 and MCL1 was increased following transfection, and it is this which is probably responsible for the induced in vitro chemoresistance.

Given their role in the aetiology, progression and survival in CLL patients and the observations that standard chemotherapy acted, at least in part, via manipulation of the BCL2 apoptotic family, more targeted approaches to the BCL2 family-dependent therapeutics have been developed, with many presently undergoing clinical evaluation. Obviously, increased apoptosis can be achieved by either reducing pro-survival or increasing pro-apoptotic proteins. The first agent used clinically to reduce pro-survival proteins was a BCL2 antisense oligonucleotide, which in preclinical studies was shown to downregulate BCL2 mRNA and protein expression, upregulate BAX protein and increase apoptosis. However, surprisingly, the extent of the reduction in BCL2 protein did not correlate with the degree of apoptosis [92]. Further studies revealed that p21 was induced, indicating a p53-dependent mechanism, and that the BCL2 antisense sensitised CLL cells to chlorambucil-mediated cell death probably by altering the apoptotic threshold [93, 94]. In the first phase I/II study of BCL2 antisense in relapsed and refractory CLL, encouraging responses in hepatomegaly (29 %), splenomegaly (41 %), lymphadenopathy (32 %) and lymphocytosis (50 %) were seen, although some adverse reactions were also observed [94]. In a larger (230 treated patients) follow-up randomised study of fludarabine/cyclophosphamide with and without BCL2 antisense therapy, complete remission/nodular partial (CR/nPR) remission was

obtained in 21 % of antisense-treated patients compared to only 7 % in the fludarabine/cyclophosphamide only arm (p = 0.02), but with no overall significant improvement in survival for the BCL2 antisense-treated arm. However, for those patients who achieved a PR/CR, the 5-year survival rate was 47 % in the antisense-treated arm, compared to 24 % in the FC-only-treated arm ($p = 0.038$), although the reason for this difference is not known [95–97]. Again, a slight surprise was that the greatest benefit with BCL2 antisense therapy was observed in patients with fludarabine-sensitive disease, which showed a 50 % reduction in the risk of death ($p = 0.004$). One would have perhaps expected that as higher BCL2 levels have in previous studies been associated with fludarabine resistance and that fludarabine therapy induces survival of high BCL2-expressing clones, this would be the group most likely to benefit from direct targeting of BCL2. Common toxicities included thrombocytopenia and, more rarely, tumour lysis syndrome and cytokine release reactions. It is unlikely given the overall lack of efficacy that antisense targeting of BCL2 will be a major therapeutic direction in the future.

The development of pro-apoptotic drugs has largely revolved around identifying drugs that mimic the action of BH3-only proteins, the so-called BH3 mimetics. The agents induce apoptosis by competing for the BH3-binding pocket of anti-apoptotic proteins, resulting in the displacement of pro-death proteins. However, a recent study suggested that with the exception of ABT-737, which is a true BH3 mimetic, many other so-called BH3 mimetics, including gossypol and GX15-070/obatoclax, actually activate the endoplasmic reticulum stress response. This results in the induction of ATF4, ATF3 and NOXA, which can then bind to and inhibit MCL1 and thereby induce apoptosis [98]. Also, many agents not only target more than one BCL2 family member but induce off-site—non-BCL2 family—mechanisms. One cannot therefore assume that the proposed mechanism of action is actually the effector mechanism in vivo. Also, off-site collateral effects are likely to increase clinical toxicity [99].

The first agent identified in this class was the cotton plant-derived phenolic gossypol isomer (AT101). This was originally reported to target BCL2, BCL-X_L and BCL-W, although in lymphoid cell lines and primary CLL cells a role for NOXA has been described [99, 100]. Preclinical studies showed gossypol-induced cell death in a caspase-independent manner. MOMP was induced resulting in activation of BAX, and cytochrome c and AIF release. Blocking of AIF led to reduced apoptotic killing, indicating a role for AIF in gossypol-mediated cytotoxicity in CLL cells [101]. Furthermore, gossypol was able to overcome the protective effects of stroma-induced MCL1 expression and retained cytotoxicity under these pro-survival conditions. Induction of MOMP also led to the production of reactive oxygen species, but the addition of antioxidants did not reduce the cytotoxicity. In a phase I study of 7 early stage, treatment naïve patients, in evaluable patients 5/6 had a reduction in lymphocytosis, 6/6 a reduction in lymphadenopathy and 5/5 a reduction in splenomegaly with common but typically only grade I/II toxicities and no haematological toxicities [102].

ABT-737 and its oral version ABT-263 are small organic BH3 mimetics, which binds strongly to BCL2, BCL-X_L and BCL-W but not to MCL1, BFL-1, BCL-B or A1, antagonising their anti-apoptotic properties in several lymphoid cell lines and

primary CLL cells [103]. ABT-737 was shown to displace BIM from the BCL2 BH3-binding pocket, thereby allowing BIM to induce BAX, and BAK oligomerisation and induce MOMP. The LC_{50} in primary CLL cells is highly variable, ranging from low nanomolar concentrations to values more than a thousand times greater [104]. However, when CLL cells were co-cultured with CD154-expressing fibroblasts in the presence of interleukin-4 to mimic the lymph node microenvironment, they developed an approximately 1,000-fold resistance to ABT-737, although this can be reversed by PI-3K blockade [47]. The underlying mechanism was identified as de novo synthesis of the anti-apoptotic proteins BCL-X_L and BCL2A1 [105]. However in another study using the same co-culture cell system, it was the NOXA/MCL1 balance that determined the sensitivity to ABT-737 [106]. This is supported by the observation that ABT 737 has poor killing against high MCL1-expressing tumour cell lines and that MCL1 inhibition increases tumour sensitivity to ABT 737 [107]. Presumably in resistant tumours, even if pro-apoptotic proteins are effectively displaced from BCL2 by ABT-737, they can be sequestered by MCL1 and BFL-1 before they can induce MOMP. ABT-737 enhanced apoptosis of CLL cells treated with the proteasome inhibitor bortezomib and synergised CLL cells to the effects of dexamethasone, etoposide, fludarabine and doxorubicin [104, 108]. There remains disagreement as to whether the expression of BCL2 family members predicts in vitro response to ABT-737 [99]. In one study there was no correlation between BCL2, BCL-W, MCL1, BAX, BIM, BCL2/BIM, MCL1/BIM, BCL2/BAX and MCL1/BAX ratios and sensitivity to ABT-737, whilst another identified a correlation between MCL1 and BFL-1/BCL2 ratio expression [104, 109]. Likewise, although ABT-737 appears to kill primary CLL cells from all prognostic groups in vitro, there is a discrepancy in p53-dysfunctional cells, where its apoptotic potential was significantly reduced (63.7 ± 2.9 % to 39 ± 7.3 %) in one study whilst another showed no influence of p53 on ABT-737 sensitivity [104, 110]. In a phase I dose escalation study in patients with advanced relapsed and refractory CLL, 19/21 patients had a >50 % reduction in lymphocytes [111]. Nine (35 %) patients receiving >110 mg/day had a partial response, with seven maintaining stable disease for over 6 months. Median progression-free survival was 25 months, with responses in all prognostic groups–including patients with fludarabine refractoriness, bulky disease and 17p deletions. Low MCL1 levels and high BIM/MCL1 and BIM/BCL2 ratios all correlated with improved response. The major dose-limiting toxicity was thrombocytopenia, which is perhaps not surprising, as platelets express high levels of BCL-X_L.

The first clinical experience of ABT-199, a new oral BCL2 inhibitor with BCL-X_L-sparing characteristics (500-fold less activity compared to BCL2), showed dose-limiting tumour lysis syndrome in the first cohort of 3 patients. However, it also induced a rapid reduction of lymphadenopathy and >95 % reduction of lymphocytosis in 2 patients. Dose reductions were undertaken in subsequent cohorts. Of the first 15 patients, 4 have discontinued therapy due to progressive disease (3) and incidental discovery of adenocarcinoma (1). In the remaining 11 patients after a median of 4 months therapy, all have had a >50 % reduction in lymphocytes and 9/15 experienced reduced lymphadenopathy. Very preliminary

formal responses showed 8 patients achieved a partial response, including 4 out of 8 with fludarabine resistance and 3 out of 5 with p53 deletions. As expected, caspase-3 activation and apoptosis induction was observed but thrombocytopenia was not clinically dose limiting [112].

GX15-070/obatoclax is a small-molecule pan-BCL2 inhibitor, which was originally reported to bind BCL2, BCL-W, BCL-X_L and MCL1 and potently interfere with the direct interaction between MCL1 and BAK in intact mitochondria, although this has recently been questioned [98, 113]. In preclinical studies, however, it inhibited the association between MCL1 and BAK and was capable of killing ABT-737-resistant cells. GX15-070 also disrupted BCL2/BIM and MCL1/BAK complexes in CLL cells, followed by the activation of the intrinsic apoptotic pathway [114]. Decrease in BCL2 phosphorylation at the serine 70 residue by extra cellular signal-regulated kinase (ERK)1/2 inhibition increased CLL cell sensitivity to GX15-070, whilst blocking BCL2 dephosphorylation using a PP2A antagonist reduced its activity. The cytotoxicity of GX15-070 was increased by co-treatment with bortezomib. However, proteasome inhibition led to the accumulation of phosphorylated BCL2, with the degree of interaction between GX15-070 and bortezomib regulated by basal pBCL2 (Ser70) levels. In a dose-escalating phase 1 study, 26 heavily pretreated CLL patients received a total of 74 infusions (1 or 3 h) of escalating doses with a maximum tolerated dose of 28 mg/m^2 over 3 h. Activation of Bax and Bak was demonstrated in peripheral CLL cells, with induction of apoptosis related to overall GX15-070 exposure, as measured by the plasma concentration of oligonucleosomal DNA/histone complexes. Although circulating lymphocyte counts were reduced in 18 of 26 patients, with a median reduction of 24 %, and 3/11 and 4/14 patients with anaemia and thrombocytopenia, respectively, had improvements in haemoglobin and platelets, only a single partial response was observed. Also neurological toxicities proved dose limiting [115].

Polyphenols in green tea have long been proposed as health-promoting substances that can reduce the risk of cancer including lymphoid malignancies. Epigallocatechin gallate (EGCG), the major catechin in tea, induces apoptotic cell death in primary CLL cells via reduction in BCL2, MCL1 and XIAP [77]. Using polyphenon E as a source of EGCG, a phase I early stage cohort study (dose of 400–2,000 mg twice daily orally) showed the maximum tolerated dose was not reached and minimal toxicity (3 % grade 3 and 4) noted [116]. One patient achieved a partial remission, with 11 patients (33 %) having a sustained reduction ($\geq$20 %) in absolute lymphocyte count (ALC) and 11/12 (92 %) of patients with palpable lymphadenopathy experiencing at least a 50 % reduction in the sum of the products of all nodal areas during treatment. A phase II study of 42 asymptomatic early stage patients using 2,000 mg twice daily again confirmed minimal toxicity with an overall response rate of 69 % [117]. Thirteen patients (31 %) experienced a sustained reduction of $\geq$20 % in the ALC and 20/29 patients (69 %) with palpable lymphadenopathy experienced at least a 50 % reduction in the sum of the products of all lymph node areas. EGCG plasma levels after 1 month of therapy were found to be correlated with reductions in lymphadenopathy and responses were seen in all prognostic groups. Nine patients experienced an adverse event and 12 developed

progressive disease that required more conventional chemo-immunotherapy. After a median follow-up of 32 months (range, 21–51 months) from the time of registration and a median follow-up of 56 months from diagnosis, the 24-month treatment-free survival rate from registration was 79 %. Unfortunately, in neither study was any data provided on the in vivo effects of polyphenon E on the BCL2 family expression.

Although great strides have been made in understanding the role of the BCL2 family in CLL, the picture is far from complete. As outlined above, the various in vitro studies have identified pathogenic roles for at least BCL2, MCL1, BAX, BAD, BIM, NOXA, PUMA and BAK, clearly indicating a very complex relationship, especially when one considers that differing compartments (blood, bone marrow and lymph node) appear to differentially regulate the various BCL2 family members. The underlying role of miRs is very incomplete, and again it is possible that miRs will be themselves differentially regulated in the three lymphoid compartments. The data is fairly compelling that chemo-immunotherapies exert their effects through the BCL2 family members and that the apoptotic threshold determines clinical response—probably by preferentially killing high threshold cells. Clearly, the addition of BH3 mimetics will allow more specific targeting of BCL2 family members, but they in many ways do not appear to be very different from old-fashioned chemo-immunotherapy, as stromal cells clearly induce resistance, and again, the BH3 mimetics are probably selectively killing the high apoptotic threshold CLL cells, potentially leaving a more resistant clone. Although hopefully they will have less off-target and hence clinical side effects, it looks unlikely that alone they will significantly alter the clinical outcome of CLL and combination with other—old and newer—agents will be required. New specific signal pathway inhibitors, including HSP90, NF-κB, COX-2 and histone deacetylase inhibitors are undergoing clinical development. These agents are predicted to indirectly alter the expression of BCL2 family proteins and in particular MCL1 [76, 118–121]. The prospect of combining these new agents to increase the apoptotic threshold should give rise to combination therapies with conventional chemotherapies, monoclonal antibodies and other non-chemotherapy signal inhibitors, as well as direct BCL2 family inhibitors (BH3 mimetics). The ultimate aim of these combinations would be to induce deeper, more sustained responses and reduce chemotherapy side effects. These are indeed exciting times in the world's most exciting cancer—chronic lymphocytic leukaemia! Forty years of basic research into apoptosis has led us to the "threshold" of a new era in the fight against this key cancer.

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Chapter 8
Richter Syndrome

Davide Rossi and Gianluca Gaidano

Abstract Transformation of chronic lymphocytic leukemia (CLL) to aggressive lymphoma is known as Richter syndrome (RS). In the CLL population considered as a whole, the prevalence of RS development ranges from 2 to 7 %. The most common pathologic phenotype at the time of RS transformation is diffuse large B-cell lymphoma (DLBCL), while, in a small fraction of cases, the transformed phase shows pathologic features mimicking Hodgkin lymphoma. *TP53* disruption and *MYC* activation cooperate as dual hits in driving DLBCL transformation. Two biomarkers (*NOTCH1* mutations and usage of the immunoglobulin VH CDR3 subset 8) may help in identifying CLL patients at risk of DLBCL transformation to be considered for close monitoring and a careful biopsy policy. In the presence of clinical features suspicious of RS, diagnosis of transformation and choice of the site of biopsy may take advantage of ^{18}FDG PET/CT. The prognosis of RS transformation is generally highly unfavorable. However, the pattern of survival is not homogeneous and may be predicted on clinical and biological grounds. RS that are clonally unrelated to the paired CLL phase are biologically and clinically different from clonally related cases, and should be considered, and probably managed, as a de novo DLBCL arising in the context of CLL. Rituximab-containing polychemotherapy represents the backbone for induction treatment in patients with clonally related DLBCL transformation. Younger patients who respond to induction therapy should be offered stem cell transplant to prolong survival.

Keywords Richter syndrome • Chronic lymphocytic leukemia • Diffuse large B-cell lymphoma • Hodgkin lymphoma • Transformation • *TP53* • *MYC* • *NOTCH1* • Immunoglobulin genes • Prognosis • Treatment

D. Rossi, M.D., Ph.D. (✉) • G. Gaidano, M.D., Ph.D.
Division of Hematology, Department of Translational Medicine, Amedeo Avogadro University of Eastern Piedmont, Via Solaroli 17, 28100 Novara, Italy
e-mail: rossidav@med.unipmn.it

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Definition of Richter Syndrome

Richter syndrome (RS) represents the clinico-pathologic transformation of chronic lymphocytic leukemia (CLL) to an aggressive lymphoma [1, 2]. At the time of RS transformation, the most common pathologic phenotype (95–99 % of cases) is diffuse large B-cell lymphoma (DLBCL). However, in a small fraction of cases (1–5 %), the transformed phase shows pathologic features mimicking Hodgkin lymphoma (HL) [1]. According to the relationship between the CLL clone and the aggressive lymphoma clone, RS may be molecularly distinguished into at least two biologically different entities: (1) transformation of CLL cells to a clonally related aggressive lymphoma and (2) development of an aggressive lymphoma that is unrelated to the CLL clone [3–11]. Transformation of CLL cells to a clonally related RS is by far the most frequent of the two conditions and represents true RS. In clonally related RS, the pathogenetic link between CLL cells and the emerging DLBCL clone is apparently obvious and is substantiated by gain of novel molecular lesions at a certain timepoint of the natural history of the CLL clone, conceivably corresponding to the time of clinico-pathologic transformation. On the other hand, clonally unrelated RS of both DLBCL and HL variants merely represents a second lymphoid cancer in the context of CLL, a disease that is prone to the development of second primary tumors. The mechanisms underlying the development of an aggressive lymphoma that is clonally unrelated to CLL cells are not fully understood and may be related, at least in part, to the condition of immune deregulation that frequently characterizes patients affected by CLL.

The development of an aggressive lymphoma may also be the consequence and complication of previous CLL treatments. In this respect, after the introduction of the anti-CD52 antibody alemtuzumab, occasional CLL patients have been reported to develop clinically aggressive lymphomas, usually clonally unrelated to the CLL phase, mostly characterized by Epstein–Barr virus (EBV) infection, and displaying several similarities to lymphomas arising in immunodeficient hosts [12–14]. These cases of alemtuzumab-associated aggressive lymphomas represent a novel type of immunodeficiency-related lymphoma developing after T-cell depleting therapies in patients already immunocompromised because of the underlying disease and/or because of previous chemotherapy. The increased risk of aggressive lymphomas and EBV-related lymphoproliferations in patients treated with alemtuzumab-based regimens has been confirmed within a recent randomized trial comparing fludarabine–cyclophosphamide–rituximab vs. fludarabine–cyclophosphamide–alemtuzumab [15].

Epidemiology of Richter Syndrome

Information about the incidence of RS, risk factors predisposing to transformation, and its time of onset after CLL presentation mainly derives from analyses of retrospective cohorts.

Table 8.1 Summary of the published reports assessing the frequency of Richter syndrome transformation[a]

References	Study design	CLL patients included in the study	Patients that developed RS	RS prevalence (%)
Maddocks-Christianson et al. [21]	Retrospective	962	14	1
Robak et al. [19]	Retrospective	1,487	15	1
Catovsky et al. [28]	Clinical trial	777	13	2
Mauro et al. [18]	Retrospective	1,011	22	2
Parikh et al. [27]	Retrospective	1,641	37	2
Tsimberidou et al. [22]	Retrospective	3,986	148	4
Fisher et al. [26]	Clinical trial	817	33	4
Alipour et al. [23]	Retrospective	465	24	5
Tabuteau et al. [17]	Retrospective	620	37	6
Keating et al. [16]	Clinical trial	174	13	7
Solh et al. [25]	Clinical trial	521	34	7
Rossi et al. [10]	Retrospective	783	69	9
Rossi et al. [24]	Retrospective	185	17	9
Thornton et al. [20]	Retrospective	101	12	12

[a]*CLL* chronic lymphocytic leukemia, *RS* Richter syndrome

The hematologist's perception of RS as a rare complication of CLL may be due, at least in part, to under-recognition of RS in the current clinical practice. Whereas re-biopsy is standard practice at each progression of follicular lymphoma, a similar attitude is not commonly followed in the case of CLL, even in patients with substantial enlargement of lymph nodes. Consistently, the incidence of RS transformation varies among series from 1 to 7 %, being higher in cohorts managed with pre-specified biopsy protocols specifically tailored at histologically documenting transformation (Table 8.1) [10, 16–28].

To date, the issue of RS epidemiology has been specifically addressed by a single prospective study. Solh et al. examined a large series of 521 previously untreated CLL patients enrolled on the CALGB 9011 trial that aimed at comparing the efficacy of fludarabine vs. chlorambucil vs. fludarabine + chlorambucil [25]. The study population was provided with a remarkably long follow-up of at least 15 years and was subjected to a long-term evaluation for the assessment of second primary tumors, including lymphomas. Overall, biopsy-proven RS developed in 7 % of cases from the CALGB 9011 trial, a rate of RS transformation that is consistent with other published retrospective cohorts.

Though the incidence of transformation increases with the number of previous regimens, it is important to underscore the notion that a fraction of RS cases have been diagnosed at the time of first progression requiring treatment or even at the time of first presentation of CLL [24]. These data suggest to take into account the possibility of RS transformation at each progression, even during the early phases of the CLL clinical history.

Following the example of other models of transformation from indolent to aggressive tumors, a fundamental question that arises is whether chemotherapy may contribute to the development of RS in patients with CLL. Fludarabine is a mainstay drug for the treatment of CLL, either alone or in combination with alkylators and monoclonal antibodies. Though concerns have been raised about the carcinogenic effects of fludarabine, analysis of large CLL cohorts has consistently shown that the risk of second primary cancers after fludarabine exposure is not higher than the overall rate expected in these patients. However, by limiting the analysis to lymphoproliferative disorders, retrospective studies of case series have postulated a potential relationship between fludarabine exposure and development of an aggressive lymphoma, though the results are somehow conflicting [21]. A systematic assessment of the risk of RS in a randomized trial comparing fludarabine monotherapy to alkylating agent-based regimens as first-line therapy for CLL found no impact of initial therapy with fludarabine compared to chlorambucil on the risk of transformation to RS [25]. Consistent with these results, in the LRF CLL4 trial comparing first-line fludarabine vs. chlorambucil vs. fludarabine + cyclophosphamide, the frequency of RS was not different across the three treatment arms [29].

Risk Factors of Richter Syndrome Development

Early recognition of RS transformation may be clinically useful in order to avoid the exposure of patients to multiple lines of therapy that, being targeted to CLL progression, are of little efficacy on the transformed clone. This concept prompts the need for a close monitoring of CLL patients harboring risk factors of RS development. Advancements in the field have unraveled a number of intrinsic characteristics of the tumor clone that predispose CLL to subsequently transform into DLBCL and include (1) disease genetics, namely, the presence of *NOTCH1* mutations [29]; and (2) expression of specific molecules facilitating the interaction between the CLL clone and the microenvironment, as exemplified by the expression of the immunoglobulin heavy variable gene (*IGHV*) 4–39 rearranged in a stereotyped fashion (the so-called VH CDR3 "subset 8") [10].

The *NOTCH1* gene encodes a class I transmembrane protein functioning as a ligand-activated transcription factor and playing an important role in a number of cellular functions, including proliferation and apoptosis [30]. After interaction with cognate ligands on an adjacent cell, two consecutive proteolytic cleavages of the NOTCH1 receptor allow its intracellular domain to translocate to the nucleus, thus leading to transcriptional regulation of multiple target genes. The signaling is switched off by proteosomal degradation of active NOTCH1 molecules mediated by the C-terminal PEST domain. *NOTCH1* mutations recur in ~10 % of newly diagnosed CLL and are almost exclusively frameshift or nonsense events clustering within the PEST domain of the protein [31–33].

CLL harboring *NOTCH1* mutations has a significantly higher cumulative probability of transforming into a clonally related DLBCL (45 %) compared to CLL without *NOTCH1* mutations (4 %) [29]. Consistent with a prominent role of *NOTCH1* mutations in driving the histologic shift of CLL are the following observations: (1) *NOTCH1* mutations are one of the most frequent genetic lesions of RS, where they occur in 30–40 % of cases [31, 32], and (2) subclones harboring *NOTCH1* mutation are progressively selected during the CLL clinical history ending in clonally related DLBCL [31, 34].

A review of all *IGHV-D-J* rearrangements reported in the literature documents a biased usage of the unmutated immunoglobulin heavy variable gene *IGHV4-39* in ~10–15 % RS belonging to the DLBCL variant [3–10]. The same gene is rarely utilized in ~2 % non-transformed CLL [10, 35]. In the DLBCL variant of RS, the *IGHV4-39* gene is frequently rearranged in a stereotypic fashion in the unmutated VH CDR3 subset 8 [10]. Consistently, CLL utilizing a stereotyped B-cell receptor (BCR) belonging to subset 8 has a very high risk of transformation into the DLBCL variant of RS (17-fold higher than cases without a stereotyped BCR), translating into a cumulative incidence of transformation of nearly 80 % at 10 years [10].

The particular aggressiveness of BCR subset 8 and its increased propensity to transform into RS may be explained by the strong and unlimited capacity of CLL harboring this BCR configuration to respond to multiple autoantigens and immune/inflammatory stimuli present in the microenvironment [36, 37]. Among stereotyped subsets, this high reactivity is specific for subset 8 and may elicit unabated stimulation throughout the natural history of these patients, leading to progressive selection of the more aggressive clonal variants.

Overall, from a clinical standpoint, these observations suggest that a close monitoring and a careful biopsy policy may be of help for early recognition of transformation in patients carrying *NOTCH1* mutations or a stereotyped VH CDR3 belonging to subset 8.

Because the incidence of CLL transformation to HL and to clonally unrelated DLBCL is very low, risk factors predisposing to these two conditions are currently unknown, though the potential contribution of immunosuppressive treatments has been postulated [11, 38, 39].

Histopathologic Features of Richter Syndrome

RS transformation involves most frequently (60–70 % of cases) the lymph nodes, but extranodal localizations are also not uncommon and may affect the gastrointestinal tract (10 %), tonsil (10 %), and bone marrow (10 %) among other sites [9–11]. Pathologically, two variants of RS can be recognized, namely, the DLBCL variant and the HL variant [1].

The DLBCL variant of RS consists of confluent sheets of large neoplastic B lymphocytes [1]. Importantly, CLL cases presenting with numerous proliferation centers and an increased proportion of prolymphocytes and paraimmunoblasts,

but lacking clear-cut features of DLBCL, should not be diagnosed as RS [1]. Morphologically, either centroblastic (60–80 % of cases) or immunoblastic (20–40 % of cases) variants are reported among patients with DLBCL transformation [9–11]. Phenotypically, although the CD5 and CD23 antigens are invariably expressed in CLL cells, their expression is frequently lost at the time of transformation [9]. Indeed, CD5 expression is present in only a fraction (~30 %) of cases, while CD23 expression is even more rare (~15 % of cases). CD20 is generally expressed by transformed cells and represents an important target for immunotherapy with anti-CD20 monoclonal antibodies [9]. Based on immunophenotypic markers of de novo DLBCL, most cases of DLBCL transformation (90–95 %) have a post-germinal center phenotype (IRF4-positivity), whereas only 5–10 % display a germinal center phenotype (CD10 and/or BCL6 expression) [9–11].

Two types of HL transformation have been described [38, 39]. Type 1 HL transformation usually mimics the pathologic features of classical HL, being characterized by the presence of mononuclear Hodgkin cells and multinuclear Reed–Sternberg cells residing in a polymorphous background of small T cells, epithelioid histiocytes, eosinophils, and plasma cells. In type 1 transformation, the Hodgkin–Reed–Sternberg cells show the typical CD30-positive/CD15-positive/CD20-negative phenotype [38, 39]. In contrast, type 2 HL transformation is characterized by the presence of Hodgkin–Reed–Sternberg-like cells in a background of CLL cells lacking the polymorphous reactive infiltrate of classic HL. In type 2 transformation, Hodgkin–Reed–Sternberg-like cells express both CD30 and CD20 but lack CD15 [38, 39].

Based on the analysis of the rearrangement of *IGHV-D-J* genes, most (~80 %) of the DLBCL variants of RS are clonally related to the preceding CLL phase, thus representing true transformation [9–11]. In contrast, only a fraction (~40–50 %) of the HL variants of RS are clonally related to CLL [9, 40–42]. Consistently, a number of RS (~20 % showing a DLBCL morphology and ~50–60 % showing a classical HL morphology) harbor distinct *IGHV-D-J* rearrangements compared to the paired CLL, representing de novo lymphomas arising in a CLL patient [9–11, 40–42].

Molecular Pathogenesis of Richter Syndrome

The molecular pathogenesis of clonally related DLBCL transformation has been characterized to some extent (Fig. 8.1). In this context, *TP53* abnormalities, including mutations or deletions of the locus, are the most frequent genetic lesion and can be identified in ~60 % of cases [11]. Analysis of CLL/DLBCL paired samples have shown that *TP53* disruption is acquired at transformation in most cases (55 %) [11]. The tumor suppressor gene *TP53* codes for a central regulator of the DNA-damage-response pathway, and its activation leads to cell-cycle arrest and apoptosis, thus mediating the antiproliferative action of several chemotherapeutic agents [43].

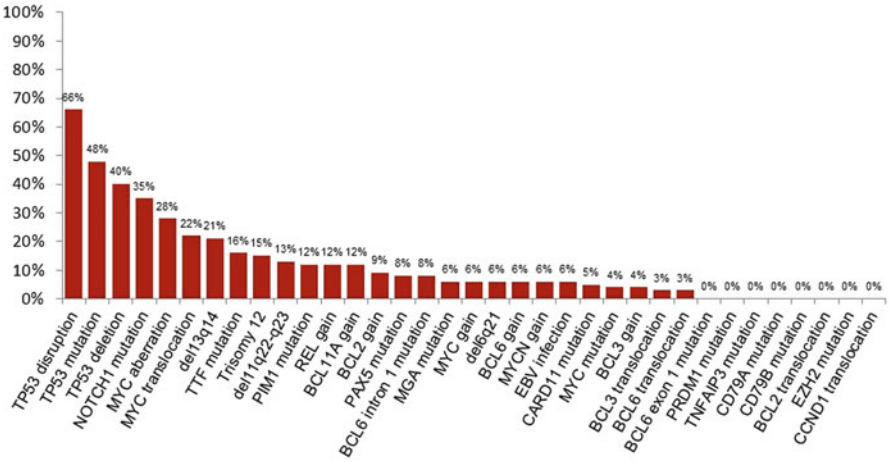

Fig. 8.1 Prevalence of genetic lesions investigated in clonally related DLBCL transformation

Consistently, *TP53* disruption is a major determinant of the chemorefractory clinical phenotype that characterizes transformation to clonally related DLBCL.

MYC deregulation characterizes a significant fraction (~60 %) of cases of clonally related DLBCL transformation and may be sustained by genetic lesions affecting the *MYC* network or by mutations affecting *MYC* trans-regulatory factors, as exemplified by mutations of *NOTCH1* [7, 11, 31, 32, 44] (Fig. 8.1). The *MYC* gene is activated by somatic structural lesions in ~30–40 % of clonally related DLBCL transformation, including translocations juxtaposing *MYC* to immunoglobulin loci, gain/amplification at 8q24, and point mutations [11]. *MYC* belongs to a transcription regulating network that also includes *MAX*, a cofactor required for DNA binding by the various members of this network, as well as a group of putative *MYC* antagonists, namely, *MNT*, *MXD1-4*, and *MGA* [45]. In physiological conditions, the MYC protein activates gene transcription through heterodimerization with MAX and then by binding to the E-box DNA recognition sequences in its target gene promoters. Conversely, heterodimers of MAX with MNT, MXD1-4, and MGA antagonize MYC-dependent gene expression by transcriptional repression of the same E-box sequences. On these bases, the MYC oncoprotein transforming ability depends also on the balance between the MYC-MAX and MNT/MXD1-4/MGA-MAX complexes [45]. In CLL, this balance may be disrupted by genetic lesions targeting *MYC* antagonists. Indeed, *MGA*, one of these MYC oncoprotein antagonists, is targeted by focal and recurrent gene deletions or truncating point mutations in a fraction of clonally related DLBCL transformation [44].

NOTCH1 mutations recur in ~30 % clonally related DLBCL transformation (Fig. 8.1) and, similar to mutations observed in unselected CLL, result in the removal of the C-terminal PEST domain of the protein that is required for switching off activated NOTCH1 signaling [31, 32]. Consistently, the removal of the PEST

domain is known to result in NOTCH1 impaired degradation and accumulation of an active NOTCH1 isoform, sustaining deregulated transcription of multiple target genes, including *MYC* activation.

Similarly to *TP53* lesions, abnormalities leading to *MYC* deregulation are also acquired at the time of transformation in a relevant fraction of cases [7, 11, 31, 32, 44]. In addition, *TP53* abnormalities and *MYC* lesions frequently co-occur in the same patient, thus suggesting that both *TP53* and *MYC* lesions are required for the development of the transformed clinico-pathologic phenotype and cooperate in a classical dual-hit mechanism of action [31, 44].

The role of EBV infection has been suggested by some studies as a potentially relevant factor for RS pathogenesis. The observation that the overwhelming majority of DLBCL transformed from CLL do not carry EBV infection in the malignant cells, however, does not favor this hypothesis [9–11, 24] (Fig. 8.1). The presence of EBV sequences has been documented in some, though not all, cases of RS with a DLBCL morphology originating in patients previously treated with fludarabine for their preexistent CLL. EBV infection in these cases has been thought to be a passenger phenomenon related to the immune deregulation caused by purine analogues [20, 46].

In contrast, EBV infection conceivably has a role in the HL variant of RS, which is EBV-positive in ~70 % of cases, as documented by staining for latent membrane protein 1 (LMP1) on immunohistochemistry or by in situ hybridization of EBV-encoded RNA transcripts [9, 38, 39].

Diagnosis of Richter Syndrome

Clinical features suspicious of RS include rapid clinical deterioration, fever in the absence of infection, rapid and discordant growth of localized lymph nodes, and sudden and excessive rise in lactate dehydrogenase (LDH) levels. However, the presence of one or more of these clinical clues of transformation is not specific for RS. Indeed, only 50–60 % of CLL patients presenting with clinical features suspicious of RS receive a final histologic diagnosis of transformation, while in the remaining cases the histopathologic assessment of the biopsy results in the diagnosis of CLL, prolymphocytic evolution, or even solid cancer [24, 47]. Therefore, histologic documentation by open biopsy is mandatory for diagnosing RS, which otherwise can only be clinically suspected, but not proven.

Fine needle biopsy or aspiration may not be acceptable for reaching the diagnosis of RS. In fact, the samples obtained with these techniques may not be entirely representative of the pathologic architecture of the tumor, especially in cases where the sheets of transformation are admixed to small cells, thus resulting in false negative results. In addition, fine needle biopsy or aspiration of an enlarged proliferation center, which may be observed in a fraction of CLL, may give rise to false positive misdiagnosis of RS transformation.

In some patients, transformation from CLL to RS does not occur simultaneously at all sites, but, at a certain timepoint, might be restricted to one single lesion, either nodal or extranodal. From a practical standpoint, this knowledge mandates that any biopsy aimed at exploring whether RS has occurred should be directed at the index lesion, i.e., the lesion displaying the largest diameter by imaging or the most rapid kinetics of progression.

In the presence of clinical features suspicious of RS, the ^{18}FDG PET/CT characteristics of the lesion, in particular the standardized uptake value (SUV), may guide the choice of whether to perform a biopsy and which site to choose for the biopsy, since sites affected by RS are expected to have SUVs overlapping those of de novo DLBCL [47]. A SUV greater than 5 has a high sensitivity (91 %) for detecting RS transformation, though its specificity is relatively low (80 %), since it may also highlight lymph nodes with expanded proliferation centers or metastases of solid tumors. Consistently, the main value of ^{18}FDG PET/CT in RS diagnosis relies on its high (97 %) negative predictive value, meaning that in the presence of a negative ^{18}FDG PET/CT, the final probability of biopsy proven RS transformation would be only 3 %. Based on these notions, in the presence of a negative ^{18}FDG PET/CT, an open biopsy may be avoided, especially if the index lesion is located in a profound and poorly accessible anatomical site [47].

Prognosis of Richter Syndrome

Overall, the prognosis of DLBCL transformation is generally highly unfavorable as documented by a median survival after transformation ranging from 8 to 16 months in the two largest available series [11, 22]. However, prognosis is not uniformly poor among transformed patients and may be predicted on clinical and biological grounds. An RS prognostic score based on five adverse risk factors (Zubrod performance status >1, elevated LDH levels, platelet count $\leq$100,000, tumor size $\geq$5 cm, and more than two prior lines of therapy) helps in predicting survival once that transformation has occurred [22]. Patients are assigned by the RS prognostic score to one of four risk groups based on number of presenting risk factors: 0 or 1, low risk (median survival: 13 months); 2, low-intermediate risk (median survival: 11 months); 3, high-intermediate risk (median survival: 4 months); 4 or 5, high risk (median survival: 1 month) [22].

Remarkably, risk factors that are relevant to the International Prognostic Index for de novo DLBCL, namely, number of extranodal sites of disease, age, and stage, were not relevant to the RS score, confirming the notion that DLBCL transformation and de novo DLBCL are very different diseases [22]. In contrast, survival prognostication after DLBCL transformation takes advantage of parameters reflecting bone marrow failure, such as thrombocytopenia, and parameters reflecting immune system exhaustion and selective pressure to chemorefractory clones, such as number of prior lines of treatment [22].

In addition to the clinical risk factors included in the RS score, survival post-transformation may also be predicted by the tumor genotype and by the clonal

relationship of the transformed phase with the preexisting CLL clone [11]. *TP53* status, response to treatment, and ECOG performance status (ECOG PS) were used to build an algorithm for classifying DLBCL transformed patients according to risk of death [11]. Low-risk patients who presented with a good ECOG PS $\leq$1, had no *TP53* disruption, and achieved CR after RS induction treatment displayed a low risk of death (median survival: not reached; 5-year survival: 70 %). Intermediate-risk patients presenting with a good ECOG PS $\leq$1, but harboring *TP53* disruption or not achieving CR after treatment despite being *TP53* wild type, had an intermediate risk of death (median survival: 24 months). Patients belonging to the high-risk category, marked by ECOG PS >1, show a very poor outcome, with a median survival of only 4 months [11]. Indeed, in these patients, the application of aggressive treatment is frequently hampered by patient frailty due to disease aggressiveness and to the sequelae of multiple lines of ineffective treatment that have a negative impact on performance status.

Though included among the various types of RS transformation, the biology and clinical course of clonally unrelated DLBCL significantly differ from those of clonally related cases. Despite similar clinical features at presentation, clonally unrelated DLBCL is characterized by a significantly longer survival (median: 62 months), that is in the range of de novo DLBCL, compared to clonally related cases (median: 14 months) [11]. Such differences in clinical outcome between clonally related and clonally unrelated DLBCL reflect differences in the genetics of the disease. Indeed, the prevalence of *TP53* disruption in clonally unrelated DLBCL is low (~20 %) and overall is similar to that of de novo DLBCL. Also, stereotyped VH CDR3, an immunogenetic feature that is frequent in clonally related DLBCL transformation (~50 % of cases) but very rare in de novo DLBCL, is virtually absent in clonally unrelated DLBCL [11]. Overall, these observations suggest that clonally unrelated DLBCL should be considered, and probably managed, as a secondary DLBCL arising de novo in the context of CLL, rather than a true RS transformation.

In the HL variant of RS, the median survival after transformation ranges from 10 to 20 months in the few available series [38, 39]. Consistent with this poor outcome, virtually all HL-transformed patients have features of high-risk disease according to the International Prognostic Index for de novo HL. The RS score, which has been developed for DLBCL transformation, is not useful to stratify the outcome of HL transformation [38].

Treatment of Richter Syndrome

Regimens that have been utilized for the treatment of DLBCL transformation reflect the evolution of therapy observed in the last few years in the management of aggressive non-Hodgkin lymphoma (Table 8.2).

Despite their promising activity in terms of complete response (CR) rate, regimens designed for the treatment of highly aggressive lymphomas are severely

Table 8.2 Summary of the published induction regimens for Richter syndrome[a]

References	Study design	Patients	RS variant	Regimen	ORR (%)	CR (%)	PFS/FFS (months)	OS (months)	Neutropenia (grade 3–4) (%)	Thrombocytopenia (grade 3–4) (%)	Infection (grade 3–4) (%)	TRM (%)
Dabaja et al. [48]	Clinical trial	26	DLBCL	Hyper-CVXD	41	38	na	10	100	79	39	14
Tsimberidou et al. [50]	Clinical trial	16	DLBCL	FACPGM	6	6	1	10	90	83	55	18
Tsimberidou et al. [49]	Clinical trial	30	DLBCL	R + hyper-CVXD + GM-CSF/R + HDM-ara-C + GM-CSF	43	27	na	8	100	40	39	22
Tsimberidou et al. [51]	Clinical trial	7	DLBCL	^{90}Y ibritumomab tiuxetan	0	0	1	na	29	71	14	0
Tsimberidou et al. [53]	Clinical trial	35	DLBCL	OFAR	40	7	3	8	67	74	19	7
Jenke et al. [52]	Clinical trial	15	DLBCL	R-CHOP	67	7	15	27	55	65	28	3
Bockorny et al. [39]	Retrospective	67	HL	ABVD (31 %), MOPP (16 %), CHOP (13 %), other (40 %)	52	27	12	20	na	na	na	na

[a]*RS* Richter syndrome; *ORR* overall response rate; *CR* complete response rate; *PFS* progression-free survival; *FFS* failure-free survival; *OS* overall survival; *TRM* treatment-related mortality; *DLBCL* diffuse large B-cell lymphoma; *HL* Hodgkin lymphoma; *hyper-CVXD* fractionated cyclophosphamide, vincristine, liposomal daunorubicin, and dexamethasone; *R+hyper-CVXD+GM-CSF/R+HDM-ara-C+ GM-CSF* rituximab, fractionated cyclophosphamide, vincristine, liposomal daunorubicin, dexamethasone, and granulocyte-macrophage colony-stimulating factor alternating with rituximab, methotrexate, ara-C, and granulocyte-macrophage colony-stimulating factor; *FACPGM* fludarabine, ara-C, cyclophosphamide, cisplatin, and granulocyte-macrophage colony-stimulating factor; *OFAR* oxaliplatin, fludarabine, ara-C, and rituximab; *R-CHOP* rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone; *ABVD* adriamycin, bleomycin, vinblastine, and dacarbazine; *MOPP* mechlorethamine, vincristine, procarbazine, and prednisone; *CHOP* cyclophosphamide, doxorubicin, vincristine, and prednisone; *na* not available

toxic in RS patients. The hyper-CVAD, a fractioned cyclophosphamide, vincristine, doxorubicin, and dexamethasone regimen, induced a response in 41 % (CR 38 %) of patients with DLBCL transformation, but the median overall survival was only 10 months [48]. This aggressive regimen was invariably complicated by severe hematotoxicity in all cases, translating into a high severe infection rate of 50 % and a treatment-related mortality of 14 % [48]. Combination of hyper-CVAD alternating with methotrexate and ara-C regimen with rituximab resulted in a response rate of 43 % (CR 38 %), but the median overall survival was 8 months [49]. This combination was highly toxic (severe hematotoxicity in all cases, severe infection rate of 39 %, treatment-related mortality of 22 %) despite the prophylaxis with granulocyte-macrophage colony-stimulating factor (GM-CSF) [49] (Table 8.2).

The combination of fludarabine, ara-C, cyclophosphamide, cisplatin, and GM-CSF (FACPGM) has been reported to have limited activity (response rate of 5 %, complete response rate of 5 %) and significant toxicity (severe hematotoxicity in 90 % cases, infection rate of 55 %, treatment-related mortality of 18 %) in DLBCL transformation [50] (Table 8.2).

Though ^{90}Y ibritumomab tiutexan is active in transformed follicular lymphoma, no responses have been documented in RS patients treated with radioimmunotherapy [51] (Table 8.2).

Less intensive chemotherapy regimens provide a good balance between activity and toxicity. R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone) resulted in a response rate of 76 % (CR 7 %) in patients with DLBCL transformation, and the median overall survival was 27 months [52]. Treatment-related mortality was low (3 %). Hematotoxicity was documented in 65 % of patients. Infections were the most common severe non-hematologic toxicity and occurred in 28 % of patients [52] (Table 8.2). The OFAR (oxaliplatin, fludarabine, ara-C, and rituximab) regimen induced a response rate of 40 % (CR 7 %), with acceptable toxicity (severe hematotoxicity in 74 % of cases, severe infection rate of 19 %, treatment-related mortality of 7 %) [53] (Table 8.2). Consistent with these results, rituximab-containing regimens, namely, R-CHOP or OFAR, currently represent the backbone for induction treatment in patients with DLBCL transformation.

Though the response rate obtained with R-CHOP and OFAR is relatively high, the long-term outcome achieved by these regimens is not satisfactory, as documented by the short progression-free survival (3–15 months) [52, 53]. On these bases, the European Group for Blood and Marrow Transplantation has retrospectively investigated the role of stem cell transplant (SCT) as post-remission therapy in RS [54] (Table 8.3). By this analysis, the outcome of patients who underwent both allogeneic and autologous SCT was encouraging. At 3 years, relapse-free survival was 27 % after allogeneic SCT and 45 % after autologous SCT. The non-relapse mortality at 3 years was 26 % after allogeneic SCT and 12 % after autologous SCT. Survival at 3 years was 36 % after allogeneic SCT and 59 % after autologous SCT. Disease activity at SCT was the main factor influencing the post-transplant outcome. Indeed, patients who underwent SCT with chemotherapy-sensitive RS had a superior survival compared with those who underwent transplantation with active and progressive disease. The major benefit

Table 8.3 Stem cell transplant in Richter syndrome[a]

References	Patients	Age <60 years (%)	Transplant	CR/PR at transplant (%)	RIC (%)	VUD (%)	3-year relapse (%)	3-year RFS (%)	NRM (%)	3-year OS (%)	Prognostic factors
Tsimberidou et al. [22]	17	52	Allogeneic	41	na	52	na	na	na	75 (if remission at transplant)	Remission at transplant
Cwynarski et al. [54]	25	60	Allogeneic	60	72	44	47	27	26	36	Remission at transplant Age <60 years RIC
Cwynarski et al. [54]	34	65	Autologous	82	–	–	43	45	12	59	None

[a]*CR* complete response, *PR* partial response, *RIC* reduced intensity conditioning, *VUD* volunteer unrelated donor, *RFS* relapse-free survival, *NRM* non-relapse mortality, *OS* overall survival

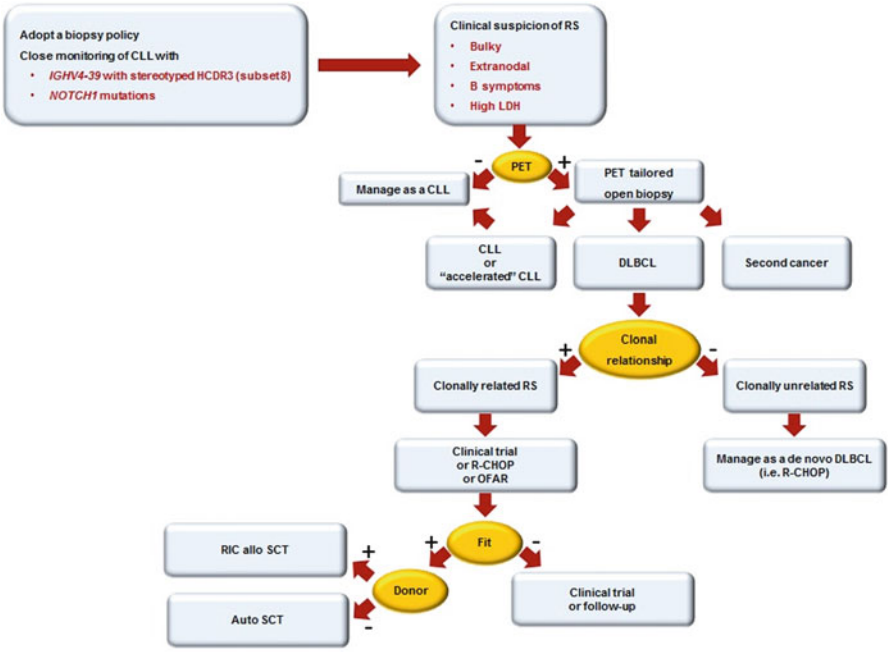

Fig. 8.2 Suggested management of patients with the DLBCL variant of Richter syndrome

of SCT was obtained in young (<60 years) patients. Among patients receiving allogeneic SCT, those conditioned with a reduced intensity regimen had the longest survival. Overall, these data suggest that both autologous SCT and reduced intensity conditioning allogeneic SCT can be effective in young patients with transformed CLL as long as a status of chemosensitivity is maintained [54].

Data from the limited series of patients so far analyzed suggest that the treatment of patients who have HL transformation is challenging (Table 8.2). The current results suggest that HL-type multiagent chemotherapy, such as ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine), is effective for response induction (response rate of ~40–50 %), but patients eventually develop recurrent disease after a short period of time [38, 39]. On these bases, SCT may be considered for patients who respond to chemotherapy.

Suggested Management of Richter Syndrome

RS remains one of the major challenges in the management of CLL patients. Current guidelines for CLL lack specific recommendations for RS, and based on the presently available published data, mostly derived from retrospective studies, it is difficult to propose a standard and optimized approach for these patients. However, at least in the setting of the DLBCL variant of RS, some suggestions can be made based on the available literature (Fig. 8.2): (1) adopt a biopsy policy

for CLL patients; (2) carefully monitor CLL cases harboring risk factors for RS transformation; (3) in the presence of a clinical suspicion of RS transformation, perform a ^{18}FDG PET/CT and tailor the open biopsy of the index lesion according to its results; (4) if the biopsy reveals an aggressive lymphoma, establish its clonal relationship with CLL by *IGHV-D-J* rearrangement analysis; (5) if the CLL and RS are clonally unrelated, treat the disease as a de novo DLBCL; and (6) if the CLL and RS are clonally related, encourage participation in prospective studies. If such studies are not available, the following approach can be considered: (1) treat with an induction regimen, namely, R-CHOP or alternatively OFAR in case the patient had received prior anthracycline-containing chemotherapy; and (2) consolidate young and fit patients with autologous or reduced intensity conditioned allogeneic stem cell transplant.

Conclusions

Advancements during the last few years have revitalized interest in Richter syndrome and on our ability to predict the development of this clinical condition. A limited but growing number of molecular and phenotypic markers have been identified that may facilitate the identification of CLL patients who are at risk of RS development at some point during the natural history of their disease. However, even with the available clinical and molecular data, we are still unable to identify at diagnosis all patients who are destined to transform.

We also lack the tools to manage this complication, either at CLL diagnosis to prevent its development in susceptible patients or when RS arises. A better understanding of the pathogenesis of RS and unraveling the mechanisms leading to transformation may provide a new rationale for the design of targeted approaches to RS treatment. In this respect, given the availability of inhibitory drugs in clinical and preclinical development, NOTCH and MYC signaling represent promising targets for molecular therapy in RS [30, 55]. In addition, in the changing scenario of CLL treatment [56, 57], it is currently unknown whether B-cell receptor signaling kinase inhibitors may be helpful to prevent RS development in patients at high risk.

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Chapter 9
Molecular Biomarkers in Chronic Lymphocytic Leukemia

Sami Malek

Abstract Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western World and remains incurable with conventional chemotherapy treatment approaches. CLL has a highly varied clinical course. The substantial clinical variability in the clinical course of CLL has motivated intense efforts at identifying molecular markers that can be used for CLL prognostication. While many such markers have been proposed, few have stood the test of time; this is due to various reasons outlined in detail in this chapter.

Of the reasons that have affected the usefulness and broad applicability of CLL biomarkers a few stand out as recurrent: lack of *independent* effects of individual markers on prognosis; the use of arbitrary cutoffs when using continuous variables; technical challenges in validity, reproducibility, and reliability (classical test characteristics); and lack of marker validation in prospectively identified CLL patient cohorts.

Nonetheless, a few useful prognostic markers (CLL interphase FISH, immunoglobulin heavy chain variable region mutation status) have been identified, and others are still in transition to widespread clinical applications (*TP53* mutations, SNP array-based elevated genomic complexity).

As CLL therapy transitions from genotoxic combination therapies to targeted therapies, it will be of importance to reestablish the usefulness of our current understanding of individual CLL traits in CLL prognosis. Finally, the identification of predictive markers remains important given the established associations of poor response rates with shortened survival and the ongoing need for more personalized approaches in CLL management.

S. Malek, M.D. (✉)
Department of Internal Medicine, Division of Hematology and Oncology, University of Michigan, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0936, USA
e-mail: smalek@med.umich.edu

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Introduction: The Conceptual Framework for CLL Biomarkers

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western World and remains incurable with conventional chemotherapy treatment approaches. CLL is a heterogeneous disease that is diagnosed by a parsimonious set of criteria, and it is likely that the term CLL constitutes an umbrella for multiple related illnesses [1, 2]. The heterogeneity in CLL biology and possible cell of origin is reflected in large patient-to-patient variations in CLL clinical presentations, including anatomic sites of disease, CLL-associated symptoms, speed of disease progression, response to therapies, and ultimately, survival times from diagnosis [3].

Given that physicians that attend to patients with CLL are challenged by the substantial heterogeneity of CLL clinical presentations and given the chronic nature of this illness, many are interested in identifying measurable CLL characteristics that could be used to more accurately counsel and manage CLL patients [4, 5]. Such counseling may include a prediction of the time from diagnosis to need of first therapy (TTFT); the anticipated remission duration, or, alternatively, the time to subsequent therapy (TTST) following each of a number of standard therapies; the fraction of patients responding to standard therapies; and anticipated overall survival (defined as either the time from diagnosis to death or from the beginning or end of first therapy to death). Complicating such justified goals are numerous challenges that are both conceptual and technical in nature that have had a substantial negative impact on the widespread use and the usefulness and applicability of biomarkers in CLL.

In this chapter, I shall briefly describe qualities of an ideal fictive CLL biomarker, followed by an in-depth discussion of the various limitations of biomarkers and biomarker research in CLL in general. These analyses will be followed by reviews of specific biomarkers in CLL and summarized in a small set of practical recommendations for future research and clinical applications.

Characteristics of a Perfect Fictive CLL Biomarker

As outlined above, a perfect CLL biomarker should inform the physician and the patient accurately about the length of time from diagnosis to first therapy, the anticipated remission duration following standard therapies, the anticipated fraction of responders to individual therapies, and overall survival. A perfect CLL biomarker should therefore have many of the characteristics listed here:

1. It should be measurable in cells or components of peripheral blood.
2. It should identify a subset of CLL that constitutes a substantial minority of all CLL.

3. It should be qualitative (all or none) and not quantitative (continuous variable).
4. The techniques used to measure the biomarker should fulfill classical test characteristics of reliability, reproducibility, and validity and should be widely available.
5. The presence or absence of the biomarker should predict for large differences in TTFT, TTST, or OS.
6. The presence or absence of the biomarker should be truly predictive or prognostic for individual patients most of the time—not just for CLL patient cohorts.
7. The predictive or prognostic effects of a perfect biomarker should not be substantially modified by the presence of other variables.
8. The output of a biomarker test should directly inform the physician and patient without need for further in-depth data review.
9. The biomarker was developed in large CLL cohorts and was validated in large CLL cohorts.
10. The biomarker was measured and developed in blood samples that were procured from CLL patients as close as possible to the CLL diagnosis date.
11. The biomarker was developed in CLL cohorts that were treated with contemporary treatment regimens.
12. The biomarker represents a biological mechanism that is directly involved in CLL cell proliferation, clonal diversification, apoptosis, or chemotherapy resistance.

It should be clear from the review of the characteristics of such a perfect biomarker that the current set of CLL biomarkers does not fulfill many of these criteria; therefore substantial technical and clinical knowledge by the treating physician is required to justify and allow for judicious clinical use of existing biomarkers in CLL.

Limitations of Current CLL Biomarkers

There are numerous limitations that partially negate the usefulness of current biomarkers in CLL; these are discussed in detail here and echoed again in subsequent sections. The purpose of such an in-depth critique is not to invalidate the judicious use of select markers but rather to inform the practitioner and to stimulate future research. Furthermore, it is of great importance that knowledge of a biomarker status in individual patients be integrated with the overall clinical situation and not be used alone for therapeutic or prognostic purposes.

Often confused is the distinction between a valuable CLL biomarker and the molecular mechanisms that influence various CLL properties that are associated with disease progression or characteristics of aggressive CLL. Many factors influence the latter and it is important to identify such factors to better understand CLL, but few of these are suitable as a biomarker that can clearly assist prediction of future events in individual CLL patients.

Limitation #1: *The biomarker test output is not all or none but reflects a continuum of values* (*continuous variable*). This is the most common of all scenarios and if stringently defined is true for all available biomarkers. The problem is that the degree of marker presence is not paired with an accurate clinical outcome estimate for the individual patient's degree of marker presence. Instead, in clinical practice, discrete cutoffs for marker positivity based on thresholds are used, paired with mean or median values for TTFT, TTST, or OS that are derived from outcome analysis of dichotomized CLL patient cohorts. This common practice results in substantial inaccuracies in patient counseling and prognostication. For instance, the dichotomized use of FACS-based ZAP-70 measurements as positive and negative (more or less than 20 % of CLL B-cells positive) defies the fact that this kinase is expressed anywhere from 0 to 100 % in CLL cells from individual patients and, importantly, that the CLL disease progression risk associated with increased expression does not demonstrate a discrete jump at a specific ZAP-70 expression value [6]. Therefore, classifying patients as ZAP-70 negative if ZAP-70 is between 0 and 20 % and positive if ZAP-70 is $\geq$20–100 % substantially overemphasizes the prognostic usefulness and precision of this marker for individuals as compared with cohorts.

For example, what is the TTFT for a patient with 15 % ZAP-70 positive CLL cells? Is it closer to the mean TTFT for the ZAP-70 negative (0–20 %) cohort or is it in fact closer to the mean TTFT of the ZAP-70 positive ($\geq$20–100 %) cohort? One way of addressing this analytical deficiency is by continuous modeling of multiplicative increases in risk for any unit increase in marker positivity. However, translating such knowledge into direct patient care is unwieldy. Another way of addressing this issue is by partitioning a large CLL cohort into many nonoverlapping but contiguous subgroups (for instance, ZAP-70 positivity of 0–5 %, 5.1–10 %, 10.1–15 %, …, 95.1–100 %) and to determine TTFT or OS for each subgroup. Such analyses would need a very large CLL discovery and validation cohort to be precise, and this has not been performed. Importantly, the issue of lack of prognostic precision for individuals is present in most if not all commonly discussed CLL biomarkers.

Biologically, the degree of biomarker presence can be a reflection of the size of subclones carrying the marker (true for recurrent CLL-associated gene mutations and CLL-associated genomic copy number aberrations), CLL cell population heterogeneity (CD38 and ZAP-70), or true inter-patient differences in the value for a marker like IgV_H mutation status, which is usually fully clonally represented in the majority of cases.

Limitation #2: *The prognostic or predictive effect of a biomarker is not independent of other markers or biological CLL traits*. All biomarkers in CLL, when analyzed in isolation, predict for survival differences of CLL cohorts. Usually, CLL cohorts are stratified by biomarker presence or absence or by marker values above or below thresholds optimized for maximal outcome differences, and outcome is determined. Importantly however, such an analysis alone does not provide information that invariably would provide prognostic value for individual patients, and therefore is of limited usefulness in the clinical setting.

The problem can be highlighted in the following example: assume a subgroup of CLL patients with a positive marker A status partially encompasses or overlaps

another CLL subgroup that carries a truly adverse biological trait B that is not measured or cannot be measured at the same time in the same patient. If such a trait B confers most or all of the observed clinical aggressiveness and if it were absent in a proportion of patients positive for the original marker A, then the subset of patients that are truly A+B− (but of unknown B marker status due to lack of measurements) would be falsely assigned to and counseled on increased risk based on the measurements of marker A. The reverse can also be true: If the presence of a marker C in cohorts identifies good risk but occasionally occurs in a subset of cases that are also B+, then measurements of C alone would miss the adverse prognosis for individual C+B+ patients, and counseling would be falsely reassuring. Similar but more complicated scenarios can be envisioned for >2 partially coexisting markers and for markers in which the good or poor effects on CLL outcome are dosage-dependent, and such scenarios do exist in the real world CLL setting.

To improve the accuracy of using any CLL biomarker test result for individuals, it is therefore important that a biomarker during preclinical development be subjected to appropriate multivariate analyses, inclusive of all other important CLL biomarkers, and that established biomarkers be retested in such models once new potentially important markers are identified. It is only in multivariate analysis that independence of effects of individual markers from other markers can be ascertained. Of course, such analyses rely on measurements of markers in the same biological specimens and rely on clinical outcome databases that are as free as possible from various biases.

Limitation #3: *Biomarkers demonstrate interactions that cannot be or have not been quantified and that cannot easily be translated into patient counseling or treatment decisions*. In proportional hazard modeling an interaction between markers may be present if the hazard associated with the presence of two or more markers substantially deviates from the product of the individual hazards. Such a statistical interaction, which is rarely measured in biomarker development (as very large patient numbers are needed to accurately measure such interactions), may be due to true biological interactions. One example of such an interaction is the observed modification of clinical aggressiveness of CLL with del17p or elevated SNP 6.0 array profiling-based genomic complexity by the IgV_H status of a patient, as outlined below. Importantly, for none of the currently employed CLL biomarkers have formal assessments of statistical interactions been published. This shortcoming complicates biomarker development in CLL in general as well as prognostication for individual CLL patients in which multiple marker determinations are available or in patients in which a marker profile is discordant (comprising good and bad prognostic marker values).

Limitation #4: *The biomarker status is not stable over time* (*longitudinal instability*). Ideally, a prognostic biomarker test is performed on the date of CLL diagnosis while a predictive biomarker test is performed prior to initiation of therapy. However, clinical reality sometimes necessitates biomarker measurements at times that deviate from these ideals. While it is rarely advisable to measure a

CLL biomarker in the relapse setting, where most markers lack confirmed value, it may occasionally be attempted to measure a marker after diagnosis but prior to first therapy. However, if a biomarker is not stable in longitudinal CLL samples (for biological reasons—not just for technical reasons) then a measurement at a time other than the diagnosis date may provide false prognostic information.

Limitation #5: *The biomarker was developed in a patient population that is not reflective of current populations or current individual patients or current therapies*. The therapeutic approach to CLL patients in need of therapy is changing. Frontline therapies for medically fit and young patients (<70 years) usually comprise chemoimmunotherapy (a monoclonal antibody together with purine analogues and/or alkylators) while the treatment for older individuals is tailored to the outcome of a risk—benefit assessment (therapy potency vs. side effects). Furthermore, the therapeutic armamentarium is widening, and novel and non-genotoxic approaches are increasingly employed. Finally, CLL patients with short remission durations following potent frontline therapies or del17p status detection are increasingly referred for allo-RIC-BMT as consolidation. Why is this important? It is important because therapy prolongs the survival of patients with CLL as opposed to no therapy, and there is evidence that more potent therapies and changes in medical supportive care have improved the survival of CLL patient cohorts over the last decades [7].

If a biomarker was not developed within the setting of contemporary therapies, then results from such a marker may not accurately predict outcome in patients treated with therapies that differ. Such a scenario applies in particular to markers that are used for prediction and prognostication, such as CLL FISH results and the mutation state of selected genes like *TP53* or *ATM*.

Limitation #6: *The biomarker test is not standardized and does not fulfill classic test criteria of reliability*, *reproducibility, and validity*. A biomarker test needs to be reliable, reproducible, and valid. Surprisingly, many CLL biomarkers do not fulfill these basic test criteria. Complicating matters is the fact that many laboratories provide test results without attempts at national or international standardizations. While the lack of reliable, reproducible, and valid assessment of ZAP-70 expression by FACS is well published, even testing for genomic aberrations by FISH can lead to false information. This arises as a result of operator inexperience, lack of controls, cell preparations, and differences in probe designs. CLL karyotyping is equally unreliable, and even gene mutation testing is not standardized and is currently performed with many different techniques. It is therefore of utmost importance that the ordering physician knows where the tests are performed and that only laboratories with high national standing and rigorous QC procedures perform testing.

What Is the Purpose of Using a Biomarker in CLL?

What CLL disease phase needs to be informed by a biomarker? The longitudinal clinical CLL dynamics can be conveniently partitioned into various disease phases [4]. Phase 1 can be defined as the time from CLL diagnosis to initiation of

first therapy (TTFT). Phase 1 is not altered by a therapeutic intervention and equals the first part of the natural history of the disease. Phase 1 in CLL is very variable and can last from months to decades. One of the most important clinical determinants of the length of phase 1 is the Rai stage at presentation: the higher the stage the shorter phase 1, with the caveat that Rai stage 4 (CLL with thrombocytopenia) is an outdated category and at times results in unnecessary therapeutic interventions [8]. As all CLL is preceded by monoclonal B-cell lymphocytosis (MBL), it is clear that a new CLL diagnosis at a Rai stage other than zero constitutes a delay in diagnosis, and as such a Rai stage of 1–4 is associated with a lead time bias and a biased shortening of phase 1 [9, 10]. The doubling time of the absolute lymphocyte count (ALC-DT) correlates inversely, albeit not linearly, with phase 1, but there is more than one discrete cutoff of ALC-DT in clinical use (<6–12 months is clinically used most often). Limiting the usefulness of these two clinical prognosticators is the fact that 90 % of all CLL are Rai stage 0–1 at diagnosis and that the ALC-DT needs longitudinal data that are not available at first or second visit (and that the ALC-DT may be modified by interventions unrelated to CLL).

Accurately predicting the length of phase 1 or TTFT is of clinical importance, as many patients wish to know what the diagnosis of CLL means to them and at what time a chemotherapeutic intervention may become necessary. Multiple biomarkers in CLL can inform phase 1, and this is one of the best characterized indications for biomarker use in CLL in general.

CLL phase 2 could be defined as the remission duration following various frontline CLL therapies. Phase 2 is therapy type-dependent, and even within a uniform therapy is dependent on CLL characteristics. For instance, one of the best predictors of a short phase 2 is the presence of *TP53* mutations/del17p or elevated SNP 6.0 array profiling-based genomic complexity. The presence of del11q or ATM dysfunction also predicts for short phase 2, but this effect is less evident in patients treated with modern chemoimmunotherapy programs (see below).

CLL phase 3 may be defined as the time from the end of frontline therapy to the patient's death. This disease phase is influenced by the length of remission durations as well as success of salvage therapies. The mean length of phase 3 is 7–8 years but substantial inter-patient variability exists. One of the best predictors of short phase 3 is the presence of a *TP53* mutation/del17p or elevated SNP 6.0 array profiling-based genomic complexity.

A Discussion of Specific CLL Biomarkers

CLL interphase FISH testing. CLL interphase FISH testing is in widespread clinical use in CLL. This test was developed to overcome difficulties in CLL karyotyping and detects the most frequent recurrent genomic copy number aberrations in CLL; these are, in order of descending frequency, del13q14 (~50 %), trisomy 12 (15–18 %), del11q (10–15 %), and del17p (7–10 %) [11–13]. Adaptations of CLL FISH tests also detect some Ig-translocations: for instance, t(14;18) and

t(14;19), and t(11;14), which is characteristic of mantle cell lymphoma. The CLL FISH test is commercially available but may produce false results if performed in inexperienced labs. For an in-depth discussion of the biology of CLL-associated acquired copy number aberrations that are detected by CLL FISH, the reader is referred to reference [14].

Historically, the importance of CLL FISH resulted from the analysis of the survival of a CLL patient cohort stratified by CLL FISH categories that was treated with chemotherapy typical for the early to mid-1990s [11]. Clear differences in survival were noted, with the shortest survival identified for del17p, followed by del11q, trisomy 12, and either sole del13q14 or normal FISH. Identification of the risk associated with del17p has subsequently resulted in the development of risk-adapted therapies in CLL and early use of RIC-allo-Tx [15–17].

However, with changes in CLL therapy and a better understanding of other factors that influence CLL outcome, the role of CLL FISH testing has diminished as the test is less able to clearly separate CLL patient cohorts treated on modern chemoimmunotherapy protocols [18–20]. Furthermore, falsely reassuring CLL FISH results have been identified (for instance, CLL FISH positive for del13q14 but harboring non-del17p-associated *TP53* mutations, or CLL FISH del13q14 harboring multiple additional second-tier aCNAs) and for the majority of CLL patients that lack either del17p or del11q (~80–85 % of all patients), little actionable information is gained from knowledge of CLL FISH.

CLL FISH by definition misses all aCNAs that are not interrogated by the commonly used FISH probes. Although the individual frequency of such second tier aCNAs is <5 %, the total number of all aCNAs in any given CLL patient is strongly negatively associated with patient survival. CLL which lacks del17p or del11q but carries a high aCNA load is clinically aggressive and is almost as frequent as CLL with del17p or del11q; this high-complexity CLL subgroup is missed by CLL FISH [21]. Finally, even del17p and del11q patients can be further stratified into lower-risk and higher-risk subgroups based on the total number of aCNAs per patient.

Of additional importance is the fact that CLL patients can acquire genomic aberrations over time, which negatively changes the outlook for such patients [13, 22, 23]. While few quantitative data are available, clonal evolution (CE) occurs in a substantial minority of patients, and more research on this subject is needed before the full impact of CE in CLL is appreciated. Given that higher-risk FISH abnormalities, including del17p and del11q, can be acquired in CLL patients with seemingly indolent FISH findings at diagnosis, some have advocated for repeat FISH testing in the CLL relapse setting. The practical importance of such longitudinal CLL FISH testing remains to be fully explored, as relapsed CLL in general is more aggressive than untreated CLL.

From a practical perspective, what can be learned from a CLL FISH test result performed in a newly diagnosed CLL patient in the year 2012? The del17p is recognized as the most important finding and continues to identify a small CLL subset with short mean survival times of 5–8 years from diagnosis [22]. However, substantial inter-patient variability exists [24–26]. Del17p patients lack a standard

of care but are prime candidates for non-genotoxic treatments or clinical trials [17, 27]. The presence of del11q is always associated with progressive disease (short phase 1): a phenomenon that is due to the almost invariate presence of unmutated IgV_H genes, high insulin receptor levels (INSR), and high TCL1 levels [28, 29]. Importantly, pending publication of data from large clinical trials, a de facto standard of care for patients with del11q has been adopted: this involves inclusion of an alkylator into the frontline CIT protocol of these patients (most commonly used is the FCR regimen for young and medically fit patients) [20, 30]. Patients with isolated trisomy 12 are neither high risk nor intermediate risk, and in fact respond well to CIT with above-average remission durations. For these patients that are almost always chemosensitive, a de-escalation of therapy to non-FCR-based CIT may be advisable. Finally, del13q14 as detected by FISH is a heterogeneous genomic category (see below) and should not be used alone to counsel patients.

SNP 6.0 array based-profiling of acquired genomic copy number aberrations in CLL. The introduction of SNP arrays to cancer genomics and application of this technology to CLL has resulted in a rather complete description of aCNAs, LOH, and copy neutral LOH (cnLOH) in CLL [21, 31–36]. CLL is characterized by (1) a relatively stable genome (most CLL have 0–2 aCNAs and $\geq$3 aCNAs are detected in ~20 % of all CLL); (2) recurrent second tier aCNAs (distinct from del13q14, del17p, del11q, and trisomy 12) positioned in various locations in the genome with individual frequencies of 1–5 %; (3) a low frequency of cnLOH (detected in sporadic cases and mostly restricted to chromosomes 13 and 17p) [34, 37]; (4) anatomic heterogeneity of del13q14 with monoallelic and biallelic subtypes and, importantly, short and long subtypes [34]; (5) rare clinically adverse cnLOH at 17p associated with homozygous *TP53* mutations [37]; (6) a lack of homozygous genomic deletions other than homozygous del13q14 subtypes; and (7) a lack of high-level genomic amplifications.

From a prognostic perspective the following SNP array-defined CLL subgroups are characterized by shorter survival than various comparator groups: (1) CLL with elevated aCNA counts (elevated genomic complexity) [21, 38], (2) CLL with cnLOH at 17p and *TP53* mutations, and (3) CLL with long as opposed to short del13q14 (del13q14 type II are long and include *RB* and del13q14 type I are short and do not include *RB*) [33, 34, 39, 40].

CLL with elevated genomic complexity ($\geq$3 aCNA) identifies a CLL subgroup comprising 20 % of all CLL and is characterized by short survival [21, 38]. Statistical modeling has identified incremental risk for short OS with each additional aCNA, as evidenced by progressively shorter OS for progressively higher aCNA thresholds. Importantly, in comprehensive multivariate analysis, inclusive of del17p/del11q, *TP53* mutations, IgV_H status, ZAP-70 status, Rai stage, and CD38 status, elevated genomic complexity emerged as the only significant and independent predictor for short OS.

With regards to the underlying mechanisms of the strong negative prognostic effect of elevated genomic complexity on survival in CLL, a strong association has been identified with the presence of *TP53* mutations: 50 % of all CLL with

≥3 aCNA and 33 % of all CLL with ≥2 aCNA harbor *TP53* mutations. Strong associations have also been described with del17p, del11q, and del13q14 type II [33, 35]. However, it is currently unclear, albeit under active investigation, what other gene aberrations and mutations associate with genomic instability in CLL, in particular in the subset that is wild type for *TP53*.

An additional important mechanism relates to the heightened ability of genomically complex CLL to evolve into more aggressive disease, potentially under the influence of chemotherapy. In fact, it is here proposed that such clonal diversity constitutes the major driver of aggressive relapse and the foundation for generation of malignant subclones that ultimately dominate the clinical presentation.

Some caveats regarding the transition of SNP array genomics into clinical care in CLL apply: (1) SNP array profiling, although highly specific, is not sensitive, and genomic lesions need to be present in >25 % of the DNA analyzed to be detected with high sensitivity, and (2) the technique requires sophisticated software and statistical analysis and standardization of such analyses which has not yet occurred. Despite these limitations it is anticipated that SNP array profiling will enter clinical applications soon, due to the superior and unbiased ability to detect aCNAs on a genome-wide scale and the unique ability to identify CLL with elevated genomic complexity.

Stimulated CLL karyotyping. Historically, CLL cell karyotyping was successful only in a minority of CLL cases, and interpretation of results was complicated by a lack of standardization and a lack of clear assignment of results to CLL cells vs. other cell types. More recently, innovative CLL culture conditions (the so-called stimulated CLL karyotypes) have been developed that now allow for generation of karyotypes for almost all cases [41]. Multiple series of stimulated karyotyping of large numbers of CLL cases have been recently published, and concerns about possible artificial induction of genomic lesions in these cultures are likely not relevant [42–47].

Stimulated karyotyping identified complex aberrant karyotypes in ~20 % of all CLL, and such CLL cases demonstrate shortened survival [48]. As expected, small genomic lesions (<5 Mb) are not routinely detected by this method, but large structural aberrations are readily detected. It is likely that a complete genomic characterization of CLL requires a combination of CLL FISH (to detect minor subclones), SNP array profiling for comprehensive genome-wide analysis of aCNA and LOH in dominant clones, and stimulated karyotyping for detection of balanced translocations, isochromosomes, and marker chromosomes [49].

Caveats regarding the routine clinical use of stimulated karyotypes in CLL clinical management are related to (1) variability in interlaboratory test performance and lack of assay standardization; (2) lack of complete separation of genomically high-risk and low-risk CLL using this technique alone due to the inability to comprehensively interrogate for genomic complexity; and (3) lack of published prospective assessments of the prognostic value of this test, including a lack of results from comprehensive multivariate analyses, which are needed to demonstrate independence of effects.

TP53 mutation analysis. Mutations in the *TP53* gene are detected in ~10 % of CLL at diagnosis and at higher frequencies in relapsed CLL [50–52]. The majority of *TP53* mutations are of the missense type and predominantly involve exons 5–9; a small fraction are nonsense, indels, or splice site mutations. *TP53* missense mutations located in the DNA-binding domain result in proteins that accumulate to high levels in the afflicted CLL cell, likely due to the inability of these mutants to induce the expression of MDM2, the major negative TP53 regulator. Mutated p53 proteins are incorporated into tetrameric p53 protein complexes, providing a credible mechanism for the transdominant negative effect of overexpressed mutant p53 proteins. The majority of CLL-associated *TP53* mutations occur on the retained allele in the setting of del17p, which invariably removes one *TP53* gene [37]. A small subset of CLL (anywhere from 1 to 5 %) carries monoallelic *TP53* mutations, and another small subset carries homozygous *TP53* mutations in the setting of cnLOH at 17p [37, 53–56].

TP53 mutations can be detected through PCR amplification of *TP53* exons templated on genomic DNA followed by direct sequencing of PCR products (direct sequencing). *TP53* mutations can also be detected using chromatographic methods, next-generation sequencing methods, indirectly through comparative p53 immunoblotting of cell lysates made from unstimulated and irradiated CLL cells, or p53 immunoblotting of cell lysates made following CLL cell treatment with an MDM2-p53 protein interaction inhibitor and functional assays of the transactivation potential of individual TP53 proteins [37]. The best method for detection of *TP53* mutations has not been established, but direct sequencing or next-gen sequencing will detect the vast majority of mutations, albeit at different levels of sensitivities.

CLL cells isolated from patients with *TP53* mutations are completely resistant to radiation-induced apoptosis ex vivo, and CLL disease with *TP53* mutations rarely responds to chemotherapy to the same degree as CLL disease with wild-type *TP53* [20, 35]. Together, these findings support a critical and nonredundant role for the p53 protein in DNA damage-induced CLL cell death.

Multiple clinical studies and cohort-based observations have demonstrated a negative survival effect of mutated *TP53* in CLL [53–59]. All CLL disease phases outlined above are shorter in *TP53* mutants. CLL patients with *TP53* mutations rarely achieve CR, even with potent up-front CIT regimens, and remission durations are short. Effective salvage therapies other than early use of RIC-allo-Tx are being developed, but it is too early to definitively judge progress in this area [60].

Limiting the transition of *TP53* mutations testing to clinical practice is the lack of assay standardization, despite the absence of major technical impediments. Furthermore, it is unclear if detection of *TP53* mutants in just a few percent of the CLL population, as can be achieved with some assays, is prognostically relevant. It is also unclear if *TP53* mutants other than known hotspot mutations or structurally damaging mutations are functionally relevant, and only large clinical-genomic correlative databases can establish this fact for individual mutations. Finally, even *TP53* polymorphisms may be functionally and prognostically relevant, but little concrete information is available. Practically, many known

cancer-associated *TP53* mutations that adversely affect p53 protein function have been catalogued in international databases (http://www-p53.iarc.fr/), and therefore *TP53* mutation testing in CLL using direct DNA sequencing should result in concrete and useful information for most patients.

Immunoglobulin heavy chain variable region (*IgV_H*) *mutation status*. The somatic mutation status of rearranged immunoglobulin heavy chain variable region genes (IgV_H) is prognostic for disease progression and survival in CLL [61–65]. IgV_H genes may either be 100 % homologous to germline sequences and therefore fully unmutated, or IgV_H genes may harbor somatic mutations. The percent of bases that are mutated may be as low as <1 % to as high as ~15–20 % (corresponding to an IgV_H homology to germline status of >99 % to ~85–80 %). Through correlation of the degree of IgV_H homology to germline (the IgV_H mutation load or status) with CLL patient survival, a cutoff of 98 % was identified that optimally separated the survival of dichotomized CLL patient cohorts. Using the cutoff of ≥98 % IgV_H homology to germline, approximately 40 % of untreated CLL patients are classified as unmutated and higher risk and 60 % are classified as mutated and lower risk.

The methods and procedures to derive an IgV_H status are not standardized, but international collaborations (for instance the European Research Initiative in CLL or ERIC) have published valuable guidelines [66–68]. Critical points to consider are (1) the source of template DNA (genomic DNA or cDNA), (2) the primers used (leader sequence primers vs. framework region 1 primers—the latter result in slightly shorter products and therefore lack nucleotides at the 5′ end of the gene) [69], (3) the sequencing technique (direct sequencing of gel purified PCR products is preferred—sequencing of a sufficiently large number of cloned PCR products is also acceptable and at times required), (4) the choice of database that is used for alignment (international immunogenetics information [IMGT; http://www.imgt.org/], V-base [http://vbase.mrc-cpe.cam.ac.uk/], or GenBank [http://www.ncbi.nlm.nih.gov/igblast/]), (5) the identification of cases that lack straightforward results (single unproductive rearrangements, double rearrangements with discordant results, cases with missing VH CDR3 anchors), and (6) the prognostic interpretation of results close to the cutoff of 98 %. Testing is best done in experienced labs that have rigorous quality controls in place.

The IgV_H status categorizes a substantial minority of CLL patients as higher risk. Importantly, the IgV_H status demarcates a CLL patient subset that is heterogeneous, and it encompasses smaller or overlapping CLL subsets that are delineated by other traits (for instance, >90 % of CLL with del11q are IgV_H status unmutated, and >80 % of IgV_H status unmutated CLL are also ZAP-70 positive). In multivariate analysis incorporating widely available factors, including clinical factors, IgV_H status unmutated is often identified as an independent predictor of shorter OS. However, given that the two most powerful predictors of shorter OS in CLL, *TP53* mutations and SNP 6.0 array-based genomic complexity, are usually not included in these analyses, it remains uncertain how much of the IgV_H status effect is independent. For instance, recent comprehensive multivariate analyses did not

identify IgV_H status unmutated as an independent prognostic factor for shorter OS, although it is not excluded that analyses of much larger CLL cohorts may still uncover such effects [21].

Mechanistically, it is not fully understood why IgV_H status unmutated CLL are more progressive or aggressive. While work has focused on the B-cell antigen receptor responsiveness to agents that mimic antigen and greater degrees of such responsiveness are observed in IgV_H status unmutated vs. mutated CLL, the mechanistic details of such heightened responsiveness are not worked out [70, 71].

Clinically, the best use of IgV_H status testing is at diagnosis, and the IgV_H status unmutated provides relatively reliable information of short initial disease stability (phase 1 or TTFT of ~3–4 years). Testing for IgV_H status at any other time in the CLL disease course is not informative and should be avoided. IgV_H status is not predictive for response to CIT regimens. One final caveat relates to the more progressive disease course of subsets of CLL with mutated IgV_H status involving VH3-21 [72].

ZAP-70 testing. The degree of expression of zeta chain-associated tyrosine kinase of 70 kD (ZAP-70) in CLL cells is inversely, albeit not linearly, proportional to short initial disease stability (phase 1 or TTFT of ~3 years) [6, 73–75]. ZAP-70 is expressed at varying levels in patients with CLL and at varying levels in CLL cells from individual patients, although formal flow-based single-cell analyses to conclusively prove this point are not available.

Given the lack of definitive information on the clonogenic cell precursors of CLL, it is impossible to judge whether ZAP-70 expression in CLL is aberrant, but it is clear that ZAP-70 is expressed at much higher levels in T-cells as compared with normal B-cells. The high expression in T-cells necessitates careful separation of T-cells from B-cells prior to quantitation of ZAP-70 in CLL cells. ZAP-70 quantitation has been achieved through cell separation followed by immunoblotting for ZAP-70 or by multicolor FACS analysis. Expression of ZAP-70 mRNA has also been measured by Q-PCR following cell separation.

Complicating the widespread clinical usefulness of ZAP-70 measurements is the fact that the available tests lack reliability and reproducibility and are also characterized by interlaboratory variance. These difficulties with accurate measurements of ZAP-70 are likely responsible for some of the discrepant results published on ZAP-70 as a predictor for progressive CLL. Therefore, routine clinical testing for ZAP-70 is not recommended. However, in research labs with tightly controlled assays, ZAP-70 testing can be done, and measurements of ZAP-70 provide a powerful prognosticator of short initial disease stability in CLL. Contrary to frequent written assessments, ZAP-70 is not a strong independent prognostic factor for short OS and also is not predictive of response rates following CIT.

Biologically, ZAP-70 expression in CLL is linked to enhanced B-cell receptor signaling, CLL cell surface receptor signaling in response to contacts in the microenvironment, and CLL cell migration [76–79]. Ultimately, ZAP-70 expression correlates with progressive CLL cell accumulation in patients, which is reflected in progressively increased tumor burden and the need for therapeutic intervention within a few years from diagnosis.

Recently, it has been proposed that the *ZAP-70* gene is differentially methylated in CLL and that the degree of methylation at specific CpG residues correlates with ZAP-70 expression [80]. If confirmed, it may be possible to measure ZAP-70 gene methylation as a surrogate for ZAP-70 expression and to develop clinical assays for ZAP-70 methylation that are more robust than those currently available.

CD38 testing. The degree of expression of CD38 on CLL cells was one of the earlier molecular markers used for prognostication in CLL [81–86]. CD38 can be measured by flow cytometry and is routinely assayed for by some hematopathology labs. Various thresholds for CD38 expression (7–30 %) have been proposed as cutoffs for dichotomization of patient cohorts and prognostication.

However, over the ensuing years various novel findings have questioned the usefulness of CD38 testing in CLL. These include (1) lack of a clear-cut, universally agreed-upon threshold for positivity, (2) longitudinal instability, and (3) lack of independence of prognostic effects when statistically modeled together with ZAP-70 or IgV_H status or the genomic prognostic factors. CD38 constitutes a good example of an important CLL biological factor that nonetheless lacks the characteristics of a good CLL biomarker.

From a practical perspective, a CD38 test result obtained or available for whatever reason should not be ignored, as high expression likely indicates progressive CLL, but in the author's opinion CD38 should not be routinely measured in CLL. However, others have incorporated CD38 measurements into multiparameter prognostic scoring systems for short TTFT and have provided data supporting its usefulness.

Various additional biomarkers in CLL. Many additional biomarkers have been proposed by various research teams as useful prognostic factors in CLL and some are in routine clinical use in some centers. These include measurements of beta2-microglobulin, serum thymidine kinase, various cytokine levels, aberrant interferon signaling, CD49 expression on CLL cells, LPL expression in CLL cells, *ATM* mutations, *NOTCH1* mutations, aberrant microRNA expression, telomere length, and others [28, 87–97]. As few if any of these have been measured in prospectively collected and analyzed CLL patient cohorts, it remains unclear how valuable each individual variable is, especially for the counseling of individual patients as opposed for research on CLL cohorts. Further, given the strong effects of *TP53* mutations or elevated SNP 6.0 array-based genomic complexity on short OS in CLL, multivariate modeling inclusive of these important traits needs to be performed before strong conclusions about these markers can be supported.

Complex prognostic scoring systems. Presumably as a response to some of the challenges in risk prognostication based on novel molecular markers faced by clinicians as outlined above, multiple authors have recently presented data on complex prognostic scoring systems using relatively easily obtainable variables. Analyses are based on the variables age, clinical stage, beta2-microglobulin, ALC, number of involved LN stations, and patient sex [98–102]. These models are improving the ability to predict disease progression in CLL patients presenting

at early clinical stage at diagnosis and therefore are useful additions to CLL prognostication when employed by a physician with experience in CLL management. It is, however, somewhat unlikely that any of these scoring systems can provide precise prognostications for individuals.

A practical albeit imperfect guide to the use of molecular prognostic factors in CLL. Here, I would like to provide a few practical suggestions for the use of individual biomarkers in CLL, realizing that others may derive slightly different conclusions after study of the available data [4]. One also likes to be cognizant of increasing health care costs and the damages inflicted from over-testing.

Routine Molecular CLL Biomarker Tests

CLL FISH: Obtain once at diagnosis or immediately prior to planned therapy, which should only be based on clinical criteria. Consider repeat CLL FISH testing in selected patients, including patients with short remission durations, sudden changes in clinical disease characteristics, or younger individuals.

IgV_H testing: Obtain at diagnosis only if counseling on short vs. long TTFT is important or if a general outlook on disease activity is planned.

ZAP-70 testing: Obtain at diagnosis only and instead of IgV_H if counseling on short vs. long TTFT is important and only if the lab has standardized the assay. Otherwise, do not test [103].

CD38 testing: Do not test but review results if routinely available.

Important Biomarker Tests Not Yet Widely Available

TP53 mutation testing: Obtain at diagnosis or immediately prior to planned therapy, which should only be based on clinical criteria. Consider repeat CLL *TP53* mutation testing in selected patients, including patients with short remission durations, sudden changes in clinical disease characteristics, or younger individuals.

SNP 6.0 array-based genomic complexity: Obtain at diagnosis or immediately prior to planned therapy, which should only be based on clinical criteria. Consider repeat CLL SNP 6.0 array-based genomic complexity testing in selected patients, including patients with short remission durations, sudden changes in clinical disease characteristics, or younger individuals.

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Sami N. Malek wrote the paper.

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Chapter 10
Critical Signal Transduction Pathways in CLL

Asish K. Ghosh and Neil E. Kay

Abstract Receptor tyrosine kinases (RTKs) are cell-surface transmembrane receptors that contain regulated kinase activity within their cytoplasmic domain and play a critical role in signal transduction in both normal and malignant cells. Besides B cell receptor (BCR) signaling in chronic lymphocytic leukemia (CLL), multiple RTKs have been reported to be constitutively active in CLL B cells, resulting in enhanced survival and resistance to apoptosis of the leukemic cells induced by chemotherapeutic agents. In addition to increased plasma levels of various types of cytokines/growth factors in CLL, we and others have detected that CLL B cells spontaneously produce multiple cytokines in vitro which may constitute an autocrine loop of RTK activation on the leukemic B cells. Moreover, aberrant expression and activation of non-RTKs, for example, Src/Syk kinases, induce resistance of the leukemic B cells to therapy. Based on current available knowledge, we detailed the impact of aberrant activities of various RTKs/non-RTKs on CLL B cell survival and the potential of using these signaling components as future therapeutic targets in CLL therapy.

Keywords CLL • Signal transduction • RTK • Non-RTK • Apoptosis • Kinase inhibitor • Therapy

Introduction

While treatment approaches in the past were based on disease control that largely employed single agents that for the most part achieved a chronic indolent disease, treatment goals nowadays are aimed at achieving long-term remissions, at least in

A.K. Ghosh, Ph.D. • N.E. Kay, M.D. (✉)
Division of Hematology, Mayo Clinic, 200 First Street SW,
Rochester, MN 55905, USA
e-mail: Kay.Neil@mayo.edu

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low-risk patients, [1] with the use of chemoimmunotherapy (CIT). The treatment of B cell chronic lymphocytic leukemia (CLL) is in the process of substantial changes as the novel therapies increasingly turn to oral drugs that attack signaling pathways in the leukemic B cell. The complex signaling pathways, particularly those transmitted via various receptor tyrosine kinases (RTKs), are responsible for the enhanced survival and apoptotic resistance in CLL [2–6]. Activation of the B cell receptor (BCR) signaling pathway, either via antigen or "tonic signaling," plays an important pro-survival role in CLL B cells, even without any somatic mutation in the immunoglobulin heavy chain variable region gene (IGHV), which encodes part of the antigen-binding domain of the BCR. However, this review is primarily focused on non-BCR RTK signaling pathways in CLL irrespective of the IGHV mutational status of the leukemic clone.

RTKs constitute one of the largest classes of signaling molecules and have long served as a model for elucidating cellular signaling networks [7]. However, oncogenic mutations and gene fusions generated by chromosomal translocations in RTKs are frequently observed in human cancers, leading to their constitutive activation [7]. Moreover, oncogenic mutations or overexpression of RTKs can promote misfolding and aggregation of these proteins and impair trafficking to the cell surface [8]. Although CLL B cells from CLL patients of various risks for progression have been reported to express a number of non-BCR RTKs [2–6], their precise role in CLL B cell biology and therapeutic applications directed at the RTKs have not been explored extensively. Here, we will focus on the expression and activation status of the RTKs and non-receptor kinases known to be expressed in CLL B cells. We will, where possible, discuss current or future approaches to target these kinases in order to treat CLL patients. Our final section focuses on potential treatment approaches to CLL using the knowledge of these RTKs.

Membrane RTKs in CLL B Cells

This section discusses relatively more well-studied membrane RTKs that have known involvement in CLL B cell survival, well-described signaling pathways, and selected in vitro and or in vivo attempts to interfere with these pathways in CLL.

Insulin-like growth factor receptor and insulin receptor: Insulin-like growth factor-I (IGF-I) produced by bone marrow stromal cells is involved, as a paracrine factor, in the differentiation of normal pro-B to pre-B lymphocytes, stimulating μ-heavy chain expression [9]. IGF-I plays a role in maintaining hematopoietic cells by increasing the proliferation of progenitor cells [10] and by preventing the apoptosis of interleukin (IL)-3-deprived cells [11]. IGF-I receptor (IGF-IR) is undetectable in $CD34^+$ cells but is expressed in committed precursors [12] and in mature B lymphocytes [13].

It is now known that IGF-I and IGF-IR are involved in the genesis of cancer. IGF-IR expression is a prerequisite for the development of several tumors because

it facilitates transformation by viral and cellular oncogenes [14]. The IGF-IR is a phylogenetically conserved RTK and belongs to the insulin receptor family, also involving the insulin receptor (IR) (see below), hybrid receptors, and the IGF-2R/mannose 6-phosphate receptor. The function of the hybrid receptor is still not well understood [15]. The IGF-2R/mannose 6-phosphate receptor is a monomeric receptor without TK activities [15]. Both IGF-IR and IR are preformed dimeric TK receptors made up of two extracellular α-subunits and two β-subunits involving a small extracellular domain, an intramembranous one, and an intracellular domain [16]. The latter includes the juxtamembranous domain, the TK domain, and the C-terminal domain. Interestingly, the IGF-IR is primarily involved in the regulation of cell proliferation, apoptotic resistance, differentiation, and cell motility, while IR is mostly involved in the control of glucose uptake and metabolism [15]. In contrast to IR, IGF-IR is ubiquitously expressed in tissues in which it plays a role in tissue growth, mostly via growth hormone, which liberates IGF-I to activate IGF-IR. However, current evidence suggests that IGF-IR is not an absolute requirement for normal growth [14].

The ligand-receptor interaction results in phosphorylation of tyrosine residues in the IGF-IR TK domain (spanning amino acids 973–1229) of the β-subunit. In the unstimulated receptor state, the activation loop (a-loop), containing the critical tyrosine (Y) residues 1131, 1135, and 1136, behaves as a pseudo-substrate that blocks the active site. However, there are numerous intracellular adaptor proteins (e.g., Shc, Grb2, CrkII, CrkL) that link receptor signaling to downstream pathways [17–21]. After ligand binding, phosphorylation of Y1131 and Y1135 destabilizes the auto-inhibitory conformation of the a-loop, whereas phosphorylation of Y1136 stabilizes the catalytically optimized conformation of the RTK [22]. In turn, phosphorylation of the adapter proteins insulin receptor substrate 1–4 (IRS-1–4) and Shc leads to activation of the phosphatidyl inositol-3 kinase (PI3K), the mitogen-activated protein kinase (MAPK), and the 14-3-3 pathways [23].

The first demonstration of IGF-IR expression in CLL B cells from a subgroup of CLL patients was reported in 2005 [6]. IGF-IR protein and mRNA were shown to be present in CLL B cells in 44 % and 59 % of CLL patients, respectively. Importantly, IGF-IR expression in CLL patients was positively correlated with the expression of the anti-apoptotic protein Bcl-2 and was involved in CLL cell survival in vitro [6]. IGF-IR expression in CLL cells has been shown to be associated with CD38 expression, a marker associated with cells with poor response to treatment and shorter patient survival. Interestingly, serum IGF-I was elevated in CLL patients, but growth hormone (GH), an inducer of IGF-I expression, was normal [6]. Therefore, local tissue site production of IGF-I by CLL B cells may account for the increased levels of serum IGF-I, independent of GH, and may be related to paracrine/autocrine control of leukemic lymphocyte survival by binding to and activating IGF-IR [6]. This information highlights the importance of this growth factor receptor signaling as a possible therapeutic target in CLL. Indeed, blocking of IGF-IR with a neutralizing antibody induces apoptosis in CLL B cells, but not in normal cells, in vitro [6]. Indeed, IGF-IR inhibition using IGF-IR antibodies and tyrosine kinase inhibitors has been reported to enhance the tumor-cell-killing effects

of numerous conventional chemotherapeutic agents such as gemcitabine, irinotecan, etoposide, carboplatin, Adriamycin, ifosfamide, Navelbine, 5-fluorouracil, and vincristine both in vitro and in vivo in various types of human malignancies [24].

More recently, detection of differential expression of the insulin receptor has been reported in CLL cases, with higher levels in the majority of CLL with 11q chromosomal abnormalities (11q-del) [25]. Indeed, a mean of about tenfold higher IR mRNA expression level was documented in CLL with 11q-del cases as compared to CLL cases with other genomic categories [25]. This study also found that the exogenous addition of insulin stimulated canonical IR signaling pathways, including AKT/mTOR and Ras/Raf/Erk in CLL B cells in vitro. Importantly, this study demonstrates a positive correlation of IR expression levels in CLL cells with shorter time to first therapy and shorter overall survival [25], suggesting a biologically meaningful link between IR expression levels in the leukemic B cells and clinical course of the disease in a subset of CLL patients.

Vascular endothelial growth factor receptors: In humans, vascular endothelial growth factor (VEGF) ligand family consists of five members, VEGF A, B, C, D, and placenta growth factor (PLGF). These ligands bind in an overlapping pattern to three RTKs, VEGF receptor (VEGFR1), VEGFR2, and VEGFR3 as well as to their co-receptors. VEGF A, B, and placental growth factor (PLGF) bind to VEGFR1, VEGF A binds to VEGFR2, and VEGF C and D bind to VEGFR3; however, proteolytic processing of the human VEGF C and D allows for binding to VEGFR2, albeit at much lower affinity than VEGFR3 [26] (Fig. 10.1). The VEGFRs are members of the RTK superfamily, and they belong to the same subclass as receptors for platelet-derived growth factor and fibroblast growth factors (FGFs). VEGFR1 is a positive regulator of monocyte and macrophage migration and has been described as a positive and negative regulator of VEGFR2 signaling capacity. Negative regulation is exerted, at least in part, by an alternatively spliced soluble VEGFR1 variant that binds to VEGF and thereby prevents VEGF from binding to VEGFR2. VEGFR2 is implicated in many aspects of normal and pathological conditions, whereas VEGFR3 is important for lymphatic endothelial cell development and function [26].

The VEGFRs contain an approximately 750 amino-acid-residue extracellular domain, followed by a single transmembrane region, a juxtamembrane domain, a split tyrosine kinase domain that is interrupted by a 70-amino-acid kinase insert, and a C-terminal tail. Interestingly, alternative splicing or proteolytic processing of VEGFRs gives rise to secreted variants of VEGFR1 [27] and VEGFR2 [28], and in humans, to a C-terminal truncated VEGFR3 [29]. Guided by the binding properties of the ligands, the VEGFRs are able to form both homodimers and heterodimers [30]. Dimerization of receptors is accompanied by activation of the receptor-kinase activity that leads to the autophosphorylation of the receptors. Phosphorylated receptors recruit interacting proteins and induce the activation of signaling pathways, including Ras, Src, PI3K, focal adhesion kinase (FAK), and phospholipase C (PLC)-γ, leading to proliferation, vascular permeability, cell migration, and cell survival [26, 31].

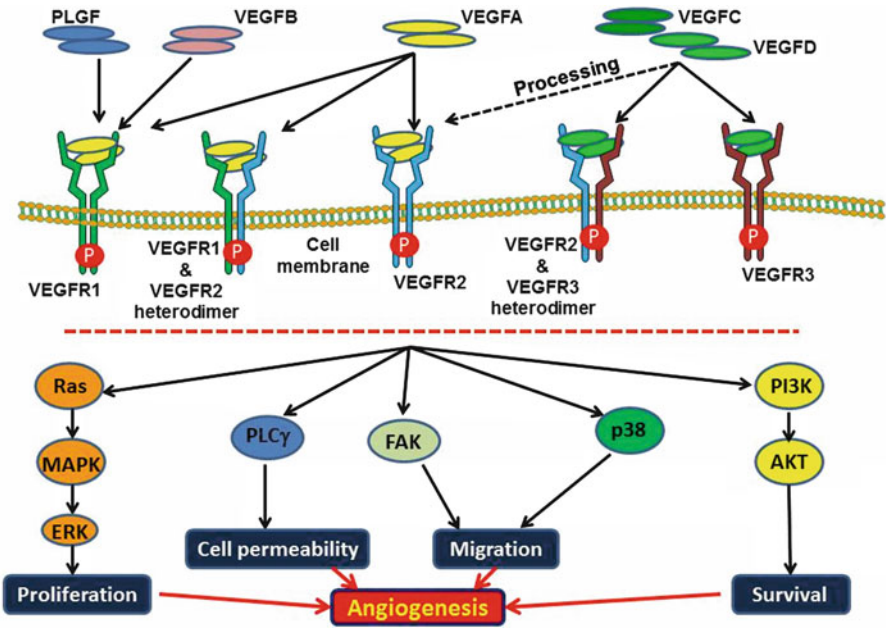

Fig. 10.1 VEGF ligand and receptor-binding properties and signaling complexes. Mammalian VEGF ligands bind to the three VEGF receptor tyrosine kinases, leading to the formation of VEGFR homodimers or heterodimers. Proteolytic processing of VEGF C and VEGF D allows for binding to VEGFR2. Upon ligation with the ligand, VEGFRs transmit signals to transcribe the target cells via various intermediate components which also depend on the cellular context. Thus, activation of the specific VEGFR (via ligand binding or activating mutation) results in cell migration, permeability, proliferation, and survival, leading to angiogenesis

In CLL, the pro-angiogenic factor VEGF (VEGF A) acts as an important survival factor for the leukemic B cells, at least in part, by activating the STAT1/STAT3 signaling pathway and upregulating the critical anti-apoptotic protein, myeloid cell leukemia-1 (Mcl-1) [5]. Indeed, in a limited number of CLL patients ($n = 88$), a strong correlation between Mcl-1 and VEGF mRNA expression levels was found [5]. Angiogenesis and signaling via angiogenic cytokines have increasingly been recognized as an important process in the growth of both solid tumors [32] and hematologic malignancies [33], including CLL [34]. This latter work has invoked the well-known "angiogenic switch" as a factor in CLL progression [35]. Early work in CLL demonstrated that the CLL B cell synthesizes and secretes pro-angiogenic molecules [36] (i.e., VEGF and basic fibroblast growth factor [bFGF]) as well as antiangiogenic molecules, but the balance favors a pro-angiogenic environment. In addition, bone marrow microvessel density, a marker of angiogenesis, correlates with CLL disease stage [37, 38] and identifies patients with a shorter progression-free survival [39]. Other reports also suggest that serum and urine levels of the pro-angiogenic factors VEGF and bFGF are increased in CLL [40]. Indeed,

increased levels of serum VEGF or bFGF have been found to be associated with disease progression in patients with early-stage CLL [41].

CLL B cells express VEGF receptors (R1 and R2) [42–44], and these receptors are constitutively phosphorylated [2]. Culture of CLL B cells with exogenous VEGF is associated with increased levels of the anti-apoptotic proteins MCL-1 and XIAP, as well as a reduction in both spontaneous and drug-induced apoptosis [2, 45]. VEGF has also been implicated in CLL B cell migration [46, 47] and can modulate the expression of BCR signaling through effects on protein kinase CβII [48]. In addition, clinical studies found that patients with early-stage CLL who had higher serum VEGF levels had significantly shorter progression-free survival [40]. Interestingly, VEGF levels in pretreatment plasma were associated with response to CIT treatment in patients with CLL [49]. While these receptors were shown to be expressed on tumor cells and are likely to be involved in both autocrine survival and/or neovascularization in tumor models, there is increasing evidence that another VEGF receptor, neuropilin-1 (NRP-1), is critical in tumor angiogenesis and most likely involved in VEGF-mediated resistance to apoptosis [50]. Aberrant NRP-1 expression has been shown in acute myeloid leukemia (AML) and associated with shortened overall survival of the AML patients [51]. Importantly, it has also been reported that a subset of CLL B cells, but not normal B lymphocytes, express NRP-1 [52]. However, since VEGF supports an autocrine pathway that promotes CLL B cell survival [2, 45, 53] and NRP-1 expression is limited to a subset of CLL patients, it will be critical to establish a relationship of NRP-1 expression with the known CLL prognostic factors. In addition, most recently our unpublished observations have detected the expression of VEGFR3 in CLL B cells, leading to the possibility that all three VEGF receptors may be part of a network that results in the enhanced survival of the leukemic B cells (unpublished observations: Kay and Ghosh). Consistent with this, we have also found that VEGF C levels in early-stage CLL (Rai stage 0) are comparable with that obtained from normal, healthy individuals but higher than in more advanced stages of CLL (Fig. 10.2a), suggesting that VEGF C could mediate disease progression in the early-stage CLL patient. Interestingly, we see a reverse trend for VEGF D, with the highest levels in the plasma of late-stage CLL (Rai stages 3/4) when compared to that in normal plasma and lower-stage CLL (Fig. 10.2b) (unpublished observations: Kay and Ghosh). Importantly, we found that VEGF A and C are both produced by CLL B cells, via ELISA assays of their culture medium (data not shown: unpublished observations).

In total, these results suggest that signaling via the VEGF receptor signaling pathway may be an important process in the pathogenesis of CLL and could provide an important therapeutic target for patients with this disease.

Although various in vitro experiments on VEGF/VEGFR axis underscore a pro-survival role of this axis in CLL in addition to in vivo correlation of serum VEGF with early-stage CLL progression, it is important to note that a phase II clinical trial using anti-VEGF agents targeting VEGF or VEGFR (single agent) in relapsed/refractory CLL patients ($n = 46$) has shown minimal clinical activity in this cohort of patients [54] (see below for detail). Information obtained from that

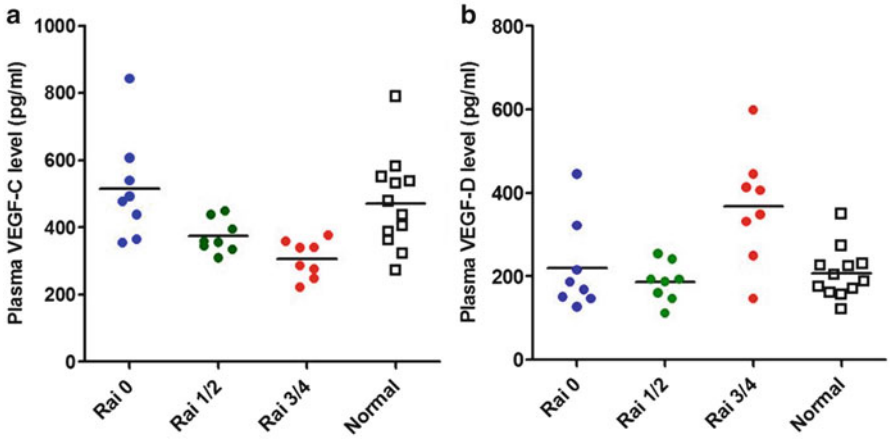

Fig. 10.2 CLL plasma contains both VEGF C and VEGF D. Plasma levels of VEGF C (*panel a*) and VEGF D (*panel b*) were measured in previously untreated CLL patients of various disease stages as indicated or age-matched healthy subjects using specific ELISA kits. Individual values are presented. Horizontal lines indicate the mean values. Although a trend of decreasing VEGF C levels was discernible with the disease progression, a sharp increase in VEGF D levels was detected in advanced stages of CLL

clinical study also suggests that VEGF/VEGFR axis may not likely be the primary or predominant pro-survival axis in CLL.

Axl: It was originally detected in 1988 from patients with chronic myelogenous leukemia (CML) as an unidentified transforming gene and later was cloned from patients with CML and chronic myeloproliferative disorders [55]. The name "Axl" was derived from the Greek word "anexelekto" which meant "uncontrolled." The human Axl gene is located on chromosome 19q13.2 [55] and encodes a protein of molecular mass between 100 and 140 kD (depending on the extent of posttranslational modifications) that contains an extracellular (N-terminal) domain and an intracellular (C-terminal) tyrosine kinase domain [56]. Axl is a highly conserved gene across species (20 exons), but has two alternative variants due to a splicing site in exon 10 within the transmembrane domain [57–59]. The promoter region of Axl is GC-rich and contains recognition sites for a variety of transcription factors, including Sp1 (specificity protein 1), AP2 (activating protein 2), and CREB (cAMP-response-element-binding protein) [60]. Indeed, Axl is regulated by the Sp1/Sp3 transcription factors and methylation of CpG sites within specific Sp1 motifs [61]. Given this, posttranscriptional regulations play a critical role in modifying and stabilizing the protein levels depending on cellular context. In addition, PKCα, PKCβ, and constitutive activation of the Erk1/2 pathway have been reported to be critical for the overexpression of Axl in tyrosine kinase inhibitor-resistant cell lines [62].

Axl is a member of the TAM RTK family that also includes Tyro3 and Mer [63]. Axl is composed of two immunoglobulin-like domains and dual fibronectin type III repeats in the extracellular region: a single transmembrane and a

cytoplasmic domain with kinase activity [55]. Axl is ubiquitously expressed in a wide variety of organs and cells, including hippocampus and cerebellum, monocytes, macrophages, platelets, endothelial cells, heart, skeletal muscle, liver, kidney, and testis [58, 64, 65]. However, Axl overexpression has been reported in several human cancers, including colon, esophageal, thyroid, breast, lung, liver, and astrocytoma glioblastoma [66–72].

Protein S and growth arrest-specific gene 6 (Gas6) are the ligands for Axl, where the latter has very high affinity to the Axl receptor [73, 74]. Axl activation and signaling have been implicated in multiple cellular responses, including cell survival, proliferation, migration, adhesion, and angiogenesis [75–79].

We identified Axl in CLL B cells during our reported work on microvesicles in CLL plasma, where we detected that CLL microvesicles carry the Axl RTK. CLL B cells from the majority of CLL patients showed expression of constitutively phosphorylated and functionally active Axl RTK [3]. Importantly, Axl RTK is physically associated with multiple non-receptor kinases and enzymes, including Lyn (a member of the Src family kinases), Syk/ZAP70, PLC-γ2, and PI3K [3]. In particular, the PI3K/AKT axis is a critical signaling pathway in many human malignancies, including CLL, and overexpression and increased activity of Lyn kinase has been reported in CLL. Interestingly, although CLL B cells express c-Src, Axl showed very little affinity to bind to c-Src but did exhibit a very high affinity towards Lyn (Fig. 2B of ref. [3]). Our study suggests that Axl RTK is likely to be the primary RTK, as inhibition of Axl induced massive cell death in CLL B cells [3].

We have examined Axl expression on the CLL B cell surface from over 200 previously untreated CLL patients and detected variable levels of Axl expression (Kay and Ghosh: unpublished observations). However, we did not find any correlation of Axl expression with the known novel cell-based prognostic factors in CLL (data not shown). In a related study most recently, we identified an miR-34a-binding site on the Axl 3′-untranslated region (UTR). Interestingly, miR-34a is a direct target of the tumor suppressor p53, which has been reported to be inactive in many human cancers, including CLL [80–82]. Indeed, findings from a series of experiments suggest that miR-34a targets Axl 3′-UTR in response to p53 activation, suggesting the existence of an inverse relationship between p53 functionality and regulation of Axl RTK expression in CLL [83].

Although Axl expression appears to be a predominant pro-survival signaling pathway in CLL, its relation or association with the CLL clinical course is yet to be established.

c-MET: The RTK c-MET, originally identified as a TRP-MET fusion gene from a human osteosarcoma cell line, encodes a prototypic member of the c-MET RTK subfamily [84]. The tyrosine kinase c-MET is the high-affinity receptor for hepatocyte growth factor (HGF)/scatter factor, a multifunctional cytokine with pleiotropic effects. The HGF/c-MET signaling pathway is one of the most frequently dysregulated pathways in human cancers. Aberrant HGF/c-MET signaling has been reported in a wide range of human malignancies, including bladder, breast, cervical, colorectal, endometrial, esophageal, gastric, head and neck, kidney, liver,

lung, nasopharyngeal, ovarian, pancreatic, prostate, and thyroid cancers, as well as cholangiocarcinoma, osteosarcoma, rhabdomyosarcoma, synovial sarcoma, Kaposi's sarcoma, leiomyosarcomas, and MFH/fibrosarcoma [85]. In addition, abnormal HGF and/or c-MET expression has also been reported in hematological malignancies such as acute myelogenous leukemia, adult T cell leukemia, chronic myeloid leukemia, lymphomas, and multiple myeloma, as well as other tumors like melanoma, mesothelioma, Wilms' tumor, glioblastoma, astrocytomas, and CLL [85, 86].

The c-MET RTK subfamily is structurally distinct from most other RTK subfamilies. The mature form of the c-MET receptor is a disulfide-linked heterodimer containing an extracellular α-chain and a transmembrane β-chain, both of which result from the proteolytic cleavage of the same precursor protein [87]. The β-chain consists of an extracellular domain, a transmembrane domain, and a cytoplasmic portion containing juxtamembrane and kinase domains, and a C-terminal tail that is essential for substrate docking and downstream signaling [88–91]. The binding of HGF ligand to functionally mature c-MET leads to receptor dimerization or multimerization, phosphorylation of multiple tyrosine residues in the intracellular region, catalytic activation, and downstream signaling through docking of a number of substrates [85] including RAS-MAPK, PI3K-AKT, STATs, PLC-γ, and c-Src [88–90, 92]. The c-Met signaling pathway has been shown to affect a wide range of biological activities, including cell motility, proliferation, and protection from apoptosis. HGF/c-Met pathway is necessary for the normal growth and development of various cell types, including hematopoietic progenitors in embryonic life and adults [93, 94]. Prior studies indicate that the signaling pathways of the HGF/c-Met system and integrin family of adhesion molecules are linked and can cross-modulate their separate functions [95].

Recently, a group of investigators has reported that CLL B cells express increased levels of c-METα and c-METβ, while no expression was detected on normal $CD19^+$ B cells. Interestingly, this increase was found to be inversely correlated with decreased expression of adhesion molecules [86]. In addition, the serum level of HGF in CLL was reported to be increased [86]. In vitro studies demonstrate that expressions of critical signaling molecules shared by adhesion molecules VLA-4 and HGF/c-MET systems, including Bcl-xL, AKT, PI3K, and phospho-BAD_{136} following HGF stimulations of CLL B cells have been found to be increased [86]. These findings suggest that c-MET activation plays an important role in enhanced survival and apoptotic resistance of the leukemic B cells. However, critical involvement of the HGF/c-MET signaling axis in CLL pathobiology or the prognostic relevance of HGF/c-MET expression in CLL B cells remains to be investigated.

Novel Membrane RTKs in CLL

This section discusses more recently discovered or less well-studied membrane RTKs that are likely involved in CLL B cell survival.

FGF receptors: The FGF family and their four RTKs, FGFR1/2/3/4, mediate multiple physiologic processes, including cell migration, proliferation, survival, and differentiation. All of the four FGFRs are encoded by distinct genes, and their structural variability is increased by alternative splicing [96]. FGFRs are expressed on nearly every cell type of hematopoietic origin, and the deregulation of FGFR gene expression and/or gene mutation has been found in hematologic malignancies [97]. Given the importance and critical roles of the FGF/FGFR signaling pathway, it is not surprising that aberrant FGFR signaling is detected in many human malignancies, including multiple myeloma, gastric, endometrial, prostate, and breast [98, 99]. For example, FGFR1 amplification in about 20 % of squamous non-small cell lung carcinoma [100] and about 10 % of breast cancers [101] has been reported. The FGFR2 gene is amplified in some cases of gastric cancer, resulting in a highly overexpressed and constitutively active RTK [102, 103]. On the other hand, t(4;14)(p16;q32) chromosomal translocation, detected in 15 % of multiple myeloma patients, often results in overexpression of FGFR3 [104–106]. The overexpressed FGFR3 is usually wild type, sensitive to ligand binding, and the activated FGFR3 has a role in myelomagenesis [107]. Amplification of FGFR4 has been detected in rhabdomyosarcoma and activating mutations characterized in 7 % of cases [108]. The affinity of bFGF with various FGFRs is different, and the downstream signaling pathways of different FGFRs are also varied [109], although the signaling domains of FGFRs are highly conserved. Several signaling pathways can be activated by FGFRs, such as the PLC-γ, Src, Crk, and SNT-1/FRS2 [110].

We and others have found that CLL B cells constitutively produce the pro-angiogenic bFGF in vitro [36, 111, 112]. Increased levels of bFGF have also been reported in the blood and urine of CLL patients [37, 111, 112]. It is likely that the leukemic cells are the primary source of bFGF in vivo. Interestingly, higher plasma levels of VEGF and bFGF (FGF-2) have been reported to be predictors of longer survival in acute lymphoblastic leukemia (ALL) [113], while Bairey et al. [114] showed that Bcl-2 expression correlates positively with serum bFGF and negatively with cellular VEGF in patients with CLL. Indeed an in vitro study using CLL-derived cell lines showed bFGF upregulates Bcl-2 expression, resulting in delaying apoptosis [115]. Interestingly, a recent study established a functional link between FGF and VEGF signaling pathways [116]. This latter finding underscores that inhibition of both bFGF and VEGF signaling pathways may be necessary to sufficiently impair CLL B cell survival.

A gene expression study using leukemic B cells from CLL patients detected FGFR1 transcript with higher expression levels in CLL B cells with unmutated IgVH status [117]. However, this study did not demonstrate any expression of FGFR2, FGFR3, or FGFR4 in CLL B cells. Most recently, our laboratory has indeed detected expression of FGFR1 and FGFR3, but not FGFR2 and FGFR4, in CLL B cells from previously untreated CLL patients by both flow cytometric and Western blot analyses (Kay and Ghosh: unpublished observations). Constitutively phosphorylated FGFRs were also detected in CLL B cells, suggesting the existence of a paracrine/autocrine loop for activation of this FGF/FGFR signaling pathway.

However, at present, whether this RTK signaling pathway is critical for CLL B cell survival and apoptotic resistance remains unknown.

ROR: Receptor tyrosine kinase-like orphan receptor (ROR) proteins are a conserved family of RTKs that function in developmental processes, including skeletal and neuronal development, cell movement, and cell polarity. Recent studies suggest that depending on cellular context, ROR proteins can either activate or repress transcription of Wnt target genes and can modulate Wnt signaling by sequestering Wnt ligands [118]. It is not surprising that deregulated RTKs cause severe developmental defects and diseases like cancers. Thus, ROR proteins are no exception, and disruption of human ROR proteins is associated with skeletal deformities and with increased incidence of leukemia [118].

Vertebrates express two ROR family members encoded by ROR1 and ROR2 genes [119]. ROR proteins are type-I transmembrane RTKs and located predominantly in the plasma membrane [120]. The extracellular region of ROR proteins contains an immunoglobulin (Ig) domain; a Cys-rich domain (CRD), also called Frizzled domain; a Kringle (Kr) domain; an intracellular tyrosine kinase domain; and a proline-rich domain (PRD) straddled by two Ser-/Thr-rich domains, Ser/Thr1 and Ser/Thr2 [119]. However, in humans, normal functions of the ROR protein are known to be related primarily for skeletal development [121–124].

Gene expression profiling studies showed a 43.8-fold increase of the ROR1 in CLL B cells [125]. ROR participates in signal transduction, cell-cell interaction, regulation of cell proliferation, differentiation, cell metabolism, and survival [119, 126]. The ROR1 gene is located on human chromosome 1p31.3, a region where chromosomal aberrations are not frequently detected in hematological malignancies [127]. The human ROR1 is expressed in heart, lung, and kidney but less in placenta, pancreas, and skeletal muscles [128]. Truncated ROR1 (t-ROR1) has also been reported in fetal and adult human central nervous system, human leukemias, lymphoma cell lines, and in a variety of human cancers derived from neuroectoderm [128]. CLL cells have been reported to express ROR1 at the mRNA and protein levels uniformly, but not in normal B lymphocytes [4, 127]. Expression of ROR1 on CLL B cells has been found to be independent of disease stages, IGVH mutational status, and B cell activation status [4, 127]. Of note, expression of ROR2 was not detected on CLL B cells [4]. In total, the unique expression pattern of ROR1 on CLL B cells, not in normal B lymphocytes, makes it an attractive target in CLL. However, whether ROR1 is critical for CLL progression or enhanced survival remains to be investigated.

Signaling in CLL B Cells via Non-receptor Tyrosine Kinases That Are Independent of BCR Stimulation

This section discusses the relevant relationships of non-RTKs and their signal events to leukemic B cell biology.

Lyn kinase: The members of Src family kinases (SFKs) consist of Src, Fyn, Yes, Lck, Hck, Fgr, Lyn, Blk, and Yrk. Each of these proteins is about 60 kD in molecular weight and has a common structure consisting of an N-terminal unique domain, followed by Src homology (SH) domain 3, SH2, and tyrosine kinase domains [129]. SFKs can act as an upstream or downstream modulator of several receptors, as well as non-RTKs, which are responsible for robustness and persistence of RTK signaling [130]. SFKs participate in the activation of various downstream signaling pathways through molecular interactions with growth factor receptors such as the epidermal growth factor receptor (EGFR) family, MET, integrin cell adhesion receptors, steroid hormone receptors, G protein-coupled receptors, FAK, and cytoskeleton components [130, 131]. SFKs can activate PI3K/AKT, growth factor receptor-bound protein 2 (Grb2)-Ras/Raf-mitogen-activated protein kinase (MAPK), Jak-signal transducers and activation of transcription (STAT), and FAK-paxillin-p130-Crk-associated substrate (Cas) cascades that are most crucial for cell cycle progression, survival, and proliferation [132–137].

Lyn, a member of the SFKs, is reported to be robustly overexpressed at the protein level in leukemic B cells from CLL patients as compared to normal B lymphocytes, with a substantial aliquot of the kinase anomalously present in the cytosol [138]. While in normal B lymphocytes, Lyn activation is dependent on BCR stimulation, in resting malignant cells, the constitutive activity of the kinase accounts for high basal level protein tyrosine phosphorylation and low responsiveness to IgM ligation, suggesting that it is independent of BCR stimulation [138]. Interestingly, the evidence that Lyn mRNA level was comparable in normal and neoplastic B cells demonstrates the anomalous protein expression was not related to differences in gene transcription and/or mRNA stability. A possible explanation for this might be deregulated protein turnover in leukemic B cells [138]. However, treatment of CLL B cells with the Lyn kinase inhibitors PP2 and SU6656 induces apoptosis, suggesting a direct correlation between high basal Lyn activity and defects in the induction of apoptosis in leukemic B cells [138]. In total, these findings support a critical role for Lyn in CLL pathogenesis and identify this non-RTK as a potential therapeutic target.

Syk kinase: The protein tyrosine kinase spleen tyrosine kinase (Syk) represents a key mediator of proximal BCR signaling, providing proliferation and survival signals in a variety of hematopoietic cells [139]. After BCR stimulation, Syk is recruited to the BCR and becomes activated by sequential phosphorylation at conserved tyrosine residues. Once activated, Syk propagates signals by associating with critical signaling intermediates such as VAV, PLC-γ2, Bruton's tyrosine kinase (Btk), and B cell linker protein. The signaling cascade then proceeds with the activation of further downstream signaling molecules, including extracellular signal-regulated kinase 1/2 (Erk1/2) and p38 [140]. Translocations involving Syk have been identified in myelodysplastic syndromes and T cell lymphoma, indicating that Syk may also function as a proto-oncogene [141, 142].

Gene expression profiling identified increased expression of Syk and downstream pathways in CLL compared with normal B cells from healthy individuals.

Western blot analysis showed increased expression and constitutive phosphorylation of Syk and its downstream PLC-γ2, STAT3, and Erk1/2 in CLL B cells as compared to normal B cells [143, 144]. Indeed, Syk has been reported to be overexpressed in CLL B cells at both mRNA and protein levels vs. normal B cells and pharmacological inhibition of Syk activity induced massive apoptotic leukemic B cell death, regardless of clinical and biological status of the CLL patients [143, 144], emphasizing the potential clinical utility of Syk inhibition in hematological malignancies like CLL.

Potential of Tyrosine Kinase Inhibitors in Future CLL Therapy

Multiple tyrosine kinases in the form of receptors and non-receptors have been detected in CLL as constitutively active and for the most part related to CLL B cell survival. We believe that constitutively active RTKs in CLL B cells constitute a network where one RTK acts as the predominant one, while others work as secondary RTKs, and that a functional interplay between multiple RTKs where a common converging signaling point is, for example, AKT. In this scenario, then, it is likely that inhibition of the primary RTK in leukemic B cells may promote activation of a secondary RTK that maintains the survival signaling in the cells, as most RTKs share the same downstream signal intermediates, like Src and PI3K/AKT (Fig. 10.3). Thus, effectively targeting multiple RTKs should have a better impact in CLL therapy. Nevertheless, we wish to describe here prior clinical trials in CLL that have used a strategy of single RTK inhibition in the trial design. Because these were usually phase 2 trials, all patients treated with RTK inhibition were relapsed/refractory CLL.

Targeting VEGF/VEGFR axis: To test the efficacy of anti-VEGF therapy in CLL, we initiated and completed separate phase II clinical testing of three different anti-VEGF therapies for patients with relapsed/refractory CLL: AZD2171 (a potent, oral, and pan-VEGF receptor inhibitor), bevacizumab (a recombinant humanized monoclonal antibody to VEGF), and sunitinib malate (a multi-targeted, small-molecule inhibitor of RTKs involved in tumor proliferation and angiogenesis including VEGFR1, VEGFR2, VEGFR3, and platelet-derived growth factor receptor [PDGFR]) [54]. Overall, 10 (71 %) patients in the AZD2171 trial, 4 (33 %) in the bevacizumab trial, and 16 (89 %) in the sunitinib malate trial experienced a grade 3 or higher adverse event attributed to study medication. In the AZD2171 trial, the most frequent grade $\geq$3 adverse events were thrombocytopenia (5/14 patients), fatigue (5/14 patients), diarrhea (3/14 patients), muscle weakness (3/14 patients), and hypertension (3/14 patients). In the bevacizumab trial, the most frequent grade $\geq$3 adverse events were proteinuria (2/12 patients) and fatigue (2/12 patients). In the sunitinib malate trial, the most frequent grade $\geq$3 adverse

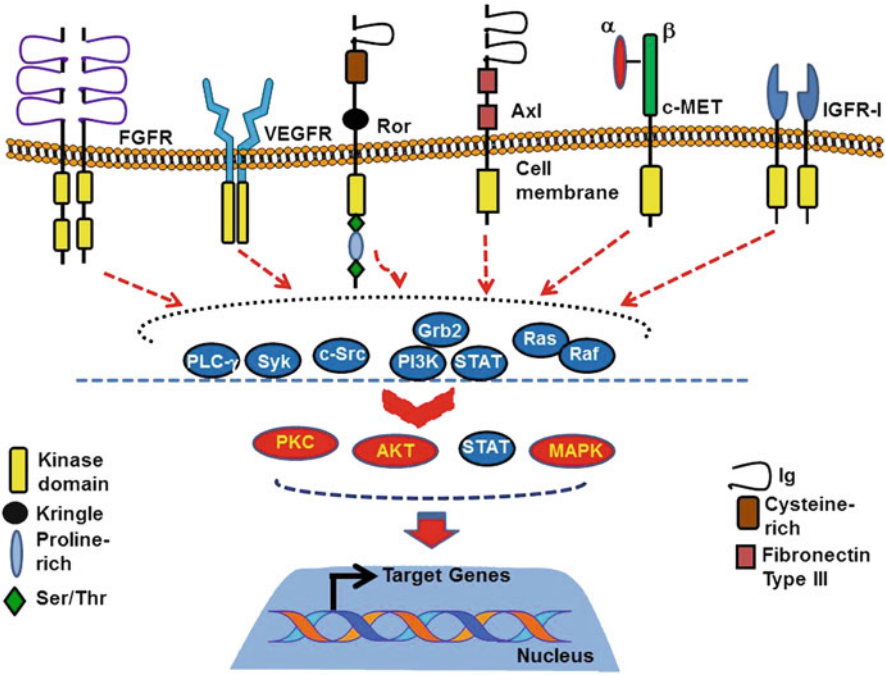

Fig. 10.3 Tyrosine kinase network in CLL. Leukemic B cells from CLL patients express multiple RTKs which may directly or indirectly participate in the "Cell Survival" signaling network. As most of the RTK signaling pathways share common intermediate signaling components, for example, Src and PI3K/AKT, we believe that in this "RTK Network," one RTK plays the role of the "Predominant RTK" while others play a secondary role, likely depending on the risk factors of the cells. In CLL, upon binding specific ligands, these RTKs may activate multiple signaling intermediates, including Src, Syk, Grb2/PI3K, Ras/Raf, and PLC-γ, leading to activation of the downstream effector signaling components: for example, AKT, MAPK, PKC, or STATs, which ultimately activates various specific target genes, resulting in cell survival, proliferation, and apoptosis resistance. However, expression of constitutively active RTKs in CLL B cells results in uncontrolled activation of the downstream signaling molecules, leading to increased cell survival and apoptotic resistance to therapeutic agents. One such constitutively active RTK in CLL we detected was Axl

events were thrombocytopenia (10/18 patients), fatigue (6/18 patients), neutropenia (5/18 patients), and anorexia (4/18 patients).

All three trials were closed early due to lack of efficacy. Although no complete or partial responses were obtained, 5/14 patients on AZD2171, 10/12 patients on bevacizumab, and 10/18 patients on sunitinib had stabilization of disease for a median duration of 2.7, 2.9, and 4.4 months, respectively. Thus, the absolute lymphocyte count (ALC) values declined by, at least, 10 % during treatment for 5/14 patients on AZD2171, 3/12 patients on bevacizumab, and 6/18 patients on sunitinib malate.

Despite the lack of clinical activity observed in these trials, our and others' work on the biology of VEGF and other related angiogenic events play a role in

CLL [34]. These include recent studies indicating that marrow vascular density is significantly higher in patients with CLL with high-risk FISH and CD38 positivity [145], a pro-angiogenic profile favors disease progression [146], circulating endothelial cells correlate with more advanced disease stage [147], pro-angiogenic molecules such as angiopoietin-2 and matrix metalloproteinase 9 are associated with progressive CLL [148, 149], and the use of combination CIT may work in part via antiangiogenic effects [150]. Newer VEGF RTK inhibitors have also recently demonstrated activity against CLL B cells in vitro as well as in a xenograft model and appear to increase the efficacy of purine nucleoside analogs against CLL on in vitro testing [151]. These observations suggest that VEGF inhibition remains a potential therapeutic target in CLL and suggest that combining anti-VEGF therapy with more traditional therapeutic agents may be a useful strategy for patients with this disease. Indeed, we and others have already initiated clinical trials exploring the benefits of this approach as part of efforts to improve outcomes for patients with CLL.

Targeting Syk: The first clinical trial targeting Syk non-RTK used fostamatinib disodium (an oral Syk inhibitor) in phase I/II studies in patients with relapsed/refractory non-Hodgkin lymphoma (NHL) and CLL [152]. The dose-limiting toxicity in the phase I portion was neutropenia, diarrhea, and thrombocytopenia, and 200 mg twice daily was chosen for the phase 2 study. In this phase of the trial the most common toxicities were reversible cytopenias, fatigue, diarrhea, and hypertension. Interestingly, 6 of 11 CLL patients (55 %) achieved a partial response, and the response rate in CLL was the highest amongst the patients with other NHL. However, to date no follow-up studies of fostamatinib in B cell malignancies have been initiated in spite of a recently completed randomized phase III study in rheumatoid arthritis that showed significant activity and good tolerability of the drug [153].

Targeting Lyn kinase: Dasatinib is an oral multikinase inhibitor targeting Src and Abl kinases which was approved for use in imatinib resistant CML. It has been reported recently that dasatinib inhibits not only Lyn kinase but also Btk at low nanomolar concentrations [154]. However, in vitro data demonstrates that dasatinib induces variable degrees of apoptosis in leukemic B cells with no correlation between response and inhibition of Lyn phosphorylation [155].

A phase II study of 140 mg dasatinib once daily in a small cohort of relapsed/refractory CLL patients ($n = 15$) reported an overall response rate of 20 % with a progression-free survival of 7.5 months [156]. However, 5 patients exhibited >50 % reduction in lymphadenopathy. Myelosuppression was the primary toxicity, with grade 4 neutropenia and thrombocytopenia occurring in 40 % and 13 % of the CLL patients, respectively [155].

Impact of Axl inhibitor in vitro: Axl RTK plays a critical role likely by regulating activity of multiple cellular kinases, including non-RTKs like Lyn and Syk and lipid kinases like PI3K and PLC-γ2, in CLL B cells to modulate survival of the leukemic B cells [3]. We believe that Axl is acting as the predominant RTK in CLL

B cells (Fig. 10.3). This hypothesis is based on the fact that Axl inhibition induces robust apoptotic cell death in CLL B cells from CLL patients with various disease stages, prognostic profiles, and risk factors at very low LD_{50} doses (0.25–2.0 μM) of the high-affinity Axl inhibitors (unpublished observations: Kay and Ghosh) [3]. Indeed, a high-affinity, oral Axl inhibitor BGB328 (BergenBio), formerly known as R428 [157], reduced breast tumors in a mouse xenograft model with favorable toxicity profiles. A single administration of the agent in female BALB/c mice by oral gavage resulted in high plasma exposures (C_{max} of approximately 2.6 and 6.8 μM/L with doses of 25 and 75 mg/kg, respectively), with linear dose proportionality up to 100 mg/kg body weight [157]. Importantly, the Axl inhibitor exhibited a long plasma half-life (4 h at 25 mg/kg; 13 h at 75 mg/kg) and distributed effectively to tissues [157]. Information from this preclinical study emphasized the potential use of the Axl inhibitor in CLL patients in future phase I/II studies.

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Chapter 11
Immunotherapies in CLL

Jae H. Park and Renier J. Brentjens

Abstract Chronic lymphocytic leukemia (CLL) is the most frequently diagnosed leukemia in the Western world, yet remains essentially incurable. Although initial chemotherapy response rates are high, patients invariably relapse and subsequently develop resistance to chemotherapy. For the moment, allogeneic hematopoietic stem cell transplant (allo-HSCT) remains the only potentially curative treatment for patients with CLL, but it is associated with high rates of treatment-related mortality. Immune-based treatment strategies to augment the cytotoxic potential of T cells offer exciting new treatment options for patients with CLL, and provide a unique and powerful spectrum of tools distinct from traditional chemotherapy. Among the most novel and promising of these approaches are chimeric antigen receptor (CAR)-based cell therapies that combine advances in genetic engineering and adoptive immunotherapy.

Keywords Cellular immunotherapy • Chimeric antigen receptor • CLL • Hematopoietic stem cell transplant

Evidence of a Graft-Versus-Leukemia Effect in CLL

The existence of a graft-versus-leukemia (GVL) effect in chronic lymphocytic leukemia (CLL) is supported by several clinical observations. First, in contrast to autologous hematopoietic stem cell transplant (HSCT) or other intensive chemotherapies where no durable remissions have been observed, the relapse incidence decreases over time following allogeneic HSCT (allo-HSCT). Second, the relapse risk is reduced in the presence of chronic graft-versus-host disease

J.H. Park • R.J. Brentjens (✉)
Department of Medicine, Leukemia Service, Memorial Sloan-Kettering Cancer Center, New York, NY, USA
e-mail: brentjer@MSKCC.ORG

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(GvHD). Third, immunomodulation via withdrawal of immunosuppression or donor lymphocyte infusion (DLI) can induce sustained deep remissions with no detectable minimal residual disease (MRD). Lastly, the long-term survival of patients with reduced-intensity conditioning (RIC) is not different from myeloablative conditioning (MAC), implying that the benefit of the transplant stems from elimination of tumor cells by alloimmune effector lymphocytes.

Allogeneic HSCT in CLL

Allo-HSCT appears particularly effective in high-risk patients, as defined by purine analog refractoriness or the presence of either unmutated IgVH or del17p. The data from a larger registry analysis and the German prospective CLL3X trial reported a 4-year event-free survival (EFS) and overall survival (OS) of 45 % and 59 %, respectively, that compares favorably to a median survival of less than 3 years reported in these patients without transplant. Furthermore, the durable MRD-negative remissions achieved in 6 of 13 patients with the plateau on the relapse curve support the role of allo-HSCT in providing long-term disease control in these high-risk patients. Table 11.1 summarizes the patient characteristics and outcomes of clinical trials of allo-HSCT published to date in CLL patients.

Early studies of MAC allo-HSCT in CLL reported high TRM, ranging from 25–50 %, mostly due to infection and GvHD. The introduction of RIC allo-HSCT at the end of the 1990s opened the possibility of delivering allo-HSCT to older patients with decreased TRM. Despite the initial concern of increased relapse rates with RIC compared to MAC, an improved outcome has been reported in RIC allo-HSCT over time due to decreases in both TRM and relapse rates in RIC patients (Table 11.1). However, the favorable relapse rate appears to be from higher percentages of patients in partial remission (PR) at the time of transplant, as a higher disease burden at HSCT has been associated with increased relapse. Because patients with CLL who require allo-HSCT are typically heavily pretreated elderly patients with comorbid conditions, and given lower TRM with comparable efficacy during the last decade, RIC has replaced MAC in CLL.

Despite the evidence of GVL in CLL, owing to the ongoing risk and toxicity of allo-HSCT, the optimal timing of transplant and the target patient population remain controversial. Because many patients often progress while waiting for transplant, the window for successful allo-HSCT may be small. Furthermore, HSCT is often delayed until patients have been multiply treated and have refractory disease with bulky lymphadenopathy, all of which have been associated with a higher risk of disease relapse or progression after RIC allo-HSCT. Therefore, allo-HSCT is most likely to achieve the best outcome if utilized early in the clinical course, before the development of refractory bulky disease and treatment-induced toxicity. The ongoing prospective randomized trial conducted by the German CLL Study Group (GCLLSG) wherein patients with purine-analog-refractory or del17p CLL will be biologically randomized to allo-HSCT versus conventional salvage

Table 11.1 Clinical trials of allogeneic HSCT in chronic lymphocytic leukemia (CLL)

Reference	Time period	No. of patients	Median age (range)	Median no. of prior Tx (range)	Prognostic factors	Disease status prior to transplant (%)	Conditioning	TRM (follow-up)	Relapse rates (%)	GvHD (%)	Outcome
Pavletic et al.	1993–1999	38	45 (26–57)	3	N/A	Refractory to prior chemo (55)	MAC	38 % (5 years)	32	85	5-year OS: 33 %
Delgado et al.	N/A	41	54 (37–67)	3 (1–6)	N/A	CR (12), PR (70), PD (17)	RIC (w/ alemtuzumab)	26 % (2 years)	29	33	2-year PFS: 45 % 2-year OS: 51 %
Schetelig et al.	1995–2006	44	54 (35–64)	3 (2–7)	del17p (100 %)	CR (13), PR (39), SD/PD (48)	RIC (89 %), MAC (11 %)	32 % (4 years)	34	68	3-year PFS: 37 % 3-year PFS: 44 %
Sorror et al.	1997–2006	82	56 (42–72)	N/A	del17p/11q/ complex (41 %)	CR (5), PR (37), SD/PD (56), bulky (24)	RIC	23 %	38	24	5-year PFS: 39 % 5-year OS: 50 %
Khouri et al.	1996–2007	86	58 (36–70)	N/A	del17p/11q (23 %)	Most in CR or low volume disease	RIC	17 % (1 year)	N/A	56	5-year PFS: 36 % 5-year OS: 51 %
Machaczka et al.	1999–2007	38	53 (42–64)	3 (1–6)	del17p/11q (21 %)	CR (11), PR (61), PD (47)	RIC	21 % (5 years)	50	47	5-year PFS: 25 % 5-year OS: 45 %
Dreger et al.	2001–2007	90	53 (27–65)	4 (1–11)	del17p (18 %), del11q (36 %)	SD/PD (24)	RIC	23 %	40	55	4-year EFS: 42 % 4-year OS: 65 %
Delioukina et al.	2001–2008	27	53 (38–68)	N/A	Poor/complex (30 %)	PR (59), SD (4), PD (37), bulky (56)	RIC	N/A	15	64	2-year PFS: 62 % 2-year OS: 64 %
Michallet et al.	2003–2008	40	54 (35–65)	N/A	del17p (7.5 %)	CR (17), PR (73), SD/PD (10)	RIC (w/rituximab)	27 % (3 years)	22	N/A	5-year EFS: 46 % 5-year OS: 55 %
Toze et al.	1991–2009	49	54 (32–68)	4 (1–14)	del17p (20 %), del11q (27 %)	Bulky LN (17)	MAC (45 %), RIC (55 %)	36 % (10 years)	N/A	63	5-year OS: 55 %
Brown et al.	1998–2009	32	48 (27–60)	3 (1–8)	N/A	CR (3), PR (56), SD (16), PD (25)	MAC	48 % (5 years)	N/A	51	5-year PFS: 36 % 5-year OS: 49 %
		76	55 (36–73)	4 (1–9)	del17p (17 %), del11q (8 %)	CR (8), PR (48), SD (22), PD (21)	RIC	16 % (5 years)	N/A	65	5-year PFS: 43 % 5-year OS: 63 %

(continued)

Table 11.1 (continued)

Reference	Time period	No. of patients	Median age (range)	Median no. of prior Tx (range)	Prognostic factors	Disease status prior to transplant (%)	Conditioning	TRM (follow-up)	Relapse rates (%)	GvHD (%)	Outcome
McClune et al.	2000–2009	26	53 (43–67)	4 (1–9)	del17p/11q/ complex (42 %)	Bulky disease (23)	RIC (12 MRD, 14 UCB)	19 % (1 year)	25	23	3-year PFS: 38 % 3-year OS: 51 %
Mortensen et al.	2000–2010	45	58 (34–70)	4 (1–9)	del17p/11q (53 %)	CR (20), bulky LN (16)	RIC	30 % (5 years)	N/A	60	5-year PFS: 38 % 5-year OS: 53 %
Jaglowski et al.	2005–2011	51	58 (37–73)	4 (1–11)	del17p/11q (57 %), complex (37 %)	CR/PR (22), SD/PD (78)	RIC	20 % (18 months)	41	N/A	18-month EFS: 48 % 18-month OS: 66 %

Tx treatment, *TRM* treatment-related mortality, *PFS* progression-free survival, *EFS* event-free survival, *OS* overall survival, *CR* complete remission, *PR* partial remission, *SD* stable disease, *PD* progressive disease, *LN* lymph node, *MAC* myeloablative conditioning, *RIC* reduced-intensity conditioning, *MRD* matched related donor, *UCB* umbilical cord blood, *N/A* not available

Rate of extensive chronic GvHD

therapy will address the role of allo-HSCT in patients with high-risk CLL. Currently, the European Bone Marrow transplantation (EBMT) guidelines recommend allo-HSCT in patients with p53 abnormalities and in younger patients with CLL who fail to respond to, or relapse within 2 years of, first-line chemoimmunotherapy.

Although allo-HSCT is a very effective tool in the treatment of CLL, high TRM and the unavailability of suitable donors limit its widespread use. However, the evidence for GVL implies that CLL can be effectively targeted by cellular immunotherapy, and has inspired the development of strategies that obviate the use of allogeneic donor cells and allow for autologous immune cells. These strategies will be reviewed in this chapter.

Genetic Modification of Autologous T Cells

Attempts to augment the antitumor immune response by activation of unmodified autologous T cells from patients with CLL have been largely unsuccessful, and it appears that the activity of these T cells was insufficient to sustain a clinical benefit. Patients with CLL have defective circulating T cells with significantly lower levels of CD154 (CD40 ligand) and aberrant gene expression profiles due to the hostile tumor microenvironment created by the immune-suppressive cytokines such as transforming growth factor (TGF)-β, IL-10, and IL-4 secreted by the CLL cells.

One promising approach to augment the antitumor efficacy of the T cells without the adverse side effects of GvHD is through genetic modification of autologous T cells to express receptors capable of recognizing cancer antigens. Two types of antitumor receptors have been studied: conventional T cell receptors (TCRs) composed of a heterodimer of α and β chains that recognize processed peptides presented on cell surface major histocompatibility complex (MHC) molecules, and chimeric antigen receptors (CARs) that contain genes encoding the single-chain variable regions of the heavy and light chains of antibodies attached to T cell intracellular signaling molecules.

T cells modified to express TCR have mainly been studied in patients with metastatic melanoma, and published data utilizing this approach in the setting of B cell malignancies is limited. Moreover, because the TCR gene transfer approach can only recognize tumor-associated antigens (TAAs) as peptides that are processed and presented by human leukocyte antigen (HLA) molecules, specificity of the TCR is restricted to specific patient HLA phenotypes and therefore lacks universal applicability. In addition, many tumor cells downregulate HLA molecules and/or have dysfunctional antigen-presenting machinery so that the targeted TAA-derived peptides are often not adequately presented on the targeted tumor cell surface.

The use of CARs can overcome these limitations of TCR gene transfer. CARs are composed of a single-chain variable-fragment (scFV) antibody specific to TAA, fused to a transmembrane (TM) domain, which is further fused to a T cell signaling moiety, most commonly either the CD3ζ or Fc receptor γ cytoplasmic

signaling domains. The CAR approach has several advantages over TCR gene-modified T cells: (1) CAR recognition of the target antigen is HLA-independent and applicable to patients of all HLA types, and is unaffected by HLA downregulation on the tumor cells; (2) CARs function in both CD4 and CD8 T cells, enabling both helper and cytotoxic tumor-targeted effector functions; and (3) CARs can be further modified to overcome the lack of co-stimulatory ligands on tumor cells to enhance modified T cell antitumor efficacy.

Method of Gene Transfer

Multiple methods can be used to introduce CARs into T cells. The most common approach uses gamma retroviruses, which efficiently and stably integrate the receptor sequence into the target cell genome. This approach has been extensively studied and validated, and has proved to be safe for transduction of mature T cells. Although concerns remain that insertional mutagenesis or immune responses against retroviral antigens could occur, recent studies have demonstrated a decade-long safety using gamma retroviruses. Another approach uses lentivirus to transduce the CAR sequence into expanding T cells. Lentiviruses, compared to retrovirus, have higher cargo capacity and reduced susceptibility to gene silencing, and there is a reduced possibility of integration into transcriptionally sensitive sites compared to retroviral vectors. Nonviral methods, such as the use of transposon-based systems and direct RNA transfection, have also been utilized but are generally associated with lower transduction efficiency. Moreover, lengthy in vitro culturing times required to select for stably transfected T cells have been shown to result in functional exhaustion and senescence of the T cells, and subsequently diminished cytolytic potential of the CAR-modified T cells over time.

Preferred T Cell Phenotype for CAR Transduction

The subtype of T cell into which the CAR is expressed may impact the ability of the T cell to proliferate and survive after adoptive transfer. Terminally differentiated T-effector memory (Tem) cells, which is a predominant phenotype seen after prolonged ex vivo cultures and expansion using anti-CD3 plus IL-2, have been suggested to have a limited proliferation and survival capacity in vivo. In contrast, some studies have demonstrated superior survival and efficacy of the CAR-modified T cells using other T cell subtypes, including T-central memory (Tcm), CD8+ native T (Tn), and T stem cell memory (Tscm) cells. Lastly, recent data also suggest a positive correlation between the efficacy of CAR-modified T cells and the number of infused CD4+ T cells, possibly by supporting the persistence of CD8+ T cells. Although CD4+ regulatory T cells (Treg) theoretically could be transduced with a CAR or develop from a non-Treg that was

CAR-transduced, culture conditions do not seem to select for this subset, and to date there is no evidence that CAR-transduced Tregs mediate clinically significant immunosuppression.

Despite these emerging data, optimal approaches for generating or maintaining these T cell subtypes during expansion are not well defined, and the T cell phenotype best suited for CAR expression remains unknown. Given the multitude of cell subsets that can be targeted with CAR therapy, and the vast array of approaches that can be used to transduce and expand the modified T cells, optimizing preclinical models to identify the preferred T cell phenotypes represents an important area for future efforts.

Targets for CAR-Modified Autologous T Cells

The identification of a tumor-specific antigen is critical for the clinical application of CAR-modified T cells to selectively target the tumor cells and to reduce detrimental effects on normal cells. In that aspect, CLL is particularly suited for CAR-modified adoptive T cell therapy since there are several suitable TAAs that have been clinically validated, such as CD19, CD20, and CD22, which appear in the early phase of B cell development and are almost invariably expressed at high levels on B-CLL cells.

CAR-modified T cells targeting CD19 and CD20 have been most extensively studied, with consistent demonstration of antitumor efficacy against B cell malignancies in animal models. However, one potential drawback of targeting these antigens is the potential prolonged elimination of normal B lymphocytes and consequent impairment of humoral immunity, although this can be mitigated with immunoglobulin repletion in patients. In order to reduce the toxicity to the normal B cell compartment, other groups of investigators have designed CARs targeting CD23, the κ or λ light chain of human immunoglobulin, which is clonally expressed on CLL cells, and ROR1, which is uniformly expressed on CLL with only transient expression on early B cell precursors in the bone marrow but not on mature B cells in the periphery. However, it remains to be seen whether CD23, κ/λ light chain, or ROR1 will prove to be a safer and more effective target for CAR-modified T cells in CLL.

Initial Clinical Experience with First-Generation CAR-Modified Autologous T Cells in CLL

Despite the promising antitumor activity of CD19- or CD20-targeted CAR-modified T cells demonstrated in animal models, only limited antitumor activity was observed in initial clinical trials with CAR-modified autologous

T cells. One potential explanation for the lack of efficacy was the limited persistence of CART cells due to the absent co-stimulatory signal in the CAR (termed first-generation CAR).

Optimal activation and proliferation of T cells require both TCR engagement and signaling (termed "signal 1"), as well as co-stimulatory signaling through co-stimulatory receptors on T cells binding to cognate ligands expressed either by the target tumor cell or by the professional antigen-presenting cells (termed "signal 2"). In the absence of such co-stimulation, the response is aborted and T cells may become anergic. To overcome the lack of T cell co-stimulation in the first-generation CARs, two different approaches have been used to provide co-stimulation to the CAR-modified T cells. One approach is to express CARs in antigen-specific T cells such as Epstein–Barr virus (EBV)-specific T cells and another is to incorporate the intracellular signaling domains of co-stimulatory molecules into a CAR (termed second-generation CAR). Several groups have adopted the latter approach, and have tested these second-generation CARs containing co-stimulatory domains such as CD28, CD137 (4-1BB), and CD134 (OX40) in animal models, where increased persistence and enhanced antitumor efficacy have been demonstrated. However, these co-stimulatory domains have not been compared in controlled clinical trials as yet, and therefore it remains unclear whether any particular co-stimulatory molecule is superior to another.

Clinical Experience with Second-Generation CAR-Modified Autologous T Cells in CLL

Since the publication of initial clinical trials with the first-generation CAR-modified T cells, promising results have been obtained in CLL using the second-generation CD19-targeting CAR-modified autologous T cells, either with CD28 or with 4-1BB co-stimulatory domains, as summarized in Table 11.2.

Investigators from the University of Pennsylvania (U Penn) initially reported the clinical outcomes of three patients with relapsed CLL who received the treatment with autologous second-generation CD19-targeted CAR-modified T cells containing 4-1BB co-stimulatory domain. They reported durable complete remissions (CRs) in two of the patients and a PR in one patient. At the 2012 American Society of Hematology (ASH) meeting, they presented an updated report of additional six patients. Of the six additional patients, CR was achieved in one patient, PR in three patients but with a short follow-up (less than 5 months), and treatment failures in two patients.

Investigators at Memorial Sloan-Kettering Cancer Center (MSKCC) have published the outcome of eight patients with purine analog refractory or relapsed CLL with bulky lymphadenopathy who received the autologous CD19-targeted CAR-modified T cells containing the CD28 co-stimulatory domain. Of the four evaluable patients, one experienced a dramatic reduction of lymphadenopathy, and

Table 11.2 Clinical trials of CD19-targeted chimeric antigen receptor (CAR)-modified autologous T cells in CLL

Institution	CAR construct	Patient ID	Age	Prior therapies	Genetic abnormalities	Disease involvement			Chemosensitive disease	Conditioning therapy	Cytokine support	CAR dose (per kg)	Response (follow-up)
						BM	PB	LN					
MSKCC	scFV-CD28-CD3ζ	01	51	FCR, PCRM	del11q	Y	Y	Bulky	N	None	N	31×10^6	PD (7 months)
		02	72	FR, RCVP, PCRM	UM	Y	Y	Bulky	N	None	N	15×10^6	PD (15 months)
		03	73	Chlorambucil, PCR, =PCRM	Normal	Y	Y	Bulky	N	None	N	15×10^6	PD (16 months)
		05	68	PCR	del11q, trisomy12	Y	Y	Bulky	N	Cy 1.5 g/m	N	5.2×10^6	LN reduction (15 months)
		06	68	RCVP, PCR, bendamustine	del11q, inv1, UM	Y	Y	Bulky	N	Cy 1.5 g/m	N	4.6×10^6	PD (3 months)
		07	62	CVP, RC, PCR, PCRM	del17p, UM	Y	Y	Bulky	N	Cy 1.5 g/m	N	8.1×10^6	SD (14 months)
		08	61	RCVP, alemtuzumab	del17p, monosomy 14/15	Y	Y	Bulky	N	Cy 1.5 g/m	N	11×10^6	SD (11 months)
		09	63	FR, RCVP, BR	Complex	Y	Y	<5 cm	N	Bendamustine	N	28×10^6	PR (12 months)
		10	46	FCR	del11q, UM	Y	Y	<5 cm	Y	Bendamustine	N	39×10^6	CR, MRD− (9 months)
U Penn	scFV-41BB-CD3ζ	01	65	FR, alemtuzumab, RCVP, lenalidomide, PCR	Normal	Y	N	1–3 cm	Possible	Bendamustine	N	16×10^6	CR, MRD− (28 months)
		02	77	Alemtuzumab, BR	Complex, del17p	Y	Y	<5 cm	N	Bendamustine	N	10×10^6	SD (24 months)
		03	64	FR, BR, alemtuzumab	del17p	Y	N	<5 cm	Possible	Pentostatin/Cy	N	0.2×10^6	CR, MRD− (27 months)
NCI	scFV-CD28-CD3ζ	03	61	N/A	N/A	N/A			Unknown	Flu/Cy	IL-2 × 2	25×10^6	CR (15 months)
		05	54	N/A	N/A				Unknown	Flu/Cy	IL-2 × 2	6×10^6	SD (6 months)
		06	57	N/A	N/A				Unknown	Flu/Cy	IL-2 × 1	55×10^6	PR (7 months)
		07	61	N/A	N/A				Unknown	Flu/Cy	IL-2 × 2	54×10^6	PR (7 months)

BM bone marrow, *PB* peripheral blood, *LN* lymph node, *Y* yes, *N* no, *UM* unmutated IgVH, *PD* progressive disease, *SD* stable disease, *PR* partial remission, *CR* complete remission, *N/A* not available, *Cy* cyclophosphamide, *FCR* fludarabine/cyclophosphamide/rituximab, *M* mitoxantrone, *RCVP* rituximab/cyclophosphamide/vincristine/prednisone, *PCR* pentostatin/cyclophosphamide/rituximab, *BR* bendamustine/rituximab

Patients have never been previously exposed to these chemotherapeutic agents

No prior chemotherapy information is provided to assess chemosensitivity of the disease

Fludarabine 25 mg/m^2 × 5 days and cyclophosphamide 60 mg/kg × 2 days

two patients had stable disease despite rapid tumor progression before therapy. Since the initial publication, two additional patients have been treated. The updated results presented at the 2012 ASH meeting reported MRD-negative CR in one patient and PR in another patient.

More recently, investigators at the National Cancer Institute (NCI) reported the outcome of four relapsed CLL patients treated with CD19-targeting CAR-modified autologous T cells containing CD28. All patients received nonmyeloablative conditioning therapy consisting of fludarabine and cyclophosphamide prior to T cell infusion, and one patient achieved a CR and three patients achieved PR.

While all these trials have utilized the second-generation CARs targeting the CD19 antigen and demonstrated very promising results with durable CRs in several patients, there are important differences among these trials as listed in Table 11.2. These factors are explained in more detail below.

Impact of Conditioning Therapy Prior to T Cell Infusion

Several preclinical studies using clinically relevant immunocompetent mouse models demonstrated that conditioning therapy prior to T cell transfer is required to improve the antitumor efficacy of the CAR-modified T cells. The importance of conditioning, also termed lymphodepleting therapy, was subsequently confirmed in clinical trials with CAR-modified T cells. In the study conducted at MSKCC, a direct comparison of CAR-modified T cell infusions with or without cyclophosphamide conditioning therapy revealed a significant improvement in persistence of modified T cells and clinical outcomes in the cohort receiving conditioning despite the lower dose of infused T cells.

However, the mechanism by which conditioning therapy improves the clinical outcomes and the relevance of tumor sensitivity to the conditioning regimen remain unclear. Initially, the benefit of conditioning therapy was attributed to lymphopenia, promotion of homeostatic cytokines (i.e., IL-7 and IL-15), and reduction of Treg numbers. These data prompted investigators at the NCI to adopt both myeloablative and nonmyeloablative conditioning, and encouraging clinical outcomes were reported. In contrast, the trials conducted at the U Penn and MSKCC included less intense conditioning and reported similar results (Table 11.2). Moreover, the updated results of the trial at MSKCC suggested a potentially greater effect of conditioning regimens through tumor burden reduction than the induction of a supportive cytokine response.

Tumor Burden and Sensitivity to Conditioning Therapy

Investigators at MSKCC observed an inverse correlation between tumor burden and detectable infused modified T cells. Similarly, in the trial conducted at U Penn, the best responses were observed in patients with low total white blood cell counts, no

peripheral lymphocytosis, and lymph nodes $\leq$3 cm in size. These findings are consistent with an enhanced antitumor efficacy mediated by CAR-modified T cells in the setting of lower tumor burden in preclinical models, and raise a question regarding the role of potential tumor sensitivity to conditioning chemotherapy in the clinical outcomes of patients treated with CD19-targeted CAR-modified T cells. Patients at MSKCC with cyclophosphamide-refractory CLL who received cyclophosphamide conditioning achieved no significant clinical benefit, but a dramatic increase in response rates was noted when the conditioning regimen was selected based on the likelihood of tumor sensitivity to the conditioning chemotherapy. Similarly, CLL patients treated at U Penn as well as the initial patient reported at the NCI were naïve to conditioning chemotherapies with likely chemosensitive disease. Combined together, these results suggest a potential correlation between clinical outcomes and tumor sensitivity to conditioning chemotherapies, and warrant further investigation.

Toxicities

Early successes with CAR-modified T cells were not achieved without toxicities. Flu-like symptoms such as fever, malaise, and myalgias, as well as capillary leak syndrome, are common after the T cell infusions, likely related to cytokine release by the infused cells. Prolonged B cell aplasia and hypogammaglobulinemia, anticipated side effects from on-target effects of the CD19-specific CAR-modified T cells, have also been observed in responding patients. However, to date, there have been no apparent genotoxic events attributed to genetically modified T cells that have been transduced by recombinant virus or electroporation.

As a result of these toxicities, there has been increasing interest to install a "safety switch" to quickly eliminate the infused cells in cases of adverse events. One of the most well studied strategies is to incorporate suicide genes, such as the herpes simplex thymidine kinases (TK) gene or an inducible caspase 9 (iCaspase9) protein, that can be activated by specific drugs and eradicate the genetically engineered cells if adverse effects occur following adoptive transfer. Of the two suicide genes, iCasp9 seems to be the most appropriate for CAR-modified T cell therapy due to its rapid onset of action and non-immunogenicity.

Clinical Experience with Allogeneic CD19-Targeted CAR-Modified T Cells

Given the promising results with autologous CAR-modified T cells in patients with CLL, the CD19-targeted CAR technology has also been applied in the setting of allogeneic T cells. Investigators at the NCI recently presented a preliminary

short-term outcome of a 65-year-old man with CLL, who relapsed after two allo-HSCTs and subsequently received a single infusion of CD19-targeted CAR-modified allogeneic T cells. His pre-T cell bone marrow showed 80–90 % involvement by CLL, but the posttreatment biopsy at day 26 showed a normocellular marrow with no evidence of CLL. While the patient developed cytokine-mediated symptoms, no GvHD exacerbation was reported, although it is unclear whether the patient had any evidence of GvHD or was maintained on GvHD prophylaxis at the time of T cell infusion. Bollard et al. used EBV/CMV/adenovirus-specific allogeneic T cells modified to express CD19-targeted CAR, and observed a transient reduction in lymphadenopathy in one patient and stable disease in three other treated patients with relapsed CLL following allo-HSCT. While longer follow-up is needed, these results encourage further development of donor-derived CAR-modified T cells as a treatment for relapsed CLL after allo-HSCT. The concern over the potential GvHD exacerbation may be mitigated by incorporation of the suicide gene, which will allow a rapid elimination of the infused T cells immediately upon recognition of adverse events.

Conclusion

That durable responses can be achieved in patients with chemotherapy-refractory and relapsed CLL supports the evidence of GVL and illustrates the potency of immune-mediated cellular therapy in CLL.

Among the most novel and promising of these approaches are CAR-based cell therapies that combine advances in genetic engineering and adoptive immunotherapy. Early clinical reports have certainly been promising, but many questions remain unanswered. Carefully designed clinical trials will be needed to assess the optimal timing for incorporating CAR-based adoptive immunotherapies in CLL, and to test the safety and efficacy of the new generation of cytokine secreting CAR cells that may have enhanced activity against the chemotherapy-refractory bulky disease.

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Chapter 12
Towards Targeted Therapy of Chronic Lymphocytic Leukemia

Carsten U. Niemann, Jade Jones, and Adrian Wiestner

Abstract The B cell antigen receptor (BCR) and its downstream pathways are pivotal in the pathogenesis of chronic lymphocytic leukemia (CLL). Recently, inhibitors of kinases in the BCR pathway have shown promising clinical activity in CLL. Based upon these results, the treatment paradigm for CLL will likely undergo major changes. The kinases essential for BCR signal transduction, which are emerging as targets for CLL treatment, and the specific inhibitors under development are the focus of this chapter. In particular, the BTK inhibitor ibrutinib and the PI3K inhibitor idelalisib (GS-1101) are two evolving targeted therapies with the most mature clinical data.

Keywords BCR (B cell antigen receptor) • Microenvironment • Targeted therapy • Lymphocytosis • Ibrutinib (PCI-32765) • idelalisib (GS-1101, CAL-101) • Everolimus (RAD001) • Navitoclax (ABT-263) • ABT-199 • Lenalidomide • Dasatinib (BMS-354825) • Fostamatinib (R788) • BTK (Bruton's tyrosine kinase) • SYK (spleen tyrosine kinase) • LYN • PI3K (phosphatidylinositol 3 kinase) • mTOR (mammalian target of rapamycin) • BCL-2

Introduction

Differences in the somatic mutational status of the immunoglobulin loci among B cell chronic lymphocytic leukemia (CLL) patients were revealed almost 20 years ago [1]. Since that time, the B cell antigen receptor (BCR) and the downstream pathways have been thoroughly investigated. In 1999, the groups of Stevenson and Chiorazzi demonstrated the prognostic significance of unmutated vs. mutated

C.U. Niemann • J. Jones • A. Wiestner (✉)
Hematology Branch, National Heart, Lung, Blood Institute, National Institutes of Health, Bldg. 10, CRC 3-5140, 10 Center Drive, Bethesda, MD 20892-1202, USA
e-mail: wiestnera@mail.nih.gov; wiestnea@nhlbi.nih.gov

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IGHV (immunoglobulin heavy chain variable gene cluster) genes among CLL patients [2, 3]. A few years later, the concept of stereotyped motifs for the IGHV gene among CLL patients [4] further underscored the significance of the BCR pathway in CLL and pointed towards CLL as a partially antigen-driven disease. Most recently, an autonomous signaling through interaction of the HCDR3 of the heavy chain of the BCR with an internal epitope (FR2) of the BCR in CLL cells was shown [5]. This finding will probably modify the understanding of the pathogenesis of CLL significantly. It may also allow for new treatment targets to be revealed [6]. Along with this growing understanding of the role of the BCR in CLL, the differences in phenotype and sensitivity to different treatments among CLL cells in peripheral blood, bone marrow, and lymph nodes have underscored the impact of the microenvironment on the course of CLL [7]. This is further emphasized by the effect of new targeted therapies for CLL which cause redistribution of CLL cells from lymph nodes to peripheral blood [8]. Furthermore, the corroboration of tissue and lymph node as the proliferating compartment compared to the predominantly resting CLL cells of peripheral blood underscores the significance of the microenvironment in CLL [9]. BCR signaling is likely a pivotal pathway activated in CLL cells in lymphatic tissue as indicated by gene expression profiling of lymph node-derived CLL cells compared to peripheral blood CLL cells from the same patient [10]. Even though the BCR pathway has been well characterized for decades, the intricate interrelation of downstream signaling cascades continues to be extended and modified. Several targets for development of new treatments in CLL have been revealed, along with new achievements in the understanding of BCR signaling [11].

Targets in CLL: Reflections of Normal B Cell Pathways

The molecules in the BCR pathway that are emerging as targets for CLL treatment are outlined in Fig. 12.1. The focus of this chapter is on molecules that are currently targeted in clinical trials or for which there are significant preclinical data. Initially, an overview of the evolving concepts of how B cells interact with the microenvironment and a discussion of different types of BCR signaling is presented.

Initiation of BCR Signaling

The conformational state of BCRs in resting B cells has hitherto not been fully outlined. Moreover, the change in the conformation of the BCR that propagates signaling upon antigen binding is not clearly understood. One model describes the BCRs of resting B cells as inhibited oligomers flowing in the plasma membrane. Upon antigen stimulation, the state of the BCR changes to an open conformation for signal propagation [12]. Another model describes oligomerization upon antigen binding that initiates signaling [13]. The detection of stereotyped BCRs in some subsets of CLL patients [4]

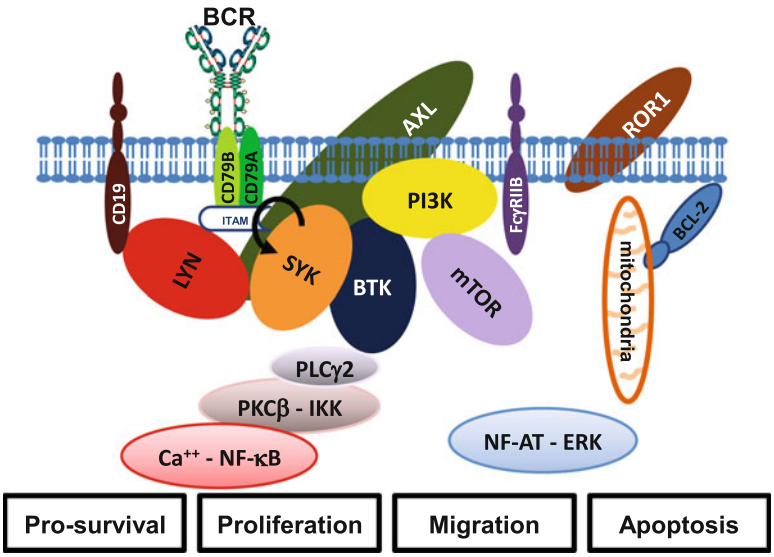

Fig. 12.1 Signaling through the B cell antigen receptor (BCR) with downstream and parallel pathways in chronic lymphocytic leukemia (CLL) outlined. Antigen binding or cell autologous interaction initiates BCR signaling, causing phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of co-receptors CD79A and B. LYN has a double function in initiating and terminating BCR signaling depending on interaction with CD19. Spleen tyrosine kinase (SYK) amplifies the initial signal by further phosphorylation of ITAMs. SYK also activates phosphatidylinositol 3 kinase δ (PI3Kδ) that in turn converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). Mammalian target of rapamycin (mTOR) relays PI3K activation further to cell cycle regulation. Bruton's tyrosine kinase (BTK) is recruited to the plasma membrane and activates PLCγ2, which in turn generates a set of second messengers to activate protein kinase C β (PKCβ). PKCβ phosphorylates IκB kinase (IKK) to activate NF-κB transcription factors that regulate gene expression of several survival factors. Signaling can be modulated by interaction with BCL-2 family members guarding apoptosis induction. FcγRIIB inhibits BCR signaling upon binding of complement. The downstream effectors can be turned towards the pro-apoptotic NF-AT–ERK pathway, depending on balancing of the signaling cascades. ROR1 signals through the alternative Wnt pathway. Only molecules of relevance for the discussion of evolving targeted therapies in CLL are included. Please see the sections on specific parts of the signaling pathways for further details

has resulted in research to determine the antigenic drive in CLL. Based on affinity to some of the stereotyped immunoglobulins, candidate autoantigens like non-muscle myosin from apoptotic cells [14] and common microbial antigens [15] have been identified as probable drivers for CLL cells [16]. However, a new understanding of dysregulated BCR signaling has been proposed. Dühren-von Minden et al. showed that the HCDR3 region of the BCR binds the FR2 epitope of the same or adjacent BCRs on the same cell [5]. Transfer of the HCDR3 region from both mutated and unmutated as well as stereotyped and non-stereotyped CLL cells to normal B cells conferred the chronic BCR signaling of the CLL cells to the normal B cells. The elucidating of this

new mechanism for autonomous signaling through the BCR and the impact on the understanding of the pathogenesis of CLL await further research [6]. Most importantly, the revealing of a pathway leading to autologous chronic activation of the BCR emphasizes the molecular basis for targeting this pathway to treat CLL.

From BCR Activation to Phosphorylation

Within the cytoplasmic part of the BCR, ITAMs (immunoreceptor tyrosine-based activation motifs) were identified more than 20 years ago as probable intracellular signal generators. ITAMs are specific preserved sequences of the CD79A/B of the BCR complex [17], which are also represented in other pathways of the immune system, like the CD3 components of the T cell receptor. ITAMs were shown to link antigen binding by BCR to intracellular signaling cascades [18]. Binding of antigen to the extracellular part of the BCR confers initiation signals to the phosphorylation of ITAMs in the cytoplasmic part of CD79A/B. Several of the kinases in the BCR pathway bind directly to ITAMs.

ITAMs are also involved in the anergic state of some B cells by monophosphorylation of ITAMs. This mechanism is part of the host's protection against autoreactivity [19]. Anergic B cells are described as being in a condition of unresponsiveness to antigen stimulation induced by chronic autoantigen occupation of a part of the BCRs [20]. Furthermore, mutation of a critical tyrosine residue in the ITAM of CD79B seems to allow abnormally active responses to chronic antigen stimulation through attenuation of LYN signaling in some B cell malignancies [21].

Signal Propagation

Propagation and amplification of BCR signaling, as well as further clustering, is dependent upon downstream signaling through spleen tyrosine kinase (SYK) and LYN [22]. Whereas LYN has a balancing effect on BCR signaling, both initializing and terminating the signaling cascade, SYK amplifies the signal through further phosphorylation of ITAMs at CD79A/B (see Fig. 12.1). Thus, SYK and LYN have been among the first targets for clinical trials of inhibition of BCR pathway kinases in CLL. On the other hand, in resting B cells, a tonic survival signal is relayed through the phosphatidylinositol 3 kinase (PI3K) pathway downstream of the BCR that does not require phosphorylation of ITAMs, SYK, or LYN [13]. It has been suggested that part of the pathogenesis of CLL involves dysregulation of the tonic signaling of resting normal B cells [11].

Balancing BCR Signaling

In normal B cells, FcγRIIB is an inhibitory co-receptor that inhibits BCR signaling in response to complement binding, thus taking part in the balancing of the BCR pathway. The inhibitory effect of activated FcγRIIB is at least in part mediated by interaction with LYN and BCR oligomer formation early in the BCR pathway [23]. These balancing effects may be dysregulated in CLL cells, thus adding support to targeting of LYN [24]. The understanding of the dynamics in the interaction of membrane-bound proteins, properties inherent to the lipid bilayer, and the cytoskeleton is evolving. It appears that FcγRIIB activation, as well as inhibition of Bruton's tyrosine kinase (BTK) and PI3K, affects the mobility of BCRs in the plasma membrane. For BTK and PI3K inhibition, the effect is through inhibition of sphingolipid glucosylation [25]. This may in part explain the effect of BTK and PI3K inhibition on abrogating the BCR signaling in CLL.

The tonic signaling through the BCR of a resting B cell is also dependent on interaction between CD79B and the actin cytoskeleton that restricts the movement of the BCR in the plasma membrane [26]. BTK directly interacts with the actin cytoskeleton during BCR signaling, causing migration of B cells and internalization of the BCR [27]. Thus, targeting BTK may modulate mobility of the BCR in the plasma membrane by interaction with both actin and sphingolipid synthesis, in addition to inhibiting downstream activation signals from the BCR.

Internalization of BCR was initially seen as a way of extinguishing the antigen activation of B cells. Strong down-modulation of surface BCR by repeated antigen stimulation contributes to lack of surface IgM and BCR signaling in normal B cells, explaining at least in part the anergic state of these cells [11, 28]. The same mechanism may be responsible for the indolent course observed for some CLL patients. Internalization of BCRs also results in a more immature N-glycosylation of surface-mannosylated IgM in normal B cells after prolonged antigen activation. This kind of immature N-glycosylation is also reported to be more prevalent in unmutated CLL [29]. This may suggest that the immaturely N-glycosylated surface IgM in part causes the more aggressive phenotype of unmutated CLL. Recent findings have revealed that BCR internalization is indeed employed in the regulation and fine tuning of the intracellular signaling cascades. Inhibition of BCR internalization was shown to result in dysregulated signaling that may reflect the state of some B cell malignancies [30].

Downstream Pathways

BCR signaling is able to switch between a proliferative, pro-survival, anti-apoptotic signaling and an anergic, apoptosis-promoting signaling. This seems to be reflected by the balancing between large biphasic calcium responses with activated NF-κB

vs. low calcium oscillation with activated ERK/NF-AT downstream in the BCR pathway [31]. These different downstream pathways convey in part the ability of normal B cells to switch between positive selection by foreign antigens and negative selection by self-antigens. A molecular signature of anergy in a subset of CLL patients (constitutively phosphorylated ERK, increased NF-AT transactivation) may be a correlate of negative selection by self-antigen and is associated with a more indolent course [32]. These downstream pathways of BCR represent ubiquitous pathways in normal cells as well as in the malignant counterparts. The ubiquitous usage of these pathways could represent a caveat in targeting them in CLL due to the expectation of multiple unwanted effects in other cell systems. Even so, the different pathways implicated in microenvironmental interaction that are so important for proliferation of CLL cells [8] converge with the BCR pathways on these kinases and transcription factors. Thus, these downstream pathways might very well point towards new targets for development of CLL treatment. Indeed, agents targeting NF-κB are in preclinical development with possible forthcoming testing in CLL.

Translation into Clinical Results

The elucidation of B cell pathways has rapidly been followed by development of new targeted therapies for CLL with impressive results. While these results are preliminary, they are promising for the treatment of CLL patients in forthcoming years. However, a caveat should be kept in mind: most of the targeting agents are actually multi-targeting drugs that inhibit several different kinases acting in different pathways. Thus, making inference from clinical effects to cellular mechanisms of action is difficult. Furthermore, the alluring concept of targeted therapies that achieve impressive results in initial clinical testing should still be rigorously studied in controlled clinical trials. It will be important to validate surrogate markers like progression-free survival (PFS) or complete/partial response (CR/PR) to overall survival [33, 34]. An additional consideration will be the quality of life with different treatment options. The current discussion of amendments to response criteria for CLL based upon the peripheral lymphocytosis seen with most of the evolving targeted therapies emphasizes the importance of reassessing response criteria [35]. At the same time, the benefits of targeted therapies should be implemented for patients as soon as possible. This is especially important, as current standards of treatment do not always meet the need for long-lasting control of CLL.

Targeting LYN

Upon activation of the BCR by antigen, LYN, an SRC family kinase, initiates BCR signaling by phosphorylating ITAMs on the cytoplasmic part of CD79A and CD79B that then recruit further components of the signaling pathway. LYN directly

phosphorylates SYK as part of the downstream pathways from the BCR complex. LYN also activates phosphatases that in turn inhibit signal transduction through the BCR [11]. By this double mode of action, LYN both activates and terminates BCR signaling. LYN-deficient mice have reduced numbers of B cells that at the same time are less responsive to acute BCR activation. These mice eventually develop a lupus-like autoimmune disease, thus pointing towards an essential function of LYN to both downregulate BCR activation and limit the expansion of autoreactive B cells [36, 37]. The balancing between activation and inhibition of the BCR pathway has been shown to depend on a close interaction between CD19 and LYN (see Fig. 12.1). Deficiency of CD19 represses the autoimmune phenotype of LYN-deficient mice [38]. Furthermore, mutations of CD79B in some lymphoid malignancies reduce LYN kinase activity, thereby promoting "chronic active BCR" signaling and constitutive NF-κB activation. These findings underscore the bidirectional function of LYN in B cells [20].

Dasatinib (BMS-354825)

Dasatinib is a dual SRC/ABL kinase inhibitor that is approved for use in CML [39]. In addition to targeting the BCR/ABL kinase in CML and several kinases from the SRC and TEC families (including both LYN and BTK involved in the BCR pathway [40], see Fig. 12.2a) it appears that dasatinib targets several other kinases in different kinase families [41]. In vitro, dasatinib induces variable degrees of apoptosis in CLL cells with no correlation between response and inhibition of LYN phosphorylation. However, the impact of dasatinib on the BCR pathway was demonstrated by in vitro apoptosis being inversely correlated with drug-induced inhibition of SYK phosphorylation. While dasatinib inhibited BCR signaling, stromal cell contact and CD40 stimulation antagonized the pro-apoptotic effect of the drug [42]. However, dasatinib has recently been shown to inhibit actin polymerization and migration in response to CXCL12 through inhibition of CXCR4 signaling [43]. This may be indicative of LYN inhibition, as mononuclear cells from LYN-deficient mice have shown impaired CXCR4-dependent migration [44].

Only one phase II study of dasatinib in relapsed/refractory CLL (15 patients enrolled) has been published to date [45]. The overall response rate (OR) for patients was 20 %. In addition, 4 patients (27 %) exhibited more than 50 % reduction in lymphadenopathy. Median PFS was 7.5 months. Myelosuppression was the primary toxicity, with grade 4 neutropenia and thrombocytopenia occurring in 40 % and 13 % of patients, respectively. As of November 2012, four active clinical studies with dasatinib in CLL (alone or in combination with fludarabine or fludarabine + rituximab) are registered at clinicaltrials.gov.

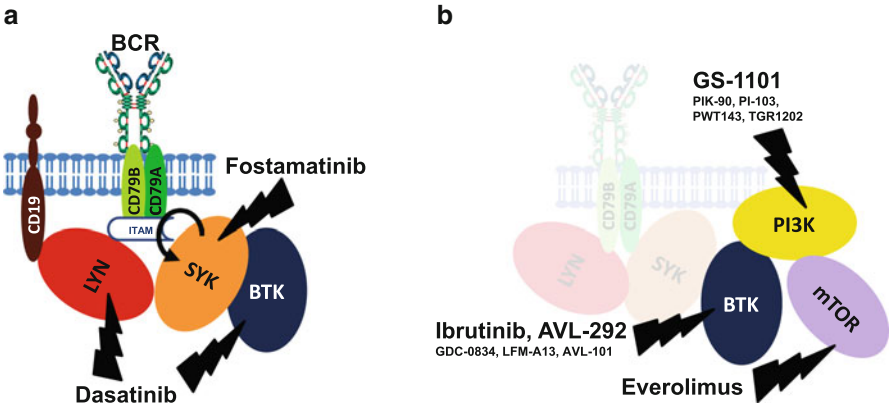

Fig. 12.2 (**a**) Inhibition early in the BCR pathway. LYN can be inhibited by dasatinib that was developed as a BCR/ABL inhibitor. BTK is also targeted by dasatinib. SYK is targeted by fostamatinib. See text and Table 12.1 for further details. (**b**) Inhibition in the intermediate part of the BCR pathway. PI3K can be inhibited by idelalisib and several other agents in preclinical development. Inhibition of BTK has been shown in clinical trials by ibrutinib and AVL-292 with other agents in preclinical development. Everolimus is an inhibitor of mTOR in clinical use for immunosuppression, being clinically tested for CLL. See text and Table 12.1 for further details

Targeting SYK (Spleen Tyrosine Kinase)

SYK is a non-receptor tyrosine kinase closely related to ZAP70 (essential for T cell receptor signaling) [46]. Mice with a genetic deletion of SYK have a severe impairment of B cell development at the pro-B cell to pre-B cell transition and lack mature B cells [47, 48]. Some redundancy between ZAP70 and SYK is indicated by ZAP70 expression during B cell development that can partially substitute for loss of SYK function. ZAP70 expression in CLL is correlated with unmutated phenotype and a more progressive disease course. Interestingly, the increased BCR response in ZAP70-expressing CLL cells is independent of its kinase activity. It appears to be mediated by inhibition of events that terminate the signaling response and/or interfere with internalization of the BCR, culminating in a more prolonged activation of SYK [10, 49].

Upon antigen binding to the BCR and through phosphorylation of ITAMs, LYN is recruited to the plasma membrane for subsequent phosphorylation of SYK. However, SYK is also directly recruited and activated by binding to two ITAMs of the BCR through its own two SH2 domains (see Mocsai et al. for a thorough review of SYK function [46]). This results in an amplification of the initial BCR signal and promotes the activation of downstream signaling cascades (see Fig. 12.1). In addition, SYK is involved in chemokine, integrin, and Fc-receptor signaling [46] and is thus an important factor in mediating multiple microenvironmental signals in CLL cells. Even though SYK is constitutively activated (pY352) in peripheral blood CLL cells, no correlations between

Table 12.1 Targeted therapies in development for CLL

Target	Function	Inhibitors	Response	PFS (median)	Toxicity	Trials
LYN	Activate and terminate BCR signaling	Dasatinib (+targets BTK)	3/15 PR + 6 nodal with lymphocytosis, phase II [45]	7.5 months	Neutropenia (40 % grade 4), thrombocytopenia (14 % grade 4), infection (14 % grade3)	Single agent, +fludarabine, +fludarabine/rituximab
SYK	Upstream amplification of BCR signaling, binds ITAM	Fostamatinib (and others)	6/11 PR, all lymphocytosis initially, phase I/II [57]		Diarrhea (54 % grade 1/2), neutropenia (25 % ≥ grade 3), thrombocytopenia (25 % grade 1/2), febrile neutropenia (8 % ≥ grade 3)	None in CLL, one in DLBCL
PI3Kδ	Intermediary in BCR pathway, also in tonic survival signaling for resting B cells	idelalisib	*n* 54, 26 % PR, 80 %: ≥50 % reduced lymph nodes, ≥50 % lymphocytosis in 58 %, phase I [72]	15 months	Pneumonia (24 % ≥ grade 3), neutropenia (24 % ≥ grade 3), thrombocytopenia (7 % ≥ grade 3), febrile neutropenia (7 % ≥ grade 3)	+Bendamustine, +/− rituximab (preliminary results [73]), +ofatumumab (preliminary results [74]), +fludarabine, +chlorambucil
BTK	Downstream of LYN/SYK in BCR signaling, also implicated in microenvironment interaction	Ibrutinib	11/16 PR (2 CR), all lymphocytosis initially, phase I [96]	26 months	Neutropenia (12.5 % ≥ grade 3), thrombocytopenia (7.2 % ≥ grade 3), respiratory (7.1 % ≥ grade 3), diarrhea (46.5 %)	+Rituximab (preliminary results [98]), +bendamustine/rituximab (preliminary results [99]), +ofatumumab (preliminary results [100]), +fludarabine/cyclophosphamide
		AVL-292	75/116 ≥ PR, +22 PR with lymphocytosis, phase Ib/II [97]		Diarrhea 54 %, all grades; respiratory 29 %, all grades	

(continued)

Table 12.1 (continued)

Target	Function	Inhibitors	Response	PFS (median)	Toxicity	Trials
mTOR	Downstream of PI3K in BCR pathway, immunomodulatory	Everolimus	4/22 PR + 8 nodal with lymphocytosis [112], minor response, lymphocytosis, phase I/II [110, 111]		Neutropenia (32 % ≥ grade 3), thrombocytopenia (50 % ≥ grade 3), infections (23 % ≥ grade 3), ARDS (5 % grade 4, class effect)	+idelalisib, +alemtuzumab, +rituximab (maintenance), +panobinostat, +bortezomib, +lapitinib, +sorafenib
		Temsirolimus	Minimal effect, phase II [113]			
CXCR4/ CXCL12	Microenvironment	Nox-A12				Single agent, +lenalidomide
		Plerixafor	5/14 PR (phase I, +rituximab) [130]			
BCL2	Mitochondria-mediated anti-apoptotic	Navitoclax	9/29 PR, phase I [134]		Neutropenia (28 % ≥ grade 3), thrombocytopenia (28 % ≥ grade 3), respiratory (21 % ≥ grade 3)	+Rituximab, +/− maintenance (preliminary results [136])
		ABT-199	Preliminary, phase I [137]		Diarrhea (24 %), infections (18 %)	
		Oblimersen	Modest effect, phase III [140]			None
		Obatoclax	Modest effect, phase I [142]			None
Cereblon	Immunomodulatory, both B and T cells	Lenalidomide	21/45 ≥ PR, tumor flare, phase II [151]		Neutropenia (70 % ≥ grade 3), thrombocytopenia (45 % ≥ grade 3), infections (5 % ≥ grade 3), febrile neutropenia (15 % ≥ grade 3)	+Cyclophosphamide, +fludarabine, +ofatumumab, +rituximab, +flavopiridol (results [152]), plerixafor, azacitidine

See text for further details and agents in preclinical development

the degree of SYK activation and clinical or biological features of more aggressive disease have been shown [50]. One possible explanation for this could be that CLL cells in peripheral blood only partially reflect the activity of signaling pathways in the tissue microenvironment [10].

Fostamatinib (R788, Oral Pro-drug of R406, the Active Metabolite)

Fostamatinib is the orally available formulation of an ATP-competitive kinase inhibitor that inhibits a number of other kinases in addition to SYK [51] (see Fig. 12.2a). Initially, fostamatinib was developed with a focus on treating inflammatory diseases. Treatment of CLL cells with fostamatinib in vitro inhibited BCR and integrin signaling, antagonized the protective effect of stromal cells, reduced migration to chemokines and adhesion to stromal components, and induced a moderate degree of apoptosis [50, 52, 53]. Thus, SYK inhibition antagonizes both BCR-dependent and BCR-independent pathways in CLL cells and thereby abrogates stimulatory input from the microenvironment. The significance of SYK inhibition in treating B cell malignancies has been supported by studies in animal models. Fostamatinib prevents disease progression both in TCL1 transgenic mice (in which antigen-dependent selection appears to play a similar role as in human CLL) and in a non-Hodgkin lymphoma model that depends on cooperation between MYC and BCR-derived signals [54, 55]. In the TCL1 transgenic mouse, SYK inhibition induced a transient increase in circulating lymphocytes, reduced the proliferation of malignant B cells, and prolonged survival of the mice [54]. The on-target effect of fostamatinib in the BCR pathway has been demonstrated by downregulation of BCR pathway-specific targets in patients treated with fostamatinib as well as downregulation of NF-κB and MYC targets. However, no correlation between inhibition of BCR signaling and response to treatment could be shown [56].

The only published clinical trial of an SYK inhibitor used fostamatinib in a phase I/II study which included 11 patients with CLL, of whom 6 (55 %) achieved a PR [57]. The response rate in CLL was the highest, ahead of diffuse large B cell lymphoma (DLBCL) (22 %), mantle cell lymphoma (11 %), and follicular lymphoma (10 %). The dose limiting toxicity was a combination of diarrhea (17 % $\geq$ grade 3), neutropenia (33 % $\geq$ grade 3), and thrombocytopenia (17 % grade 1/2). In the phase II portion of the trial the most common adverse events were reversible cytopenias (anemia: 13 % $\geq$ grade 3, neutropenia: 25 % $\geq$ grade 3), fatigue (50 % grade 1/2), diarrhea (54 % grade 1/2), and hypertension (29 %, 4 % $\geq$ grade 3). There is one ongoing study testing fostamatinib in DLBCL. However, there are currently no active trials in CLL. Late stage clinical trials in rheumatoid arthritis [58] are ongoing. Preclinical data on two more specific SYK inhibitors (PRT318 and P505-15) have recently been published [59].

Targeting PI3K (Phosphatidylinositol 3 Kinase)

The PI3Kδ isoform primarily expressed in leukocytes together with the ubiquitously expressed PI3Kα isoform is essential for B cell development. Mice lacking PI3Kδ/α show a virtual absence of B1 B cells and marginal zone B cells but still have follicular B cells [60]. The significance of the PI3K pathway downstream of the BCR is emphasized by rescue from apoptosis of resting B cells that lack BCR by introducing constitutive active PI3K into these cells [61]. However, PI3Kα and PI3Kδ fulfill somehow redundant functions in tonic BCR signaling, as demonstrated by constitutively active PI3Kα also being sufficient to rescue B cells that have lost BCR expression [62]. The PI3K pathway furthermore mediates migration signals in B cells, thus pointing towards an important role in the interaction of CLL cells with the microenvironment [60, 63]. The p85 subunit of PI3K binds via SH2 domains to tyrosine kinases or adaptor molecules, while the p110 subunit phosphorylates phosphatidylinositol substrates, thus generating PIP3. However, the mechanisms of activation/inhibition seem to be more complex, as one of the SH2 domains is in part inhibitory. This explains the oncogenic effect of a deletion of this domain identified in a Hodgkin lymphoma cell line [64]. PIP3 is a pivotal scaffold, recruiting the components for the functional signaling complex downstream of the BCR, including BTK, PLCγ2, and AKT (see Fig. 12.1). While BTK can be tyrosine-phosphorylated in the absence of PI3Kδ, downstream effects such as calcium mobilization and cell proliferation are severely impaired in the absence of PI3K [65].

Idelalisib (GS-1101, CAL-101)

Idelalisib has been shown to inhibit the PI3Kδ isoform and induce apoptosis in CLL cells [66] (see Fig. 12.2b). Microenvironmental signals relayed through the BCR and alternative pathways, including interaction with CD40L, BAFF, TNFα, and fibronectin, are inhibited by idelalisib [67, 68], resulting in inactivation of AKT and ERK. Moreover, idelalisib inhibits secretion of cytokines and chemokines from CLL cells in a dose-dependent manner. In vivo, CLL patients treated with idelalisib showed a rapid decrease in CCL3 and CCL4, previously shown to be upregulated in CLL cells in a BCR-dependent manner [10, 68, 69]. T cell viability is not affected by idelalisib, although T cell secretion of some inflammatory and anti-apoptotic cytokines seems to be inhibited [67]. Thus, T cell modulation may play a role in the function of idelalisib in CLL as well.

Safety and activity of idelalisib in hematologic malignancies were evaluated in a phase I study. Fifty-four patients with CLL were enrolled. The OR by IWCLL criteria [70] was 26 % [71, 72]. However, 80 % of patients had a reduction in lymphadenopathy by $\geq$50 %. Many of these patients did not meet criteria for response by IWCLL criteria due to a transient increase in the absolute lymphocyte count. As discussed recently by Cheson et al., the peripheral lymphocytosis seen

with most targeted drugs in CLL may warrant amendment of the response criteria for CLL [35]. PFS was not reached at >11 months and responses were independent of classic risk factors including response in patients with 17p deletion. Grade ≥3 adverse events included pneumonia (24 %), neutropenia (24 %), thrombocytopenia (7 %), neutropenic fever (7 %), anemia (6 %), and increased liver enzymes (6 %). Studies in which idelalisib is combined with bendamustine and/or rituximab, fludarabine, ofatumumab, chlorambucil, and chlorambucil + rituximab maintenance are currently being performed. Preliminary results from some of these studies have reported OR and 1-year PFS rates between 74 and 88 % (+bendamustine and/or rituximab) [73]. The combination with ofatumumab reported preliminary results with an OR of 82 % [74]. No major safety concerns have hitherto been reported for any of the combinations. However, it should be noted that these are very preliminary data.

Other PI3K Targeting Agents

Several PI3K inhibitors are in preclinical and early clinical studies in hematologic malignancies. Specificity of different inhibitors for specific isoforms of PI3K seems to confer specific effects, i.e., the PI3Kα inhibitors PIK-90 and PI-103 were more effective than PI3Kδ or PI3Kβ/δ-specific inhibitors at inhibiting CLL cell migration to CXCL12 and in antagonizing stromal cell-mediated survival signals [75]. Rigosertib, another PI3Kα/β inhibitor currently explored for myelodysplastic syndrome, induced apoptosis in CLL cells cultured in contact with stromal cells [76]. Despite the attractive option of selectivity, selective inhibitors of PI3K isoforms may be bypassed due to different PI3K isoforms assuming redundant functions [77]. Other PI3Kδ-specific inhibitors are also in preclinical and early clinical trials in CLL, including PWT143 and TGR1202 (recently reported preclinical findings [78, 79]). SAR245408 is a pan-PI3K inhibitor that is well tolerated in patients with solid tumors [80, 81].

Targeting BTK (Bruton's Tyrosine Kinase)

BTK is a member of the TEC kinase family that also includes TEC (B cells/T cells/liver cells), IL2-inducible T cell kinase (ITK), and BMX/ETK (bone marrow, endothelia, epithelia). Loss of BTK causes X-linked agammaglobulinemia with the absence of mature peripheral B cells and low serum immunoglobulin levels [82, 83]. BTK is a non-receptor tyrosine kinase recruited early in the BCR signaling cascade and closely linked to SYK, PI3Kδ, PLCγ, calcium signaling, and NF-κB activation [84]. Upon activation of the BCR pathway, BTK attaches to the plasma membrane through its pleckstrin homology domain that binds to PIP3 [85]. BTK appears to be essential only in B cells and is required for

BCR-induced calcium release, cell proliferation, and activation of the NF-κB pathway [86, 87]. BTK is shown to regulate actin dynamics and antigen processing during BCR activation [27]. A downstream target of BTK is PKCβ, which in turn phosphorylates IKK, resulting in release and translocation of NF-κB transcription factors to the nucleus [88]. The on-target effect of BTK inhibition has been confirmed by downregulation of BCR signaling targets and NF-κB activity in tumor cells from both the peripheral blood and lymph nodes of CLL patients treated with ibrutinib [89]. It has also been shown that BTK is involved in B cell trafficking through the pathways of CXCR4/CXCR5 chemokine receptors [90]. Using in vitro and in vivo models, CLL homing to CXCL12 and CXCL13, as well as secretion of chemokines upregulated by BCR and/or NF-κB activation (CCL3 and CCL4), was shown to be decreased by ibrutinib [91]. In the murine TCL1 transgenic model of CLL it was shown that lack of BTK activity slowed the development of CLL [92]. The lymphocytosis seen in some patients upon inhibition of BTK may be explained in part by inhibition of pathways involved in B cell trafficking and adhesion to stroma.

Ibrutinib (PCI-32765)

Ibrutinib binds covalently to the cysteine Cys-481 of BTK and thereby irreversibly inactivates the kinase [86, 93]. In addition to blockage of BCR signaling, it is reported that integrin-mediated adhesion as well as signaling, adhesion, and migration to CXCL12, CXCL13, and CCL19 is inhibited in primary CLL cells [94]. Herman et al. showed that ibrutinib not only inhibits BCR signaling but also disrupts the protective effect of stromal cells, and inhibits CD40, BAFF, TLR, and cytokine signaling [95]. Furthermore, ibrutinib decreases adhesion to stromal elements such as fibronectin and VCAM1 [91]. Human activated B cell (ABC)-like DLBCL cell lines with constitutive active BCR signaling were selectively inhibited by ibrutinib as well as by knockdown of BTK [21]. Trials in mice and dogs showed inhibition of BCR signaling with no effect on T cell receptor signaling. Levels of circulating autoantibodies were reduced in a mouse model of autoimmune disease and objective clinical responses were described in dogs with spontaneous non-Hodgkin lymphoma [87].

In the first clinical trial reported with ibrutinib treatment, an OR of 60 % across different B cell malignancies was reported. Out of 16 patients with CLL, 11 were categorized as responders, including two CRs [96]. More recently, OR of 71 % for treatment-naïve patients, 67 % for relapsed or refractory patients, and 50 % for high risk patients has been reported [97]. If PR with lymphocytosis is included according to the proposed amendment to CLL response criteria [35], the response rates increase to 81 %, 87 %, and 79 %, respectively. The estimated PFS at 26 months was 75 % for the relapsed/refractory cohort and 96 % for treatment-naïve patients, demonstrating a remarkable duration of response with single-agent therapy. Preliminary results from combination therapy with ibrutinib and

rituximab in high risk patients showed 17 out of 20 evaluable patients achieving PR, with the last 3 patients achieving a nodal PR with persistent lymphocytosis. Adverse events are reported to be manageable [98]. Another study investigating a combination of ibrutinib with bendamustine and rituximab showed preliminary OR in the same range without major toxicity [99]. A third study with preliminary results on the combination of ibrutinib and ofatumumab confirms the efficacy of targeting CD20 and the BCR pathway: an approximate 100 % OR in heavily pretreated patients [100].

Other BTK Inhibitors

Another selective, orally available BTK inhibitor, AVL-292, has been tested in early clinical trials. Preliminary data showed stable disease in 8 of 8 CLL patients, with a median decrease in lymph node size of 28 % and initial augmented peripheral blood lymphocytosis in most patients [101]. Several other BTK inhibitors are in preclinical testing (GDC-0834, LFM-A13, AVL-101) [102], with no clinical studies registered at clinicaltrials.gov.

Targeting mTOR (Mammalian Target of Rapamycin)

mTOR is an ubiquitously expressed serine/threonine kinase. It is a downstream mediator of BCR signaling (through PI3K/AKT) as well as a cell cycle regulator at the transition from G1 to S phase [103, 104]. Rapamycin (sirolimus, used as an immunosuppressant in organ transplants, isolated from *Streptomyces hygroscopicus*) was initially identified as a fungicide and later as an antitumor substance [105]. mTOR was identified as the primary target of rapamycin through binding of rapamycin to FKBP-12 [106]. The cell cycle arrest at G1 phase brought about by rapamycin treatment is dependent upon inhibition of cyclin-dependent kinase 2 (CDK2). In addition, survivin, which is expressed in proliferation centers of CLL cells in vivo, is downregulated, and cyclin D3, cyclin E, and cyclin A levels are reduced due to rapamycin inhibition of mTOR [104]. Preclinical data show that rapamycin-treated cells lacking functional p53 go into apoptosis, whereas p53 wild type cells arrest in G1 phase but remain viable [107]. Apoptosis induction mediated through mTOR inhibition, which depends on a lack of p53 function (del(17p) and p53 mutations), thus points towards a treatment option for CLL patients with the most dismal prognosis. In addition to the direct effects in B cells, inhibition of mTOR may deplete the oligoclonal, activated T cells found in CLL. Furthermore, it may block angiogenesis and VEGF, which is reported to be upregulated in cycling CLL cells [108]. The importance of mTOR signaling in CLL has been demonstrated in a mouse model. CLL cells arising in the TCL1 transgenic mouse (TCL1 overexpression is a coactivator of AKT, through which mTOR is activated) were transplanted into syngeneic mice.

Inhibition of mTOR signaling in this model was shown to both prevent and delay CLL development. At the same time, downstream targets of mTOR were shown to be inactivated in the CLL cells [109].

Everolimus (RAD001)

The development of everolimus, which has improved stability and oral availability compared to rapamycin, pioneered the way for trials of mTOR inhibitors in CLL [110]. In the first clinical trial of everolimus [111] in CLL, 4 out of 8 CLL patients were reported to have a reduction in adenopathy despite no objective response according to IWCLL criteria [70]. The second trial [110] showed signs of severe toxicity from immunosuppression and infectious complications (50 % $\geq$ grade 3, including two fatalities). At the same time, a tumor flare syndrome was seen. The immunosuppressive effect of everolimus in CLL was substantiated by a third trial [112] that also reported severe infectious complications (23 % $\geq$ grade 3, including two fatalities). In addition to general immunosuppression, pneumonitis was revealed as a class effect of mTOR inhibitors [113]. However, 4 out of 22 patients in this study achieved a PR. Additionally, 8 patients achieved a median decrease in lymph node size of 75 % while demonstrating increased peripheral lymphocytosis. Therefore, the phenomenon of mobilization of CLL cells from bone marrow and secondary lymphoid tissue to peripheral blood, similar to that reported for other targeted therapies, was also shown with mTOR inhibitors. The effect of everolimus treatment on redistribution of CLL cells to the peripheral blood was interpreted by the authors as a proof of effect. The reported peripheral lymphocytosis suggests an effect in combination therapy despite modest single agent activity [112]. However, the use of everolimus in combination therapy for CLL will await further clinical trials. Future trials also have to address strategies to handle the immunosuppressive effect of everolimus in CLL. At least eight active studies (excluding those in allogeneic stem cell transplantation) of everolimus combined with idelalisib, alemtuzumab, rituximab (maintenance), panobinostat, bortezomib, lapitinib, and sorafenib are registered at clinicaltrials.gov.

Other Drugs Targeting mTOR

Temsirolimus (CCI-779) has been evaluated for treatment of CLL in one single agent phase II study [113]. There are currently no ongoing trials registered at clinicaltrials.gov for CLL. Minimal single-agent effect in CLL was reported from the study. Other mTOR inhibitors are in preclinical and clinical development for other malignancies and immunosuppression, but no trials are registered for CLL.

Targeting of Other Tyrosine Kinases in CLL

AXL is a receptor tyrosine kinase recently identified in a constitutively phosphorylated state in microvesicles from CLL cells [114]. Further investigation identified AXL in a complex with several molecules of the BCR pathway, including LYN, SYK, PI3K, and PLCγ2 [115]. Preclinical studies on more or less specific inhibition of AXL by BMS777607 and LDC2636 showed effects on viability, polarization, and migration of CLL cells. Homing of CLL cells was also abrogated by AXL inhibition in a mouse model [116]. BMS777607 is currently being tested in clinical trials for other solid tumors. R428 is another specific AXL inhibitor with only preclinical data. Bosutinib (SKI-606) is an SRC/ABL kinase inhibitor developed for CML that also inhibits AXL. Both R428 and bosutinib have been shown to induce apoptosis in CLL cells [115]. These preclinical data point towards AXL as a new target in CLL.

ROR1 (receptor tyrosine kinase-like orphan receptor family member) is another receptor tyrosine kinase expressed in CLL cells. It functions as a receptor in the noncanonical Wnt pathway [117, 118]. In addition to targeting ROR1 by immunotoxins [119] and monoclonal antibodies [120], which results in apoptosis, a high throughput screening approach has recently identified a small molecule inhibitor of ROR1 (KAN0438063). Preliminary preclinical data demonstrate a selective apoptotic effect in CLL cells compared to normal peripheral blood mononuclear cells [121]. Thus, a new pathway for targeting CLL is being revealed. More research addressing the significance of ROR1 in microenvironmental interaction and intracellular signaling is warranted to advance clinical development in CLL.

PKCβ (protein kinase C β) functions downstream of BTK in the BCR pathway relaying signals further downstream through IKK to activation of NF-κB transcription factors. PKCβ has been targeted in preclinical and clinical trials for different B cell malignancies by enzastaurin. However, the clinical results have been disappointing and no further clinical trials in CLL are expected [122]. Nevertheless, clinical and preclinical experiences from targeting PKCβ may prove helpful in further exploring the BCR pathway and guide combination therapy approaches [123]. In regard to the targeting of IKK, only preclinical results with no translation into clinical studies have been reported, despite the strong preclinical rationale for targeting IKK as part of the BCR pathway that conveys signals to NF-κB [124].

NF-κB is constitutively activated in many cancers, including B cell malignancies and CLL. This is in part due to NF-κB being a major downstream point in the BCR pathway conveying BCR activation to pro-survival, proliferation, and migration signaling [11, 125]. NF-κB is one of the final signaling mediators of the BCR (and several other) pathways downstream of IKKα and PKCβ. NF-κB upregulates the anti-apoptotic BCL-2 family members [88]. PBS-1086 was recently shown in vitro and in xenograft models, of multiple myeloma to inhibit NF-κB. Apoptosis was induced in the models and synergism with bortezomib cytotoxicity was demonstrated [125, 126]. Thus, preliminary preclinical data point towards an anti-apoptotic

effect of NF-κB inhibition in CLL. Other NF-κB inhibitors like BAY 11-7082 have not been advanced into clinical development despite promising preclinical results [127]. Still, modulation of this key effector downstream of the BCR may be entering clinical development within the near feature.

Other Approaches to Target Tumor–Microenvironment Interactions

The effects of the above-described targeted therapies on the redistribution of CLL cells from lymph nodes to peripheral blood underscore the importance of microenvironmental factors in CLL. The outlining of both chemokines secreted by CLL cells (CCL3, CCL4, CCL22, IL-8) and chemokine receptors expressed on CLL cells (CXCR3, CXCR4, CXCR5, CCR7) warrants further research into modulation of these axes [128]. Preclinical and early clinical studies are currently addressing these pathways in the cellular microenvironment of CLL cells. The interaction of CXCL12 (SDF1α) with CXCR4 and CXCR7 is targeted by several agents, including Nox-A12 (an oligonucleotide-based, designed inhibitor of CXCL12, in clinical development) and plerixafor (AMD3100, a CXCR4 antagonist currently registered for mobilization of stem cells). Currently, one study of plerixafor in combination with lenalidomide is registered for the indication of CLL [129]. Plerixafor has also shown clinical effect in combination with rituximab (5 out of 14 patients achieved PR) [130]. Further clinical and preclinical data are awaited for outlining whether modulation of this chemokine pathway is feasible and beneficial in combination therapy for CLL.

Apoptosis Inducing Drugs Targeting the BCL-2 Family

The BCL-2 family of proteins govern the entry to the mitochondrial apoptotic pathway. Both pro- and anti-apoptotic family members exist in a delicate balance for decision-making regarding whether the CLL cells (or other cancer cells/normal cells) will live or die [131]. The apoptotic signals can originate from several cellular events, including DNA damage, growth factor activation, oncogene activation, or directly through dysregulation of BCL-2 expression, e.g., by t(14;18) translocation in follicular lymphomas [132]. Due to the plentitude of pro-apoptotic factors in most malignant cells, the anti-apoptotic BCL-2 family members balancing these pro-apoptotic factors are often occupied to capacity. This is contrary to the situation in normal cells, where the anti-apoptotic factors outnumber the pro-apoptotic factors. Therefore, pro-apoptotic drugs may more easily cause apoptosis in malignant cells than in normal cells [131].

Navitoclax (ABT-263, an Orally Available Analog of ABT-737)

Navitoclax is a BH3 mimetic that binds and inhibits the anti-apoptotic BCL-2 family members BCL-2, BCL_{-x} (BCL2L1), and BCL_{-W} (BCL2L2). Thereby, the repression of the pro-apoptotic BAX and BAK BCL-2 family members is relieved, and subsequently apoptosis is induced [133]. The first phase I trial of navitoclax in relapsed or refractory CLL patients showed an OR of 31 % (9 patients with PR out of 29 patients) [134]. Furthermore, nodal disease was reduced in 21 of 29 patients, splenomegaly resolved in 5 of 13 patients, and peripheral blood lymphocytosis was reduced by at least 50 % in 19 of 21 patients. Thrombocytopenia (28 % $\geq$ grade 3) was the dose limiting toxicity. In addition, gastrointestinal adverse events (76 % grade 1–2), neutropenia (28 % $\geq$ grade 3), and one possibly related event of progressive multifocal leukoencephalopathy were reported. A possible biomarker for response to navitoclax treatment was identified in the study. A high BIM:MCL1 ratio was statistically significantly associated with response among the subset of patients tested. This may be explained by displacement of BIM from BCL-2 by navitoclax, thus releasing BIM to antagonize pro-survival signaling through MCL1 that is not directly inhibited by navitoclax [135]. Currently, no actively recruiting phase II or further studies are registered for navitoclax at clinicaltrials.gov. Preliminary results from an open label phase II study randomizing between rituximab, rituximab + navitoclax, and rituximab + navitoclax + navitoclax maintenance showed 70 % OR in the arm with navitoclax maintenance vs. 35 % for the rituximab-only arm [136]. Bone marrow suppression, gastrointestinal symptoms, and increased laboratory values for liver enzymes were the common adverse events in the navitoclax-treated arms.

Other BCL-2 Targeting Drugs

Another BCL-2 inhibitor (ABT-199) with higher specificity than navitoclax for BCL-2 is being tested in phase I trials [137, 138]. Preliminary results are reported with effect in B cell malignancies.

The first strategy to target the BCL-2 protein used an antisense oligonucleotide [139]. A phase III trial showed only modest clinical activity of oblimersen (G3139 antisense) in lymphoid malignancies, including CLL [140]. However, the ability of oblimersen to lower BCL-2 protein levels in vivo has been questioned [131].

Another BH3 mimetic with pro-apoptotic effects through inhibition of pro-survival BCL-2 family members is obatoclax (GX015-070). In addition to the direct pro-apoptotic effect through the BCL-2 pathway, an indirect effect through NF-κB pathway-dependent apoptotic mechanisms has also been reported [141]. A phase I study reported very modest single-agent effect in advanced CLL patients,

with significant but transient infusion-related neurological adverse events probably caused by on-target effects [142]. No active studies for obatoclax in CLL are currently registered at clinicaltrials.gov.

Lenalidomide

Lenalidomide was developed as a derivative of thalidomide with antiangiogenic, antitumorigenic, and immunomodulatory activities. Despite activity in CLL and other hematological malignancies, the significance of the different mechanisms of actions for lenalidomide in different settings has not been fully resolved [143]. A few years ago, cereblon (CRBN) was identified as the intracellular binding partner for thalidomide and lenalidomide responsible for the teratogenic effect [144]. The interaction of lenalidomide with cereblon and the significance for T cell modulation was recently shown [145]. Multiple effects of lenalidomide on T cell signaling through the T cell receptor as well as co-stimulatory pathways seem to be implicated in the effect of the drug in CLL [146]. Through modulation of actin dynamics, lenalidomide restores the immunological synapse between T cells and CLL cells [147]. The inhibition of proteasome activity by binding of cereblon to the 20S core proteasome subunit β type 4 [148] and the identification of interferon regulatory factor 4 (also downstream of NF-κB) as a downstream target of cereblon [149] add to the complexity. Through induction of cytokine secretion (especially IL2 and INFγ) from both CLL cells and T cells, tumor recognition by the adaptive immune system is enhanced. Additionally, antibody-dependent cytotoxicity by NK-cells is reported to be enhanced by lenalidomide in CLL patients. Preliminary data showed that the level of cereblon expression pretreatment in CLL cells was not related to lenalidomide response [150]. The evolving understanding of the mechanisms of action for lenalidomide will guide development of combination approaches with lenalidomide for CLL.

The first clinical study of lenalidomide in CLL patients was published in 2006 [151]. An OR of 47 % was achieved among patients with relapsed or refractory CLL. For 9 % of the patients CR was achieved. Tumor flare reaction and tumor lysis syndrome were concerns in this as well as in subsequent trials, with slow-dose escalation and prednisolone investigated to ameliorate the side effects [152]. Tumor flare reaction is described as an initial tender swelling of CLL nodes with overlying erythema, sometimes seen as peripheral lymphocytosis or verified by CT scans for internal nodes. The tumor flare reaction is interpreted as a result of lenalidomide-driven modulation of the immune microenvironment. Studies have indicated that tumor flare reaction may predict for better responses; however, no differences in PFS were shown [153, 154]. Expression of co-stimulatory molecules on CLL cells that are in part responsible for the tumor flare reaction induced by lenalidomide can be abrogated by inhibition of PI3Kδ [155].

Based upon reported enhancement of antibody-dependent cytotoxicity by lenalidomide, combinations with rituximab were examined. Results from these studies indicate a possible superiority of the combination approach despite concerns for CD20 downregulation by lenalidomide [152]. Several other combinations with conventional chemotherapy (cyclophosphamide, fludarabine), ofatumumab, and flavopiridol (alvocidib, HMR-1275) have been reported, with OR between 46 and 90 % [152]. Preliminary data from the combination of lenalidomide and ofatumumab in relapsed CLL patients, including patients with del(17p), showed an OR of 68 % with less tumor flare reaction than in trials of single agent lenalidomide [156]. Several studies are registered at clinicaltrials.gov testing maintenance after conventional therapy or after transplantation, combinations with plerixafor, azacitidine (demethylation), chimeric IL2/CD40 cells, and NK-cell cord blood transplantation.

Peripheral Blood Lymphocytosis Due to Targeted Therapy in CLL

Among CLL patients treated with targeted drugs for CLL, peripheral lymphocytosis that typically resolves over weeks to months is commonly reported. Discussions whether this warrants the response criteria for CLL to be amended are ongoing [35]. Furthermore, this phenomenon may reveal new approaches for combination therapy. Disruption of microenvironment signaling by tyrosine kinase inhibitors or immunomodulatory drugs may sensitize the malignant cells to chemotherapy or indicate synergistic effects from targeting of additional pathways [8, 157]. Preliminary results from the combination of idelalisib with bendamustine show reduced initial lymphocytosis compared to single agent idelalisib [73]. Further preclinical and clinical trials are needed to address these aspects of CLL combination therapy.

Experimental Findings Guiding Future Approaches to Combination Therapy

A recent in vitro study on the impact of kinase inhibitors on sphingolipid metabolism points towards new combination therapies to overcome some types of chemoresistance in CLL. Lipoprotein lipase has been identified as a prognostic factor in CLL. Inhibition of lipases by orlistat was shown to induce apoptosis in CLL cells at a much lower concentration than in healthy B-lymphocytes [158]. Inhibition of BTK and PI3K has now been shown to inhibit glucosylation of ceramides and the mRNA levels of UDP-glucose ceramide glucosyltransferase (UGCG) in primary CLL cells. Furthermore, inhibition of UGCG directly or by BTK or PI3K inhibitors was shown to increase the sensitivity of primary CLL cells to apoptosis

induced by inhibition of BCL2 by navitoclax [25]. Thus, a possible synergy between BTK/PI3K inhibition and BCL-2 targeting through interaction with sphingolipid metabolism has to be tested in animal models or clinical trials.

Preliminary results on migration and chemokine secretion from ibrutinib-treated high risk CLL patients point towards redundancy in the BCR pathway in vivo [159]. As previously published [91], plasma levels of CCL3/CCL4 and secretion of these chemokines by CLL cells from ibrutinib-treated patients were significantly reduced. However, even though ibrutinib abrogated cell survival after anti-IgM stimulation of the BCR in pretreatment samples, this was not the case in posttreatment samples. This is in part conflicting with recent data demonstrating downregulation of BCR target genes in patients treated with ibrutinib [89]. It raises the question whether part of the IgM-triggered pro-survival signaling can bypass BTK inhibition, thus suggesting that attacking different points in the BCR pathway could be beneficial in CLL.

Different pathways work in parallel downstream of the BCR and microenvironmental signaling. As emphasized above, targeting only one kinase in the pathways may result in resistance due to redundancy in the signaling cascades, upregulation of parallel pathways, or mutations in the kinase. In addition to combination approaches, these preclinical considerations provide a rationale for the development of dual activity tyrosine kinase inhibitors like SAR245509 that targets both PI3K and mTOR (as well as several other kinases) [160]. The price of more broadly targeting kinase inhibitors may be increased toxicity. This remains to be explored during clinical development.

Combinations of lenalidomide and PI3K inhibitors are supported by preclinical data. Upregulation of co-stimulatory molecules on CLL cells involved in the tumor flare reaction described during lenalidomide treatment was decreased upon PI3Kδ inhibition [155]. The exploration of mechanisms of action for lenalidomide in CLL has further revealed the importance of interaction between CD4+ T cells that secrete IFNγ and CLL cells in the microenvironment of lymph nodes [153]. These indices of cross talk between immunomodulatory pathways and the BCR pathway should be further addressed both at the molecular biology level and in clinical trials.

Conclusion

The extraordinarily positive early results from phase I/II trials of targeted therapies in CLL bring a hitherto unseen optimism among clinicians, patients, and researchers. The concomitant development of a multitude of promising targeted therapies in CLL poses special challenges for the collaboration between academic researchers and pharmaceutical companies: the different combination approaches should be rigorously addressed in randomized trials. Comparison should be made to the current gold standards in treatment of different subsets of CLL patients. At the same time, the benefits of new treatments should be transferred to patients as soon as possible.

To address these commitments, several issues have to be addressed. The slow clinical course for most CLL patients emphasizes the need for validating surrogate endpoints like PFS, OR, and negativity for minimal residual disease in addition to overall survival and quality of life. The validation of these surrogate endpoints should be tested by systematic approaches [34]. Moreover, the evolving targeted therapies question the current response criteria for CLL [35]. Also, examination of whether new treatment regimens up front would be better than watchful waiting in terms of quality of life and OS is warranted [161]. New strategies for the design of clinical trials may address some of these issues. Ongoing incorporation of new treatment options into a randomized trial has been evoked for AML [162]. While continuously including new treatment options, those that do not meet predefined efficacy end points are discarded. The goal for this study design is to "pick a winner" from among several promising treatment options. This kind of study design may prove helpful in bringing new treatment options to patients while maintaining sound scientific testing of new targeted therapies.

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Chapter 13
Inherited Susceptibility to CLL

Helen E. Speedy, Georgina Sava, and Richard S. Houlston

Abstract Chronic lymphocytic leukaemia (CLL) is the most common lymphoid malignancy in Western countries, accounting for around a quarter of all leukaemias. Despite a strong familial basis to CLL, with risks in first-degree relatives of CLL cases being increased around sevenfold, the inherited genetic basis of CLL is currently largely unknown. The failure of genetic studies of CLL families to provide support for a major disease-causing locus has suggested a model of susceptibility based on the co-inheritance of multiple low-risk variants, some of which will be common. Recent genome-wide association studies of CLL have vindicated this model of inherited susceptibility to CLL, identifying common variants at multiple independent loci influencing risk. Here we review the evidence for inherited genetic predisposition to CLL and what the currently identified risk loci are telling us about the biology of CLL development.

Keywords Chronic lymphocytic leukaemia • Family • Genetic • Susceptibility

Chronic lymphocytic leukaemia (CLL) is an indolent malignancy resulting from the accumulation of slowly proliferating CD5-positive neoplastic B-cells. The disease accounts for about a quarter of all leukaemias and is the most common form of lymphoid malignancy in Western countries [1]. While family and epidemiological studies have consistently provided evidence for the role of inherited genetic susceptibility to CLL, genetic analyses have only recently begun to identify predisposition loci. The identification of these loci is now providing insight into the biological basis of CLL development.

H.E. Speedy • G. Sava • R.S. Houlston (✉)
Division of Genetics and Epidemiology, Institute of Cancer Research,
Sutton, Surrey SM2 5NG, UK
e-mail: richard.houlston@icr.ac.uk

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Descriptive Epidemiology of CLL

While CLL can affect young individuals, CLL is generally a malignancy of later life, with the median age at diagnosis in most European populations being around age 70 [2]. Two features of CLL have hampered the acquisition of descriptive data on the disease. Firstly, CLL is often encountered as a chance diagnosis, which in turn may simply reflect health-care provision, leading to an apparent disparity in disease incidence between countries. Secondly, many studies have failed to distinguish between CLL and other low-grade B-cell malignancies. Even accepting these caveats, it is, however, apparent that CLL is nearly twice as common in men as in women, and disease incidence rates vary considerably throughout the world.

The incidence of CLL is highest in Europe and in individuals of European descent worldwide. Low incidence rates typify South and East Asia and sub-Saharan Africa populations, with the lowest recorded rates being reported in the Japanese. Migrant studies of CLL have shown that the incidence of CLL remains low in Asians, even in those born in the United States and in subsequent generations resident in the United States, providing evidence for genetic susceptibility to CLL rather than differences in incidence rates simply being a reflective of lifestyle or environmental risk factors [3, 4].

The Cellular Origin of CLL

The cellular origin of CLL is ambiguous. B-cells develop from haematopoietic stem cells in the bone marrow; the B-cell receptors (BCR), which mediate B-cell signalling in response to antigenic stimulation, are generated through rearrangement of variable (*V*), diversity (*D*) and joining (*J*) immunoglobulin heavy chain (*IGH*) gene elements [5]. Naïve, immature B-cells exit the marrow and continue their maturation in the spleen, either becoming marginal zone B-cells or follicular B-cells [6]. Upon antigenic stimulation, both marginal zone B-cells and follicular B-cells can undergo a rapid burst of proliferation and develop into plasma cells that secrete immunoglobulin. These rapidly responding plasma cells are generally short-lived and undergo apoptosis in situ. Some follicular B-cells can form a germinal centre, and these acquire *IGHV* point mutations. Plasma cells exiting the germinal centre mainly have somatically mutated, high-affinity BCRs and have the potential to become long-lived [7, 8].

Approximately 50 % of CLL carry somatic *IGHV* mutations, comparable to the frequency seen in normal B-cell development [9, 10]. The presence of somatic mutations is consistent with these CLL cells having undergone antigenic stimulation and being derived from germinal centre cells. Conversely, CLL cells without mutated *IGHV* genes are thus likely to be derived from naïve B-cells. It has, however, been shown that all CLL cases, regardless of their *IGHV* mutation status, show the hallmarks of BCR-mediated stimulation [11–13].

In CLL there is biased *IGHV* usage, and subsets of patients can be identified with the so-called stereotyped or homologous complementarity-determining region 3 sequences [14]. Because the probability of two individual B-cells expressing identical BCRs is extremely low, the observation that around 25 % of unrelated CLL cases carry stereotyped BCRs is significant [15]. Moreover, it suggests a central role for the recognition of a limited set of structurally similar epitopes in the selection and proliferation of leukaemic clones. While the presence of polyreactivity, in at least a proportion of CLL cases, underscores the possibility that specific self-antigens are involved in the activation of the leukaemic clone, the existence of a specific, foreign antigen responsible for cell stimulation cannot be excluded.

Inherited Susceptibility to CLL

Over the last 7 decades, case reports of over 100 families have appeared in the literature in which clustering of CLL has been documented [16]. It has often been suggested that even very striking familial clustering can be ascribed to ascertainment bias. This is a statistical fallacy. For example, a family with three siblings affected with CLL would be expected to occur by chance approximately once every 100 years in the UK. Hence striking families, such as the one reported by Videbaek in 1947, provide unambiguous evidence for familial risk [17]. While clustering is not necessarily exclusively a consequence of genetic predisposition, it provides strong indirect evidence for inherited genetic factors playing a role in disease development. In a number of families, CLL has been reported to segregate with other B-cell malignancies, including Hodgkin lymphoma (HL) and multiple myeloma, suggesting that part of the familial predisposition to CLL may be mediated through pleiotropic genetic susceptibility [17–19].

Case–control and cohort studies have systematically estimated the risk of CLL and other B-cell malignancies in the relatives of CLL patients [18–25]. Despite differences in design, all studies have consistently demonstrated a significantly elevated risk of CLL in relatives of CLL cases. The largest and most comprehensive of the studies conducted to date was based on an analysis of 9,717 CLL cases using Swedish Cancer Registry data [25]. This study showed that the familial relative risk associated with CLL is amongst the strongest for any cancer, with the risk in first-degree relatives of cases being increased 8.5-fold. Furthermore, the study substantiated the familial correlation between CLL and other B-cell malignancies including non-Hodgkin lymphoma (NHL) [25] suggested by the anecdotal case reports.

An earlier age of onset and increased risk of second tumours is a classical feature of many familial cancers. While one survey of 28 CLL families found that familial cases tended to present around 10 years earlier than sporadic cases [26], more recent studies provide little support for this observation [27]. Anticipation, the phenomenon of intensified clinical severity and earlier age of onset with each successive

generation, has variously been reported for CLL [28–30]. However, findings were based on families ascertained for genetic analyses, which tend to be enriched for younger cases in the most recent generations. Bias is therefore likely to be introduced through censoring or cohort effects. In a study using Swedish registry data where corrections were made for possible sources of bias, there was little evidence to support anticipation in CLL [31].

It is possible that CLL development may be influenced by antigenic recognition or selection through the BCR. Under such a model, familial CLL would be associated with a more restricted phenotype with respect to ontogenic development than sporadic disease. Two studies have compared CLL phenotypes between familial and sporadic CLL [27, 32]. Stage at diagnosis, need for treatment and overall survival were reported to be comparable [32]. However, the sex ratio of familial CLL was found to be more equal than that of sporadic CLL [27, 32]. Females affected by CLL might therefore have more predisposition alleles. The relatives of affected females probably share the same predisposition genes, which increase their genetic liability, accounting for the higher proportion of familial cases among females compared to males. The frequency of mutated CLL was higher among familial CLL cases, and there was evidence of intrafamilial concordance in mutation status. The repertoire and frequency of *IGHV* usage was, however, not significantly different between familial and sporadic CLL, and *IGHV* usage was not correlated between affected members of the same family [27]. These observations indicate familial CLL is essentially indistinguishable from sporadic CLL and favours a multifactorial basis to disease development in general.

Notwithstanding these data, the repertoire of *IGHV* genes expressed by B-cells in CLL patients is, however, biased when compared to that of normal B-cells [33]. Asymmetric usages of the immunoglobulin genes have been well characterised in CLL, with notable overrepresentation of various genes, including *IGHV1-69* and *IGHV4-34* [34]. Such preferential usage of certain *IGHV* genes could indicate selective drive on a B-cell population via a superantigen and lends support to the hypothesis that selection by a common antigen could contribute to disease pathogenesis. Preferential stimulation of B-cells expressing the *IGHV4-34* gene occurs in a number of infections, including those caused by Epstein–Barr virus (EBV) and cytomegalovirus (CMV) [35]. The first evidence linking latent or persistent infection by EBV and CMV with CLL cases expressing *IGHV4-34* was recently published [36], signifying the possible involvement of these pathogens in disease aetiology.

Models of Inherited Predisposition in CLL

The effect size of the familial risk for CLL is compatible with a wide range of genetic models of inheritance. Although rare, large families with multiple individuals affected with CLL have been interpreted as providing support for a role of high-penetrance susceptibility to the disease. To date, five linkage scans of

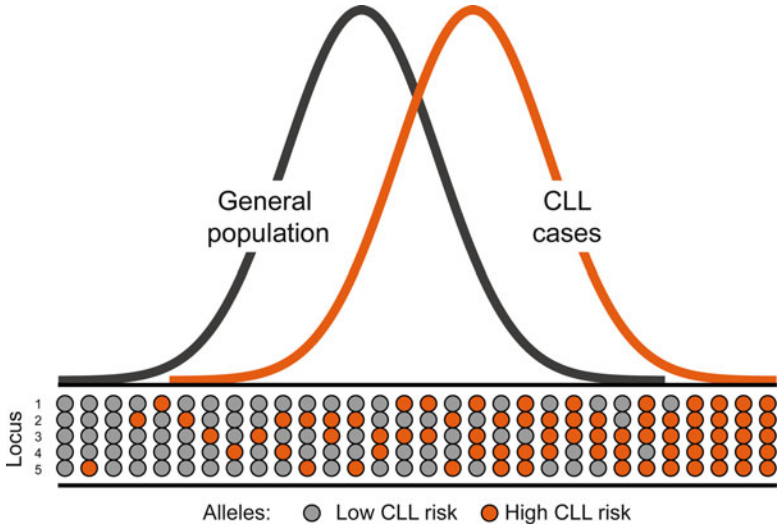

Fig. 13.1 Polygenic model of susceptibility to CLL. The distribution of alleles at CLL risk loci follows a normal distribution in cases and in the general population. CLL cases carry a higher number of high-risk alleles compared to the general population. For illustrative purposes, only five risk loci are shown

CLL families have been performed seeking to identify a major disease locus [37–41]. The largest of these studies, in terms of number of families genotyped, was based on an analysis of 206 CLL families [38]. While the inheritance patterns of CLL in many families has suggested dominant transmission of disease, the best evidence for linkage in this study was paradoxically obtained at 2q21 under a recessive model.

Genetic heterogeneity inevitably erodes study power, and the failure to identify a disease locus by linkage scans may have been a consequence of limited power. To circumvent the issue of heterogeneity, two linkage searches on single, large CLL families have been performed. The first was based on analysis of a family comprising 11 affected members [40]. The second linkage scan involved the genotyping of a multigenerational family in which seven members had been diagnosed with CLL [41]. Neither study, however, provided statistically significant evidence for a single major locus conferring susceptibility to CLL.

The failure to identify a disease-causing gene for CLL through linkage has led to a reappraisal of the assumption of Mendelian predisposition, with the conclusion that a polygenic model of inheritance must, in part, play a role in defining disease risk (Fig. 13.1). Under this model, multiple, relatively common, low-risk variants, conferring relative risks of 1.1–1.5, make an important contribution to the overall familial risk. Although such alleles confer small effects individually, they could contribute significantly to disease susceptibility in the general population. Furthermore, by acting in concert they have the capacity to generate a high risk of CLL in a subset of the population [42].

Table 13.1 CLL susceptibility loci identified by GWAS and replication studies

SNP	Chromosome	Nearest gene	OR	RAF	References
rs17483466	2q13	*ACOXL*, *BCL2L11*	1.39	0.20	[43]
rs13397985	2q37.1	*SP140*, *SP110*	1.41	0.19	[43]
rs757978	2q37.3	*FIR*	1.39	0.15	[44]
rs210142	6p21.33	*BAK1*	1.40	0.70	[45]
rs872071	6p25.3	*IRF4*	1.54	0.54	[43]
rs2456449	8q24.21	–	1.26	0.36	[44]
rs735665	11q24.1	*GRAMD1B*	1.45	0.21	[43]
rs7169431	15q21.3	*RFX7*, *NEDD4*	1.36	0.12	[44]
rs7176508	15q23	–	1.37	0.37	[43]
rs783540	15q25.2	*CPEB1*	1.17	0.39	[48]
rs305061	16q24.1	*IRF8*	1.22	0.33	[44]
rs11083846	19q13.32	*PRKD2*, *STRN4*	1.35	0.22	[43]

Odds ratios quoted are taken from the largest association study
OR odds ratio, *RAF* risk allele frequency

The search for low-risk alleles for CLL has, until recently, centred on association studies of candidate genes, where the frequencies of variants, usually single nucleotide polymorphisms (SNPs), are compared in cases and controls. Most of these studies have evaluated only a restricted number of polymorphisms, such as those influencing methylation or carcinogen metabolism, and no definitive susceptibility alleles have emerged from such candidate gene analyses. The inherent statistical uncertainty of case–control studies involving just a few hundred cases and controls seriously limits the power of such studies to reliably identify genetic determinants conferring modest but potentially important risks. Furthermore, without a clear understanding of the biology of predisposition, the definition of what truly represents a candidate gene for CLL is inherently problematic, making an unbiased approach to loci selection highly desirable.

Following completion of the Human Genome Project, more than 20 million SNPs have been catalogued in addition to smaller numbers of insertion/deletion and copy number variations. The high resolution linkage disequilibrium (LD) maps and comprehensive sets of tagging SNPs available through the international HapMap initiative, coupled with the development of highly efficient analytical platforms, have allowed genome-wide association studies (GWAS) to be conducted efficiently and cost-effectively. This approach is unbiased and does not depend on prior knowledge of the function or involvement of any gene in disease causation. Furthermore, important variants in previously unstudied genes or even in non-coding regions can be identified.

Recently conducted GWASs of CLL [43–45] have vindicated the hypothesis of common susceptibility to CLL, identifying SNPs at 12 novel risk loci (Table 13.1). These associations are robust, having been replicated in multiple independent case–control series [46–48]. Importantly, none of the genes implicated by these GWAS have previously been evaluated in targeted association studies, emphasising that the candidate gene approach was severely limited by inadequate knowledge of the biology of CLL.

While the risks of CLL associated with these SNPs are modest, with relative risks of 1.2–1.7 per allele, as predicted by the polygenic model, their contribution to CLL incidence is high, since a large proportion of the population are carriers of these risk alleles (Fig. 13.1). Moreover, the risk of CLL increases with increasing numbers of variant alleles, and for the 2 % of the population who carry 13 or more risk alleles the risk is increased ~8-fold [44].

Monoclonal B-Cell Lymphocytosis

The recognition that common variants influence the risk of CLL raises the possibility that, while clinically diagnosed CLL may be relatively uncommon in the population, progenitor lesions may be far more common. Intriguingly this assertion is supported by the observation that CLL-phenotype B-cells (i.e. CD5+, CD23+, $CD20_{low}$, $sIgM_{low}$) of monoclonal B-cell lymphocytosis (MBL) are detectable in ~3 % of adults in the general population. These cells are essentially indistinguishable from CLL cells in terms of chromosomal abnormalities and *IGHV* mutation status [49].

Studies have shown that MBL develops into CLL at a rate of 1.1 % per year [50]. These data coupled with the observation that approximately 15 % of relatives of familial CLL patients have MBL [51, 52] are compatible MBL being a surrogate marker of genetic predisposition.

Integrating Genetics and Biology

Findings from the GWASs of CLL provide evidence that variation in a number of B-cell developmental genes, including *BAK1*, *SP140*, *IRF4* and *FIR*, influences the risk of developing the disease (Fig. 13.2) [43–45]. *IRF4* is a strong candidate gene for CLL susceptibility a priori, being a key regulator of lymphocyte development and proliferation. Moreover, *IRF4* expression is involved in the development of CLL and multiple myeloma. Through interaction with transcription factors, including PU.1, IRF4 controls the termination of pre-BCR signalling and promotes the differentiation of pro-B-cells to small B-cells. Furthermore, IRF4 controls the transition of memory B-cells, thought to be the precursor cell type for CLL, to plasma cells [8, 53, 54]. The observation that genotype is associated with *IRF4* expression in a dose-dependent fashion in EBV-transformed lymphocytes is consistent with a model in which the causal variant influences risk by arresting transition of memory B-cells through decreased *IRF4* expression [43]. Interestingly, it has also been found that EBV transformation of human B-cells in vitro requires the presence of high levels of *IRF4* [55]. Sunlight has immunosuppressive properties and a link between malignant melanoma and CLL has been reported, raising the possibility of a common biological basis to the two diseases

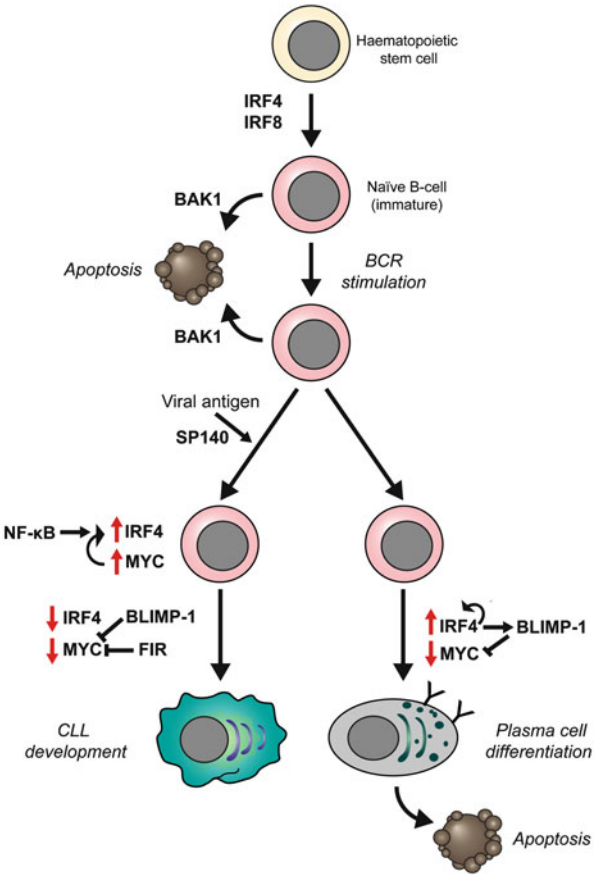

Fig. 13.2 Model of normal B-cell versus CLL development. Biological functions of BAK1, IRF4, IRF8 and MYC in B-cell development are indicated. Possible roles for FIR and SP140 are also shown. BCR, B-cell receptor [8, 53, 79]

[56, 57]. Such an assertion is supported by the observation that genetic variation in *IRF4* has been associated with skin pigmentation [58] as well as risk of CLL [43] and melanoma [59].

BAK1 is a member of the BCL-2 protein family, which is vital for the maintenance of B-cell homeostasis, regulating apoptosis at checkpoints in the B-cell developmental pathway. BAK1 promotes apoptosis by facilitating the formation of pores in the outer mitochondrial membrane, through which pro-apoptotic factors are released [60, 61]. As the CLL risk allele is associated with decreased *BAK1* transcript levels [45], it is possible this facilitates proliferation through avoidance of apoptosis.

SP140 is the lymphoid-restricted homolog of *SP100* expressed in all mature B-cells and plasma cell lines, as well as some T-cells [62, 63]. SP100 is a major mediator of EBV-encoded nuclear antigen leader protein co-activation, which is important for establishment of latent viral infections and B-cell immortalisation [64]. As *SP140* expression has been implicated in host response to immunodeficiency virus type 1 [65], it is possible that *SP140* influences CLL risk by affecting response to viral challenge.

The association signal at 2q37.3 provides evidence for a role of the *FARP2* gene in the aetiology of CLL. *FARP2*, also known as FBP-interacting repressor (*FIR*), was originally isolated as a poly(U) binding splicing factor that together with the splicing factors p54 and U2AF promotes RNA splicing [66]. FIR is also an important regulator of *MYC* gene activity which, by interacting with far upstream element (FUSE) and FUSE binding protein (FBP), represses *MYC* transcription [67, 68].

The 19q13.32 association implicates variation in *PRKD2* in CLL. Low levels of *PRKD2* expression and autophosphorylation have been reported to be a feature of a number of B-cell tumours, including mantle cell and Burkitt's lymphoma and ~50 % of CLL/small lymphocytic lymphomas [69]. Variation in *IRF8* is a strong candidate for the association with CLL risk, as IRF8 regulates α (alpha)- and β (beta)-interferon response. Moreover, *IRF8* is involved in B-cell lineage specification, immunoglobulin rearrangement and regulation of the germinal centre reaction [70]. The association signal at 15q21.3 is flanked by *NEDD4* and *RFX7*. Although there is no evidence for a direct role of *NEDD4* in CLL, it represents a credible candidate gene because of its role in regulating viral latency and pathogenesis of EBV. Specifically, NEDD4 regulates EBV-LMP2A, which mimics signalling induced by the BCR, thereby altering B-cell development [71].

The probable basis for the 2q13, 11q24.1 and 15q23 associations identified may be less straightforward than a regulatory effect on candidate gene expression, perhaps favouring a position effect through long-range LD with a functional variant mapping outside the gene locus.

The 8q24.21 association is intriguing, as GWASs of other cancers have shown that the 128–130 Mb genomic interval at 8q24.21 harbours multiple independent loci with different tumour specificities: prostate [72], breast [73], colorectal-prostate [74, 75], prostate [76] and bladder cancer [77]. The genomic regions defining these loci are, however, distinct from the 8q24.21 CLL association signal. The 8q24.21 region to which the cancer associations map is bereft of genes and predicted transcripts. The colorectal-prostate cancer locus has been shown to affect TCF4 binding to an enhancer for *MYC*, providing a mechanistic basis for this 8q24.21 association [78]. It is possible that the effect of the other 8q24.21 cancer risk loci is via *MYC* through similar long-range *cis*-acting mechanisms. If the 8q24.21 locus influences risk through differential *MYC* expression, the association is highly relevant, because MYC is a direct target of IRF4 in activated B-cells (Fig. 13.2) [79]. This, together with the fact that FIR plays an important regulatory role in *MYC* expression, might indicate a central role for MYC in CLL development.

Collectively, these data show that common low-penetrance susceptibility alleles contribute to the risk of developing CLL and implicate genes involved in transcriptional regulation and differentiation of B-cell progenitors as the biological basis of predisposition. The observation that the risk SNPs for CLL are also associated with MBL [80] suggests that the biological basis of these associations is mediated through propensity to disease rather than impacting on progression.

The testing of SNPs individually for an association in GWAS necessitates the imposition of a very stringent *P*-value to address the issue of multiple testing. While this reduces false positives, real associations may be missed and therefore any estimate of the total heritability will be negatively biased. By considering all typed SNPs simultaneously, it has been calculated that 59 % of the total variation in CLL risk can be ascribed to common genetic variation [81]. These findings suggest that common variation rather than a restricted number of associations influence CLL and provide further support for a polygenic basis for susceptibility to the disease. It is therefore likely that additional common, low-risk variants remain to be discovered and should be eminently harvestable in new larger GWAS or through further pooling of additional existing datasets. How much of the unaccounted heritable risk is truly embodied in a long tail of association is currently unknown but will impact on the ability to fully understand the genetic and ultimately biological basis of CLL predisposition.

Immune Dysfunction and Genotype

Intuitively, links between genetics and immune dysfunction as a possible basis for CLL development are highly attractive. The clinical course of CLL is dominated by events associated with immune dysfunction, manifesting as susceptibility to infection and autoimmunity. Autoimmune complications occur in 10–25 % of patients [82], the most common of which are haemolytic anaemia and immune thrombocytopaenia. The pathogenesis of autoimmunity in CLL is unknown but may be related to the ability of CLL cells to both process and present antigen.

There is currently no compelling evidence linking infection by human T-cell lymphotropic virus, human immunodeficiency virus or immunosuppression following organ transplantation with CLL pathogenesis [83]. A variety of prior medical conditions have been reported to confer an increased risk of CLL. Scarlet fever, bronchitis and rheumatoid arthritis are some examples [23]. Two Scandinavian studies [84, 85] have suggested episodes of pneumonia might serve as a potential trigger for CLL development, although the pneumonia could simply be a consequence of relative immune deficiency as an early manifestation of CLL, prior to diagnosis. Overall, no consistent association has yet emerged and these associations must be considered as unreliable.

The observation of an increased risk of HL and NHL in relatives of CLL patients has suggested a common aetiology to B-cell malignancy through HLA variation. Moreover, as B-cell proliferation is part of an adaptive immune response, which can be initiated by major histocompatibility complex-restricted T-cell activation, a possible influence of HLA on CLL pathogenesis is plausible. A recent comprehensive analysis of association between the HLA allelotypes and risk of CLL has recently provided compelling evidence for an HLA-class I association and specifically that HLA-A*0201 is associated with an increased CLL risk [86].

There is increasing evidence that T-cell dysfunction in CLL may contribute to disease aetiology. Specifically, T-cells in CLL may be unable to start, maintain and complete an immune response to the malignant B-cell and other antigens and are involved directly in sustaining the tumour. In addition, in the context of T-cell crosstalk, CD4 T-cells in CLL have been identified in the pseudofollicle/proliferation centres on the tissues involved, and their physical contact with CLL cells suggests an important role in the activation and survival of CLL cells [87]. A role for the HLA-A*02 allele in evoking an effective immune response is supported by the observation that HLA-A*02 is associated with reduced persistence of hepatitis B viral infection [88] and the finding of an underrepresentation of HLA-A*02 in patients with tuberculosis [89]. The HLA-A*02 allele has also been consistently shown to afford protection against multiple sclerosis [90]. HL displays a strong HLA-class I association, with underrepresentation of HLA-A*02 associated with EBV-positive disease [91]. The CLL association with HLA-A*0201 is, however, analogous to that shown in nasopharyngeal carcinoma (NPC), whereby an increased risk of NPC is associated with HLA-A*0201 carrier status [92]. The non-random usage of variable domain elements of *IGHV* provides evidence of selection by chronic antigen stimulation or selection through the BCR. The absence of a strong association between specific HLA-A*0201 genotype and *IGHV* subtype in CLL cases, however, argues against a simple environmental basis for disease development.

In terms of impact, HLA variation has a weaker effect on CLL risk than the recently identified non-HLA loci; this is in stark contrast to HL, a disease which is primarily defined by HLA. Finally, although speculative, the reciprocal HLA-A*02 associations seen for HL and CLL raise the possibility of differential response to viral infection, such as EBV, also playing a role in the development of CLL.

Conclusions

While it has been well recognised for some time that CLL has a strong familial basis, it is only in recent years that direct evidence for inherited genetic susceptibility has been proven. Our knowledge of inherited genetic susceptibility to CLL is now rapidly developing. Moreover, the observation that MBL represents a progenitor lesion for CLL offers considerable opportunities for understanding the key events in the early development of CLL.

The advent of analytical platforms which allow comprehensive interrogation of the genome is enabling researchers to identify variants that influence an individual's susceptibility to develop CLL. This will provide further insight into the biology of CLL, and this may lead to the development of aetiological hypotheses regarding non-genetic risk factors. Presently, there is increasing data to implicate a viral basis to CLL development. Finally, a greater understanding of the biological basis of the disease should lead to the development of novel therapeutic interventions.

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Chapter 14
miR Deregulation in CLL

Veronica Balatti, Yuri Pekarky, Lara Rizzotto, and Carlo M. Croce

Abstract B-cell chronic lymphocytic leukemia (CLL) is the most frequent human leukemia and it occurs in two forms, indolent and aggressive. Although clinical features and genetic abnormalities in CLL are well documented, molecular details underlying the disease are still under investigation.

MicroRNAs are small noncoding RNAs involved in a variety of cellular processes and expressed in a tissue-specific manner. MicroRNAs have the ability to regulate gene expression. In physiological conditions, microRNAs act as gene expression controllers by targeting the mRNA or inhibiting its translation. Their deregulation can lead to an alteration of the expression level of many genes which can induce the development or promote the progression of tumors.

In CLL, microRNAs can function as oncogenes, tumor suppressor genes, and/or can be used as markers for disease onset/progression. For example, in indolent CLL, 13q14 deletions targeting *miR-15*/*16* initiate the disease, while in aggressive CLL *miR-181* targets the critical *TCL1* oncogene and can also be used as a progression marker.

Here we discuss the foremost findings about the role of microRNAs in CLL pathogenesis, and how this knowledge can be used to identify new approaches to treat CLL.

Keywords CLL • MicroRNA • miR-15/16 • Tcl1

V. Balatti • Y. Pekarky • L. Rizzotto • C.M. Croce (✉)
Department of Molecular Virology, Immunology and Medical Genetics,
Comprehensive Cancer Center and the Wexner Medical Center,
The Ohio State University, Columbus, OH, USA
e-mail: Carlo.Croce@osumc.edu

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CLL: Characteristics and Outcomes

Chronic lymphocytic leukemia (CLL) is the most common human leukemia, accounting for ~30 % of all cases of adult leukemia. In the United States, almost 15,000 new cases are diagnosed each year [42]. CLL is mostly a disease of elderly people, and the incidence increases linearly with each decade [12]. This disease occurs in two forms, aggressive and indolent, both characterized by the progressive accumulation of functionally incompetent B-lymphocytes expressing CD5 antigen on their surface [12]. More than 90 % of the leukemic cells are nondividing and are at the G0/G1 phase of the cell cycle [12]. However, several reports showed that high lymphocyte count in CLL patients is also triggered by the presence of proliferating cells from the bone morrow, spleen, or lymph nodes [22, 52, 79]. CLL cells are also quite resistant to apoptosis [12].

The clinical course of CLL is highly variable, but several prognostic markers have been identified so far to facilitate the clinical management of CLL patients, such as the mutational status of the immunoglobulin heavy-chain variable-region gene (IgH V_H), the expression levels of the 70 kD zeta-associated protein (ZAP-70), and the presence of different chromosomal alterations [58, 70]. CLLs with unmutated IgH V_H gene and high expression of the ZAP-70 usually have an aggressive course, whereas patients with mutated V_H clones and low ZAP-70 expression have an indolent course [23]. Genomic alterations in CLL are also important independent predictors of disease progression and survival [29]; however, the molecular basis of these associations was largely unknown until recently. Genomic aberrations are detected by fluorescence in situ hybridization (FISH) in over 80 % of CLL cases and include 13q, 11q, 17p, and 6q deletions and trisomy 12 [29]. The incidence of these genetic abnormalities is ~50 % for deletion of 13q14, ~10 % for deletion of 11q23, ~15 % for trisomy 12, 7–10 % for deletion of 17p, and 2–5 % for deletion of 6q [56, 81]. Prognosis is worst in patients with 17p deletion, followed by 11q deletion, trisomy 12, and normal karyotype (negative FISH panel), while patients with deletion of 13q as the only abnormality have the best prognosis [56, 95]. Cytogenetic abnormalities can be used to identify subsets of patients with different clinical course, time to progression, and survival rates. According to recent studies, three risk groups can be differentiated: (1) low-risk: patients with a normal karyotype or isolated 13q deletion; (2) intermediate-risk: subjects with 11q deletion, trisomy 12, or 6q deletion; and (3) high-risk: patients with 17p deletion or a complex karyotype [54]. Approximately one third of patients never require treatment; in another third the initial indolent phase is followed by progression of the disease, and the remaining third has aggressive disease at the onset and needs immediate treatment [27]. Because several CLL cases show discordant prognostic factors, the identification of new parameters able to relate disease activity and clinical outcome is essential for patient management.

Signatures of MicroRNAs in CLLs

The miRNAs are a large family of highly conserved noncoding genes thought to be involved in temporal and tissue-specific gene regulation [3]. miRNAs represent an evolving class of gene products with generally unknown function and are usually excised from 70- to 80-nt stem-loop RNA precursor structures. Derived from transcripts transcribed by RNA polymerase II [14], microRNAs are made via a two-step processing mechanism from a primary transcript (pri-miRNA) through an intermediate 60–90 nucleotide stem-loop structure (pre-mRNA) to the final mature microRNA. Dicer and Argonaute family members are required for the miRNA precursor processing reaction [2]. In mammals, single-stranded microRNA binds specific messenger RNA (mRNA) through sequences that are significantly, though not completely, complementary to the target mRNA, mainly to the 3′ untranslated region (3′ UTR) [2]. By a mechanism that is not fully characterized, the bound mRNA remains untranslated, resulting in reduced levels of the corresponding protein; alternatively, the bound mRNA can be degraded, resulting in reduced levels of both the corresponding transcript and consequently the protein. It was estimated that there could be from 300 to 1,000 microRNA genes in the mammalian genome (~1–3 % of known genes are represented by microRNAs). The function of most microRNAs is not known. However, recent reports revealed functions of several microRNAs: hematopoietic B-cell lineage fate (*miR-181*), B-cell survival (*miR-15a* and *miR-16-1*), cell proliferation control (*miR-125b* and *let-7*), brain patterning (*miR-430*), pancreatic cell insulin secretion (miR-375), and adipocyte development (*miR-375*), reviewed in [38]. Recently, several microRNAs were also linked to several types of cancer [6] and DNA methylation [32]. Moreover, microRNAs can modulate gene expression in a tissue-specific manner and are able to bind target mRNAs, either inhibiting their translation or promoting their degradation [41].

Since the first association between microRNAs and cancer has been demonstrated by Calin et al. [16], it was clear that these genes could play a role in the clinical management of cancer patients. Numerous reports further confirmed that microRNAs are differentially expressed in cancers, thus suggesting that their deregulation could play tumor suppressor or oncogenic roles in cancer pathogenesis [90].

MicroRNA expression profiles revealed several remarkable outcomes that could be applied to the clinic. MicroRNA profiles can be used to distinguish normal B-cells from malignant CLL cells and, more importantly, they are associated with prognosis, progression, and drug resistance in CLL [33]. In particular, a signature profile was reported, describing 13 microRNAs that differentiate aggressive and indolent CLLs [18]. Another report showed that the expression profile of 32 microRNAs is able to discriminate between cytogenetic subgroups [88]. For instance, patients with high levels of *miR-21* had a higher risk of death compared to patients with low expression levels [74]. Likewise, high expression of *miR-155* was reported in the aggressive form of CLL [19]. Intriguingly, we recently found that

expression levels of *miR-181b* can not only distinguish between indolent and aggressive cohorts of patients but also predict time to treatment, acting as a biomarker of the disease progression. We studied serial time points derived from the same patients and found that expression of *miR-181b* decreases along with the severity of the disease. These new findings highlight the importance of *miR-181b* in clinics, suggesting that expression levels of microRNAs can be used not only to classify patients according to the gravity of the pathology but also for tracking the disease course [89]. Moreover, microRNA signature can be also used to predict refractoriness to fludarabine treatment in CLL [33]. To clarify if microRNAs are directly involved in the development of fludarabine resistance, Ferracin et al. analyzed the expression of microRNAs before and after fludarabine therapy in patients classified as responder or refractory and identified a microRNA signature able to distinguish between these two classes. Expression levels of several microRNAs were also able to predict fludarabine resistance in an independent test cohort. Among these microRNAs, *miR-148a*, *miR-222*, and *miR-21* exhibited a significantly higher expression in nonresponders either before or after treatment. Recently, Zenz et al. found that fludarabine refractory CLLs are frequently characterized by lower levels of *miR-34a* [96], and low expression of miR-34a was associated with fludarabine resistance even in the absence of p53 aberrations [96].

To conclude, microRNA expression levels can distinguish normal B-cells from CLL, discriminate between indolent and aggressive CLL forms, indicate the progression of the disease, and separate responder and refractory cohorts of patients. These findings provide new roles for microRNAs as markers for CLL development/sensitivity to treatment [33] and potential predictors of time to treatment [89].

Role of MicroRNAs in CLL

Besides using microRNAs' expression levels as tools to discriminate different CLL forms or to keep track of disease progression, researchers have recently focused on the molecular impact of microRNA deregulation in CLL. Interestingly, the *miR-15/16* cluster, *miR-29*, *miR-181* family members, and *miRs-34b/c* were found as the most deregulated microRNAs in CLL. The same microRNAs were found to regulate gene expression patterns, helping to clarify molecular steps that lead to the onset of the disease or drive its progression.

MicroRNA 15a/16-1. In CLL, deletion at chromosome 13q14.3 is the most frequent genomic aberration (about 50 % of cases) and is associated with the longest treatment-free interval [29]. The first attempts to identify tumor suppressor genes at the 13q14 locus by using positional cloning and sequencing of a region of more than 1 Mb failed [13, 53]; moreover, none of the known genes in this region were found to be down-regulated in CLL by deletions or mutations [13, 51, 53, 73]. In 2001 we generated somatic cell hybrids using mouse and CLL cells carrying

13q14 deletion and translocations, and we identified a 30-kb region of deletion between exon 2 and exon 5 of the *LEU2* gene [16, 62]. Interestingly, the translocation breakpoint was mapped to the same region [16, 62]. Since *LEU2* had previously been sequenced and excluded as a candidate tumor suppressor gene in 13q14 [13, 51, 53, 92], we continued to investigate that region and finally discovered a cluster of two noncoding microRNA genes, *miR-15a* and *miR-16-1*, located exactly within the deleted region and near the translocation breakpoint [16]. Accordingly, the *miR-15a/16-1* cluster was found deleted or its expression down-regulated in ~66 % of CLL cases [16, 80]. In contrast, expression levels of the other genes in the region (*DLEU1*, *DLEU2*, and *RFP5*) were not affected by the 13q14 deletions [13, 53, 62].

The first genetic manipulation in mice that confirmed the importance of *miR-15a/16-1* deletion in CLL was carried out by Dr. Dalla-Favera and colleagues [45]. These authors designed a model with conditional alleles that resembled either the loss of the minimal deleted region (*Mdr*), already characterized in human CLL and entirely spans the *DLEU2* gene [53], or the specific *miR-15a/16-1* cluster deletion, without altering Dleu2 expression [45]. Both *Mdr* and *miR-15a/16-1* knockout strains at 1 year of age presented approximately 50 % of $CD5^+$ $B220^+$ B-cells among mononuclear cells in the peritoneum vs. 15 % in control animals. In total, mice with CLL were 27 % of *Mdr* KO and 21 % of *miR-15a/16-1* KO, while some type of clonal B-cell proliferation affected 42 % of *Mdr* KO and 26 % of *miR-15a/16-1* KO mice between 15 and 18 months of age. *Mdr* KO animals lived less than WT siblings and eventually succumbed to leukemias, while the differential survival between *miR-15a/16-1* and their WT littermates was not statistically significant, providing evidence that the latter were affected by a phenotype milder than the former. Because of the more aggressive disease shown by *Mdr* KO mice, it is likely that other elements included in the *Mdr* locus, like the *DLEU2* gene itself, may participate to CLL tumor suppression [45]. Mechanisms leading to B-cell proliferations were investigated with different approaches. *MiR-15a/16-1* KO B-cells were shown to begin DNA synthesis earlier than WT B-cells [45]. The authors also analyzed levels of phosphorylated retinoblastoma (pRb) protein, an indicator of entry into the cell cycle, in mitogen-stimulated B-cells isolated from *miR-15a/16-1* KO or *Mdr* KO and WT animals. PRb was produced in both KO B-cells at earlier time points than in WT B-cells. Individual contributions of *miR-15a/16-1* cluster vs. *DLEU2* gene to the lympho-proliferation were dissected, generating an inducible system where these two genetic elements underwent separate in vitro re-expression in a human cell line derived from a 13q14 KO CLL. These findings demonstrated that impaired proliferation occurred in *miR-15a/16-1* expressing cells, with a higher fraction of cells in G0/G1 phase, but not in those expressing Dleu2, thus suggesting a possible control of the inhibition of G0/G1 phase transition by *miR-15a/16-1* [45].

The importance of the *miR-15a/16-1* cluster in CLL was confirmed in a study of CLL development in New Zealand black (NZB) mice, the only mouse strain that naturally develops CLL [72]. In NZB mice, CLL arises late in life, with an autoimmune phenotype and B-cell hyper-proliferation followed by slow progression to late-onset CLL [71, 93]. Older NZB animals show a clonal expansion of the

subpopulation of B-1 B-cells similar to that found in human CLL [71, 93]. Linkage analysis has found that the mouse genomic region homologous to 13q14 is one of the *loci* associated with CLL development. Subsequent DNA sequencing resulted in the identification of a point mutation in *miR-15a/16-1* precursor causing a decrease of *miR-16-1* expression in NZB lymphoid tissues, accompanied by elevated levels of Bcl-2 [72]. Accordingly, lymphoid tissues from NZB mice were analyzed for the levels of mature *miR-16-1* and showed reduced expression of this microRNA. Finally, delivery of exogenous *miR-16-1* to a NZB malignant cell line led to cell cycle alterations such as decrease in S phase cells and G1 arrest [72]. Other strains of mice, including the NZW strain, the closest relative of NZB, did not show the mutation in *miR-15a/16-1* precursor.

B-cell lymphoma 2 (*BCL2*) is a central player in the genetic program of eukaryotic cells, promoting survival by inhibiting cell death [26]. Over-expression of Bcl2 protein has been reported in many types of human cancers, including leukemias, lymphomas, and carcinomas [76]. In follicular lymphomas and in a fraction of diffuse large B-cell lymphomas, *BCL2* is activated due to the translocation t(14,18)(q32;q21), which places the *BCL2* gene under the control of Ig heavy-chain enhancers, resulting in the over-expression of the gene [83, 84]. In CLL, malignant B-cells over-express Bcl2 [44]; however, with the exception of less than 5 % of cases, in which the *BCL2* gene is juxtaposed to Ig loci [1], no mechanism has been discovered to explain *BCL2* up-regulation in CLL. *MiR-15a* and *miR-16-1* expression is inversely correlated to Bcl2 expression in CLL and these microRNAs negatively regulate *BCL2* at the posttranscriptional level [25]. Since *BCL2* is a predicted target of both *miR-15a* and *miR-16-1*, the down-regulation of these microRNAs in a leukemic cell line resulted in an increase of Bcl2 expression with consequent inhibition of apoptosis [25]. Interestingly, *miR-15a/16-1* expression also resulted in growth inhibition of tumor engraftment of leukemic cells in nude mice, confirming the tumor suppression properties of these microRNAs [15]. In summary, Bcl2 over-expression driven by down-regulation of *miR-15a* and *miR-16-1* seems to be a regulatory mechanism involved in the pathogenesis of a large part of human CLL. These studies determined that the *miR-15a/16-1* cluster functions as a tumor suppressor in CLL by inhibiting Bcl2, and deletions at 13q14 represent an initializing step in CLL development [25]. In this respect *miR-15a/16-1* have promise to be used as a drug for CLL.

Since the indolent form of the disease is often characterized by 13q14 deletion, it is likely that up-regulation of *BCL2* plays a major role in this subset of CLLs. Evidence for this hypothesis came from Dr. Reed and colleagues, who used two previously described mouse models, one with Bcl2 over-expression in the lymphoid system [43] and the second with up-regulation of a specific isoform of *TRAF2* (tumor necrosis factor (TNF) receptor-associated factor 2) in B- and T-cells [47]. *TRAF2* can bind to the TNF receptor family and mediate the activation of NF-kB by TNF proteins [24]; TNF-mediated signaling increased lymphocyte proliferation and survival [36].

TRAF2 transgenic mice failed to develop a frank leukemia, but showed an increased number of B-cells accompanied by lympho-adenopathy and splenomegaly [47]. *BCL2* transgenic animals, which were designed with a construct

mimicking the t(14;18) translocation, juxtaposing *BCL2* gene with the immunoglobulin heavy-chain locus at 14q32 as reported in human follicular lymphomas, did not develop malignancies either, presenting only prolonged in vitro B-cell survival and in vivo polyclonal B-cell expansions [43].

TRAF2DN-BCL2 double transgenic mice, on the other hand, displayed severe splenomegaly, and most animals were affected by a CLL-like disease with high B-cell blood count [94]. While single transgenics showed a normal lifespan, the double ones survived only between 6 and 14 months. Because of their complex features, it was not clear whether *TRAF2DN-BCL2* transgenics were a model of indolent or aggressive CLL [67].

Based on these findings, 13q14 deletions could induce CLL development by a molecular mechanism resembling the oncogenic events in *TRAF2DN*/*BCL2* transgenics[60]. In fact, in addition to *miR-15a*/*16-1*, the 13q14 region deleted in indolent CLL contains the *DLEU7* gene, located telomeric to *miR-15a*/*16-1*, [59]. Our report showed that *DLEU7* is a cooperating tumor suppressor along with miR-15a/16-1, and we recently confirmed that *DLEU7* deletions result in the induction of TNF signaling through TRAFs, while *miR-15a*/*16-1* deletions cause a constitutive increase of Bcl2 expression.

DLEU7 was previously identified as a candidate tumor suppressor gene at 13q14 [37]. Recently, Ouillette et al., by using microarray technology, have displayed that the minimal deleted region at 13q14 in CLL contains *DLEU7* gene [59]. Since *DLEU7* is the only protein coding gene located within the reported minimal deleted region at 13q14, we investigated whether *DLEU7* can cooperate with *miR-15a*/*16-1* [60]. Sequencing of *DLEU7* coding exons failed to find mutations in CLL samples, although a previous study reported hyper-methylation of *DLEU7* promoter, with consequent silencing of this gene in 61 % of CLL cases [37]. Real time RT-PCR experiments confirmed that expression of *DLEU7* in CLL samples is decreased when compared to normal $CD19^+$ B-cells. *MiR-15a*/*16-1* were also found down-regulated in the same CLL samples [60].

Since recent studies confirmed a significant role for the NF-kB pathway in the pathogenesis of CLL [67], we examined whether Dleu7 might function as an inhibitor of NF-kB. In the inactive state, NF-kB proteins are bound to IκB proteins in the cytoplasm; after stimulation, IκB is degraded and NF-kB translocates the nucleus [11, 21, 34]. Induction of NF-kB can be driven by a variety of stimuli, including exposure to members of the TNF superfamily, chemotherapy, and ionizing radiation [7, 85, 91]. Activation of NF-kB prevents B-cells from undergoing apoptosis and regulates growth and differentiation [7, 85, 91]. In B-cells, it has been shown that transgenic expression of the TNF ligand APRIL resulted in an expansion of $B220^+$ $CD5^+$ cells [68]. APRIL binds BCMA (B-cell maturation antigen) and TACI [36], which stimulate the NF-kB pathway, thus suggesting that NF-kB activation through TACI and BCMA is important in the pathogenesis of CLL [60]. Moreover, nuclear factor of activated T-cells (NFAT) can also be activated by TACI and BCMA [48]; NFAT was previously reported as a hallmark of unstimulated CLL cells [8, 78].

Since *DLEU7* is located within the 13q14-deleted region and NF-kB/NFAT activation can be critical in CLL pathogenesis, we studied whether Dleu7

expression has an effect on NF-kB and NFAT activation by TACI and BCMA. Our experiments showed that Dleu7 expression inhibits NF-kB activation by BCMA over fivefold, while activation by TACI was inhibited over fourfold [60]. Also, Dleu7 expression can inhibit NFAT activation by TACI and BCMA approximately eightfold. Thus, we concluded that Dleu7 functions as NFAT and NF-kB inhibitor [60].

In conclusion, *miR-15a/16-1* deletion is an initializing step in CLL development, eliciting control on Bcl2 expression level and cooperating with DLEU7 in promoting the activation of NF-kB and NFAT via TACI and BCMA. Moreover, we also recently discovered a *miR-15a/16-1-TP53* feedback circuitry, in which p53 directly transactivates *miR-15a/16-1* promoter, while *miR-15a/16-1* cluster targets *TP53* expression [31].

MicroRNA 34b/c. It is currently unknown how the 11q, 17p, and 13q deletions contribute to CLL pathogenesis and progression [29]. However, it has been proved that the loss of the long arm of chromosome 11 includes the region where the *miR-34b/c* cluster is located [5], while deletion of 17p leads to abrogation of the p53 tumor suppressor [50] and 13q deletion involves *miR15a/16-1* down-regulation. To establish the possible existence of molecular interactions between these chromosomal alterations, we investigated if the *miR-15a/16-1* cluster, tumor protein p53, and *miR-34b/c* cluster are connected in a molecular pathway that could explain the prognostic implications (aggressive vs. indolent form) of 11q, 17p, and 13q deletions in CLL [31].

Several *TP53* binding sites were found upstream of the *miR-15a/16-1* on chromosome 13 and of the *miR-34b/c* on chromosome 11. Chromatin immunoprecipitation analysis revealed that *TP53* directly binds to its predicted binding sites on both chromosomes 13 and 11. Thus, *TP53* can induce the expression of both these microRNAs [31]. On the other hand, *miR-15a/16-1* target *TP53*, while a binding site for the *miR-34* family was predicted in ZAP-70 mRNA [31]. These interactions could lead to different outcomes via feedback circuits involving protein coding genes and microRNAs [31]. In this model, *TP53* (on chromosome 17p) represents the molecular connection between *miR-15a/16-1* (on chromosome 13q) and *miR-34b/c* (on chromosome 11q) [31].

In 13q-deleted patients, the loss of *miR-15a/16-1* expression shifts the balance not only toward higher levels of the anti-apoptotic protein Bcl2 [15, 25] but also toward higher levels of the tumor suppressor protein p53. Consequently, in 13q patients, while the number of apoptotic cells may decrease because of the increased levels of Bcl2, the p53 tumor suppressor pathway remains intact, thus keeping the increase in tumor burden relatively low. This finding could explain how 13q deletions are associated with the indolent form of CLL. Moreover, increased p53 levels in patients with 13q deletions are associated with transactivation of *miR-34b/c* and with reduced levels of ZAP-70 [70], and further supporting the indolent course of CLLs carrying 13q deletions.

CLL patients with 11q deletion, instead, express significantly lower levels of *miR-34b/c* and significantly higher levels of ZAP-70, both at mRNA and protein levels.

These patients show poorer overall survival than patients with normal cytogenetic profiles and lower levels of ZAP-70. In these patients, TP53 is not upregulated because *miR-15a*/*16-1* are not deleted, and this condition is associated with lower control on apoptosis [31].

In conclusion, we demonstrated that a microRNA/TP53 feedback circuitry is associated with the pathogenesis of CLL. These results also showed that restoring expression of *miR-15a*/*16-1* indirectly affects expression of the *miR-34* family by modulating levels of TP53 expression. Moreover, the *miR-34* family is a downstream target of p53, and its over-expression can cause p53-like effects on apoptosis or cell cycle arrest [31].

MicroRNA 29. In both indolent and aggressive CLLs, *miR-29* is over-expressed compared to normal B-cells, but its role in development/progression of CLLs is still unclear. In addition, expression levels of *miR-29* are higher in indolent than in aggressive CLLs [17, 66, 77]. These results prompted us to evaluate the role of this microRNA in CLL. The up-regulation of *miR-29* in indolent CLL compared to normal B-cells implies an oncogenic function for this microRNA, initiating or at least significantly contributing to the pathogenesis of CLL [17, 66, 77]. On the other hand, we showed that expression levels of *TCL1* and *miR-29* are inversely correlated, and that *miR-29* targets *TCL1* expression [66], thus suggesting a possible tumor suppressor function for *miR-29* in aggressive CLL. Furthermore, a microRNA signature was published with 13 microRNAs that differentiate aggressive and indolent CLLs [18]. Intriguingly, of the four down-regulated microRNAs in aggressive CLL, three are different isoforms of *miR-29* (*miR-29a-2*, *miR-29b-2*, and *miR-29c*) [18], strongly suggesting that deregulation of *miR-29* can play a role in the pathogenesis of aggressive CLLs. In addition, expression of members of the *miR-29* family could discriminate between CLL samples with good and bad prognosis [17].

In order to study the role of *miR-29* in B-cell leukemias, we designed a transgenic mouse characterized by over-expression of *miR-29* in B-cells. In splenocytes from these transgenics we reported an increase in $CD5^+$ $CD19^+$ IgM^+ B-cell populations, a hallmark of CLL [77]. Eighty-five percent of *miR-29* animals showed a marked growth of $CD5^+$ B-cells that, between 12 and 14 months of age, represented up to 50 % of total B-cells. Only 20 % of the transgenics died because of leukemia between 24 and 26 months of age. These data led us to conclude that *miR-29* mice mimicked the indolent form of CLL. In fact, the percentage of leukemic cells increased with age, from 20 % of all B-cells in mice below 15 months of age to more than 65 % in mice above 20 months of age, indicating a gradual progression of indolent CLL [77]. Using BrdU incorporation experiments to measure the proliferative capacity of leukemic cells, we confirmed a significantly increased proliferation in *miR-29* transgenic B-cells compared to wild type $CD19^+$ cells, where no proliferation was found. Thus, *miR-29* over-expression seems to play a role in promoting B-cell proliferation. Furthermore, since immune incompetence and progressive hypogammaglobulinemia are typical features of human CLL, immune response to SRBC antigen and serum levels of

immunoglobulins were analyzed in *miR-29* mice and their wild type littermates. Both parameters were drastically decreased in transgenic animals, confirming that *miR-29* transgenics mimic the indolent course of human CLL [77].

In aggressive CLLs, the down-regulation of *miR-29* appears to be involved in Tcl1 over-expression, along with *miR-181* [66]. Activation of the *TCL1* oncogene is a central initiating event in the pathogenesis of aggressive CLL. *TCL1* (T cell leukemia/lymphoma 1) was originally identified as a target of translocations and inversions at 14q32.1 in T-cell prolymphocytic leukemias (T-PLL) [87]. High Tcl1 expression in human CLL correlates with aggressive phenotype [40]. Tcl1 functions as a promoter of the PI3K–Akt(PKB) oncogenic pathway [46, 64], activating Akt, driving its nuclear translocation and leading to an increased proliferation, inhibition of apoptosis, and transformation [64]. At the same time, Tcl1 activates NF-kB, inhibits AP-1 [65], and restrains *DNMT3a* [61], which is involved in epigenetic deregulation of gene expression. This leads to defects in cell death, increased survival, and CLL pathogenesis.

Recently we investigated whether *TCL1* expression in CLL is regulated by microRNAs [66]. *MiR-29b* and *miR-181b* are down-regulated in aggressive CLLs with 11q deletions and are predicted to target Tcl1 [66]. Interestingly, *miR-181* is differentially expressed in B-cells, and *TCL1* is mostly a B-cell-specific gene [69], thus suggesting that Tcl1 might be a target of *miR-181* not only in CLL cells but also in normal B-lymphocytes. We therefore proceeded to verify if these microRNAs really target Tcl1 expression. Our experiments revealed that co-expression of Tcl1 with *miR-29* and *miR-181* significantly decreased Tcl1 expression [66], and we consequently concluded that *miR-29b* and *miR-181b* target *TCL1* expression on mRNA and protein levels [66]. Concordantly, we found an inverse correlation between *miR-29b* and *miR-181b* expression and Tcl1 protein expression in CLL samples, which further supports the idea that Tcl1 expression in CLL is, at least in part, regulated by *miR-29* and *miR-181* [66].

Since *TCL1* expression is regulated by microRNAs, like *miR-29* and *miR-181*, that target the 3′ UTR region of the gene, we generated transgenic mice of Eμ-*TCL1* Full Length (Eμ-*TCL1* FL), including both the 3′ and 5′ UTRs of *TCL1* under a B-cell-specific promoter [30]. These animals showed the development of a CLL-like leukemia between 16 and 20 months of age and a population of $CD5^+$ $CD23^+$ B-cells accumulated in spleens and lymph nodes of these mice. Immunological abnormalities like hypoimmunoglobulinemia, impaired immune response, and abnormal levels of cytokines were also found in Eμ-*TCL1* FL animals and were similar to those observed in human CLL [30]. In conclusion, both classical Eμ-*TCL1* and Eμ-*TCL1* FL transgenic mouse models of CLL displayed important biological similarities with their human counterpart that went beyond the simple resemblance between the two leukemias. Our study demonstrated that *TCL1* up-regulation in mouse B-cells results in aggressive CLL [9].

In conclusion, the current idea of the role of *miR-29* in CLL is associated with its effect on Tcl1 expression levels in both indolent and aggressive forms. Since *TCL1* is generally not expressed in indolent CLL [66], it likely does not play an important

function in indolent CLL, and its down-regulation due to *miR-29* over-expression does not slow indolent CLL development. Up-regulation of *miR-29* expression is not sufficient to cause aggressive CLL; on the other hand, up-regulation of Tcl1 is absolutely required for the initiation of the aggressive form of CLL. Down-regulation of *miR-29* expression in aggressive CLL (compared to the indolent form) contributes to up-regulation of Tcl1 and development of aggressive CLL [63].

Effects of Polymorphisms and Epigenetic Regulation on microRNAs Expression

The complexity of the pathways involving microRNAs in CLL development/progression was found to extend beyond their ability to directly regulate gene expression. MicroRNA expression can respond to the presence of single nucleotide polymorphisms (SNPs) and can also be altered by transactivator factors [4]. Moreover, deregulation of epigenetic processes can modify microRNA expression, leading to a diverse progression of the disease and a different prognosis [75].

A good example of SNPs being involved in altered microRNA expression is offered by *miR-34a* [4]. *MiR-34a* has been implicated in the CLL response to DNA damage through a p53-mediated induction [28, 55, 96]. TP53 protein transactivates *miR-34a* on chromosome 1p36, inducing tumor suppressor effects, enhancing apoptosis and cycle arrest [10, 20, 39, 82]. The presence of a SNP 309 in the intronic region of the promoter of ubiquitin ligase *MDM2* leads to increased expression of *MDM2*, which binds p53 [4]. In patients with intact p53, it has been reported that the presence of this SNP inhibits p53 transactivation effects on *miR-34a* and can induce down-regulation of *miR34a* [4]. In many types of cancer this SNP has been associated with accelerated tumor formation and poor prognosis [35, 49, 57]. Asslaber et al. have shown that the GG-genotype of *MDM2* SNP 309 is associated with reduced overall survival and treatment-free survival in CLL. CLL cells of patients with the GG-genotype had a significantly lower mean expression of *miR-34a* as compared with the TT-genotype, suggesting attenuation of the p53 pathway by the SNP 309. *MiR-34a* levels in cells with the heterozygous GT-genotype were found between those with the GG- and the TT-genotype. Thus, the presence of this SNP restrains p53 activity on *miR-34a* expression in CLL patients without p53 deletion/mutation [4].

MicroRNAs can be also involved in epigenetic gene regulation with positive and negative feedback circuits [75]. The histone deacetylases (HDACs) are chromatin-modulating enzymes that catalyze the removal of acetyl groups on specific lysines around gene promoters [86]. Moreover, they can trigger the demethylation of lysine 4 on histones (H3K4me2/3), thus promoting chromatin compaction and leading to epigenetic gene silencing [86]. Recent data established that HDACs can also silence microRNAs. In particular, it has been observed that *miR-15a*/*16-1* are silenced by

epigenetic mechanisms in 30–35 % of CLL samples, therefore cooperating with 13q14 deletion to account for the low expression levels of these microRNAs in CLL [75]. Indeed, it has been found that HDAC1–3 are over-expressed in CLL but not in normal lymphocytes, hence identifying an independent mechanism for the silencing of *miR-15a/16-1* [75].

In samples with monoallelic 13q14 deletion it has been observed that the HDACs repressed *miR-15a/16-1* expression on the residual allele, providing an example of functional cooperation between a genetic and an epigenetic mechanism to achieve gene repression. Induction of *miR-15a/16-1* in response to HDAC inhibition is associated with activation of cell death. Future prospective trials should evaluate the specific impact of epigenetic silencing of *miR-15a/16-1* on disease behavior and progression that could represent a new therapeutic strategy to antagonize an important survival mechanism in cells. CLL patients who exhibit such epigenetic silencing may represent a group that will possibly benefit from HDAC inhibitor-based therapy [75].

Conclusions

CLL is a heterogeneous disease. Karyotypic aberrations are strongly prognostic of survival, as well as IgH V_H mutational status and ZAP-70 expression. Lately, microRNA expression has been considered as a new important tool in the management of the disease. In the order of highest to lowest risk, the genomic categories so far identified are: 17p deletion, 11q deletion, trisomy 12, normal FISH, and 13q deletion. Patients with 17p deletion respond poorly to treatment while patients with 11q deletion CLL show a better response to treatment, even if it progresses early. Moreover, unmutated IgH V_H/ZAP-70-positive patients have increased rates of progression and reduced remission durations.

MicroRNAs are differentially expressed in cancers, and their deregulation could play tumor suppressor or oncogenic roles in cancer pathogenesis. MicroRNA expression profiles have been found to be useful tools to distinguish normal B-cells from malignant CLL cells and can be correlated with prognosis, progression, and drug resistance of CLL. MicroRNAs modify gene expression, and their deregulation involves downstream effects on cell cycle and proliferation. Deletion of *miR-15a/16-1* has been correlated to Bcl2 up-regulation in indolent CLL, while down-regulation of *miR-29* and *miR-181* has been correlated to Tcl1 up-regulation in aggressive CLL. On the other hand, over-expression of *miR-29* in B-cells results in development of indolent CLL. *MiR-34* family members are involved in a finely regulated feedback circuitry with p53 and *miR-15a/16-1* in 13q-deleted CLL, thus suggesting that the interplay between microRNAs and genes is bidirectional.

Deregulation of microRNAs can be a consequence of chromosomal alteration, epigenetic modulation, or interaction with other genes. In fact, microRNAs can be epigenetically silenced, suggesting a new cooperating system of abnormal regulation of these molecules. The study of these mechanisms can clarify the

role of microRNAs in the development and progression of CLL and allow the identification of new targets for therapy.

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