

Entry

CRISPR/Cas9 in Chronic Lymphocytic Leukemia

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Definition: Genome-editing systems such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology have uncovered new opportunities to model diseases such as chronic lymphocytic leukemia. CRISPR/Cas9 is an important means of advancing functional studies of Chronic Lymphocytic Leukemia (CLL) through the incorporation, elimination and modification of somatic mutations in CLL models.

Keywords: leukemia; CRISPR/Cas9; editing

1. Introduction

B-cell lymphoproliferative disorders are a clonal expansion of the various stages of B lymphocytes in bone marrow, blood, or other tissues. The World Health Organization (WHO) has described more than 30 different entities in the category of mature B-cell neoplasms [1]. Both lymphomas and lymphoid leukemias are included in this classification in which you could find Burkitt Lymphoma, Diffuse Large B-cell Lymphoma, Follicular Lymphoma, and Mantle Cell Lymphoma, as well as Chronic Lymphocytic Leukemia or Hairy Cell Leukemia.

Among the leukemias, **Chronic Lymphocytic Leukemia (CLL)** is the most common adult leukemia in Western countries, with an incidence of 4.2 cases per 100,000 people per year [2]. Its median age at diagnosis ranges from 70 to 72 years, being more frequent in men (2:1). This hematological malignancy is characterized by the presence of mature clonal B lymphocytes that accumulate in the blood, bone marrow, and other lymphoid tissues [1,3,4]. The diagnostics is mainly based on laboratory techniques, namely blood count, morphology, and immunophenotyping [2,3]. Specifically, the diagnosis of CLL is mainly defined by the presence of more than 5×10^9 /L B lymphocytes in peripheral blood for at least three months. The clonality of the circulating B-lymphocytes needs to be confirmed by flow cytometry [2–4]. The immunophenotype of CLL distinguishes it from other B hematological malignancies by the expression of B cell markers such as CD19, CD23, and weak CD20, along with CD5, a T cell antigen, and low expression levels of surface membrane immunoglobulin [5]. In terms of morphology, the CLL cells found in the blood smear are characteristically small and mature lymphocytes with a narrow border of cytoplasm, and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin. Large atypical cells, cleaved cells, or prolymphocytic cells, which may be up to 55% of the blood lymphocytes, could also be observed [6]. Gumprecht nuclear shadows, or smudge cells, found as cell debris are other characteristic morphologic features in CLL.

The natural course of CLL is highly heterogeneous, ranging from asymptomatic with no need for therapy to an aggressive disease associated with therapeutic resistance and short overall survival [7]. CLL patients are generally diagnosed with an asymptomatic disease by blood tests performed during a routine physical exam. However, other patients showed symptoms such as fatigue, fever, lymphadenopathy, hepatomegaly, splenomegaly,



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bone marrow failure, recurrent infections, and/or weight loss [8,9]. In recent decades, the improved understanding of CLL pathogenesis has resulted in the identification of a great number of prognostic markers (clinical systems, serum markers, genetic alterations, et.), significantly improving patient stratification [10]. Prognostication in CLL remains an active research field in order to define not only the prognostic markers able to predict the clinical course at diagnosis but also the predictive markers able to predict the response to treatment in the era of targeted therapies [11].

Numerous studies have demonstrated that the clinical variability is a clear consequence of marked biological diversity [12,13]. During the last decades, Next-Generation Sequencing (NGS) technologies have uncovered a great number of genetic alterations in CLL [14,15], with a long tail of hundreds of genes mutated only in a short fraction of CLL patients [16]. The biological impact of some of CLL genetic abnormalities has been partially understood thanks to CLL models [17–20]. Since CLL disease is the result of a complex interaction of different lesions, novel models are required to study the biological effects of single and multiple genetic lesions to gain insight into the mechanisms underlying the clonal evolution as well as the treatment response. In this line, models applying genomic engineering will serve as valuable tools to study the effects of CLL drivers on cellular fitness.

The development of genome-editing technologies has broadened new possibilities to model diseases such as CLL. The development of genome editing technologies has opened up the possibility of directly targeting and modifying genomic sequences in almost all eukaryotic cells. The first approaches used in the field of genome-editing were based on the zinc-finger nucleases (ZNFs) and transcription activator-like effector nuclease (TALENs) [21,22]. The discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems as genome-editing technologies has overcome many of the limitations of the earlier strategies, allowing us to create disease models in a rapid, efficient, and cheap way [23]. Since 2013, when it was first applied in mammalian cells as a tool to edit the genome [24,25], the versatile CRISPR/Cas9 technology has been rapidly expanding its use in modulating gene expression, ranging from genome sequence changes to epigenetic and transcriptional modifications.

CRISPR/Cas9 genome editing technology is based on inducing DNA double-strand-breaks (DSBs) that stimulate the cellular DNA mechanisms: error-prone non-homologous end joining (NHEJ) and homologous recombination (HR). This system—derived from a bacterial adaptative immune system—relies on two key components: the nuclease Cas9 and the single-guide RNA (sgRNA) [24,25]. The sgRNA molecule is complementary to the target region of interest and directs Cas9 to the genomic region of interest, leading to the generation of DSBs [26]. Consequently, on one hand, NHEJ is an error prone repair process that joins broken ends, generally resulting in the introduction of small indels (insertions and deletions), and therefore, the presence of frameshift mutations which generate premature stop codons and mimic loss-of-function (LoF) mutations, which can be useful to generate knock-out models. On the other hand, HR happens in the presence of a donor DNA template, allowing specific DNA edition such as gain-of-function (GoF) mutations, being the ideal strategy for generating knock-in models [27,28].

Besides the application of the generation of isogenic models with LoF or GoF mutations, CRISPR/Cas9 technology allows us to generate chromosomal rearrangements by the introduction of two distant DSBs within the same chromosome to produce chromosomal inversion or deletion, or the induction of two DSBs in different chromosomes leading to a chromosomal translocation [29–32].

CRISPR-gene editing system also provides a powerful way to switch gene expression on or off at the transcription level [33,34]. In this line, a nuclease-deactivated form of Cas9 termed deadCas9 (dCas9), which is unable to cleave DNA, is necessary. A fused dCas9 with silencer agents or transcriptional activators can bind to the promoter region to efficiently repress or activate gene expression, respectively [35,36].

The possibility to perform multiplex mutagenesis by CRISPR has opened a range of functional genome-screening approaches. These approaches could reveal key genes associated with drug resistance or identify vulnerable target genes for the development of targeted drugs [37,38].

2. Applications of CRISPR Technology in CLL

The development of genome-editing tools based on CRISPR systems has revolutionized the possibilities of modelling CLL biology, mimicking the somatic mutations with disease relevance observed in patients [39]. In the last decade, some studies have emerged to understand the effects of genetic alterations on tumoral cells. The present review summarizes the main results published in the field of CLL modelling using CRISPR/Cas9 technology.

In 2017, Arruga et al. used CRISPR technology to emulate *NOTCH1* mutations in an in vitro CLL model [40]. Using MEC-1 cell line, they successfully generated cells with different *NOTCH1* variants. They reported that *NOTCH1* can modulate the expression of the tumor suppressor *DUSP22* since *NOTCH1* affects the methylation of *DUSP22* promoter through a nuclear complex that affects the activity of *DNMT3A*. Consequently, the downregulation of *DUSP22* in *NOTCH1*-mutant cells decreases MAP kinases signaling and STAT3 activation which impaired growth and chemotaxis. In xenograft models, *NOTCH1*-mutant cells displayed a unique homing behavior localizing preferentially to the spleen and brain. These findings related to the migratory properties of these leukemic cells could explain the association of *NOTCH1* mutations with a more aggressive disease process and unfavorable prognosis in CLL patients [40].

FBXW7 gene is another recurrently mutated CLL gene involved in *NOTCH1* pathway. Close et al. published a study applying CRISPR/Cas9 technology to generate *FBXW7*-knockout cells in HG3 cell line [41]. The induction of truncation of *FBXW7* resulted in an increase of activated *NOTCH1* intracellular domain and c-MYC protein levels as well as elevated hypoxia-inducible factor 1- α activity. Therefore, since the expression of *NOTCH1* target genes was elevated in *FBXW7*-mutant cells similarly to what happens in *NOTCH1*-mutant cells, they postulated that CLLs with *FBXW7* mutations could be functionally included into the group of CLL patients with dysregulated *NOTCH1* signaling [41].

Deletion on 17p (del(17p)) is a common chromosomal alteration in CLL patients [42]. This deletion compromises the tumor suppressor gene *TP53*, which is mutated in approximately 10% of CLL cases at diagnosis [43]. Moreover, *TP53* mutations can be frequently found in the remaining allele of del(17p) patients, leading to a biallelic inactivation of *TP53* [44]. *TP53* dysfunction has been widely associated with chemoimmunotherapy relapse or refractory in CLL [15,45,46]. Therefore, these patients were the first one in who targeted inhibitors such as ibrutinib or venetoclax were approved [45,46]. For these reasons, CRISPR-edited CLL models with *TP53* mutations are very useful as preclinical models. In 2017, Amin et al. generated *TP53*-knockout cells using different CLL in vitro models (HG3 and PGA1) and a B-cell lymphoblastoid cell line (PG-EBV cell line) to test ibrutinib (BTK inhibitor) as well as to assess the effects of *TP53* disruption on BCR signaling [47]. In 2019, Shen et al. tested IBL-2020 (PI3/PIM kinase) in combination with venetoclax in *TP53*-deficient OSU-CLL cells generated using the CRISPR/Cas9 system. In 2020, Mancikova et al. not only were able to generate *TP53* disruption but could also disrupt *ATM* using CLL-derived cell lines [48]. They exposed the edited cells to CAR T cells and evaluated the in vivo efficacy of anti-CD19 CAR T cell therapy in NSG mice injected with CRISPR-generated knockout cell lines. Interestingly, they observed early disease onset, high-tumor burden, and inefficient T cell engraftment, associated with *TP53*-knockout tumors, ultimately led to poorer response to CAR T cell treatment.

RPS15 has been described as a novel CLL driver thanks to genomic studies from larger cohorts of CLL patients [14,15]. In 2018, Bretones et al. implemented CRISPR/Cas9 technology to understand the function of this gene [49]. They revealed that *RPS15* mutants alter ribosomal activity and induce proteome-wide changes. Other studies have also implemented this technique to gain insight into the role of other proteins (14-3-3 ζ ,r ADAR)

that could play an important role in CLL biology, specifically, in Wnt signaling or RNA editing processes [50,51].

In CLL, among the driver alterations, some have exhibited as being highly co-occurring, whereas others appear to have absence of co-occurrence [14,15]. While interactions between genetic alterations could synergistically act to enhance tumor growth, lack of co-occurrence could indicate that alterations could have highly similar downstream effects, and hence the lack of further advantage to the tumor cell to have redundant functional effects [52–54]. However, the number of CLL models accurately reflecting the heterogeneity of the disease and assessing the effects of multiple mutations is still very limited. Up to date, functional studies largely address only effects of single mutations, as mentioned in previous paragraphs. In 2020, ten Hacken et al. performed, for the first time, multiplexed CRISPR editing to model simultaneously common LoF CLL mutations (*TP53*, *ATM*, *BIRC3*, *CHD2*, *MGA*, *SAMHD1*) in the murine interleukin 3 (IL-3) dependent pro-B cell line Ba/F3 [55]. By single cell resolution, it was demonstrated that co-transduction using six sgRNAs could generate several combinations of gene modifications, allowing us to study gene interactions in CLL.

Deletion on 11q (**del(11q)**) is one of the most prevalent in CLL, ranging from 12% to 20% of CLL cases at diagnosis [42,56–58]. The size of this deletion can be variable and commonly includes *ATM* and/or *BIRC3* gene. In addition, LoF mutations of *ATM* or *BIRC3* are associated with del(11q) cases, aggravating the outcome of del(11q) CLL patients. CRISPR/Cas9 system has been used to successfully establish CLL models that recapitulate the biology of del(11q) as well as concurrent mutations in *ATM*, *BIRC3*, and *TP53* genes [59–61]. In 2020, CRISPR/Cas9 technology was used to generate the typical del(11q) observed in CLL patients, creating for the first time a CLL in vitro model with this common alteration [59]. The presence of the monoallelic del(11q) favored genomic instability by impaired the repaired of DSBs, facilitating the acquisition of other mutations, especially those on *ATM* and *BIRC3*. In terms of *ATM* dysfunction, biallelic inactivation of *ATM* led to a higher dysregulation of the signaling of DNA Damage Response (DDR), resulting in higher genomic instability [59]. In the case of *BIRC3*, truncation of this gene constitutively activated the non-canonical NF-κB signaling with an overexpression of anti-apoptotic BCL2 family proteins. This could explain the enhanced clonal advantage observed in in vitro and xenograft models [61]. Moreover, del(11q) could also co-occur with *TP53* alterations in CLL patients. In 2021, Quijada-Álamo et al. demonstrated that *TP53* dysfunction contributed to enhanced cellular fitness as well as clonal advantage of cells with del(11q) [60]. The outcome of del(11q)/*TP53* mutated CLL patients is poor and may be related to a cooperative dysregulation of the DDR and loss of cell-cycle checkpoints, resulting in an increased genomic instability in their tumoral cells.

Apart from getting a deeper knowledge of the biological role of del(11q)-related alterations in CLL, the CRISPR-edited isogenic in vitro models presented in the previous paragraph are also useful to perform novel pre-clinical approaches. For instance, del(11q)/*ATM* mutated HG3 cells were hypersensitive to PARP inhibitors such as olaparib [59]. Interestingly, the combination of olaparib and BTK inhibitor ibrutinib was synergistic and especially effective in CLL cells with biallelic *ATM* loss. Moreover, del(11q)/*BIRC3* mutated cells were more sensitive to BCL2 inhibitor venetoclax or BCL-xL inhibitor A133185 [61].

Thus, CRISPR/Cas9 system has successfully used to generate del(11q)-related CLL in vitro models using HG3 and MEC1 cell lines. In combination with xenograft in vivo models and ex vivo primary CLL cultures, the specific contribution of each different genetic backgrounds has been elucidated, being extremely important for the understanding of CLL biology and treatment response.

Epigenetic alterations such as promoter hypermethylation may drive cancer through tumor suppressor gene inactivation. Recently, Pan et al. have generated CRISPR-edited DNA methylation CLL driver cells [62] performing DNA methylation modification. They have successfully silenced the expression of three candidate drivers (*DUSP22*, *RPPM*, and *SASH1*) in HG3 cell line, modifying promoter methylation by dCas9-guide approach.

Advances in the understanding of CLL biology have resulted in the development of new targeted therapeutic approaches. In fact, there has been an impressive explosion of new approaches for CLL patients during the last decade [63,64]. Chemotherapeutic-based regimens were the standard of care for many years but have taken a backseat, with targeted agents and their combinations occupying first place due to their excellent efficacies [65]. In this current chemotherapy-free era, BCR and BCL2 inhibitors have changed the management of CLL patients, clearly improving their prognosis and quality of life [11]. Venetoclax is an approved BCL2 inhibitor for the treatment of CLL [46]. Despite its potent clinical activity, even in CLL cases failing with chemotherapy regimens such as those carrying disruption of *TP53* [46], relapse disease following venetoclax is an emerging therapeutic challenge [66,67]. To identify determinants of **drug resistance**, Guèze et al. conducted parallel genome-wide screens of the BCL-2-driven OCI-Ly1 cell line after venetoclax exposure along with integrated expression profiling and functional characterization of drug-resistant and engineered cell lines [68]. Based on genome-wide CRISPR library tools, they identified regulators of lymphoid transcription and cellular energy metabolism as drivers of venetoclax resistance in addition to the known involvement by BCL-2 family members, which were confirmed in CLL patient samples. These insights support the implementation of combinatorial therapy with metabolic modulators to address venetoclax resistance.

3. Conclusions and Prospects

NGS techniques have provided enormous amount of genomic data to interpret, generating the need to translate those data into functionally and clinically relevant knowledge that allows the researchers to determinate how genotype influences phenotype in tumoral cells. Over the past decade, the integration of genome editing systems has enabled researchers to directly manipulate any gene in a diverse range of cell types and organisms.

Here, it is shown that the recent availability of genome-editing tools such as CRISPR-Cas9 are an important means of advancing functional studies of CLL through the incorporation, elimination, and modification of somatic mutations in CLL models. As mentioned in the Section 1 (Introduction), CLL is a genetically heterogenous disease with a large number of driver alterations present in a low percentage of patients. In the near future, there will probably be an explosion of CRISPR-based studies with novel CLL models to gain insight into the biological effects of CLL driver alterations that have been detected thanks to NGS.

Understanding the impact of CLL genetic alterations requires testing their effects within a B cell context. Most of the published studies have been based on the implantation of CRISPR approaches using in vitro CLL models and xenograft models injecting the CRISPR-edited cell lines. However, any cell line has potentially already undergone numerous alterations which may mask the effect of engineered driver mutations. To overcome this limitation, researchers should improve the capacity of CRISPR-mediated genome editing in primary CLL cells, increasing the efficiency of transfection or transduction to introduce the CRISPR/Cas9 machinery into CLL cells. Up to now, few studies have shown efficient editing approaches in primary human B cells [69–71] which may be translated in B tumoral cells from CLL patients.

CRISPR/Cas9 has been frequently used to generate knockout models in CLL. Much attention in recent studies has focused on understanding the phenotypic consequence of LoF mutations. Nevertheless, we still lack knowledge about the impact of GoF mutations. This could be due to the difficulty to generate models with point mutations since HR occurs at lower rates and only in the presence of a donor template. A lot of efforts have been made to increase the efficiency of CRISPR systems to generate knock-in models. Its implementation in the study of CLL will allow us to explain the consequences of several missense mutations such as mutations in *SF3B1*, *XPO1*, *IKZF3*, *MYD88*, and *RAS*-pathway genes.

Elucidating the functional impact of both LoF and GoF mutations will help to distinguish driver variants from passenger mutations. This is important to understand the mechanisms underlying the process of initiation, progression, and relapse evolution [15,72,73].

The development of CLL is usually preceded by a premalignant state called monoclonal B-cell lymphocytosis [1]. At the other end of the spectrum, CLL may undergo histologic transformation into an aggressive B-cell lymphoma termed Richter's syndrome [74]. Further studies using CRISPR-edited models introducing early or late genetic events will help to explain the transformation processes previously mentioned.

CLL genetically engineered models are crucial for prioritizing variants and their resultant therapeutic liabilities. In addition, these models are quite useful for preclinical testing. They will help to make progress towards the implementation of personalized medicine in CLL since they could be used to evaluate drug response attending to the genetic background and to uncover therapeutic vulnerabilities based on synthetic lethal combinations between different genetic lesions.

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