

Nuevos retos en la estudio de las resistencias en pacientes con LMC

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Introduction

The fusion gene *BCR::ABL1*, resulting in the Philadelphia chromosome, is detected in all patients with chronic myeloid leukemia (CML). This genetic alteration leads to the constitutive activation of the tyrosine kinase, resulting in the clonal expansion of differentiated cells of the myeloid lineage. Imatinib was the first rationally designed inhibitor of the *BCR::ABL1* oncoprotein, competing for the ATP binding site, and selectively inhibiting ABL1's tyrosine kinase activity. Besides imatinib, there is currently a range of TKIs available for the treatment of chronic phase CML (CML-CP), including three second-generation TKIs (dasatinib, nilotinib, and bosutinib), and one third-generation TKI (ponatinib).

TKI treatment-the current panorama

Targeted TKI therapy has revolutionized the treatment of CML patients, significantly improving long-term outcomes. However, the development of mutations in the kinase domain can confer resistance to TKIs, affecting up to 10%-15% of patients receiving imatinib and less than 10% of patients receiving a second-generation TKI as first-line treatment, according to the European LeukemiaNet (ELN)¹.

A sixth TKI, asciminib, has recently received approval for patients who have received at least 2 prior TKI lines. Asciminib is an allosteric STAMP inhibitor that differs from previous TKIs due to its distinct mechanism of action. This inhibitor does not target the ATP

binding pocket but instead targets the vacant pocket commonly occupied by the N-terminal myristoyl group of ABL1, a 14-carbon saturated fatty acid that modifies the glycine residue at position 2. This results in the selective inhibition of ABL1's kinase activity by inducing a change in the protein's conformation to an inactive "off" state.

Consistent with its unique mechanism of action, asciminib has a distinct resistance profile compared to other TKIs. This means that mutations located in the ATP binding site that confer resistance to other TKIs, including the T315I mutation, theoretically will not affect asciminib. Therefore, this drug has generated a lot of excitement because it can provide another treatment option for multi-resistant patients. However, results from the Phase I clinical trial of patients resistant/refractory to ≥ 2 TKIs reported that patients treated with asciminib developed *ABL1* KD variants that specifically affect the myristoyl pocket, so not in the sites usually detected for TKI resistance, such as P223S, G463S, P465S, and V468F but also more common variants including A337V/T and F359V/I/C mutations². Specifically, mutations were identified in two out of 20 patients showing progression (10%) and two out of 66 patients at the 12-month mark without progression (3%). More patients are beginning to receive treatment with asciminib so the laboratory needs to be aware of and our next-generation sequencing (NGS) protocols must be optimized to detect the new spectrum of KD mutations that can confer reduced sensitivity to asciminib.

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The importance of compound mutations

The detection of KD mutations is very important since they are detected in 70%-80% of patients in the blast phase (CML-BP). Moreover, we observe a higher frequency of compound mutations (more than one mutation found in the same *BCR::ABL1* allele) in patients who progress to BP. The detection of compound mutations is important because cells that express them require a higher dose for TKI inhibition compared to cells with single mutations. Cells harboring some compound mutations may exhibit resistance to ponatinib and asciminib, limiting treatment options.

With conventional NGS, the ability to detect compound mutations depends on several factors. These include the distance between the two variants and, crucially, the length of the amplicon being sequenced. Meanwhile, the quantification of compound mutations requires the use of unique molecular identifiers. Not all sequencing companies offer this technology, and it can increase the cost of the NGS analysis. For this reason, third-generation sequencing techniques, such as Oxford Nanopore and Pacific Biosciences SMRT sequencing, hold promise due to their long-reading capability.

New long-read sequencing technologies

PacBio SMRT sequencing uses dsDNA to prepare libraries by ligation to adapters, creating a circular template. The circularized DNA library is then immobilized in a well on a flow cell and the polymerase incorporates nucleotides in a continuous long read mode, which can easily read 20,000 bases, and up to 50 kb, which is much more than the 75 or 150bp we are used to with most current sequencing technologies. Oxford Nanopore sequencing devices use flow cells which contain an array of tiny holes or nanopores. Each nanopore is connected to a channel and a sensor to measure the electrical current that flows through the nanopore. When a DNA or RNA molecule passes through a nanopore, the current is disrupted, and this alteration in the current can be decoded to determine the nucleotide in the DNA sequence in real time.

In CML, these new long-read sequencing techniques are very promising. As sequencing is done at the single-molecule level, they can provide information about the clonal composition of co-occurring mutations and so can be particularly useful for detecting compound mutations³. These technologies require no PCR amplification steps before sequencing, so fewer errors are

introduced during library preparation. Long reads are also better at identifying structural variants such as insertions, deletions, and complex rearrangements that are often missed by short-read sequencing. Hence, they are also useful for characterizing breakpoints of atypical variants, essential to design primers for the monitoring of “individual molecular response” during follow-up by qPCR or digital PCR⁴.

Finally, as the bases are called in real-time, results can be reviewed immediately, with one group able to confidently identify the *BCR::ABL1* transcript type within 15 min of sequencing. The major disadvantage of long-read sequencing techniques is the need for bioinformatic support, which will be the main caveat to the introduction of this technology in routine clinical practice. Nevertheless, open source software is being developed and many scripts are freely available online.

Methylation in CML

Cytosines within CpG sites are the main targets of methylation. CpG sites are spread throughout the genome, although most regions are found in gene promoters. In general, this methylation results in transcriptional silencing due to a closed chromatin configuration. Both Pacific SMRT and Oxford Nanopore approaches can be used to detect 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) base modifications. One study employed long-read sequencing, with a slight modification to select for regions of interest (Nanopore Cas-9 targeted sequencing, nCATS), in this case, *BCR::ABL1* transcripts, and demonstrated that it was possible to simultaneously detect KD mutations and the methylation status of the BCR promoter with this technique, both associated with imatinib response⁵.

Indeed, progression from CP to BP is associated with epigenetic changes including aberrant methylation of the *ABL1* promoter as well as other regions. Specifically, patients in CP generally lack methylation of the *ABL1* promoter a (Pa), which is retained in the *BCR::ABL1* fusion gene, whereas samples from BP patients were shown to be methylated at this region⁶. In a study by Heller and colleagues, 897 hypermethylated genes were identified, including common tumor suppressor genes, such as *CDKN2B*, with hypermethylation present at BC but not at diagnosis⁷. Importantly, the expression of these genes could be rescued by the use of epigenetic drugs such as azacytidine⁷.

BCR::ABL1-independent resistance mechanisms

The constitutive activation of *BCR::ABL1* induces genomic instability, ultimately leading to the acquisition of additional genetic aberrations, such as those previously described. Mechanisms of genomic instability include the induction of reactive oxygen species (ROS), whose activity results in oxidative DNA damage in the form of double-strand DNA breaks⁸. Moreover, DNA damage surveillance is affected in CML, further intensifying the mutagenic effect of the *BCR::ABL1* oncoprotein, and resulting in the accumulation of somatic mutations and chromosomal alteration⁸.

Although the *BCR::ABL1* gene fusion is characteristic of the disease, CML is often falsely regarded as having a unique initiating molecular event. However, molecular studies have revealed a wide genetic heterogeneity in CML patients that could explain at least some of the variation observed in terms of the clinical outcomes to TKI treatment. Patients may harbor atypical transcripts of *BCR::ABL1*, with uncommon breakpoints detected in < 5% of patients, some of which may be “cryptic,” in other words not visible in the karyotype (with a sensitivity > 5 Mb). Often, atypical translocations may give a negative result in FISH and PCR analysis and can make molecular monitoring by qPCR challenging.

KD mutations are the most common and best-known cause of TKI resistance, but other *BCR::ABL1* independent pathways of mechanism also exist. For example, according to the 2024 NCCN clinical practice guidelines, NGS with a myeloid mutation panel should be considered for patients with who lose a major molecular response but have no identifiable KD mutations due to the possible association between somatic mutations affecting cancer-associated genes, such as *ASXL1*, *RUNX1*, and *IKZF1*, and TKI resistance and/or disease progression⁹. Indeed, patients with a poor outcome were found to have more somatic mutations at diagnosis compared to optimal responders (33% vs. 11%), and patients who acquired mutations during TKI treatment were significantly more likely to fail TKI treatment¹⁰.

Moreover, 5% of CP patients may also present with additional chromosomal alterations (ACA) at diagnosis. Detection of ACAs is important since some are considered to be high risk, that is, are associated with a poorer response to TKI treatment, a higher risk of progression, and worse overall survival¹¹. High-risk ACAs include +8, a second Ph-chromosome, i(17q), +19, -7/7q-, alterations at 11q23 or 3q26.2, and a complex

karyotype. Importantly, ACA is detected in 45% of patients with TKI resistance, increasing to 60%-80% in CML-BP, with their appearance in follow-up considered as “failure” to TKI, being indicative of clonal evolution and disease progression. In particular, monosomy 7 (del 7q) is associated with progression to myelodysplastic neoplasm and acute myeloid leukemia. A new technology known as “optical genome mapping”, with over 10,000 times higher resolution than cytobanding, may help detect novel ACAs in CML patients associated with TKI resistance or progression to BP. The identification of high-risk patients at diagnosis could warrant the use of more aggressive first-line treatment, such as allogeneic hematopoietic stem cell transplantation or entry into a clinical trial¹².

In addition, the cumulative negative impact of *ASXL1* and *BCOR* mutations with certain ACA (complex karyotype, +21, i(17q)) on overall survival should be considered, according to the prognostic stratification model developed by Ochi and colleagues¹³. Patients who progressed to BP had a significantly higher frequency of cancer-related somatic variants at diagnosis (54% vs. 16%) compared with patients who responded optimally to TKI treatment¹⁴.

Other *BCR::ABL1*-independent resistance mechanisms include those that affect the metabolism or intracellular concentration of TKIs, such as the cytochrome P450 enzymes and membrane transporters. The activity of these factors can influence treatment response by affecting TKI availability. For instance, patients that harbor the GC polymorphism in the promoter of the *SLC22A4* gene (that encodes an imatinib transporter) had a better response to first-line imatinib than those with the GG genotype¹⁵. Future pharmacogenomic studies will help clinicians to adapt TKI dose in an individualized manner.

Finally, the activation of many other signaling pathways, including the PI3 kinase/mTOR or the MEK/ERK pathways, has been suggested to play a role in TKI resistance. Precisely how these mechanisms drive resistance to TKIs is still unknown and is an important area for further investigation.

Novel therapies

The hypermethylation of key genes has been identified as a possible target for directed therapy, with hypomethylating agents, such as azacitidine, currently being investigated in clinical trials for use in CML. There are many other novel treatment options in

Table 1. List of targeted agents in development in clinical trials and some examples of mutations to genes that can confer resistance

Agent	Mechanism	NCT	Resistance mutations
5-azacytidine	DNA methyltransferase inhibitor	NCT03895671	<i>TP53</i>
Venetoclax	BH3 mimetic	NCT02689440 NCT02115295 NCT04188405	<i>BCL2</i> , e.g. G101V
Nivolumab	PD-1 inhibitor	NCT02011945	<i>PTEN, SETD1^B</i>
Avelumab	Anti-PD-L1 antibody	NCT0276706	<i>JAK1, B2M</i>
Tazemetostat	EZH2 inhibitor	ISRCTN68270067	<i>EZH2</i> , e.g. C663Y and Y726F
Ruxolitinib	JAK/STAT inhibitor	NCT03654768	
MK-0457 (tozasertib)	Aurora kinase inhibitor	NCT00405054	
PRI-724	Wnt/ β -catenin pathway inhibitor	NCT01606579	<i>FBXW7</i>
Everolimus	mTOR inhibitor	NCT00081874, NCT00093639	<i>KRAS, BRAF, PIK3CA</i> , e.g. E542K
BEZ235	mTOR inhibitor	NCT01756118	
Rapamycin (sirolimus)	mTOR inhibitor	NCT00776373	<i>FKBP12</i>
Pioglitazone	Peroxisome proliferator-activated receptor gamma agonist / Hedgehog inhibitor	NCT02889003	<i>PARP1, BRCA2</i>

development, including an EZH2 inhibitor and various inhibitors of the mTOR pathway (Table 1).

The development of novel CML therapies has two key aims. First, to inhibit other pathways upregulated by *BCR::ABL1* signaling through the combination of TKIs with other agents. Second, to target leukemia stem cells (LSC) that are not eliminated by TKIs. These LSCs have been suggested to play an important role in TKI resistance and are thought to be the main causes for relapse after treatment discontinuation. For example, activation of the JAK2/STAT3 signaling pathway promoted the re-entry of LSC into the cell cycle, thus decreasing the pool of quiescent LSCs¹⁶.

As more new agents are approved, molecular biologists will need to be aware of mutations to genes that can confer resistance, since the pressure exerted by targeted therapies has led to resistance to virtually all targeted therapies to date¹⁷. For example, in the case of venetoclax, a BH3 mimetic, it will be important to monitor the emergence of *BCL2* mutations.

Just a few of the novel agents in development are listed in table 1, but many others are in the pipeline, including agents for the treatment of patients in BP, many in combination with TKIs. Understanding these mechanisms of resistance is vital for optimizing treatment strategies as, although TKI treatment has

significantly improved the prognosis of CML patients, approximately 5% of patients diagnosed in CP will progress to BP, with a high associated mortality. Combination therapies that target multiple aspects of the disease may provide more robust and durable responses in patients with resistant disease.

Future challenges/Conclusions

It is clear that the *BCR::ABL1* kinase activity results in multiple genetic events-including ACA, somatic mutations, and epigenetic changes, such as hypermethylation-that accumulate over time and promote clonal evolution toward BP. The incorporation of genomic biomarkers into risk stratification algorithms, which are still currently based exclusively on clinical factors, would greatly improve the prediction of CML patients with a greater risk of progression.

In terms of TKI resistance, it is clear that we also need to consider *BCR::ABL1*-independent mechanisms of resistance, and particularly focus on the LSC that persist in CML patients. Furthermore, with each new TKI or alternative targeted therapy that reaches approval, we need to be aware of the gene mutations profile that can confer treatment resistance.

The use of artificial intelligence will allow us to decipher and apply information compiled from multiple genetic tests, including complex “omics” data, to help inform clinicians on the best drug or drug combination for each patient based on the patient’s genetic profile, as we move toward a more “personalized” therapy for CML patients.

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