Published in final edited form as:

Blood Rev. 2010 May ; 24(3): 135–141. doi:10.1016/j.blre.2010.03.004.

Biologic and clinical significance of molecular profiling in Chronic Lymphocytic Leukemia

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Abstract

CLL is extremely heterogeneous in its clinical course, with some patients living decades with no need for treatment whilst others have a rapidly aggressive clinical course. A major focus of research has been to try to identify those biological factors that influence this heterogeneity. The goal of therapy has been to maintain the best quality of life and treat only when patients become symptomatic from their disease. For the majority of patients this means following a “watch and wait” approach to determine the rate of progression of the disease and assess for development of symptoms. Any alteration to this approach will require identification of criteria that define patients sufficiently “high-risk” that they gain benefit by introduction of early therapy. The use of molecular profiling to suggest particular therapies is currently appropriate only in defining the treatment of the minority of patients with 17p deletions or p53 mutations and in all other circumstances remains a clinical trial question.

Keywords

B-Cell Chronic Lymphocytic Leukemia (CLL); Prognostic markers; 17 p deletion

Introduction

Chronic Lymphocytic Leukemia (CLL) is the most frequent leukemia in the western world¹ and affects predominantly elderly individuals with only one third of patients being under the age of 60 at presentation. CLL follows a highly variable clinical course. Approximately 25% of patients require therapy at diagnosis due to bone marrow failure or to symptoms such as bulky adenopathy, organomegaly, fatigue or B-symptoms such as fevers, night sweats, weight loss or extreme fatigue. There is a wide range of initial presenting features, most commonly painless lymphadenopathy, followed by splenomegaly and or hepatomegaly. Only 5% of patients present with lymphadenopathy without evidence of leukemic infiltration, and in this situation the disease is known as small lymphocytic lymphoma (SLL).

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Conflicts of Interest

T. Butler has no declared conflicts of interest.

J.G. Gribben has received honoraria for advisory boards from Roche, Celgene, Genzyme, and Mundipharma.

Table 1-Diagnosis of CLL

CLL cells are monomorphic small round B lymphocytes, with only rare prolymphocytes seen and the diagnostic criteria are shown in Table 1. The diagnosis is made by the detection of a clonal population of small B lymphocytes in PB or BM, or by LN biopsy showing cells expressing the characteristic morphology and immunophenotype. CLL cells express CD19, dim CD20, dim CD5, CD23, CD79a and weakly express surface IgM and IgD. Expression of CD38 is variable and has prognostic significance in this disease,^{2,3} and for this reason CD38 should be included in the immunophenotypic panel in this disease. A scoring system has been proposed,⁴ and in difficult cases, particularly those in which there is an atypical immunophenotype, the detection of specific cytogenetic and molecular features can be helpful in making the definitive diagnosis. The international workshop on Chronic Lymphocytic Leukemia (iwCLL) revised guidelines require a lymphocytosis of greater than 5000/ μ l maintained for more than 3 months with the cells expressing the diagnostic immunophenotype for the diagnosis of CLL.⁵ CLL is increasingly diagnosed in asymptomatic patients when a lymphocytosis is found at the time of a routine blood count. Intriguingly, the increasing use of immunophenotyping led to the identification of individuals with circulating clonal B cells, often with the characteristic phenotype of CLL, but below the 5000/ μ L threshold demanded by CLL guidelines. These cases have been catered for with the creation of a new entity, monoclonal B cell lymphocytosis (MBL). The prevalence of MBL in the population is high, with estimates of 3–5%.⁶ It was noted that these patients often had a very indolent disease course, and the obvious hypothesis was that a minority of these patients would go on to develop full-blown CLL (though not necessarily need treatment), analogous to the link between monoclonal gammopathy of undetermined significance (MGUS) and myeloma. This hypothesis seems to have been supported by the finding of preceding MBL in the stored samples of patients latterly diagnosed with CLL.⁶

Treatment

Some individuals diagnosed at an early stage may remain asymptomatic for the rest of their lives, with their lifespan unaffected by CLL, while others may progress to aggressive disease over time. Treatment guidelines state that therapy should be reserved for those with advanced, symptomatic or progressive disease with treatment being considered palliative due to the incurable nature of the disease with conventional chemotherapeutic agents and the often advanced age of the patient. Several trials involving more than 2,000 patients with early disease have shown no survival benefit to treating patients with alkylating agents with early versus deferred therapy.⁷

More recently, there has been improvement in the overall survival of CLL, particularly since the 1980s and in older age groups. This is not explained by lead-time bias due to the greater use of automated blood counters picking up early-stage disease.¹ There has been significant improvement over the past decade in the results of treatment of CLL with the use of combination chemotherapy and chemo-immunotherapy. This has resulted in improvements in response rates, complete remission (CR) rates and in progression free survival (PFS), but has not yet been demonstrated to result in improved overall survival (OS).

Chlorambucil was the standard of care for many years, and can induce partial remissions (PR) but few CRs. Survival with this agent has been 3–5 years, with no gains in OS with treatment.⁸ The subsequent introduction of purine analogues such as Fludarabine in the 1980s improved response rates and PFS, particularly in combination with other agents such as Cyclophosphamide and Mitoxantrone. However, improvements in OS using these newer agents have proven more difficult to establish. The use of monoclonal antibodies such as alemtuzumab and rituximab is improving response rates further. Early analysis of the German CLL Study Group (GCLLSG) –led CLL8 study comparing the use of fludarabine and

cyclophosphamide with rituximab, fludarabine and cyclophosphamide has shown that patients treated with chemoimmunotherapy achieved a significant improvement in PFS compared to patients treated with chemotherapy using fludarabine and cyclophosphamide alone. Newer agents are in development, and have shown promise in the laboratory, and in initial trials. These new treatments are being driven by an increasing understanding of the biology of the disease. The use of allogeneic stem cell transplantation has led to cure in the minority of patients for whom this approach is indicated. The efficacy, toxicity and cost of the various treatment regimes are highly variable.

Assessing response to treatment

The assessment of response to treatment in CLL has become somewhat more complicated than in other hematological diseases. Clinical experience in acute leukemia and lymphoma indicated the value of CR to chemotherapy as a significant surrogate marker of long-term survival. In these diseases, CR can be relatively simply assessed by the eradication of leukaemic blasts from bone marrow or resolution of lymphomatous masses as assessed radiologically. In CLL, the criteria for CR and partial response (PR) are based on similar criteria. CR requires no disease on physical examination and microscopic examination of blood and bone marrow, and recovery of peripheral blood counts. Additional groupings of nodular PR (as CR, but with residual nodules of CLL in bone marrow) and PR exist.⁹ Recently, more sensitive tests to detect residual disease have become available, specifically multicolor flow cytometry¹⁰ and polymerase chain reaction (PCR) for clones defined by immunoglobulin variable gene sequences. The two techniques have comparable sensitivities. In some patients achieving conventional CR, one or both of these methods of detecting minimal residual disease (MRD) can be positive. Patients who have no MRD demonstrated by these methods have a longer remission duration and survival.^{11,12} In contrast, differences in PFS between nodular PR and conventional PR appear to be minimal, questioning the value of morphological criteria. The hypothesis is now that if we could improve the proportion of patients attaining a CR, and improving the quality of the CR by eliminating MRD, then we could improve the survival of patients with CLL.¹³ As yet, there is no evidence that altering therapy on the basis of MRD results has a clear role in guiding therapy, though the strategy is attractive, and has precedents in other diseases. This is being investigated in a number of ongoing clinical trials

Whilst improvements in CR rates between fludarabine (20–30%) versus chlorambucil (5–10%) and other alkylator-based regimens have translated into longer PFS, it has been difficult or impossible to detect improvements in OS.⁸ The heterogeneity of patient groups, the common use of fludarabine for relapsed patients post alkylator treatment, and the long periods of remission associated with CLL have made interpretation of trial outcomes far from straightforward. Improvements in CR and OS continue to be made by the use of combination chemotherapy using fludarabine and cyclophosphamide (FC), immunotherapy (rituximab and alemtuzumab), and stem cell transplantation (both autologous and allogeneic).

In parallel with new therapeutic maneuvers, there has been dramatic progress in the understanding of the basic biology of CLL, and the development of a panoply of prognostic factors. The knowledge gained with use of these prognostic factors has not directly led to clarification of the wisest plan for treatment of individual patients. Here, we shall first discuss the various markers of prognosis and subsequently examine their role in management of the disease. Prognostic markers may be clinical characteristics of the patient, features of the leukemic cells themselves, or other biological variables.

Conventional Prognostic Factors

The Rai and Binet staging systems were based upon their prognostic significance and the stage of disease remains perhaps the most useful prognostic factor in CLL. They are based on readily

available parameters that seem to reflect the biology of the disease. The staging system developed by Rai assumes that CLL cells first proliferate in the bone marrow and blood, followed by the lymph nodes, then spleen and liver, and finally anemia and thrombocytopenia develops, caused by high tumor burden in the bone marrow. Binet modified his system based on further multivariate analyses of patients.

Table 2 –Staging systems in CLL

These staging systems define early (Rai 0, Binet A), intermediate (Rai I/II, Binet B) and advanced (Rai III/IV, Binet C) stage disease with median estimated survival times of greater than 10, 5 to 7, and 1 to 3 years, respectively. The prognostic value of these stages based only on clinical examination and a simple blood count has been confirmed by numerous studies. However, there is heterogeneity within a single group, particularly early stage disease.

Binet stage A disease comprises 60–80% of cases, has a median age at diagnosis of 64 years, and an expected survival of >10 years, close to the life expectancy of an age matched non-CLL population. However over 25% of these ‘indolent’ cases die of causes related to CLL, 40% progress to advanced stages, and 50% require treatment.¹⁴ Therefore the staging systems of Rai and Binet are unable to predict accurately which patients among the majority early stage group will develop progressive disease. They also do not predict the likelihood of response to treatment in an individual with advanced disease. Furthermore, in a multivariate analysis, clinical staging was not retained as an independent prognostic marker when other prognostic markers were included in the model.¹⁵ To develop risk-adapted therapies in this heterogeneous disease, better information that predicts disease behavior at diagnosis is required. A variety of biologic markers have been evaluated and have shown mixed utility.

Markers of tumor burden and serum markers in the routine clinical laboratory

As well as clinical staging, other parameters accessible to the typical clinical laboratory have been evaluated that reflect tumor burden. To be useful in clinical practice, a test has to be routinely available and be validated (reproducible, etc). These laboratory tests are presumed to reflect aspects of disease biology, and some are easily employed for day-to-day use. Absolute lymphocyte count is an independent risk factor,¹⁵ as is the lymphocyte doubling time. Though correlated with stage, the doubling time has independent prognostic value: a doubling time of less than 12 months identified a population of patients with poor prognosis, even among early stage disease patients.^{16,17} Another measure of disease burden is the histological pattern of bone marrow infiltration by the disease. Cases with diffuse infiltration have a poorer prognosis than those with a nodular pattern.¹⁷ Bone marrow histology is also used as a criterion of response to treatment in most studies, though there is an increasingly prevalent view that assessment of minimal residual disease (MRD) may provide a more useful guide to treatment than histological evaluations (see above). Lactate dehydrogenase (LDH) is an intracellular enzyme present in all tissues and is commonly elevated in CLL and other lymphomas, reflecting the high rates of lymphocyte turnover. It can also reflect the hemolysis that can accompany CLL and its treatment. Elevated levels have been shown to have prognostic value.¹⁸ Thymidine kinase (TK) is a cellular enzyme involved in DNA synthesis, and serum levels are probably related to the number of dividing leukemia cells, again reflecting tumor burden. TK has been shown to correlate with the proliferative activity of CLL cells¹⁹, with prognostic utility in early stage disease.²⁰ Beta-2-microglobulin (β_2 M) is an extracellular protein noncovalently associated with the class I major histocompatibility complex (MHC), and expressed by nucleated cells. Serum levels show positive correlation with clinical staging systems, adverse prognostic features, and short survival.²¹ β_2 M may be a more powerful predictor of survival than clinical staging, and can predict response to chemotherapy.¹²

Cytogenetics

Unlike many of the other low-grade B cell malignancies, non-random reciprocal chromosomal translocations occur rarely in CLL/SLL, including translocations involving the immunoglobulin loci. However, a number of recurrent abnormalities have been identified. The poorly dividing CLL cells are reluctant to provide metaphase spreads for classical karyotyping, and the use of agents such as 4-phorbol-12-myristate-13-acetate (PMA) to stimulate mitosis can increase metaphase yields. These studies identified recurrent abnormalities that were used to design fluorescence in situ hybridization (FISH) probes, which further increased sensitivity. Aberrations detected by FISH have been shown to have strong prognostic significance and can be detected in most cases of CLL. In a comprehensive study chromosomal abnormalities were detected by FISH in 268 of 325 CLL patients studied (82%).¹⁸ This study demonstrated convincingly that genomic aberrations in CLL are important independent predictors of disease progression and survival. The most common recurrent chromosomal abnormalities observed include deletion of the long arm of chromosome 13 (del 13q), del 11q, trisomy 12, del 17p and del 6q.^{15,18}

The nature of these recurrent abnormalities points to the loci of candidate genes involved in pathogenesis. The most common abnormality is del 13q14, which occurs in 55% of cases, and has been associated with a favorable prognosis. The first report linking microRNAs to cancer was in CLL, where it was demonstrated that two microRNA clusters, mir-15a and mir16-1, were located within the deleted region at 13q14.²² The next most common cytogenetic abnormality is del 11q, seen in approximately 20% of cases of CLL. This deletion is associated with a distinct clinical presentation including younger age, male sex, bulky lymphadenopathy and poor prognosis. The *Ataxia Telangiectasia Mutated (ATM)* gene is located within the minimal region of loss at 11q23, suggesting that alterations in DNA repair pathways may be involved in the pathogenesis of the disease. This is further supported by the finding that mutations in the *ATM* gene are associated with poor prognosis, and may not always correlate with deletions of 11q.²³ Trisomy 12 occurs in up to 20% of cases of CLL/SLL, but the molecular mechanism by which this genetic abnormality contributes to leukemogenesis is unknown. Although less common and occurring in less than 10% of patients at diagnosis, del 17p is associated with rapid progression of disease, poor response to therapy and short survival. The deletion involves the p53 locus at 17p13, and it is clear that mutations in the p53 gene can contribute to disease progression and alter the sensitivity of CLL cells to chemotherapy agents. Mutations in p53 may be detected in up to 23% of patients without 17p deletion as assessed by FISH, and confer a similarly poor prognostic impact.²⁴ Ongoing studies are assessing the impact of specific cytogenetic abnormalities in response to particular therapeutic approaches.

Immunoglobulin Heavy Chain (IgHV) Rearrangements and IgHV Usage

An important advance in the understanding of CLL was made with the demonstration that 50% of CLL cases have somatic hypermutation (SHM) in the variable regions of the immunoglobulin heavy chain (IgHV) genes and that this has prognostic significance, with cases exhibiting SHM (M-CLL) having a more indolent clinical course and longer survival than those without SHM (UM-CLL).^{2,25} The levels of somatic hypermutation in particular B cells is evaluated by comparison of the sequence of the rearranged variable region gene with germline sequences, and guidelines have been reported for analysis of IgHV rearrangements from the working group of European Research Initiative in CLL guidelines.²⁶ Sequences with less than 98% homology to germline are considered to have undergone somatic hypermutation. UM-CLL cells are presumed to have not passed through a germinal centre in the generation of an antibody response (naïve), or alternatively to have responded to T cell independent antigens. The finding that CLL cases can be divided into mutated (M) and unmutated (UM) groups implied that the two groups may be diseases that arise from different normal cellular counterparts.²⁷ Cases of CLL that have undergone SHM are presumed to have experienced

this in the germinal center, and perhaps therefore arise from antigen experienced memory B cells, although this suggestion was not supported by gene expression profiling studies²⁸.

As well as informing us about the possible pathophysiology of the disease, it has become clear that the mutation status of a particular case of CLL is one of the most powerful predictors of prognosis, with UM-CLL cases typically progressing rapidly and having shorter survival times than M-CLL.^{2,25} There is an association between unfavorable cytogenetic aberrations (del 17p and del 11q) and UM-CLL, whilst del 13q is more frequent in M-CLL. Studies have shown that VH status and cytogenetics are independent prognostic factors, and when incorporated into models, clinical staging (Rai and Binet) loses independent prognostic value²⁹.

Other data indicate the centrality of the immunoglobulin B Cell Receptor (BCR) to the biology of CLL. UM-CLL appears to express higher levels of surface immunoglobulin, and displays a greater ability to respond to stimulation of the BCR, with M-CLL tending to resemble anergic cells in producing lack of response^{30,31}. Analysis of variable region sequences demonstrated that CLL cells utilize a biased repertoire of V genes characterized by over-representation of selected Ig gene segments, in particular *IGHV1-69*, *IGHV4-34*, *IGHV3-7*, and *IGHV3-21*³². SHM does not occur uniformly among *IGHV* genes: *IGHV1-69* is more prevalent than expected in UM-CLL, with *IGHV4-34* increased in M-CLL. There is also bias of use of *IGHV3-21* in both UM-CLL and M-CLL³³, and also bias in the use of particular genes in light chains. An apparent exception to the generalization that mutated CLL cases have good prognosis is in the subgroup of patients with CLL cells that use *IGHV3-21* since these patients have relatively aggressive disease even when the expressed *IGHV3-21* is mutated.³⁴

Not only is the immunoglobulin gene repertoire expressed by CLL cell biased, it is also notable for the existence of subsets with near identical (stereotyped) CDR3 sequences of their BCRs, which constitute the main antigen-binding region and therefore imply the recognition of structurally similar epitopes is likely to be positively selecting the leukemic clones. The presence of such stereotypic rearrangements may also have prognostic significance.^{35,36}

These findings have led some authors to suggest a microbial antigen or autoantigen as a stimulus to CLL growth, and the immunoglobulin expressed by some CLL clones has been shown to have autoantibody activity.^{37,38}

Surrogates for mutation status

It is not possible to perform IgHV mutational status on a routine basis in clinical laboratories and attempts have therefore been made to identify surrogate markers for mutational status and its powerful role as a prognostic tool. In particular, expression of two proteins, ZAP70 and CD38, which both have prognostic significance, have been examined. CLL cells demonstrate a continuum of expression of these proteins, and it is necessary to determine a cut-off point at which a case is deemed to be positive or negative, leading to difficulties in standardization since different laboratories assess individual cases as being positive or negative for expression.

In a study comparing the gene expression of UM-CLL with M-CLL, it was found that only a small number of differentially expressed genes separate the two groups,³⁹ the most specific being a gene encoding a 70 kDa zeta-associated protein (ZAP-70)⁴⁰. Most mutated cases are ZAP-70 negative and unmutated cases ZAP70 positive. ZAP70 expression can be measured by number of conventional methods including western blotting, reverse transcriptase-PCR, immunohistochemistry, and flow cytometry.⁴⁰⁻⁴³ Levels of expression are higher in T cells and NK cells than in CLL cells, and it is important that effective multiparametric gating strategies are used to ensure that expression is being measured in the CLL cells. A variety of cut-offs for expression level have been adopted, with a consensus that 20% threshold may prove the most useful.⁴⁴ ZAP-70 expression is also stable over time⁴⁵.

The relationship of ZAP-70 positivity and UM-CLL is not absolute, however, with discrepant cases ranging from 8–25%.^{44,45} These discordant cases may have other biological features with poor prognostic implications such as del 17p, del 11q or use of *IGHV3-21*.⁴⁶ Some studies have suggested that ZAP70 status is more useful as a predictor of time to progression than mutation status, but this remains controversial.⁴⁷ Difficulty with standardization has plagued studies measuring ZAP-70, and impaired its translation into a standard clinical tool.

CD38 is a surface marker associated with CLL, and easily determined using standard flow cytometric methods. It was initially found to correlate with IgHV mutation status,² however the relationship is not absolute, and there is a concern that CD38 expression may vary over time.³ Also, it has become clear that CD38 and VH mutation status are independent prognostic variables.² The field is somewhat confused by a variety of cut-offs being used to define a case as being CD38 positive (5%, 7%, 20% or 30% in different series)^{48–50}, and it has been suggested that CD38 should be evaluated by its modal expression by flow cytometry, or by antigen density. It may be that CD38 and ZAP-70 provide complementary prognostic information, with expression of both markers conferring a poor prognosis, lack of both a good prognosis, and an intermediate group with discordant expression.⁵¹ Originally valued as mere surrogates for mutation status, it may be that ZAP-70 and CD38 status are ultimately accepted as complementary prognostic markers with independent value.

Other surrogates of mutation status have been suggested including expression of thymidine kinase, activation-induced cytidine deaminase, lipoprotein lipase A and ADAM29.^{52–54} MicroRNA arrays have revealed a 13 gene signature found to correlate with ZAP-70 status, UM-CLL and disease progression,²² and two of the most differentially expressed microRNAs (miR15a and miR 16) are located at the 13q locus commonly deleted in CLL. Recent work has suggested that this altered microRNA expression regulates expression of genes regulating apoptosis and cell-cycle progression.⁵⁵ Epigenetic alterations in cancer are well described, and aberrant methylation has been demonstrated in CLL, both globally⁵⁶ and for specific genes.^{57,58} The use of drugs as epigenetic therapy in CLL is being evaluated in ongoing trials. Whilst array-based analyses of different subgroups may well lead to novel insights into biology, their routine use in the clinical setting is currently not possible.

Table 3- The impact of molecular markers on prognosis in CLL

Molecular profiling in clinical practice

It is obvious that the diagnostic methods and prognostic tools relating to CLL have expanded in accompaniment to knowledge of the underlying pathophysiology of the disease. However, this has not translated into straightforward recommendations as to when and how to treat an individual patient. What we do know is that the molecular profile of CLL provides insight into the underlying pathogenesis of the disease and provides predictors of time to progression, response to therapy and overall survival. High-risk features include the high-risk cytogenetic features del 11q and del 17p, IgHV unmutated status, use of the *IGHV3-21* gene segment, expression of ZAP70 and expression of CD38. It is tempting to speculate that these markers can now be useful in clinical practice, but a number of questions remain. There is no evidence to date that if a patient presents with "high-risk" disease features that there is any benefit in offering early therapy. This question is now being addressed in ongoing and planned clinical trials; until the results of these studies are available, patients should not be offered treatment on the basis of any molecular marker until the standard criteria for treatment are reached⁵.

With the finding that a number of molecular markers have prognostic significance it is not surprising that many of these factors are correlated. However, there are still discrepancies, with many cases having some high-risk and other low-risk molecular features, and more than 50% of IgHV unmutated cases have no unfavorable cytogenetics⁴⁶. There is also an association

between unfavorable cytogenetic aberrations (del 17p and del 11q) and unmutated CLL, while 13q deletion is more frequent in mutated CLL. A recent study involving more than 1000 CLL patients examined the relative value of ZAP70, CD38 and IgHV mutation status and found that ZAP70 expression was the strongest predictor of time from diagnosis to requirement for treatment.⁵⁹ It may be that high-risk cytogenetics, IgHV mutational status, ZAP70 and CD38 provide complementary prognostic information, with expression of both markers conferring a poor prognosis, lack of both a good prognosis, and discordant expression an intermediate prognosis.^{44,46,59}

Most of the modern prognostic markers were validated by retrospective analysis, often from single-center studies, but have now been applied to prospective randomized clinical trials. These studies suggest that the same molecular markers that identify patients with more aggressive disease also impact on outcome after treatment. This finding is not surprising since these same factors have been predictive of overall survival in retrospective studies, where it would have been expected that the same treatment options would have been offered to patients regardless of risk factors, known and unknown. Predicting subsets of patients who will respond to a given therapy may be useful to avoid unnecessary toxicity and avoid the development of resistance. Deletions of 11q correlate with a lower response to fludarabine and early relapse after autologous stem cell transplantation,⁶⁰ whilst high serum $\beta 2M$ levels are correlated with inferior response to chemo-immunotherapy.¹² Studies have been published examining the impact of these factors on response in prospective randomized trials in previously untreated patients with CLL,^{61–63} and these results have been confirmed in a number of other studies that have been reported in abstract format only. These findings suggest poor-risk features for CLL are largely also predictive of poor response.

Clinical trials are underway to evaluate the utility of using molecular profiling. The German and French CLL Study Groups CLL7 trial is randomizing patients in Binet stage A with high-risk markers to either watch-and-wait or FCR therapy. A UK-based trial (RESPECT) is evaluating the use of lenalidomide in a similar high-risk patient group, and similar trials are recruiting or planned. Outside the setting of clinical trials, there is not yet any evidence to change recommendations as to when treatment should be initiated. Once patients become symptomatic or fulfill the criteria for need for treatment there is not yet sufficient data to suggest that the detection of any of these markers should alter which therapy is offered with the exception of those patients who present with del 17p or mutations of p53. Again it should be stressed that even the detection of this poorest of the prognostic markers is not an indication for earlier treatment in asymptomatic patients. Treatment should be initiated only when indicated by standard criteria, i.e. when they are symptomatic. However, we know that these abnormalities are unique in guiding clinicians as to which therapies are indicated (or rather which are not indicated due to lack of efficacy). Deletion of 17p and/or p53 gene mutations have been associated with failure after treatment with alkylating agents, purine analogues, and rituximab.^{46–64} Evidence is accumulating on the efficacy of anti-CD52 monoclonal antibody therapy (alemtuzumab) in CLL with p53 gene abnormalities.^{65–67} Although this represents only a small group of previously untreated patients, these patients should be treated preferably in clinical trials, examining agents that have efficacy in patients without functional p53. Since responses are likely to be of short duration, and patients who are young enough to consider allogeneic stem cell transplant and in whom suitable donor can be found are then eligible for clinical studies examining the role of reduced intensity conditioning allogeneic stem cell transplants in first response. There is insufficient data available presently to alter therapy for any other group of patients. Nonetheless, the ongoing characterization of novel prognostic factors provides an insight into the biology of this disease and may identify new therapeutic targets.

So, does the clinician benefit from obtaining these tests in routine clinical practice? Several of the factors cannot readily be obtained including IgHV mutational status and the current assays for expression of ZAP70 are unreliable, with no clear guidelines on the established methodology or what expression level designates ZAP70 positive or negative. There is considerable ongoing debate regarding the clinical utility of CD38 expression and its stability over time. Cytogenetics assessed by FISH appears to be robust and reproducible and provides prognostic information for the clinician and patients. Since cytogenetic abnormalities also change over time with the evolution of new genetic changes, it may make more sense to perform this analysis at the time of institution of therapy. Clinicians may be better served using cheaper and more established markers of disease including lactate dehydrogenase and beta-2 microglobulin, which can be incorporated into nomograms to assess risk of progression.⁶⁸

Therefore, decision making in clinical practice should be made on the basis of clinical features of the disease, and the use of molecular profiles in the management of CLL remains a clinical trial question only. There are two important questions that molecular profiling might plausibly provide an answer to: When should we initiate therapy, and with what regimen? There is not yet any evidence that early treatment is beneficial in patients that are defined as high risk. Ongoing and future trials will hopefully provide some answers to this question. Until we have the results of these trials, treatment should be initiated only when guidelines indicate, generally speaking when an individual patient is symptomatic of disease. This advice also applies to those with p53 abnormalities. As to the second question, there is currently a dearth of evidence to suggest a change of first-line regimen based on molecular markers of disease. The only clear exception to this at present is in symptomatic patients with 17p deletion or p53 abnormalities, in whom efforts should be made to treat these patients with agents that may act independently of p53.

Practice Points

- The treatment and survival of CLL has advanced in parallel to our understanding of the biology of the disease.
- Many new prognostic markers have been identified, some of which are powerful predictors of survival and response to therapy.
- There is insufficient evidence to advise departures from current NCI guidelines based on these prognostic factors. Patients should generally be treated only when symptomatic.
- The only prognostic marker that should influence the therapeutic regimen is the presence/absence of 17p deletions or p53 mutations.

Research Agenda

- Prospective, randomized clinical trials are ongoing, and are the only way to establish the true utility of molecular profiling.
- The identification of prognostic markers gives us clues to biology, and the further understanding of pathophysiology should provide us with new targets for therapy.

Acknowledgments

Funding sources

T. Butler is funded by a Cancer Research UK Molecular Pathology of Cancer grant. The sponsor had no role in the writing of the manuscript.

J.G. Gribben is funded by Program Grants from The National Cancer Institute and from Cancer Research UK and from CLL Global Alliance.

References

1. Kristinsson SY, Dickman PW, Wilson WH, Caporaso N, Bjorkholm M, Landgren O. Improved survival in chronic lymphocytic leukemia in the past decade: a population-based study including 11,179 patients diagnosed between 1973–2003 in Sweden. *Haematologica* 2009;94:1259–1265. [PubMed: 19734417]
2. Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood* 1999;94:1840–1847. [PubMed: 10477712]
3. Hamblin TJ, Orchard JA, Ibbotson RE, et al. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. *Blood* 2002;99:1023–1029. [PubMed: 11807008]
4. Matutes E, Owusu-Ankomah K, Morilla R, et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia* 1994;8:1640–1645. [PubMed: 7523797]
5. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood* 2008;111:5446–5456. [PubMed: 18216293]
6. Landgren O, Albitar M, Ma W, et al. B-cell clones as early markers for chronic lymphocytic leukemia. *N Engl J Med* 2009;360:659–667. [PubMed: 19213679]
7. Chemotherapeutic options in chronic lymphocytic leukemia: a meta-analysis of the randomized trials. CLL Trialists' Collaborative Group. *J Natl Cancer Inst* 1999;91:861–868. [PubMed: 10340906]
8. Rai KR, Peterson BL, Appelbaum FR, et al. Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia. *N Engl J Med* 2000;343:1750–1757. [PubMed: 11114313]
9. Cheson BD, Bennett JM, Rai KR, et al. Guidelines for clinical protocols for chronic lymphocytic leukemia: recommendations of the National Cancer Institute-sponsored working group. *Am J Hematol* 1988;29:152–163. [PubMed: 3189311]
10. Rawstron AC, Kennedy B, Evans PA, et al. Quantitation of minimal disease levels in chronic lymphocytic leukemia using a sensitive flow cytometric assay improves the prediction of outcome and can be used to optimize therapy. *Blood* 2001;98:29–35. [PubMed: 11418459]
11. Provan D, Bartlett-Pandite L, Zwicky C, et al. Eradication of polymerase chain reaction-detectable chronic lymphocytic leukemia cells is associated with improved outcome after bone marrow transplantation. *Blood* 1996;88:2228–2235. [PubMed: 8822943]
12. Keating MJ, O'Brien S, Albitar M, et al. Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. *J Clin Oncol* 2005;23:4079–4088. [PubMed: 15767648]
13. Montserrat E. Treatment of chronic lymphocytic leukemia: achieving minimal residual disease-negative status as a goal. *J Clin Oncol* 2005;23:2884–2885. [PubMed: 15738532]
14. Dighiero G, Maloum K, Desablens B, et al. Chlorambucil in indolent chronic lymphocytic leukemia. French Cooperative Group on Chronic Lymphocytic Leukemia. *N Engl J Med* 1998;338:1506–1514. [PubMed: 9593789]
15. Krober A, Seiler T, Benner A, et al. V(H) mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. *Blood* 2002;100:1410–1416. [PubMed: 12149225]
16. Montserrat E, Sanchez-Bisno J, Vinolas N, Rozman C. Lymphocyte doubling time in chronic lymphocytic leukaemia: analysis of its prognostic significance. *Br J Haematol* 1986;62:567–575. [PubMed: 3954968]
17. Rozman C, Montserrat E, Rodriguez-Fernandez JM, et al. Bone marrow histologic pattern--the best single prognostic parameter in chronic lymphocytic leukemia: a multivariate survival analysis of 329 cases. *Blood* 1984;64:642–648. [PubMed: 6466871]
18. Dohner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. *N Engl J Med* 2000;343:1910–1916. [PubMed: 11136261]

19. Kallander CF, Simonsson B, Hagberg H, Gronowitz JS. Serum deoxythymidine kinase gives prognostic information in chronic lymphocytic leukemia. *Cancer* 1984;54:2450–2455. [PubMed: 6498737]
20. Hallek M, Langenmayer I, Nerl C, et al. Elevated serum thymidine kinase levels identify a subgroup at high risk of disease progression in early, nonsmoldering chronic lymphocytic leukemia. *Blood* 1999;93:1732–1737. [PubMed: 10029603]
21. Di Giovanni S, Valentini G, Carducci P, Giallonardo P. Beta-2-microglobulin is a reliable tumor marker in chronic lymphocytic leukemia. *Acta Haematol* 1989;81:181–185. [PubMed: 2502891]
22. Calin GA, Dumitru CD, Shimizu M, et al. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A* 2002;99:15524–15529. [PubMed: 12434020]
23. Austen B, Powell JE, Alvi A, et al. Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with B-CLL. *Blood* 2005;106:3175–3182. [PubMed: 16014569]
24. Rossi D, Cerri M, Deambrogi C, et al. The prognostic value of TP53 mutations in chronic lymphocytic leukemia is independent of Del17p13: implications for overall survival and chemorefractoriness. *Clin Cancer Res* 2009;15:995–1004. [PubMed: 19188171]
25. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* 1999;94:1848–1854. [PubMed: 10477713]
26. Ghia P, Stamatopoulos K, Belessi C, et al. ERIC recommendations on IGHV gene mutational status analysis in chronic lymphocytic leukemia. *Leukemia* 2007;21:1–3. [PubMed: 17167526]
27. Kuppers R, Klein U, Hansmann ML, Rajewsky K. Cellular origin of human B-cell lymphomas. *N Engl J Med* 1999;341:1520–1529. [PubMed: 10559454]
28. Klein U, Tu Y, Stolovitzky GA, et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. *J Exp Med* 2001;194:1625–1638. [PubMed: 11733577]
29. Oscier DG, Gardiner AC, Mould SJ, et al. Multivariate analysis of prognostic factors in CLL: clinical stage, IGVH gene mutational status, and loss or mutation of the p53 gene are independent prognostic factors. *Blood* 2002;100:1177–1184. [PubMed: 12149195]
30. Lanham S, Hamblin T, Oscier D, Ibbotson R, Stevenson F, Packham G. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. *Blood* 2003;101:1087–1093. [PubMed: 12393552]
31. Nedellec S, Renaudineau Y, Bordron A, et al. B cell response to surface IgM cross-linking identifies different prognostic groups of B-chronic lymphocytic leukemia patients. *J Immunol* 2005;174:3749–3756. [PubMed: 15749915]
32. Fais F, Ghiotto F, Hashimoto S, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest* 1998;102:1515–1525. [PubMed: 9788964]
33. Tobin G, Soderberg O, Thunberg U, Rosenquist R. V(H)3–21 gene usage in chronic lymphocytic leukemia--characterization of a new subgroup with distinct molecular features and poor survival. *Leuk Lymphoma* 2004;45:221–228. [PubMed: 15101705]
34. Tobin G, Thunberg U, Johnson A, et al. Somatically mutated Ig V(H)3–21 genes characterize a new subset of chronic lymphocytic leukemia. *Blood* 2002;99:2262–2264. [PubMed: 11877310]
35. Stamatopoulos K, Belessi C, Moreno C, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. *Blood* 2007;109:259–270. [PubMed: 16985177]
36. Ghia EM, Jain S, Widhopf GF 2nd, et al. Use of IGHV3-21 in chronic lymphocytic leukemia is associated with high-risk disease and reflects antigen-driven, post-germinal center leukemogenic selection. *Blood* 2008;111:5101–5108. [PubMed: 18326815]
37. Chu CC, Cattera R, Hatzi K, et al. Chronic lymphocytic leukemia antibodies with a common stereotypic rearrangement recognize nonmuscle myosin heavy chain IIA. *Blood* 2008;112:5122–5129. [PubMed: 18812466]
38. Stevenson FK, Caligaris-Cappio F. Chronic lymphocytic leukemia: revelations from the B-cell receptor. *Blood* 2004;103:4389–4395. [PubMed: 14962897]

39. Rosenwald A, Alizadeh AA, Widhopf G, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. *J Exp Med* 2001;194:1639–1647. [PubMed: 11733578]
40. Wiestner A, Rosenwald A, Barry TS, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. *Blood* 2003;101:4944–4951. [PubMed: 12595313]
41. Catherwood MA, Matthews C, Niblock R, Dobbin E, Morris TC, Alexander HD. ZAP-70 mRNA quantification in B-cell chronic lymphocytic leukaemia. *Eur J Haematol* 2006;76:294–298. [PubMed: 16519700]
42. Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. *N Engl J Med* 2003;348:1764–1775. [PubMed: 12724482]
43. Carreras J, Villamor N, Colomo L, et al. Immunohistochemical analysis of ZAP-70 expression in B-cell lymphoid neoplasms. *J Pathol* 2005;205:507–513. [PubMed: 15685592]
44. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med* 2004;351:893–901. [PubMed: 15329427]
45. Bosch F, Muntanola A, Gine E, et al. Clinical implications of ZAP-70 expression in chronic lymphocytic leukemia. *Cytometry B Clin Cytom* 2006;70:214–217. [PubMed: 16906580]
46. Krober A, Bloehdorn J, Hafner S, et al. Additional genetic high-risk features such as 11q deletion, 17p deletion, and V3–21 usage characterize discordance of ZAP-70 and VH mutation status in chronic lymphocytic leukemia. *J Clin Oncol* 2006;24:969–975. [PubMed: 16418492]
47. Del Principe MI, Del Poeta G, Buccisano F, et al. Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. *Blood* 2006;108:853–861. [PubMed: 16601244]
48. Montillo M, Hamblin T, Hallek M, Montserrat E, Morra E. Chronic lymphocytic leukemia: novel prognostic factors and their relevance for risk-adapted therapeutic strategies. *Haematologica* 2005;90:391–399. [PubMed: 15749671]
49. Seiler T, Dohner H, Stilgenbauer S. Risk stratification in chronic lymphocytic leukemia. *Semin Oncol* 2006;33:186–194. [PubMed: 16616065]
50. Boonstra JG, van Lom K, Langerak AW, et al. CD38 as a prognostic factor in B cell chronic lymphocytic leukaemia (B-CLL): comparison of three approaches to analyze its expression. *Cytometry B Clin Cytom* 2006;70:136–141. [PubMed: 16568475]
51. Schroers R, Griesinger F, Trumper L, et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. *Leukemia* 2005;19:750–758. [PubMed: 15759031]
52. Heintel D, Kroemer E, Kienle D, et al. High expression of activation-induced cytidine deaminase (AID) mRNA is associated with unmutated IGVH gene status and unfavourable cytogenetic aberrations in patients with chronic lymphocytic leukaemia. *Leukemia* 2004;18:756–762. [PubMed: 14961036]
53. Nuckel H, Huttman A, Klein-Hitpass L, et al. Lipoprotein lipase expression is a novel prognostic factor in B-cell chronic lymphocytic leukemia. *Leuk Lymphoma* 2006;47:1053–1061. [PubMed: 16840197]
54. Oppezzo P, Vasconcelos Y, Settegrana C, et al. The LPL/ADAM29 expression ratio is a novel prognosis indicator in chronic lymphocytic leukemia. *Blood* 2005;106:650–657. [PubMed: 15802535]
55. Calin GA, Cimmino A, Fabbri M, et al. MiR-15a and miR-16-1 cluster functions in human leukemia. *Proc Natl Acad Sci U S A* 2008;105:5166–5171. [PubMed: 18362358]
56. Wahlfors J, Hiltunen H, Heinonen K, Hamalainen E, Alhonen L, Janne J. Genomic hypomethylation in human chronic lymphocytic leukemia. *Blood* 1992;80:2074–2080. [PubMed: 1382719]
57. Hanada M, Delia D, Aiello A, Stadtmayer E, Reed JC. bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. *Blood* 1993;82:1820–1828. [PubMed: 8104532]
58. Raval A, Lucas DM, Matkovic JJ, et al. TWIST2 demonstrates differential methylation in immunoglobulin variable heavy chain mutated and unmutated chronic lymphocytic leukemia. *J Clin Oncol* 2005;23:3877–3885. [PubMed: 15809452]

59. Rassenti LZ, Jain S, Keating MJ, et al. Relative value of ZAP-70, CD38, and immunoglobulin mutation status in predicting aggressive disease in chronic lymphocytic leukemia. *Blood* 2008;112:1923–1930. [PubMed: 18577710]
60. Dreger P, Michallet M, Schmitz N. Stem-cell transplantation for chronic lymphocytic leukemia: the 1999 perspective. *Ann Oncol* 2000;11:49–53. [PubMed: 10707779]
61. Byrd JC, Gribben JG, Peterson BL, et al. Select high-risk genetic features predict earlier progression following chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: justification for risk-adapted therapy. *J Clin Oncol* 2006;24:437–443. [PubMed: 16344317]
62. Grever MR, Lucas DM, Dewald GW, et al. Comprehensive assessment of genetic and molecular features predicting outcome in patients with chronic lymphocytic leukemia: results from the US Intergroup Phase III Trial E2997. *J Clin Oncol* 2007;25:799–804. [PubMed: 17283363]
63. Catovsky D, Richards S, Matutes E, et al. Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial. *Lancet* 2007;370:230–239. [PubMed: 17658394]
64. el Rouby S, Thomas A, Costin D, et al. p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression. *Blood* 1993;82:3452–3459. [PubMed: 8241511]
65. Stilgenbauer S, Dohner H. Campath-1H-induced complete remission of chronic lymphocytic leukemia despite p53 gene mutation and resistance to chemotherapy. *N Engl J Med* 2002;347:452–453. [PubMed: 12167696]
66. Lozanski G, Heerema NA, Flinn IW, et al. Alemtuzumab is an effective therapy for chronic lymphocytic leukemia with p53 mutations and deletions. *Blood* 2004;103:3278–3281. [PubMed: 14726385]
67. Gribben JG, Hallek M. Rediscovering alemtuzumab: current and emerging therapeutic roles. *Br J Haematol* 2009;144:818–831. [PubMed: 19183194]
68. Wierda WG, O'Brien S, Wang X, et al. Prognostic nomogram and index for overall survival in previously untreated patients with chronic lymphocytic leukemia. *Blood* 2007;109:4679–4685. [PubMed: 17299097]

Table 1**Diagnosis of CLL**

Clonal expansion of abnormal B lymphocytes in Peripheral Blood.
at least 5×10^9 B lymphocytes/L (5000/ μ L)
< 55% atypical/immature lymphoid cells
Low density of surface Ig (IgM or IgD) with κ or λ light chains
B-cell surface antigens (CD19, CD20 [dim], CD23)
CD5 surface antigen

Table 2

Staging systems in CLL

Rai Stage	Characteristics	Treat in practice?
0	lymphocytosis $> 1.5 \times 10^9/L$	No
I	lymphocytosis + lymphadenopathy	Symptomatic patients only
II	as above +/- splenomegaly +/- hepatomegaly	Symptomatic patients only
III	as above + anaemia Hb $< 11g/dL$	Yes
IV	thrombocytopenia $< 100 \times 10^9/L$	Yes

Binet Stage	Characteristics	Treat in practice?
A	fewer than 3 areas of lymphadenopathy	Symptomatic patients only
B	3 or more areas of lymphadenopathy	Symptomatic patients only
C	anaemia Hb $< 11g/dL$ and/or thrombocytopenia $< 100 \times 10^9/L$	Yes

Table 3
Impact of molecular markers on prognosis in chronic lymphocytic leukemia (CLL).

Marker		Frequency, %	TTT, mo	OS, mo	Reference
Cytogenetics	del 13p	55	92	133	Dohner et al 2000 ¹⁸
	Normal	18	49	111	
	Trisomy 12	16	33	114	
	del 11q	13	13	—	
IgVH	del 17p	9	9	—	
	Mutated	47	110	300 [*]	Rassenti et al 2004 ⁴⁴
	Unmutated	53	42	115 [*]	Hamblin et al 1999 ²⁵
	Negative	54	110	NS	Rassenti et al 2004 ⁴⁴
ZAP70	Positive	46	35	NS	
	Negative	67	94	193 [*]	Rassenti et al 2008 ⁵⁹
CD38	Positive	33	40	109 [*]	Hamblin et al 2002 ³

^{*} Hamblin et al 1999,²⁵ 2002³

Abbreviations: TTT, time to treatment; OS, overall survival