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Dimitar G. EFREMOV[1],[2]

THE PATHOGENESIS OF RICHTER TRANSFORMATION IN CHRONIC LYMPHOCYTIC LEUKEMIA

Abstract

Chronic lymphocytic leukemia (CLL) is the paradigm of intrinsic and extrinsic tumor heterogeneity with respect to both cellular and interpatient variance and response to treatment. The multiple genetic, epigenetic, functional and microenvironmental alterations underlying the pathophysiology of CLL limit the efficacy of currently applied treatments. Moreover, single-agent treatments present a high risk of tumor resistance, with a substantial proportion of patients relapsing during treatment or undergoing Richter transformation. The latter condition represents the transformation of CLL into a secondary high-grade and aggressive lymphoma and is considered the most important unmet clinical need in CLL because of the lack of any effective treatment option. The development of more effective treatments for Richter transformation requires a more complete understanding of the molecular mechanisms that drive this condition. This paper will describe some novel insights in the pathogenesis of Richter Transformation that may potentially provide the basis for the development of personalized therapeutic approaches for this condition in the future.

Keywords: chronic lymphocytic leukemia, diffuse large B cell lymphoma, Richter transformation, B-cell receptor, cell cycle, targeted therapy.

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[1] Dimitar G. Efremov, MD, PhD, Head, Molecular Haematology Unit, International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149 Trieste, Italy, Tel: +39-040-3757212, E-mail: efremov@icgeb.org; https://www.icgeb.org/dimitar-efremov/

[2] Member of Macedonian Academy of Sciences and Arts, Skopje, North Macedonia

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in Europe and America, accounting for more than 40% of all adult leukemia diagnoses. The disease is approximately 10 times less frequent in Asian countries, which is believed to reflect different prevalence of inherited risk factors. The risk of developing CLL increases with age, with a median age at diagnosis ranging from 70 to 72 years (1).

CLL is characterized by a highly variable clinical course and outcome. Approximately 60% of the patients have a relatively indolent disease and many of them remain symptom-free and fully active for decades, whereas the remaining patients experience a rapidly progressive disease that requires treatment soon after diagnosis, and this might result in early death due to disease-related complications (2).

The diagnosis of CLL is established based on the presence of ≥5,000 per μl clonal B cells with a distinct immunophenotype consisting of co-expression of CD5, CD19 and CD23 and a weak expression of CD79B, CD22 and surface immunoglobulin (2). This finding in most patients is the only sign of the disease at diagnosis. However, patients that progress will subsequently develop lymphadenopathy, splenomegaly and hepatomegaly, whereas anemia and thrombocytopenia occur in the most advanced stages of the disease. In addition, CLL patients have progressive defects in both cell-mediated and antibody-mediated immunity, including hypogammaglobulinemia and B cell and T cell quantitative and functional defects. These defects result in an increased risk of infections, which are the main cause of death. Another serious complication of CLL is the transformation into an aggressive lymphoma, which is a syndrome known as Richter transformation (RT) or Richter syndrome (RS). This complication occurs in 5-10% of the patients, with an annual incidence of 0.5-1.0%, and currently represents the most important unmet clinical need of CLL (3).

This review will focus on the pathogenetic mechanisms that drive Richter Transformation and on recently developed *in vivo* models that have been used to study novel combinatorial treatments for this condition. Considering the close relationship between CLL and Richter Transformation, the first part of the paper will cover the genetic lesions and microenvironmental signals that drive the pathogenesis of CLL, whereas the second part will describe the genetic lesions most frequently associated with Richter

transformations and their capacity to induce transformation in *in vivo* murine models of the disease.

Pathogenesis of CLL: Genetic lesions

Table 1 lists the most frequent genetic lesions in CLL. By far the most frequent abnormality is the 13q14 deletion, which can be detected by fluorescence in situ hybridization (FISH) in approximately 50-60% of patients at diagnosis. This deletion represents an early event in the malignant transformation, considering that it is typically present in all or most cells of the malignant clone. The deletion invariably involves the microRNAs mir-15a and mir-16-1, which are two small noncoding RNAs that negatively regulate the expression of several important apoptosis and cell-cycle regulatory proteins (4). In particular, mir-15a and mir-16-1 have been shown to negatively regulate BCL-2. BCL-2 is an antiapoptotic protein that is strongly overexpressed by the malignant B cells, and it is believed to be the main reason for the greater apoptosis resistance of CLL compared to normal B cells (5, 6). In addition, these microRNAs downmodulate the expression of several genes that control cell-cycle progression, including the cyclins CCND2 and CCND3 and the cyclin-dependent kinases CDK4 and CDK6 (7). The increased expression of these genes as a consequence of the 13q14 deletion provides the leukemic cells with a greater capacity to respond to proliferative signals.

Table 1. Major genetic lesions and affected pathways in chronic lymphocytic leukemia

Genetic lesion	Frequency	Cellular pathway
13q14 deletion / miR-15/16 deletion	50%-60%	Apoptosis (BCL2) and cell cycle (CCND2, CCND3, CDK4, CDK6)
trisomy 12	15%-25%	Unknown
11q22 deletion and ATM mutations	15%-25%	DNA damage response
17p13 deletion and TP53 mutations	10%-15%	DNA damage response
NOTCH1 mutations	10%-15%	NOTCH1 signaling
SF3B1 mutations	10%-15%	RNA processing
BIRC3 mutations	4%-5%	NF-kB signaling
NFKBIE mutations	3%-6%	NF-kB signaling
MyD88 mutations	3%-5%	TLR / NF-kB signaling

Another frequent genetic lesion is trisomy 12, which can be detected by FISH in approximately 15-25% of patients at diagnosis. This lesion is also frequently clonal and is typically mutually exclusive with the 13q14 deletion. The mechanism how this abnormality contributes to the pathoge-

nesis of CLL is still unknown, although recent data indicate that this may be related to increased expression and activity of the receptor EDRNB and the kinase IRAK4, which transduce signals through the endothelin- and Toll-like receptor pathways, respectively (8).

Mutations or deletions of the ATM and TP53 genes, which are located on chromosome 11q22 and 17p13, respectively, are present in 10-25% of cases at diagnosis and are typically subclonal, suggesting that they are acquired at later stages of the disease (9). Importantly, both ATM and TP53 are tumor suppressors that are involved in DNA damage response. ATM and TP53 preserve the genomic integrity and stability of the cells by activating DNA repair proteins upon DNA damage recognition and arresting cellular growth by holding the cell cycle at the G1/S regulation point, or initiating apoptosis if the DNA damage proves to be irreparable. Consequently, deficiency of ATM or TP53 results in increased genomic instability and greater risk for acquisition of additional genetic lesions. In addition, deficiency of ATM and particularly TP53 is associated with resistance to cytotoxic agents that function by inducing DNA damage. For these reasons, the frequency of TP53 and ATM genetic lesions increases in more advanced stages of the disease and in patients that have become refractory to chemotherapy.

Mutations in NOTCH1 are present in 10-15% of patients at diagnosis, but are more frequent in patients at advanced stages of the disease and, as will be discussed later, in patients with Richter transformation. NOTCH1 acts as a transcription factor that induces the expression of various proteins involved in cell survival, proliferation, chemotaxis and homing (10). These include the antiapoptotic proteins MCL-1, c-IAP2, BCL-2 and XIAP, the cyclin CCND3, the proto-oncogene MYC, the B cell receptor pathway components LYN, SYK, BLK and BLNK, the chemokine receptor CXCR4 and the integrin CD49d (11-13). In addition, NOTCH1 signaling has also been reported to downregulate surface expression of CD20, which might explain why such patients derive limited benefits from anti-CD20-based immunotherapies. Interestingly, NOTCH1 mutations in CLL do not result in constitutive activation of this pathway, but rather cause prolonged signaling once NOTCH1 has been activated by binding to one of its ligands, which are various members of the SERRATE/JAGGED or DELTA-like families that are expressed on adjacent cells. Activated NOTCH1 is normally rapidly

degraded because of the presence of signal sequences for ubiquitination and proteasomal degradation in its C-terminal PEST domain. This domain is either truncated or entirely removed by the NOTCH1 mutations, resulting in reduced degradation and prolonged biological activity of NOTCH1 (11).

SF3B1 is another frequently mutated gene, with a mutational frequency of 10-15% in different studies (14, 15). The SF3B1 gene encodes a protein that acts as a component of the spliceosome machinery and is involved in the binding of the spliceosomal U2 small nuclear ribonucleoprotein (snRNP) to the branch point of 3’ intronic splicing sites. SF3B1 mutations promote the use of an alternative branch point, leading to the inclusion of 3’ intronic sequences in the mature RNA (16) and, therefore, to splicing changes affecting the structure and coding potential of gene transcripts across multiple pathways, including the DNA damage response, NOTCH1 signaling, apoptosis, and cell proliferation (17). SF3B1 mutations are also typically subclonal at diagnosis and have been associated with more aggressive disease and unfavorable overall survival.

Genetic abnormalities that affect members of the NF-kB pathway are also common in CLL. The two most frequent abnormalities are loss-of-function mutations in NFKBIE and BIRC3, which are negative regulators of the canonical and non-canonical NF-kB pathway, respectively. Mutations in NFKBIE and BIRC3 are seen in approximately 3-6% of cases at diagnosis (18,19). However, BIRC3 is also frequently affected by the 11q22 deletion, which, in 80% of the cases, involves both ATM and BIRC3. Deficiency of NFKBIE or BIRC3 has been associated with increased activity of the various members of the NF-kB family of transcription factors, which play an essential role in regulating several cardinal cellular processes, including cell survival and proliferation. NFKBIE and BIRC3 mutations are enriched in cases with advanced disease and are associated with poor-prognostic markers, suggesting their likely involvement in disease progression.

Another genetic abnormality that results in activation of the NF-kB pathway are mutations in MyD88, which is an adaptor protein that is involved in signaling downstream of various Toll-like receptors (TLRs). Mutations in MyD88 have been observed at a relatively low frequency in CLL (3-5% of cases), but are considerably more frequent in some other B cell malignancies, such as diffuse large B cell lymphoma (DLBCL, 30% of cases) and Waldenstrom's macroglobulinemia (90% of cases) (20,21). The

most common recurrent MYD88 mutation (p.L265P) is a missense mutation, which leads to increased binding of MYD88 to the downstream kinase IRAK1 (22). MyD88 mutations are typically clonal and have been associated with a favorable prognosis.

In addition to these genetic lesions, at least 50 other genetic lesions have been identified in putative CLL driver genes at frequencies ranging from 1 to 5 percent. Although the exact mechanisms as to how these lesions contribute to the pathogenesis of CLL are still unknown, most of them would be expected to affect one of the cellular pathways that were previously mentioned, including cell cycle regulation (e.g., CCND2, CDKN1B, CDKN2A/CDKN2B, MGA), DNA damage response (e.g., POT1), NOTCH1 signaling (e.g., FBXW7, SPEN), RNA processing (e.g., DDX3X, XPO1, U1 snRNA, RPS15), chromatin remodeling (e.g., SETD1A, SETD2, CHD2) and signaling through the NF-kB, TLR- and B cell receptor (BCR) pathways (e.g., TRAF3, IRF4, TLR2, IRAK1, EGR2)(23).

Pathogenesis of CLL: Microenvironmental signals

In addition to genetic lesions, signals from the microenvironment have been shown to play a major role in the pathogenesis of CLL. Evidence for this stems from the fact that CLL cells die rapidly by spontaneous apoptosis *in vitro*, whereas their survival in the blood and lymph nodes of patients has been estimated by *in vivo* labelling studies to be in the order of several months (24-27). Moreover, CLL cells do not proliferate when placed in culture, although a substantial proportion of them proliferates in the lymph nodes within specialized structures called proliferation centers (28). At these sites, CLL cells have been shown to interact directly with various other cell types, including T helper cells, macrophages, stromal cells, and follicular dendritic cells. Interaction with these cells has been shown to increase the survival of the leukemic cells *in vitro* and, in some cases, induce their proliferation. These interactions are mediated by various cell surface and soluble ligands, such as CD40L, IL-4 and IL-21 (expressed by T helper cells), BAFF, APRIL and IL-15 (expressed by macrophages) or the NOTCH1-ligand Jagged and the integrin-ligand VCAM-1 (expressed by stromal cells).

CLL cells receive additional growth-promoting signals from the BCR, which is a signaling complex composed of a surface immunoglobulin and a heterodimer of the proteins CD79A and CD79B. The BCR is expressed on all B cells, both normal and malignant. However, in contrast to normal B cells, where the BCR is only transiently activated following stimulation with a microbial antigen, the BCR in CLL and other malignant B cells is frequently chronically activated (29).

Initial evidence for a role of the BCR in the pathogenesis of CLL was provided by immunogenetic studies showing that CLL BCRs have a restricted usage of immunoglobulin heavy chain variable (IGHV) and immunoglobulin light chain variable (IGLV) genes, indicating selection for particular antigen-binding properties. BCRs encoded by these IGHV/IGLV combinations and having particular HCDR3 structures have been named "stereotyped BCRs" and have been identified in approximately one-third of the CLL cases, whereas they are rarely seen in normal B lymphocytes (30-32). Subsequent studies have shown that freshly isolated CLL cells express high levels of BCR target genes and constitutively activated BCR signaling molecules, such as the kinases LYN, SYK, PI3K and BTK, suggesting that the BCR pathway is chronically activated in these cells (33-36). Definite evidence for a major role of the BCR pathway in the pathogenesis of CLL came from clinical studies showing that drugs that inhibit signaling through this receptor induce clinical responses in the majority of CLL patients. Most of these drugs target the kinases BTK or PI3K, although inhibitors of SYK, SRC family kinases, and mTOR have also demonstrated activity in clinical trials (29).

Mechanisms of BCR pathway activation in CLL B cells

Studies conducted during the previous decade have identified two main mechanisms of BCR activation in CLL cells (Figure 1). The first mechanism involves binding to low-affinity external autoantigens that are typically generated during apoptosis or oxidation (37, 38). This mechanism is primarily seen with CLL cells belonging to the more aggressive, IGHV unmutated (U-CLL) subset, and would be predicted to result in intermittent activation of the BCR pathway in CLL-infiltrated lymphoid tissues, where apoptotic cells are commonly present (39, 40). The second mechanism is a peculiar mechanism that involves binding of CLL BCRs to internal motifs

located in neighboring immunoglobulin molecules, resulting in BCR-BCR interactions that occur in the absence of any external ligand (41). These cell-autonomous BCR-BCR interactions have been detected with both IGHV-mutated (M-CLL) and U-CLL BCRs and would be expected to generate a continuous low-intensity BCR signal in all tissue compartments (39).

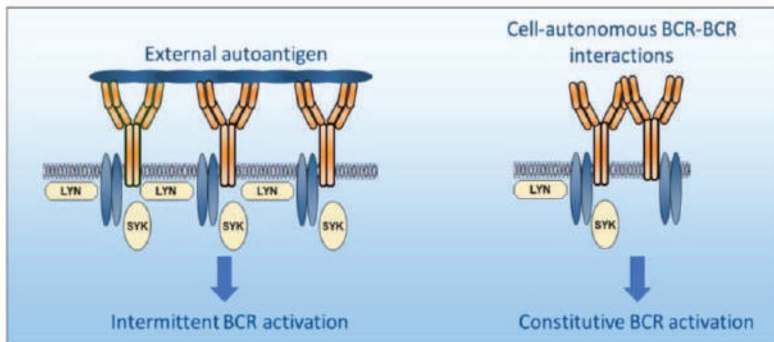

Figure 1. Mechanisms of B cell receptor activation in chronic lymphocytic leukemia

The pathogenetic role of these two mechanisms of BCR activation was explored in a study conducted by our group, in which we investigated the capacity of different antigen-BCR and BCR-BCR interactions to induce leukemia in the well-established Eμ-TCL1 transgenic mouse model of CLL [42]. These mice, which are predisposed to develop CLL because of targeted overexpression of the TCL1 oncogene in the B cell compartment, were bred with mice that expressed transgenic BCRs with different antigen specificity. Prolonged follow-up of these mice showed that only B cells that expressed BCRs with cell-autonomous activity or BCRs reactive with low-affinity self-antigens enter into the leukemogenic process and become CLL cells. By contrast, B cells that expressed BCRs that did not react with any antigen, or that reacted with high-affinity antigens, did not undergo malignant transformation, regardless of antigen form (soluble or membrane-tethered) or presentation (foreign or self). These findings provided direct *in vivo* evidence that self-reactivity is a major driving force in CLL pathogenesis and suggested that only BCR signals of certain quality can promote the growth of the malignant cells.

The exact nature of the cellular processes regulated by the two different mechanisms of BCR pathway activation has still not been fully

elucidated, because most of the studies so far have evaluated only responses induced by external ligands. However, these studies identified several BCR-driven processes that could contribute to the expansion and accumulation of the malignant clones, including increased apoptosis resistance (mediated by induction of the antiapoptotic protein MCL-1 and downregulation of the proapoptotic proteins BIM and HRK), increased adhesion to stromal cells and the extracellular matrix (mediated by activation of integrins and induction of adhesion molecules) and recruitment of T cells and macrophages (mediated by induction of the chemokines CCL3, CCL4 and CCL22) (35, 43-48). In addition, BCR engagement with an external ligand has been shown to induce CLL cells to enter into the G1 phase of the cell cycle, although, unlike normal B cells, this did not result in subsequent progression into the S phase of the cell cycle (49-51). A possible explanation for this inability of BCR signals to drive CLL cell proliferation was provided by a recent study from our group, showing that BCR stimulation of human and murine CLL cells simultaneously induces the expression of both the positive regulators MYC, CCND1, CCND2 and CDK4, which induce cell cycle entry, and the negative regulators CDKN1A, CDKN2A and CDKN2B, which inhibit CDK4 and CDK6 and block G1/S phase progression (52). This could explain why CLL cells require co-stimulatory signals from T cells (i.e., CD40L + IL-4 + IL-21) or TLR ligands (i.e., unmethylated DNA) for their proliferation (53-55).

Richter Syndrome: Clinical and diagnostic features

Richter syndrome is defined in the World Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissues as the development of a secondary and aggressive lymphoma arising on the background of CLL or small lymphocytic lymphoma (SLL) (57). The first case was described in 1928 by Dr Maurice Richter as a “reticular cell sarcoma of lymph nodes” arising in the context of “lymphatic leukemia” (58), and was nominated in his honor in 1964 by Lortholary et al. (59).

Richter Syndrome occurs in approximately 5–10% of CLL patients during their lifetime, with an annual incidence of 0.5-1.0% (60). It may present as two different pathologic entities: the diffuse large B cell lymphoma (DLBCL) variant, which accounts for approximately 90% of the cases, and the Hodgkin lymphoma (HL) variant, which accounts for the remaining cases (61).

The main clinical signs of Richter syndrome are new onset B symptoms, rapidly-growing and/or asymmetrical lymphadenopathy or extra-nodal masses, and rapidly rising LDH levels or new onset hypercalcemia. These signs and symptoms should raise suspicion of Richter syndrome and prompt an excisional biopsy, which is best guided by the results of an 18-fluorodeoxyglucose (18FDG) positron emission tomography/computed tomography (PET/CT) scan. The lesions that display the most avid 18FDG uptake with the highest standardized uptake value (SUV) should be selected for bioptic sampling (62). In contrast, lesions that have a SUV<5 are unlikely to be transformed and should not be biopsied.

The diagnosis is established based on the results of the histological analysis, which, in the case of the HL variant, requires the presence of classical Reed-Sternberg cells harboring a CD30+/CD15+/CD20- phenotype in a polymorphous background of T cells, epithelioid histiocytes, eosinophils and plasma cells. In most cases the Reed-Sternberg cells are EBV positive and have a different IG gene rearrangement from the CLL clone, suggesting that they represent *de novo*, EBV-driven lymphomas (61).

The diagnosis of the DLBCL variant is more complicated and requires an experienced pathologist, because it may mimic aggressive/accelerated CLL, which is characterized by expanded and confluent proliferation centers (57,62). The diagnosis is based on the finding of a diffuse infiltration of large B cells with a nuclear size equal to or larger than macrophage nuclei or more than twice the size of a normal lymphocyte. The tumor cells invariably express the CD20 antigen, whereas CD5 expression is maintained in only a fraction (~30%) of cases, and CD23 is expressed even less frequently (~15% of cases) (63). Most tumor cells express the Ki67 antigen, suggesting that they are actively proliferating.

Immunogenetic studies have shown that in more than 80% of the cases the tumor cells are clonally related to the CLL cells, as evidenced by the presence of identical IG gene rearrangements (63). The remaining cases carry IG gene rearrangements that are different from those of the CLL cells and therefore represent *de novo* lymphomas. The determination of the clonal relationship between the lymphoma and CLL cells is critical for the proper management of Richter syndrome, as patients with clonally-related and clonally-unrelated RT respond differently to treatment and have a different prognosis. In particular, patients with clonally-related RT are resistant to

chemoimmunotherapy and have an average survival time of less than 12 months, whereas patients with clonally-unrelated DLBCL respond well to standard DLBCL treatment and have a similar survival as *de novo* DLBCL, which is approximately 65 months.

Richter Syndrome: Genetic lesions

Another difference between clonally-related and clonally-unrelated Richter syndrome is the genetic profile of the malignant cells. The clonally-unrelated tumors carry the same genetic lesions as *de novo* DLBCL, such as inactivating mutations in the acetyltransferase genes CREBBP and EP300 and the histone methyltransferase gene KMT2D, or translocations of the BCL2 and BCL6 oncogenes. These genetic lesions do not occur in the clonally-related tumors, further suggesting that DLBCL transformed from CLL and *de novo* DLBCL represent distinct disease entities.

In clonally related RT-DLBCL, the most frequent genetic abnormalities are mutations or deletions of TP53, which occur in 60% to 80% of the cases and can be either present or acquired at the time of transformation (Table 2)(63). The second most frequent abnormality is the 9p21 deletion, which disrupts the previously mentioned cell cycle inhibitors CDKN2A and CDKN2B. Deletion of CDKN2A and CDKN2B has been reported in over 30% of Richter syndrome tumors and typically occurs at the time of transformation, whereas it has been detected in only 1.7% of unselected CLL cases (64-66). Aberrant MYC expression or activation is also frequently acquired at the time of transformation and is usually due to somatic structural alterations, such as the t (8;14) translocation or the 8q24 amplification, which are detected in approximately 30% of cases and result in MYC overexpression, or by truncating mutations and deletions of the MYC-antagonist MGA, which occur in approximately 10% of the cases and result in increased MYC activity (64,65, 67, 68). In addition, MYC is overexpressed in an additional 30% of cases because of gain-of-function NOTCH1 mutations.

Table 2. Major genetic lesions and affected pathways in Richter Syndrome

Genetic lesion	Frequency in CLL	Frequency in RT	Cellular Pathway
TP53 mutation and/or deletion (del17p13)	10%-15%	**60%**	DNA damage response and cell cycle
CDKN2A/CDKN2B deletion (del9p21)	1.5%	**35%**	Cell cycle
MYC abnormalities: t(8;14); 8q24 amplification	5-7%	**30%**	Cell cycle (increased MYC expression)
MGA deletion (del15q)	4%	**10%**	Cell cycle (increased MYC activity)
NOTCH1 mutations	10%-15%	**30%**	NOTCH1 signaling (increased MYC expression)

Based on their genetic profiles, two major RT-DLBCL subsets have been identified, each accounting for approximately one third of the cases: 1) a subset characterized by CDKN2A/CDKN2B deletions that are always associated with TP53 mutations/deletions and are frequently associated with MYC abnormalities, and 2) a subset characterized by NOTCH1 mutations that, in most cases, are associated with trisomy 12. Cases belonging to the latter subset frequently express BCRs belonging to the stereotyped subset 8, which is characterized by broad polyreactivity to multiple autoantigens. In one study, usage of a subset 8 stereotyped BCR increased the risk for development of Richter Syndrome by 24-fold, suggesting that BCR signaling may represent an important driving force during Richter transformation (69).

Richter Syndrome: *In vivo* murine models

The capacity of the above-mentioned genetic lesions to induce Richter transformation was investigated in several recent studies using the previously described Eμ-TCL1 transgenic mouse model of CLL. Crossing these mice with TP53 mice to mimic the effects of TP53 deficiency resulted in the development of aggressive lymphomas with features of Richter syndrome in 15% of the cases. This suggests that TP53 abnormalities increase the risk for Richter transformation but are unable to drive this complication on their own (70). In another study, Eμ-TCL1 transgenic mice were crossed with mice overexpressing MYC in the B cell compartment (71). These mice developed aggressive lymphomas in all cases, but the tumors were derived from B cells at an earlier maturation stage and had distinct immunogenetic features compared to the CLL cells, suggesting that they represent clonally-unrelated lymphomas. More recently, Eμ-TCL1 transgenic mice were cros-

sed with mice with B cell-restricted overexpression of the NOTCH1 intracellular domain to investigate the effects of constitutive NOTCH1 activation (72). Although these mice developed aggressive lymphomas with a high penetrance, this only occurred in mice at an advanced age, suggesting that NOTCH1-activating mutations are not sufficient to drive Richter transformation on their own and that additional genetic lesions need to be acquired for this to occur.

My own group recently investigated whether combined genetic lesions in CDKN2A, CDKN2B and TP53 can drive Richter transformation in the Eμ-TCL1 transgenic model (52). This combination piqued our interest because of the previously mentioned observations that CDKN2A/CDKN2B deletions are almost always acquired at the time of Richter Transformation and always associated with TP53 mutations or deletions. Further rationale to investigate this combination was provided by our recent finding that stimulation of CLL cells through the BCR simultaneously induces the cell cycle inhibitors CDKN1A, CDKN2A and CDKN2B. Because CDKN1A is a transcriptional target of TP53, we reasoned that genetic lesions in TP53, CDKN2A and CDKN2B may prevent the upregulation of these cell cycle inhibitors in BCR-stimulated CLL cells and, consequently, allow them to proliferate in the absence of co-stimulatory signals (Figure 2).

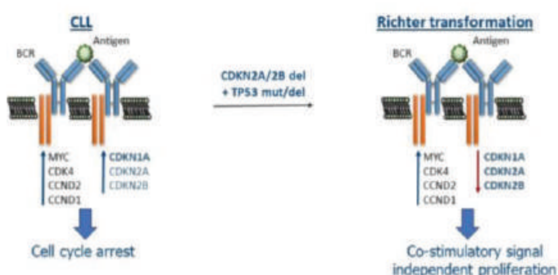

Figure 2. Mechanism of Richter Transformation in cases with combined TP53, CDKN2A and CDKN2B abnormalities.

To test this hypothesis, we simultaneously targeted the TP53, CDKN2A and CDKN2B genes in primary Eμ-TCL1 leukemia cells by CRISPR/Cas9 genome editing. The targeted and control leukemia cells were then transplanted in syngeneic mouse recipients and the impact of combined TP53, CDKN2A

and CDKN2B genetic lesions was assessed by comparing the *in vivo* growth of the leukemic cells. Mice that received TP53/CDKN2A/CDKN2B-targeted cells showed accelerated leukemia growth and developed splenic tumors with morphological features of Richter transformation, including more diffuse infiltration, larger and more pleomorphic cells, and a higher proliferative rate compared to the control leukemia cells (Figure 3). Importantly, *in vitro* culture experiments with the targeted cells showed that they had acquired the capacity to proliferate in the absence of any co-stimulatory signals, which was not the case for cells in which only TP53 or CDKN2A and CDKN2B had been disrupted. Moreover, the spontaneously proliferating cells displayed homozygous defects in all three genes, suggesting that biallelic inactivation of TP53, CDKN2A and CDKN2B is required for co-stimulatory signal-independent proliferation. These cells, however, remained dependent on BCR signals, as evidenced by their reduced growth and gradual disappearance upon CRISPR/Cas9-mediated knockdown of the BCR or treatment with the BCR inhibitors ibrutinib, idelalisib, entospletinib or fostamatinib. The latter finding was further exploited to investigate the therapeutic activity of combinations of BCR inhibitors with other drugs, which resulted in the identification of a highly potent combination of ibrutinib with the CDK4/CDK6 inhibitor palbociclib. Collectively, these data provide evidence that BCR signals are directly involved in driving Richter transformation and suggest that frequently co-occurring genetic lesions in TP53 and CDKN2A/2B contribute to Richter transformation by allowing for BCR-dependent/costimulatory signal-independent proliferation. In addition, they suggest that the combination of a BCR and a CDK4/6 inhibitor could represent an effective treatment for Richter syndrome patients with combined TP53 and CDKN2A/2B abnormalities and provide the preclinical rationale to evaluate this possibility in the clinic.

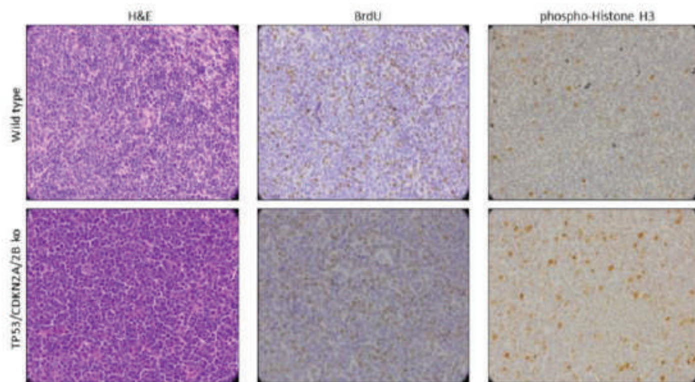

Figure 3. Histology of tumors with wild type or mutated TP53, CDKN2A and CDKN2B. Splenic sections were stained with Haematoxylin and Eosin (H&E), anti-BrdU or anti-phospho-Histone H3 antibodies. Scale bars 20 μm; 60x objective.

In summary, data from the murine models confirm the pathogenic role of TP53, CDKN2A/CDKN2B, MYC and NOTCH1 abnormalities in Richter transformation. These models, therefore, represent invaluable tools for preclinical testing of novel drugs and drug combinations that target the molecular programs that are altered in patients with Richter transformation, and these findings should also facilitate the development of personalized therapeutic approaches for this condition.

Acknowledgements and Dedication

I would like to thank the numerous members of my research group for their most valuable contributions to the studies that I described in this paper. The paper was written to celebrate the 70th birthday of my dear friend and colleague, Academician Prof. Dr. Zivko Popov, to whom I wish that he continues with the same pace, enthusiasm and productivity for many years to come.

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