(RESEARCH ARTICLE)

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New clinical treatment plan for acute lymphoblastic leukemia after whole genome sequencing: An update of the development of new technologies for genome editing

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Abstract

Acute lymphoblastic leukemia (ALL) is now being reclassified into newly identified subtypes due to genetic abnormalities, Philadelphia chromosome-like B-lineage ALL is one of the new high-risk subtypes characterized by genetic alterations that activate various signaling pathways, including those involving cytokine receptors, tyrosine kinases, and epigenetic modifiers. Philadelphia chromosome-like ALL is essentially heterogeneous; however, deletion mutations in the IKZF1 gene encoding the transcription factor IKAROS underlie many cases as a key factor inducing aggressive phenotypes and poor treatment responses. WGS (whole genome sequencing) studies of ALL patients and ethnically matched controls also identified inherited genetic variations in lymphoid neoplasm-related genes, which are likely to increase ALL susceptibility. On the contrary, Genome editing technologies offers new opportunities for tackling diseases, and genome-editing tools such as CRISPR-Cas9 are an important means of advancing functional studies of ALL through the incorporation, elimination, and modification of somatic mutations. These findings are directly relevant to clinical hematology, and further studies on this aspect could contribute to accurate diagnosis, effective monitoring of residual disease, and patient-oriented therapies.

Keywords: Acute lymphoblastic leukemia; Genome editing; Drug targets; Chimeric antigen receptors

1. Introduction

Based on engineered or bacterial nucleases, the development of genome editing technologies has opened the possibility of directly targeting and modifying genomic sequences in almost all eukaryotic cells (Table 1). Genome editing has extended our ability to elucidate the contribution of genetics to disease by promoting the creation of more accurate cellular and animal models of pathological processes and has begun to show extraordinary potential in a variety of fields, ranging from basic research to applied biotechnology and biomedical research.

The development of new techniques of genome editing such as TALENs or CRISPR-Cas9 has made it possible to produce powerful animal genetic models that recapitulate the cooperating oncogenic lesions affecting genes with an established role in the proliferation and establishment of the leukemic clone [1]

However, genome editing techniques have gone a step further and they have been used with therapeutic and clinical approaches. Their use has facilitated the design of new therapies such as chimeric antigen receptors (CARs) and have allowed the study of genes involved in the evolution of pathogenesis [2,3].

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Table 1 Genome Editing

DNA repair Mechanisms	Applications
Non Homologous end joining	
DELETION	Frameshift
	Stop codons
INSERTION	Chromosomal rearrangement
	Others
Homologous recombination	
	Precise gene knock ins
	Point mutations
DONOR TEMPLATE	Tags
	Reporters
	Conditional alleles
	Gene correction
	Others

1.1. Genome editing and targeted in ALL

Next generation sequencing (NGS) techniques are used to interpret enormous amounts of data generated by WGS . These data are required to translate into functionally relevant data from which clinically relevant knowledge can be derived and the physicians can understand the influence of genotype on phenotype.

Genome editing system is based in the utilization of engineered nucleases composed of sequence-specific DNA-binding domains fused to a non-specific DNA cleavage module (4,5). These chimeric nucleases inducing DNA double-strand-breaks (DSBs) that stimulate the cellular DNA mechanisms, including error-prone non-homologous end joining (NHEJ) and homologous recombination (HR) (4). Several approaches have been used in the last few years as genome editing technologies (Figure. 1). The combination of simplicity and flexibility has hurtled zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and short palindromic repeats interspersed with regular intervals (CRISPR) to the forefront of genetic engineering (Figure. 1) (5,6).
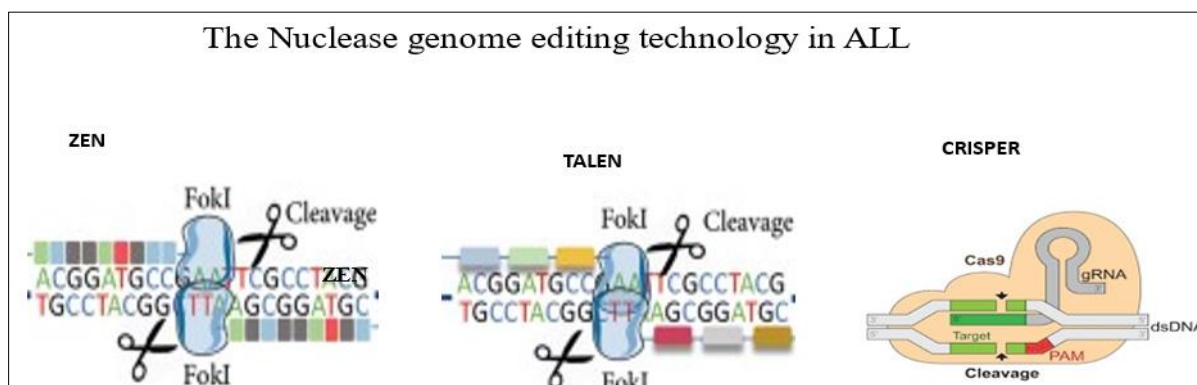

Figure 1 The nuclease genome editing technologies in ALL. The three types of nucleases are used: (1) programmable nucleases like Zinc Finger Nucleases (ZFNs), (2) transcription activator-like effector nucleases (TALENs) and (3) CRISPR systems (Clustered Regularly Interspaced Short Palindromic Repeats). These nucleases were capable of inducing double-strand breaks (DSBs) in the target followed by the activation of DNA repair mechanisms [2,3]. Induction of DSBs or nicks at targeted regions or repairing is done by either Non-homologous end joining (NHEJ) or Homologous recombination (HR) pathway. NHEJ is an error prone repair mechanism for joining of broken ends , which generally

results in heterogeneous indels (insertions and deletions) whereas HR is a precise repair method in which homologous donor template DNA is being used in repair DNA damage target site. HR is the ideal strategy for generating knock in models [5,6].

ZFNs and TALENs were first used to generate knock-out rats in 2009 and 2011, respectively [5, 6]. TALENs system was first used in human cells in the same year [7]. CRISPR-Cas9 system, discovered as part of the prokaryotic adaptive immune system at the end of 1980s [8], was introduced some years later. This was proposed as a genetic modification system in 2005 [9] but was not tested until 2012 [10].

CRISPR-Cas9 is presented as a faster, cheaper, simpler system with the potential for multiplex genome editing [11]. The method is based on generating a directed cut in the double strand of DNA by the Cas9 nuclease. This is driven by a single 20-nucleotide RNA strand, which marks the exact breakage point. After DNA cutting, the DNA repair machinery of the host cell leads to repair errors and thereby promote a modification of the original sequence by a mutation such as an insertion, deletion or inversion, among others [12]. Based on CRISPR-Cas9 system, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) emerged. CRISPRi uses a catalytically inactive version of Cas9 (dCas9) lacking endonucleolytic activity in combination with an sgRNA designed with a 20-bp complementary region to any gene of interest to silence a target gene [13], while CRISPRa uses fusions of dCas9 to activator domains to activate gene expression in Table 2. [11].

Table 2 Applications of genome editing systems in ALL

	Targeting gene	SSN technique	Modification type
Targeting Transcriptional factors	TAL1	CRISPR-Cas9	HR (Point mutation (insertion/deletion))
	PAX5	CRISPR-a	NA (Repress expression)
	NR3C1	CRISPR-Cas9	NHEJ (Knock out)
	TXNIP		
	CB2		
	LMO2	CRISPR-Cas9	NHEJ (Knock out)
Targeting gene fusion expression	MLL/AF4	TALEN	NHEJ (Chromosomal rearrangement)
	MLL/AF9		
	MLL/AF4 AF4/MLL	CRISPR-Cas9	HR (Knock in)
	MLL/ENL	CRISPR-Cas9	NHEJ (Chromosomal rearrangement)
	MLL/AF9	TALEN	NHEJ (Chromosomal rearrangement)
	AF9/MLL		
	ETV6/RUNX1	CRISPR-Cas9	HR (Knock in)

Note : This table shows the main genetic editing studies carried out in ALL, classified according to the target. The different columns indicate: the outcome of edition, the target of edition (highlighted in bold), the technique used, the type of modification, and the cell type.

Genome editing strategies have been used in several organisms, including *Drosophila* [12], *Caenorhabditis elegans* [13], zebrafish [14], mouse [15], rat [16], plants and humans [17-18] and has allowed a large number of functional studies to be carried out, based on the generation of animal and plant models. The use of genetically modified cell lines and animal models to understand the functions of genes and their pathogenesis in diseases conditioned by molecular genetics could be of help and provide insights to better understand cancer. The method used until now to generate these animal models, especially mice, is tedious and time-consuming, but CRISPR-Cas9 makes the procedure easier and more efficient [19].

Genome editing technologies, such as CRISPR-Cas9, have already been applied to the study of many diseases, including hematological diseases [20]. As exemplified by some very recent studies in Fanconi anemia (FA), a genetic DNA repair-deficient human disorder that results from mutations in FA genes [21] or the study of BCR-ABL oncogene in chronic

myeloid leukemia [22]. Specifically, most of the genetic modification studies in ALL have been with CRISPR-Cas9, more than 20 articles since 2015. The vast majority had the purpose of knocking out genes, either by introducing mutations, insertions, or deletions.

1.2. Targeting transcriptional factors

A common mechanism in the pathogenesis of human cancer, in leukemic cells, is the deregulation of Transcription factors (TFs). Genes encoding TFs are often amplified, deleted, rearranged via chromosomal translocation, or subjected to point mutations that result in a gain- or loss-of-function. .As a result , targeting of TFs can be highly effective in treating ALL. TFs such as *PAX5* and *IKZF1* were altered in nearly 80% of patients with B-ALL [23, 24]. These alterations affected glucose metabolism and energy supply, whereby the transcription factors act as metabolic repressors by limiting the amount of ATP available. A CRISPR-Cas9-based screen of *PAX5* and *IKZF1* transcriptional targets identified some target genes such as *NR3C1*, *TXNIP* and *CB2* as central effectors of B-lymphoid restriction of glucose and energy supply and therefore new targets for treating B-ALL [23].

In human T-cell ALL (T-ALL) cells, a CRISPR-Cas9 editing tool was used to disrupt *TAL1* (*SCL*) [4] or *TRIB1* (*TRB1*) [25] genes to delineate their biological functions. *TAL1* is one of the oncogenes most frequently deregulated in T-ALL [26]. This deregulation is produced by t (1;14) (p34;q11) (1–2%) or *SIL(STIL)-TAL1* deletions (del(1)(p32)) (15–20%), although there is still a large group of patients in whom the gene is deregulated but not altered. Epigenetics may therefore play an important role in these patients [27]. CRISPR-Cas9 was used in a cell line to reproduce two known alterations in *TAL1* (insertion and deletion) and it was observed how these alterations triggered their expression. Furthermore, a change in methylation or acetylation of H3K27 was observed, suggesting a causal relationship between mutagenesis, epigenetic modulation and expression of *TAL1* [28].

LMO2 is another gene deregulated in T-ALL. It is a potent oncogene that is essential for the formation of a large transcriptional complex in which genes such as *TAL1*, *LDB1*, *GATA1*, *GATA2*, *GATA3*, *RUNX1*, *ETS1*, and *MYB* intervene. Furthermore, its overexpression has been associated with the development of T-ALL. However, the reasons why this gene is overexpressed remain unclear because a few mutations have been described. Mutations targeted to the non-coding region of *LMO2* were introduced in a T-ALL cell line by CRISPR-Cas9 and proved to be a possible cause of the deregulation of *LMO2* expression [29].

1.3. Targeting gene fusion expression in ALL with chromosomal rearrangements

Chromosomal translocations are very frequent in ALL and can be used to separate different risks of ALL patients. The occurrence of *MLL* rearrangements is well known in a small percentage of B-ALL patients, but they are associated with very poor prognosis.

Several studies have proposed that *MLL* rearrangements are an initiating event in leukemic transformation, unlike ETV6-RUNX1 and BCR-ABL translocations, in which second events are necessary to initiate leukemia [19,20, 30, 31]. This was done by a homologous recombination knock-in approach by CRISPR-Cas9 to introduce the cDNA encoding of *RUNX1* exons 2–8 into the native *ETV6* locus of hIPSC . ETV6-RUNX1 expression induced a partial block of the maturation of B lymphocytes, when the second events required for leukemia development occurs [32].

MLL-AF4 and MLL-AF9 translocations were generated by genetic modification in primary hematopoietic stem and progenitor cells (HSPCs) to test the oncogenic potential as initiation event of the *MLL* translocations. Strategy was based on the double-stranded DNA breakage at specific positions of two chromosomes could lead to translocation [33].The authors used TALENs to generate cuts directed at specific positions of MLL-AF4 and AF9, based on the breakage points described in patients. In vitro, the cells that acquired the translocation showed a proliferative advantage over the others but were not able to transform completely because they eventually disappeared from the culture [34].

Shortly after, strong evidence for the formation of true t(11;19)/MLL-AF9 translocations in vitro and in vivo by CRISPR-Cas9, no full transformation was observed in liquid cultures or methylcellulose-based in vitro assays using CD34+ HSPC, while in vivo assays demonstrated that endogenous t (11;19) can initiate a monocytic leukemia-like phenotype. This study is in line with the previous study, which emphasizes the importance of environmental cues for the oncogenic transformation in *MLL*r leukemias [35].

Recently, scientists managed to generate t (9;11) chromosomal translocations encoding MLL-AF9 and reciprocal AF9-MLL fusion products in CD34+ human cord blood cells by TALENs. Transplantation of these cells into immune-compromised mice induced myeloid leukemias with absence of secondary lesions studied by targeted exome sequencing and RNA seq [33].

The prevailing theory is that *MLL* rearrangements occur in the uterus due to exposure to certain chemicals during pregnancy that cause errors in DNA repair, as has been demonstrated in vitro and in vivo [37]. *MLL*-AF4 protein and its reciprocal, AF4-MLL were induced in the AAVS1 locus of the HEK293 cell line by CRISPR-Cas9. They subsequently induced DNA damage by exposing the cells to etoposide and ionizing radiation (IR), with no differences in repair between WT cells and those expressing proteins. Thus, they demonstrated that the expression of the fusion proteins caused by *MLL* rearrangements, did not influence susceptibility to DNA damage or repair mechanisms [36].

1.3.1. IKZF1 alterations mediate therapy resistance

The presence of *IKZF1* gene lesions in *BCR-ABL1*-positive B-ALL results in inferior treatment outcome and mouse xenograft models suggest that IKZF1 loss contributes to resistance to tyrosine kinase inhibitor-based therapy(37,38). Reactivation of cell adhesion pathways by perturbation of IKZF1 function leads to elevation of key adhesion molecules, such as integrins (ITGA5) and CD90, and adhesion regulators, such as FAK, as well as increased phosphorylation of FAK itself, which permits relocalization of leukemic cells to the bone marrow niche. Indeed, FAK inhibition resensitizes *BCR-ABL1* leukemic cells to tyrosine kinase inhibitor therapy.[109] Similar results are observed after treatment with retinoids, specifically retinoid X receptor agonists, which induce expression of wild-type IKZF1, but not IK6, thereby abrogating expression of stem cell and adhesion molecules (38). Although these studies have provided important clues about how *IKZF1* deletions alter treatment response especially in the context of *BCR-ABL1*-positive ALL, alternative mechanisms of therapy resistance may exist besides protection through cell interactions within the bone marrow microenvironment.

Synthetic glucocorticoids, such as prednisolone, constitute essential drugs in the treatment of ALL patients and glucocorticoid resistance remains a substantial problem in the treatment of BCP-ALL. There is accumulating evidence that *IKZF1* deletions mediate prednisolone resistance *in vivo (39, 40)* but different mechanisms have been proposed. IKZF1 actively represses genes of the phosphatidylinositol-3 kinase pathway including PIK3CD and PIK3C2B (41).

Disruption of IKZF1 function, and subsequent activation of the PI3K/AKT/mTOR pathway can promote glucocorticoid resistance (42,43). IKZF1 controls expression of several genes involved in glucose and energy supply (44). This metabolic program may alter the threshold for responses to glucocorticoids in BCP-ALL. Specifically, the glucocorticoid receptor *NR3C1* was reported to be a target of IKZF1 in pre-B ALL cells, and downregulation of NR3C1 protein levels could be observed upon expression of IK6. However, studies performed in murine *Ikzf1+/−* B cells and human BCP-ALL cell lines with short hairpin-mediated IKZF1 knockdown have demonstrated that loss of IKZF1 function induces glucocorticoid resistance independently of altered NR3C1 mRNA and protein expression (40). Indeed, IKZF1 itself appears to regulate NR3C1-dependent gene transcription. The transcriptional regulator BTG1 has been identified as a modifier of IKZF1-mediated resistance to glucocorticoid therapy and the combined loss of BTG1 and IKZF1 leads to an even stronger inhibition of glucocorticoid-induced cell death (44). Finally, IKZF1 target gene *EMP1 (45)*, which itself represents a poor prognostic factor in pediatric ALL, was shown to regulate the response to prednisolone, but also, on the other hand, to affect normal leukemic cell viability and proliferation (46). Collectively, these findings demonstrate that IKZF1, through modulation of different signaling pathways and acting directly on glucocorticoid target genes, alters treatment response, thereby mediating therapy resistance in BCP-ALL (Table 3).

Table 3 IKZF1 gene deletion/ mutations

IKZF1 gene deletions/ mutations	IKZF1 (Loss of IKZF! Function)	TK resistance	Cell Adhesion
			Metabolic Pathways
			EMP 1
		Glucocorticoid resistance	Glucocorticoid receptor target gene regulation

Note:. *IKZF1* alterations mediate therapy resistance. IKZF1-affected pathways contributing to tyrosine kinase (TKI) inhibitor resistance and glucocorticoid (GC) resistance are shown. Enhanced cell adhesion due to loss of IKZF1 function has been shown to contribute to both TKI and GC resistance. Deregulation of metabolic pathways, such as LKB1/AMPK signalling and glucose metabolism, attenuated glucocorticoid receptor (GR) target gene regulation and upregulation of epithelial membrane protein EMP1 have been implicated in mediating GC resistance of *IKZF1*-deleted BCP-ALL. Green boxes indicate activated targets or pathways, while yellow boxes define attenuated pathways. Targets within the metabolic pathway can either promote or inhibit GC resistance.

1.4. BCR-ABL1–like ALL

To identify the genetic basis of non–CRLF2-rearranged *BCR-ABL1*–like ALL, the COG, as part of the US National Cancer Institute Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative performed mRNA-seq and whole-genome sequencing (WGS) of diagnostic and matched remission DNA and/or RNA for 15 BCR-ABL1–like cases. This identified a range of novel rearrangements, copy number alterations, and sequence mutations activating kinase signaling, including rearrangements of *PDGFRB*, *ABL1*, *JAK2*, and *EPOR*, as well as deletion/mutation of *SH2B3* and the *IL7R*.(47) Several of these alterations confer growth factor independence in murine Ba/F3 and Arf$^{-/-}$ cell lines that is attenuated with currently available TKIs such as the ABL1/PDGFRB inhibitors imatinib, dasatinib, and dovitinib and the JAK2 inhibitor ruxolitinib. Moreover, primary leukemic cells from patients with *BCR-ABL1*–like ALL exhibit activation of these signaling pathways on phosphoflow cytometric signaling analysis, and xenograft studies have shown that the engrafted tumors are sensitive to appropriately targeted TKI therapy (48) These exciting genomic and preclinical findings strongly suggest that patients with BCR-10BL1–like ALL, many of whom are at high risk of relapse and fail current maximal therapy, may be successfully treated with TKIs. Furthermore, these data indicate that concomitant lesions that disrupt hematopoietic development and drive proliferation are common in ALL, as proposed previously for AML (33-40,48).

1.5. CRLF2 rearrangements and JAK mutations in ALL

CRLF2 rearrangements, identified by FISH and SNP array profiling, is seen in approximately 7% of childhood ALL cases and 50% of cases associated with Down syndrome (DS-ALL).[13,14] *CRLF2* is located in the pseudo autosomal region (PAR1) at Xp22.3/Yp11.3 and encodes cytokine receptor–like factor 2 (also known as the thymic stromal lymphopoietin receptor). With IL7 receptor alpha (IL7Rα), CRLF2 forms a heterodimeric receptor for thymic stromal lymphopoietin. CRLF2 is rearranged by translocation into the immunoglobulin heavy chain locus (*IGH@-CRLF2*) or, more commonly, by a focal deletion upstream of *CRLF2* that results in expression of *P2RY8-CRLF2* that encodes full-length CRLF2. Both rearrangements result in aberrant overexpression of CRLF2 on the cell surface of leukemic lymphoblasts that may be detected by immunophenotyping. Less commonly, a p.Phe232Cys CRLF2 mutation results in receptor overexpression.

CRLF2 alterations are associated with the presence of activating mutations in the JAK genes *JAK1* and *JAK2*,[49-51] which, except for T-lineage ALL, are otherwise uncommon in ALL. The JAK family comprises 4 members, JAK1, JAK2, JAK3, and TYK2. The JAK/STAT pathway mediates signaling from cytokine, chemokine, and growth factor receptors via the JAK nonreceptor tyrosine kinases and the STAT family of transcription factors. The JAK mutations are most commonly missense mutations at R683 in the pseudo- kinase domain of JAK2 and are distinct from the JAK2 V617F mutations that are a hallmark of myeloproliferative diseases. Less common are activating mutations in the kinase domain of JAK1 and JAK2. Like the JAK2 V617F mutation, the JAK1/2 mutant alleles observed in ALL are transforming in vitro.(52,53). Activating JAK1/2 mutations are in 50% of *CRLF2*-rearranged cases, whereas concomitant rearrangements of *CRLF2* are almost all cases of B-ALL with JAK1/2 mutations. Transformation can be seen by coextrusion of CRLF2 and JAK1/2 mutations in vitro, which suggests that these 2 lesions are main in lymphoid transformation. Half of *CRLF2*-rearranged cases lack a JAK mutation and the nature of alternative kinase signaling mutations in these cases is unknown. In non–DS-ALL, *CRLF2* alterations and JAK mutations are associated with *IKZF1* deletion/mutation, a gene-expression profile similar to BCR-ABL1$^+$ ALL (54). The associations between CRLF2 and outcome have varied between studies and cohorts in part due to differences in the cohorts studied.[55-57] The association is most consistent in non–DS-ALL cohorts of high-risk B-progenitor ALL. However, it should be emphasized that few studies have performed comprehensive analysis of *CRLF2* rearrangement and expression, *JAK1* and *JAK2* mutational screening (including both pseudo kinase and kinase domain mutations), and detection of *IKZF1* deletions and sequence mutations.

Studies performed by the Children's Oncology Group (COG) have shown that *CRLF2* and *IKZF1* alterations are associated with inferior outcome in multiple cohorts and that elevated *CRLF2* expression in the absence of rearrangement is also an adverse prognostic feature [58].

1.6. Submicroscopic genetic alterations in ALL

The nature and frequency of genetic lesions is subtype dependent. *MLL*-rearranged leukemias bear very few additional structural or sequence alterations (27,28). In contrast, *ETV6-RUNX1* and *BCR-ABL1* ALL harbor more alterations. Emerging experimental data have shown that several of these alterations cooperate in leukemogenesis. Deletion of *Pax5* and *Ikzf1* accelerates the onset of leukemia in retroviral BM transplantation, transgenic models of *BCR-ABL1* ALL, and chemical and retroviral models of leukemia. (6)

Although many of these alterations are enriched in specific cytogenetic ALL subtypes, a notable exception is alteration of the ETS-family transcription factor *ERG* (ETS-related gene), which occurs exclusively in patients lacking known chromosomal rearrangements and is a hallmark of a novel subtype of B-ALL with a distinct gene-expression profile and generally favorable outcome. The *ERG* deletions involve an internal subset of exons resulting in loss of the central inhibitory and pointed domains and expression of an aberrant C-terminal ERG fragment that retains the ETS and transactivation domains and functions as a competitive inhibitor of wild-type ERG (10).

1.7. Intrachromosomal amplification of chromosome 21

Intrachromosomal amplification of chromosome 21 (iAMP21) occurs in up to 2% of B-progenitor ALL patients, and in the United Kingdom childhood ALL trials have been associated with older age and poor outcome. (55). AMP 21 is defined by gain of at least 3 copies of the region of chromosome 21 containing RUNX1. The amplification is often large and complex and accompanied by deletion of the sub telomeric regions of chromosome 21. The basis of generation of iAMP21 and the mechanistic contribution of this abnormality to leukemogenesis are unclear, but identification may be performed to identify cases of ALL at increased risk of relapse.

1.8. Hypodiploid ALL

Hypodiploidy with less than 45 chromosomes is associated with a very high risk of treatment failure, the genetic basis of which has been poorly understood.(58,59) Hypodiploid ALL may subclassified by degree of aneuploidy into near haploid (NH-ALL, 24-31 chromosomes) and low hypodiploid (LH-ALL, 32-44 chromosomes) cases. In conjunction with the COG, we performed a detailed genomic analysis of more than 120 hypodiploid ALL cases, including WGS or exome sequencing of more than 40 cases.(59) This sequencing showed that NH-ALL has a very high frequency of deletions and sequence mutations that activate Ras signaling, including recurring novel alterations of *NF1*, and that NH- and LH-ALL have a high frequency of inactivating alterations of the IKAROS genes *IKZF2* (HELIOS) and *IKZF3* (AIOLOS) that are otherwise rare in ALL. With transcriptional profiling, these results indicate that NH- and LH-ALL are distinct diseases. Moreover, scientists demonstrated Ras pathway activation by biochemical and phosphosignaling analysis, suggesting that therapeutic targeting of this pathway may represent an important novel treatment outcome in this high-risk leukemia (59)

1.9. Sequence mutations in ALL

Candidate gene-sequencing studies, in which genes have been selected for sequencing based on a prior knowledge of the role of a gene product in lymphoid development, tumorigenesis, or the knowledge that a gene is involved by structural genetic alterations, have identified multiple targets of mutation in ALL (e.g., *PAX5*, *BCL11B*, *FBXW7*, *IKZF1*, *LEF1*, *WT1*, *PTEN1*, and *NF1*). In general, these studies have shown that DNA copy number alterations are more common than sequence mutations in ALL. However, detailed analysis of sequence mutations in large cohorts of ALL has been costly.

Characterizations of sequence variation in ALL and further exploration the genetic basis of relapse, investigators performed Sanger sequencing of 300 genes from 24 B- and T-lineage ALL patients at diagnosis, relapse, and remission (60). The frequency of sequence mutation was low (0-5 per case), as observed for DNA-copy number alterations ; many harmful mutations present at diagnosis were no longer evident at relapse, including mutations in the Ras signaling pathway (e.g., *NRAS*, *KRAS*, *PTPN11*, and *NF1*) and B-cell development (e.g., *PAX5*, but not *IKZF1*, deletions/mutations of which were always preserved at relapse or acquired as new lesions). Deletion or mutation of *CREBBP*, encoding the transcriptional coactivators and acetyltransferase CREB binding protein (also known as CBP), were present in almost 20% of relapsed ALL cases and appeared to be particularly enriched in relapsed hyperdiploid ALL, a subtype normally associated with favorable outcome (61). The mutations identified are enriched in the histone acetyltransferase (HAT) domain and attenuated the normal HAT activity of murine CREBBP. Moreover, CREBBP mediates the transcriptional response to glucocorticoid therapy and the mutations were shown to disrupt the normal transcriptional response to glucocorticoids. Therefore, *CREBBP* mutations may represent an important mechanism underlying treatment failure in ALL and may be targeted with agents that modulate the level of histone acetylation in leukemic cells, such as histone deacetylase inhibitors. Recent studies have also shown that mutations in *TP53* (p53) are also enriched at relapse and associated with poor outcome (62).

1.10. Drug targets discovery and therapy

The targets against which a drug acts must be identified and combined with the data provided by the NGS. This may sometimes identify patients with mutations in genes associated with some type of resistance. It can also help to generate other new drugs, when there is prior knowledge of the altered pathway we wish to attack (Table 4). For example, ibrutinib has recently been proposed for the treatment of pre-BCR and *TCF3*-r-positive cases. Ibrutinib is an inhibitor

kinase targeted to those ALL subtypes with affected BCR signaling. In order to understand the mechanism of action of ibrutinib in this ALL subtype, Bruton tyrosine kinase (*BTK*) KO, B lymphocyte kinase (*BLK*) KO and *BTK / BLK* KO cells have been generated by CRISPR-Cas9 [8].The importance of *BTK* in the pathogenesis of chronic lymphocytic leukemia, diffuse large B-cell lymphoma, and other mature B-cell malignancies is well established [57,58,59], while there is less information about the role of *BTK* in ALL. Early studies reported unaltered levels of *BTK* in childhood ALL cells, whereas frequent *BTK* deficiency due to aberrant splicing was reported later [61, ̶1̶62]. *BLK* and *BTK* were the only kinase genes overexpressed in this subtype of ALL, as revealed by arrays [62]. Only the elimination of the expression of both kinases managed to reduce the proliferation in a similar way to ibrutinib. However, these should not be the only targets of ibrutinib since the decrease in proliferation was still greater when the drug was used. To confirm that *BTK* and *BLK* were drug targets, ibrutinib was tested in cell lines generated with KO genes. This indicated that ibrutinib requires the presence of both kinases for maximum effectiveness (63-64).

Table 4 Drug target discovery and therapy

	Targeting gene	SSN Technique	Modification type
Drug targets discovery and therapy	*CB1*	CRISPR-Cas9	NHEJ Knock out
	CB2		
	BTK	CRISPR-Cas9	NHEJ (Knock out)
	BLK		
	XPO1	CRISPR-Cas9	HR (Knock in)
	ABCB1	CRISPR-Cas9	NHEJ (Knock out)
Modification of CARs	*CD19*	CRISPR-Cas9	NHEJ (Knock out)
	CD19	CRISPR-Cas9	NHEJ (Knock out)
	PAX5		
	EBF1		
	TRAC	CRISPR-Cas9	HR (knock out)
	CD19		
	CD52	TALEN	NHEJ (knock out)
	TCR ab		
	TCR b	CRISPR-Cas9	NHEJ (knock out)
	CD7	CRISPR-Cas9	NHEJ (knock ~~outy) }~~ }

In a subsequent study, Thomas Vercruysse and coworkers focused on exportin 1 (*XPO1*). *XPO1* plays an important role in the transport through the nucleus of cycle regulatory proteins and tumor suppressor proteins, among others. The overexpression of this gene is associated with several types of cancer, and with poor patient outcome [65, 66,67]. *XPO1* inhibitors act by binding to the reactive cysteine residue located at position 528, preventing the export of charged proteins to the cytoplasm [65,66]. To verify that the drug binds specifically to act against *XPO1,* a point mutation was inserted at residue 528 by CRISPR-Cas9. When this occurred, the drug was not able to act, and the cells became resistant. Therefore, this study demonstrated that the drug is highly specific to *XPO1* and is potent against ALL [67].

More recently, Dronabinol (Tetrahydrocannabinol, THC), a US Food and Drug Administration-approved cannabinoid receptor (CNB) agonist for the treatment of chemotherapy-induced nausea and vomiting, was found to induce apoptosis in acute leukemia cells, as evidenced by the abrogation of pro-apoptotic effects of CRISPR-mediated knockout of *CB1* or *CB2* following THC treatment [7, 68].

Furthermore, new drugs are being proposed as an alternative to current therapy. An example is Carfilzomib (CFZ), as a substitute of proteasome inhibitor Bortezomib (BTZ), who demonstrated favorable clinical outcomes for refractory

childhood ALL. CFZ showed significantly higher activity than BTZ in vitro, except for the P-glycoprotein-positive t (17;19) ALL cell lines. Takahashi et al. generated a knock-out of *ABCB1*, who codes for P-glycoprotein, by genome editing with a CRISPR-Cas9 system and sensitized P-glycoprotein-positive t (17;19) ALL cell line to CFZ [69].

1.11. Modification of CAR

Chemotherapy and/or radiotherapy have been standard treatments for ALL to date. However, immunological therapies have gained importance. These work by harnessing the immune system of patients to fight the disease. One example is chimeric antigen receptors (CARs), which are proteins genetically engineered to allow T cells to recognize a specific antigen in tumor cells. It had already been proposed as a standard therapy for ALL patients in 2013 by Rosenberg. In this case, the CARs were directed against CD19, an antigen of B cells [130] Its efficacy had already been demonstrated in cases of refractory or relapsed ALL [70.71, 72].

CRISPR-Cas9 may be key to carry out this genetic modification through the combination of knock-out and knock-in (72). On the one hand, these investigators interrupted the TRAC locus, and added a CAR directed to CD19, inserting it in the AAVS1 locus. They compared responses to CD19 antigens from these cells with those from others in which CAR had been randomly integrated. This helps to demonstrate that targeted CAR integration under the control of endogenous regulatory elements is much more effective, reduces tonic signaling, avoids the differentiation and accelerated depletion of T cells, and increases the therapeutic potential of these cells [73].

The use of TALEN-modified T lymphocytes in two infants with refractory B-ALL was shown by another group of investigators (74). They generated universal T-cells against CD19 (CAR19), targeting the TALENs against the T-cell receptor (TCR) and simultaneously transfecting with non-human leukocyte donor cell antigens. As treated cells, they disrupted the CD52 gene, the target of the drug alemtuzumab, by TALEN, and disrupted the expression of the αβ T cell surface receptor (TCR αβ), which kept down the risk of graft-versus-host disease (GVHD). The newborns were treated with lymphoplasty, chemotherapy and anti-CD52 serotherapy before infusion of CAR19. The results were very positive, yielding remissions within 28 days before allogeneic stem cell transplantation [73].,

Despite the good results with CAR19 therapy, 10–20% of treated patients suffer relapses due to partial loss of the CD19 epitope [71, 74]. Andrei Thomas-Tikhonenko and his group have provided evidence that epitope loss is closely linked to alterations in exon 2 of CD19, detected in some samples from patients with relapses. These alterations include frameshift-type mutations and the total loss of the exon, resulting from an alternative splicing event that encodes a deficient isoform of exon 2. To assess the relevance of the detected isoforms, they eliminated CD19 expression by CRISPR-Cas9 from ALL cell lines, and then reconstituted them with different isoforms. They observed that the depleted isoform of exon 2 was located mostly in the cytosol, which could explain its mechanism of escape in front of CAR19. Thus, these deleterious mutations and the selection of isoforms resulting from alternative splicing could be the cause of this mechanism of resistance [75]. essential for 9-O-acetylation [75] .

Second mitochondrial-derived caspase-activators (SMACs) act by inhibiting inhibitors of apoptosis proteins (IAPs). One of the possible causes of resistance is revealed by the action of these proteins, which act to counteract the effects of drugs. These are also overexpressed in many types of cancer [76,77]. The main mechanism of action of IAPs is the inhibition of apoptosis through proteins such as caspases [78] or receptor interaction of protein kinase 1 (*RIP1*), a potent activator of death [79]. In this study, they set out to demonstrate that SMAC acted by reactivating apoptosis of these cells, mediated by *RIP1.* They used CRISPR-Cas9 system to knock out this gene in vivo in xenograft models, and thereby eliminate its expression. The results showed that *RIP1* was necessary for the induction of cell death by SMAC [79].

CXCR4 encodes a membrane receptor whose function is to attract and confine the stromal cells of the bone marrow stromal cells (BMSCs). This interaction with BMSCs gives B cells a degree of protection, associated with increased survival, resistance to treatment, relapse and worse prognosis [78]. *CXCR4* is highly expressed in B-ALL cells and has also been correlated with poor patient outcome 79] . Inhibitors of *CXCR4* have already been examined in the preclinical setting, in vitro and in vivo [79, 80] and may be *CXCR4* antagonists or agonists. To test whether the efficacy of these compounds was due to the inhibition of *CXCR4* and not to their own activity as agonists, they generated a B-ALL cell line with *CXCR4* knock-out by CRISPR-Cas9. They demonstrated that the agonistic activity of *CXCR4* antagonists did not affect antitumor activity. In addition, in vivo *CXCR4* knock-out models reduced the burden of leukemia and disease progression. In this way, the importance of *CXCR4* in the pathogenesis of B-ALL and in its use as a therapeutic target to fight drug resistance is demonstrated 10]

2. Evolution of pathogenesis

Although there have been great advances in the treatment and cure of ALL, there is still a large group of patients who experience relapses, persistent minimal residual disease, and drug resistance, and who ultimately have a poor prognosis [81, 82]. Efforts have therefore focused on trying to understand why these resistances occur, to counteract them, and to look for new, more personalized drugs that avoid resistance (Table 5).

In ALL, survival and drug resistance of lymphoblasts critically depend on 9-O-acetylation of sialic acids (Sais) [83, 84]. Baumann AM et al., generated a *CASD1* knock-out cells by CRISPR-Cas9-mediated genome editing and demonstrated that *CASD1* is essential for 9-O-acetylation [84].

Table 5 Evolution of pathogenesis

	Targeting gene	SSN Technique	Modification type
Evolution of pathogenesis	CASD1	CRISPR-Cas9	NHEJ Knock out
	RIP1	CRISPR-Cas9	NHEJ Knock out
	CXCR4	CRISPR-Cas9	NHEJ (Knock out)
Others	FLT3	TALEN	NHEJ (Knock out)
	NUDT15	CRISPR-Cas9	NHEJ (Knock out)
	DCK	CRISPR-Cas9	NHEJ Knock out)
	PTCH1	CRISPR-Cas9	NHEJ (Knock out)

The St Jude Children's Research Hospital–Washington University Pediatric Cancer Genome Project performed WGS of tumor and matched nontumor DNA from 12 children with ETP-ALL and examined the frequency of novel and recurring genetic alterations in an additional 54 ETP- and 42 non–ETP-ALL patients.(85,86). This showed a marked diversity in the nature and frequency of somatic sequence and structural alterations and showed that 3 pathways are commonly mutated in ETP-ALL:

- Hematopoietic development,
- Ras and/or cytokine receptor/jak-stat signaling, and
- Histone modification.

Several other studies have also identified mutations in several of these genes and pathways using non-WGS approaches.(86). Fifty-eight percent of ETP cases (compared with 17% of non-ETP T-ALL cases) had loss-of-function or dominant-negative mutations in developmental genes, including *RUNX1*, *IKZF1*, *ETV6*, *GATA3*, and *EP300*. Several of these genes are known targets of mutation in hematopoietic malignancies (e.g., *RUNX1* in myeloid disorders and *ETV6* and *IKZF1* in B-progenitor ALL) but have not previously been implicated in T-ALL. Activating signaling mutations were identified in 67% of cases (compared with 19% of non-ETP T-ALL cases), including mutations in *NRAS*, *KRAS*, *JAK1*, *NF1*, and *PTPN11* and novel mutations in *JAK3*, *SH2B3* (encoding LNK, a negative regulator of JAK2 signaling), and *IL7R*.

Other studies have now identified mutations in *IL7R* in both T-lineage and B-progenitor ALL.(87,88). The IL7R mutations are in the transmembrane domain and commonly introduce a cysteine that results in receptor dimerization and constitutive JAK-STAT that is abrogated by pharmacologic JAK inhibitors such as ruxolitinib.

 A high frequency of mutations in epigenetic regulators was seen in ETP-ALL, the most frequent of which were components of the polycomb repressor complex 2 (PRC2), a H3K27 trimethylase that normally induces transcriptional repression and antagonizes the transcriptional activating effects of MLL. The most mutated gene was *EZH2*, which encodes the catalytic component of the complex. *EZH2* is also mutated in follicular lymphoma, but, in contrast to the highly recurrent Y641 mutations observed in FL that are gain of function (87,88), the mutations in T-ALL occur in other sites in *EZH2* and are predicted to disrupt the catalytic SET domain and result in loss of function.

These pathways—hematopoietic development, signaling, and epigenetic regulation—are also frequently mutated in AML. In addition, the transcriptional profile of ETP-ALL is highly similar to that of normal hematopoietic stem cells, and that of high-risk AML but *not* the normal human ETP. This suggests that ETP-ALL may represent a stem cell or progenitor leukemia. Recent data on bi- phenotypic leukemias have identified similar mutations in a small number of cases (88). This indicates· that the entity of ETP-ALL may extend beyond leukemias nominally of T-cell (e.g., cCD3$^+$) lineage and suggest that non-ALL regimens, whether myeloid directed, targeted, or epigenetic therapies, should be pursued in this disease.

Abbreviations

- **ALL: Acute lymphoblastic leukemia**
- AML: Acute myeloid leukemia
- BCR: B-cell receptor signaling.
- BMSCs: Bone marrow stromal cells
- CARs: Chimeric antigen receptors
- CRISPR: Short palindromic repeats interspersed with regular intervals
- DSBs: Double-strand breaks
- HiPSC: Human induced pluripotent stem cell
- HR: Homologous recombination
- HSPCs: Hematopoietic stem and progenitor cells
- IAPs: Inhibitors of apoptosis proteins
- IR: Ionizing radiation
- MMEJ: Micro-homology-mediated end-joining
- NGS: Next generation sequencing
- NHEJ: Non-homologous end-joining
- PBMC: Peripheral blood mononuclear cell
- Sias: Sialic acids
- SMACs: Second mitochondrial-derived caspase-activators
- TALENs: Transcription-activating type nucleases
- TCR: Transcription-activating type nucleases
- THC: Tetrahydrocannabinol
- ZFNs: Zinc finger nucleases

3. Conclusion

Genome editing technologies offers new opportunities for tackling diseases such as acute lymphoblastic leukemia (ALL) that have been beyond the reach of previous therapies.

Recent availability of genome-editing tools such as CRISPR-Cas9 are an important means of advancing functional studies of ALL through the incorporation, elimination and modification of somatic mutations and fusion genes in cell lines and mouse models. These tools not only broaden the understanding of the involvement of various genetic alterations in the pathogenesis of the disease but also identify new therapeutic targets for future clinical trials.

Furthermore, in the clinic, genome editing systems could facilitate the rapid screening of new drugs and will promote the development of personalized medicine, connecting genomics, disease phenotypes and therapeutic goals. The use of these technologies will broaden our understanding of the mechanism of action of these novel drugs and enable the identification of novel mechanisms of acquired resistance to pathway target therapeutics. However, translating genome editing technologies to the clinical setting requires two main concerns to be addressed: the safety and efficacy of treatments. The off-target effect remains one of the major obstacles of this technology. Research will need to improve our genetic tools to eliminate any off-target effects and to improve gene edition efficiency in the future. Despite this, genome editing offers new opportunities for tackling diseases such as ALL that have been beyond the reach of previous therapies. We identified at least one potential driver mutation in nearly all AML samples and found that a complex interplay of genetic events contributes to AML pathogenesis in individual patients. The databases from this study are widely available to serve as a foundation for further investigations of AML pathogenesis, classification, and risk stratification.

Cancer genome sequencing is still largely restricted to large academic sequencing centers, although a wide applicability is seen in this technology. All these tools will need to be refined and deployed in formats that can be used outside of

these specialized centers. Clinicians particularly pathologists, will need to be retrained to address the interpretation of analyzed genomic data from sequencing-based assays. Regardless, the potential power of these techniques to transform the clinical approach to AML is considerable, and hopefully, the techniques may become routine practice within a few years.

Compliance with ethical standards

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