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Selected Topics in Chronic Lymphocytic Leukemia Pathogenesis

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1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in Western countries mainly affecting individuals older than 50 years. It follows an extremely variable course, with survival ranging from months to decades. Available treatments often induce remissions, though almost all patients relapse and CLL remains an incurable disease [1]. However, recent advances in molecular biology have enabled us to better understand the disease physiopathology and together with the development of new therapeutic agents have made the management of the disease more rational and more effective.

2. Epidemiology

The annual incidence of CLL varies with the age and sex structure of the population. Whereas in the USA it has been estimated at 3.5 per 100,000 (males 5.0: females 2.5) [2], in the UK estimates of 6.15 per 100,000 have been reported [3]. However, since in a majority of patients diagnosis is established because of an incidental blood count performed for irrelevant reasons and because of increasing life expectancies, the prevalence should augment in the future. The median age for diagnosis is 70 for males and 74 for females. Caucasian populations have a clearly higher incidence when compared to Japanese and Chinese population, even among patients having migrated to the USA, which suggests that genetic influences are stronger than environmental factors in the pathogenesis of the disease. The nature of this genetic predisposition remains unknown as yet.

CLL may rarely occur in families [4, 5]. First-degree relatives of patients are three times more likely also to have CLL or another lymphoid neoplasm than the general population [6]. Using a four color flow-cytometric assay, Rawstron et al discovered that 3.5% of normal individuals over the age of 40 have a population of monoclonal lymphocytes (MBL) with the immune phenotypic characteristics of CLL cells in their blood at levels below the $3.5 \times 10^9/L$ [7], and that in first degree relatives of patients with familial CLL the prevalence of such cells is between 13.5% and 18% [8, 9]. The relationship of this subclinical CLL with the full blown disease is a matter of intense investigation in several laboratories. MBL has been proposed as a precursor state of CLL, since MBL clones often carry typical CLL genetic

lesions and may represent pre-malignant cells. In approximately 2% of the cases, MBL progresses to CLL, and there is evidence that CLL is generally preceded by MBL [10, 11].

3. Selected topics in CLL pathogenesis

Clinical course of CLL is variable. Recently, progress has been made in the identification of biological markers that could predict disease progression. Particularly, the expression of unmutated Ig genes, some cytogenetic abnormalities like 17p and 11q deletions and the expression of the zeta-associated protein 70 (ZAP-70) are associated to a poor prognosis. A major scientific goal is to find a biomolecular explanation for CLL prognosis heterogeneity that can provide clues in the understanding of disease etiology and pathogenic mechanisms which favor the onset of the disease, as well as its progression and evolution into aggressive variants (Richter's lymphoma or prolymphocytoid leukemia) [12]. Given the important advances operated during recent years in CLL understanding, a full review of these topics is not possible within the space confines of this article. Hence, we will concentrate in 3 major topics: the genetic abnormalities, the B cell receptor and the balance between proliferation and apoptosis.

3.1 Genetic abnormalities

The nature of genetic predisposition for CLL remains unknown. None of the reported genetic aberrations is constant and it is presently unclear whether they constitute initial events or occur during evolution. In contrast with what is observed in other B cell malignancies, which typically exhibit balanced chromosomal translocations, in CLL the most frequent abnormalities are mutations, deletions or trisomies. Reciprocal balanced chromosomal translocations involving the heavy and light chain are very rare in CLL as compared to B-NHL [13, 14, 15], and aberrant somatic hypermutation, frequently present in DLBCL, is not observed in CLL [16]. This is consistent with the concept that CLL B-cells have non-active mechanisms involved in Ig class switch recombination and somatic hypermutation [15]. Thus, the transformation of the CLL precursor is likely to occur after the antigen-driven B-cell maturation. In the case of hairy cell leukemia, which correspond to an antigen-experienced post-GC B cells [15, 17], there is also a lack of reciprocal balanced chromosome translocations [18, 19]. Overall, these tumor malignancies form a group of B-cell tumors that originate from the transformation of antigen-experienced B cells.

Progress in cytogenetic techniques and the advent of fluorescence in situ hybridization (FISH) allowed important progress in this field. Döhner et al demonstrated in a series of 325 CLL patients that chromosomal aberrations can be detected in 82% of cases [13]. In these conditions, 13q deletions are observed in 55% of patients, followed by trisomy 12 (18%) and the 11q deletion (16%). A deletion on chromosome 17p including a monoallelic deletion of TP53 tumor suppressor gene, and very frequently mutations in the remaining allele [20] is less frequently seen (7%)

Deletions in 11q22-q23, typically involve the ataxia telangiectasia (ATM) gene [20] which causes a genomic instability that prevent correct DNA-damage reparation, allow the accumulation of mutations and thus may contribute to CLL pathogenesis. Interestingly, the presence of a 17p or 11q deletion is associated with poor prognosis and predominates among advanced stages of the disease and among patients displaying unmutated VH genes, whereas the 13q deletion or a normal karyotype are associated with good prognosis, early

disease and mutated VH genes. The genetic lesions associated with deletions of the short arm of chromosome 17 (del17p13) encoding the p53 tumor suppressor gene and the long arm of chromosome 11 (del11q23) encoding the ataxia telangiectasia mutated (ATM), a kinase that regulates p53 gene, result in a loss of function of the p53 gene. p53 is an anti-oncogene which, when strand breaks occur in DNA, triggers apoptosis or cell-cycle arrest. By controlling the repair or elimination of cells with damaged DNA, p53 maintains the integrity of the genome and prevents clonal progression. Many cytotoxic drugs require this pathway to be intact for them to be effective. Defects on this pathway constitute the strongest independent predictor for resistance to standard therapy [21, 22].

The pathogenic implications of trisomy 12 in CLL remain unresolved [23]. It is proposed that a putative proto-oncogen (CLLU1) may have an elevated gene dosage due to trisomy.

The most frequent chromosomal abnormality in CLL is deletion of 13q14, being monoallelic in 76% of cases, and biallelic in 24% [13, 14, 24]. This deletion, also detected in MBL [11] occurs at a much lower frequency in multiple myeloma, DLBCL, mature T-cell lymphomas, and in several solid tumors [25-29]. A minimal deleted region (MDR) has been defined in a large number of CLL cases with monoallelic 13q14 deletion. This region contains the long non-coding RNA deleted in leukemia (DLEU)-2, and the first exon of the DLEU1 gene [30, 31]. Two microRNAs (miR-15a and miR-16-1) were present within intron 4 of DLEU2 [32, 33] and are expressed by using DLEU2 promoter region. It has been also reported that downregulation of DLEU2 and miR-15a/16-1 expression in CLL cases without 13q14 deletion [32], could be explained by suppressive epigenetic mechanisms [34]. Overall, the available data suggested that DLEU2 and/or miR-15a/16-1 are candidate tumor suppressor genes. *In vitro* assays were performed by introducing DLEU2 mRNA into a 13q14-homozygous deleted cell, but failed to produce any effects on cell death or proliferation [35].

Micro-RNAs (miRNAs) play an important role in the regulation of gene expression. Using a microarray methodology, Calin et al demonstrated significant differences in miRNA expression between CLL B cells and normal CD5+ cells. Particularly, they could substantiate the absence of two miRNA (miR15 and miR16) associated to a mutated profile of Ig genes and with deletions in the 13q14 region [36]. Fulci et al [37] also found an overexpression of miR-150, miR-223 and miR-29b, and miR-29c in the *IgVH* mutated CLL compared to the *IgVH* unmutated cases. Marton et al confirmed these findings and found a significant downexpression of MiRs 181, let-7a and MiR 22 [38].

By using biostatistical algorithms it was possible to identify miR-15a/16-1 binding sites in a number of mRNAs encoding gene products involved in regulating proliferation and apoptosis [37-44]. In summary, miR-15a/16-1 are clearly involved in critical cellular processes, and their disruption may contribute to lymphomagenesis.

Two transgenic mice were developed in order to analyze the human 13q14-MDR region. The first model mimicked the MDR, and the second contained a deletion of miR-15a/16-1. Both mouse lines developed mostly indolent clonal lymphoproliferative diseases with low penetrance.

Interestingly, the IGVH-CDR3 expressed by clonal lymphoproliferative B-cells were highly similar (BCR stereotypy), suggesting that an antigen-driven process could be involved in the clonal proliferation of specific tumor cell precursors.

Transgenic mice overexpressing the TCL1 proto-oncogene develop lymphoproliferations similar to those arising in MDR and miR-15a/16-1-deleted mice [45, 46]. TCL1 mRNA expression is upregulated in most human CLL cases but the underlying mechanism is not known as yet [47, 48].

In summary, the DLEU2/miR-15a/16-1 tumor suppressor locus plays a role in regulating the expansion of the mature B-cell pool, by preventing the entry into G0/G1-S transition. The impairment of this cell cycle control in MDR-deleted cells may allow them to proliferate after BCR stimulation by foreign or self antigens.

In these conditions, a model for the pathogenesis of CLL with 13q14 deletion based on the presumptive cellular origin of the tumor cell precursor can be proposed.

The putative CLL precursor could be an antigen-experienced CD27+ B cell, expanded either in the course of a GC B-cell T-dependent or T-independent response by chronic antigen-stimulation through extrinsic or autoantigens. Over time, genetic abnormalities may accumulate in the genome of these chronically stimulated B cells and lead to the outgrowth of clones with MBL phenotype. Additional genetic aberrations may be incorporated in the course of proliferation leading to the oncogenic hit that transform these precursor in bona fide CLL cells.

Despite clinical and molecular differences, global gene expression profile analysis demonstrated that all CLL show a homogeneous gene expression profile irrespective of their IgV mutational status and differing from other lymphoid cancers, which suggests a common cellular precursor [49, 50]. These analyses in addition revealed that the gene expression profile of all CLL is related to that of antigen-experienced B cells, which in the human are defined by expression of the CD27 cell surface antigen, and that include classical memory B cells and marginal zone B cells which can be somatically mutated or unmutated [51, 52].

However, despite sharing a common signature CLLs expressing mutated and unmutated *IgVH* genes differentially express more than 100 genes. Among these, over-expression of genes encoding zeta-chain-associated protein 70 (ZAP-70), lipoprotein lipase (LPL), BCL-7a, dystrophin and gravin are observed in the aggressive unmutated cases, while stable mutated cases over-express *Wnt3*, *CTLA-4*, *NR1P1* nuclear receptor gene, *ADAM29* and the transcription factor *TCF7* [53]. These results suggest that indolent mutated and aggressive unmutated CLLs constitute two variants of the same disease. The reasons accounting for these striking differences in clinical outcomes of these two variants remain unsolved.

Genome-wide association studies have detected some loci influencing CLL risk [54, 55] and a recent whole-genome sequencing study identified 46 somatic mutations plus four recurrent mutations in the genes NOTCH1, XPO1, MYD88 and KLHL6 [56].

3.2 B-cell receptor (BCR) characteristics in CLL

Three main phenotypic features define B-CLL: the predominant population shares B-cell markers (CD19, CD21, and CD23) with the CD5 antigen, in the absence of other pan-T-cell markers; the B cells are monoclonal with regard to expression of either κ or λ light chains and the B cells characteristically express surface immunoglobulin (slg), CD79b, CD20 and CD22 with low density. These characteristics are generally adequate for a precise diagnosis

of CLL, and they also distinguish CLL from other disorders such as prolymphocytic leukemia, hairy-cell leukemia, mantle-cell lymphoma and other lymphomas that can mimic CLL [57-59].

The BCR is a multimeric complex formed by the assembly of surface immunoglobulin (SIg) and the noncovalently bound heterodimer Ig α /Ig β (CD79a/CD79b). Low expression of the BCR is the hallmark of the B-CLL lymphocyte [60, 61].

The mechanisms accounting for poor expression of the BCR in CLL remain elusive. There is no evidence of genetic defects in the BCR components [62, 63] and in contrast with their poor expression at the membrane level, transcription and intra-cellular synthesis of BCR components are normal [63, 64]. However, they cannot be assembled and transported from the endoplasmic reticulum to the cell surface because of a folding and glycosylation defect of the μ and CD79a chains though not of the CD79b chain. The poor expression of the CD22 molecule in B-CLL cells, was also found to result as a consequence of a folding defect occurring in its α chain [65].

One unsolved issue concerns the role of the clonal B-cell receptor (BCR) in disease progression. Despite the fact that low expression of the BCR correlates with reduced induction of protein tyrosine kinase activity and defective intracellular calcium mobilization and tyrosine phosphorylation [66] this receptor conserves the capacity of antigen recognition and signaling, controlling thereby key behaviors of tumor cell, like proliferation and cell survival. Individual patients have different responses to IgM ligation which are related to *VH* gene status. In a majority of cases, CLL cells expressing unmutated *IgVH* genes showed a better response than cases expressing mutated *IgVH* genes [67].

The vast majority of B-CLL cells express a CD5+ and IgM/IgD mantle zone-like phenotype of naive cells, which, in normal conditions express unmutated Ig genes [68]. However, 50%-70% of CLL harbor somatic mutations of *IgVH* genes [69] as if they had matured in a lymphoid follicle. Interestingly, the presence or absence of somatic mutations is associated with the use of particular *IgVH* genes. For instance, alleles of the *V1-69* [70] gene and the *V4-39* gene display an unmutated profile [71].

Two reports demonstrated that the clinical behavior of CLL is related to the mutational status of immunoglobulin (Ig) genes [72, 73]. CLLs with mutated Ig genes display a good prognosis and those with unmutated Ig genes a poor prognosis. This observation has been extensively confirmed [74, 75] and it is well established that the mutational status of Ig genes constitutes a strong prognostic indicator in CLL. The mutational profile of Ig genes delineates prognostic groups within all Binet's stages [76]. Interestingly, the rearrangement of a specific *IgVH* gene, *V3-21*, has been associated with poor prognosis whether mutated or not [77].

Evidence for the notion that CLL is a tumor of antigen experienced B cells comes from the structure of the rearranged IgV genes. Analyses of large panels of CLL cases revealed that certain IgV gene family members, which could be hypermutated or unmutated, were expressed significantly more frequently in CLL than would be expected from their expression in the IgV gene repertoire of normal B cells [69]. Of note, it was confirmed that the CLL-characteristic IgV gene repertoire does not simply reflect its known restriction during the aging process. These findings suggest that all CLL express restricted sets of

BCRs, and led to the conclusion that many if not all CLL originate from the malignant transformation of B cells previously stimulated by antigen. This concept was virtually proven to be true when it emerged that more than 20% of CLL cases from unrelated patients can have extremely similar, sometimes even identical antigen receptors [78–82]. The use of almost identical BCRs in 1.3% of CLLs provided compelling evidence that the Igs expressed by CLL B cells are highly selected. It would be statistically unexpected to find 2 cases with such similar BCRs in 1 million patients [83]. This finding, known as BCR ‘stereotypy’, occurs at various levels, including IgV gene usage, VD-J junctional regions (heavy chain complementarity determining region-3; CDR3), and combination of certain heavy chain CDR3s with light chain CDR3s [84].

These results strongly suggest that a common antigen epitope is recognized by these highly homologous molecules. Concerning the epitope recognized, it has been shown that unmutated CLL cells express highly polyreactive antibodies while most mutated ones do not [85, 86]. Indeed, ‘CLL antigens’ have recently been identified which represent autoantigens derived from cells normally destined for apoptosis; some of the recognized epitopes appear to be highly similar to microbial antigens [87–89]. While signaling through the BCR, either in a tonic or antigen-mediated fashion, is generally assumed to play a role in the pathogenesis of B-cell lymphomas with few exceptions (i.e. Hodgkin lymphoma which expresses ‘crippled’ BCRs) [90], the BCR stereotypy unique for CLL demonstrate that antigen as such seems to have a decisive role in the etiology of this disease.

Results from microarray and flow cytometric studies have revealed the unexpected expression among tumoral CLL cells, of molecules involved in cell activation like the zeta associated protein 70 (ZAP-70), the CD38 molecule, the activation induced cytidine deaminase (AID) and the lipoprotein lipase (LPL).

Thus, high levels of ZAP-70, usually found in T and NK cells but not in normal circulating B cells, are detected in the majority of unmutated CLLs [50]. CLL B cells that express ZAP-70 are more likely to respond to IgM cross-linking with increased tyrosine phosphorylation and calcium flux than ZAP-70 negative CLL B cells. This effect could occur because following BCR ligation ZAP-70 undergoes tyrosine phosphorylation and becomes associated with surface immunoglobulin and CD79b [91] and/or because ZAP-70 mediates inhibition events that terminate the signalling response [92] and/or because ZAP-70 expression is associated with advantageous survival responses [93]. Altogether, expression of ZAP-70 in CLL allows more effective IgM signaling in CLL B cells, which might be responsible for a more aggressive course. The apparently anomalous expression of ZAP-70 in CLL cells is not completely explained. Recent data revealed that ZAP-70 is expressed at initial stages of B cell maturation and in other B-cell malignancies, like acute lymphoblastic leukemia [94].

Another unexpected molecule expressed by a subset of CLL B cells is CD38. This molecule is present during B-cell development when cell-to-cell interactions are crucial to development [95]. Examples include an early bone marrow precursor cell, cells in the germinal center and plasma cells [96]. In CLL, expression of this molecule predominates among those with unmutated *IgVH* genes and is associated to poor prognosis [97].

Interestingly, the activation induced cytidine deaminase (AID), a B cell-restricted enzyme, required for somatic mutation and isotype switching, is upregulated in unmutated CLL cells

[98-100]. While there is evidence that AID expression could be confined to a small proportion of the clone [101], it appears to be functional, since unmutated CLL cases can generate isotype-switched transcripts and proteins and mutations in the pre-switch μ region [98]. Upregulation of AID may be associated with loss of target specificity resulting in mutations in non-immunoglobulin genes such as *BCL-6*, *MYC*, *PAX-5* and *RHOH* which are associated with more aggressive disease [102, 103].

In a previous work from our group, we reported that expression of the lipoprotein lipase (LPL) gene at the RNA level was clearly associated to an unmutated profile of Ig genes and a clinical poor outcome in CLL [104]. LPL is normally produced by parenchymal cells in several tissues, with the largest expression found in adipose tissue, cardiac and skeletal muscle and lactating mammary gland. In addition, LPL can augment interaction between cells where it has been shown to form a bridge between monocyte and endothelial cell surface heparan sulfate-proteoglycans. However, LPL expression has never been previously reported in the case of normal B cells. For this reason, its infidel expression in CLL B cells, constitutes a suitable marker to study disease prognosis.

3.3 The balance between proliferation and apoptosis in CLL

CLL can be defined as a low-grade B-cell tumor with antigen experienced monoclonal CD5+ B cells that, having escaped programmed cell death and undergone cell cycle arrest in the G0/G1 phase [105], relentlessly accumulate in lymphoid organs (lymph nodes, spleen and bone marrow) and circulate into the peripheral blood. This leukemic B cell accumulation results from a complex balance between activation of cell proliferation and inhibition of apoptotic death. Interestingly, circulating CLL B-lymphocytes are quiescent cells in the G0/G1 phase of cell cycle. Thus, CLL B cells are characterized by high expression of the anti-apoptotic BCL-2 protein in the absence of specific translocations and by high expression of the p27^{kip} protein, which blocks progression into cell cycle. Given the key role of this protein in cell cycle progression, its over-expression in CLL cells could account for the accumulation of B cells in early phases of the cell cycle. In addition, other members of the BCL-2 family such as anti-apoptotic proteins BCL-XL, BAG-1 and MCL-1 are over-expressed, while pro-apoptotic proteins like BAX and BCL-XS are under-expressed [106, 107]. Taken together, these data suggest that CLL is a disease resulting from accumulation rather than from proliferation.

As opposed to *in vivo* results, apoptosis occurs after *in vitro* culture, which suggests a role of the microenvironment in CLL cell survival [108, 109]. In agreement with this hypothesis are results indicating that apoptosis *in vitro* is prevented by exposure to interleukin-4 (IL-4) as well as by stimulation via surface CD40 [109].

Most scientific work focusing in CLL uses circulating leukemic cell samples obtained from peripheral blood. However, it is reasonable to propose that the most important physiopathological events presumably occur in tissues [110] where leukemic cells: (i) are activated by antigen - BCR stimulation; (ii) are regulated and expanded by T-cell signals; (iii) proliferate in pseudofollicular centers, and (iv) interact with stromal cells that favor cell accumulation.

In vivo, inhibition of apoptosis may occur in pseudo-follicles observed in the lymph nodes and in the cell clusters described in the bone marrow [111]. These pseudo-follicles include in

close contact with proliferating B cells increased numbers of CD4-T cells expressing CD40L. These activated CD4-T cells could be recruited by tumor B cells since they constitutively express the T cell-attracting chemokines CCL17 and 22 [112, 113]. CLL lymphocyte localization depends on sequential engagement of adhesion molecules and chemokine receptors (CXCR3, CXCR4, and CXCR5) that may direct leukemic cell chemotaxis *in vitro* [110]. In addition, CLL cell apoptosis can be prevented by interactions with stromal and nurse-like cells [114].

The interaction between CD38 and CD31 also favors the survival of leukemic cells [115]. Furthermore, interleukin-4 and CXCL13/SDF-1 might expand CLL clones by up-regulating the expression of anti-apoptotic genes including *BCL2*, *SURVIVIN*, and *MCL1*. These findings suggest that different subsets of T-cell may influence malignant B-cell to proliferate and that different stromal and accessory cells may favor prolonged survival and accumulation [110].

Toll-like receptors (TLR), concomitantly with the BCR, may also play a role in the co-stimulation of CLL cells [116]. Antigen stimulation and inflammation signals could be involved in the initial steps and in the progression of different B-cell chronic lymphoid malignancies. It has been recently reported that an inflammatory microenvironment, including TLR signaling, is at the basis of the CLL cell survival support provided by stromal accessory cells. CCL2 was reported to be induced in monocytes by the presence of CLL cells *in vitro* and increased levels of CCL2 were also detected in serum from CLL patients [117]. CCL2 binds to the chemokine receptors CCR2 and CCR4 [118], has chemotactic activity for monocytes and basophils, recruits memory T cells and dendritic cells to the sites of inflammation, and has also been implicated in the migration and localization of follicular lymphoma cells [119]. Taken together, these results could be in agreement with a model of selective survival of clones which would receive survival signals in these particular sites.

By using a non-radioactive, stable isotopic labelling method to measure CLL kinetics, Messmer et al showed that B-CLL is not a static process that results simply from accumulation of long-lived lymphocytes, but a disease where a dynamic process in which cells proliferate and die, often at appreciable levels ranging from 0.08% to 1.7% of the clone [120]. This finding is in conflict with the dogma that CLL is a disease characterized almost exclusively by cell accumulation due to a defect in apoptosis. It is clear that most, if not all, proliferative events occur in the tissues where leukemic cells are able to exploit microenvironment interactions in order to avoid apoptosis and acquire tumoral growing conditions. This mechanism may compensate for the clonal decrease that could occur in the periphery by apoptosis and depending on its importance could play a major role in the regulation of the tumor burden.

4. Conclusions

Considerable progress has been achieved in recent years in the comprehension of CLL pathogenesis. We are starting to understand which genes, molecules and accessory cell subsets are involved in CLL cell/microenvironment interactions and what roles they play. However, we still have to elucidate the molecular mechanisms through which these cells promote the accumulation of leukemic cells. Particularly, the role of cytokines, chemokines and chemokine receptors in shaping a supportive microenvironment is still poorly understood as well as the respective role of stromal cells and different T cell subsets.

The BCR appears to play a major role in CLL pathogenesis. However, we cannot provide a plausible explanation of the mechanisms leading to its poor expression at the membrane level and why the mutational profile of Ig genes plays such a major role in CLL prognosis.

Considerable progress has been achieved in the identification of the genetic lesions involved in CLL, particularly in the case of the 13q deletion, for which transgenic mouse models have provided important information on its role in CLL pathogenesis. However, the definitive role of these genetic lesions in CLL pathogenesis remains elusive as yet.

Space dictates that this review be limited in scope. We are aware that there are many other aspects of this fascinating disease which we have not covered.

5. References

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B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancytopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL's cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

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