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Commentary

Compound mutations in CML—imaginary bogeyman or real arch-nemesis?

It has been almost two decades since the introduction of the tyrosine kinase inhibitors (TKI), into routine clinical practice for Philadelphia positive leukaemias. Whilst many patients benefit over the long term with TKI treatment, a substantial number still encounter treatment resistance. This is most often seen in Philadelphia positive acute lymphoblastic leukaemia and advanced phase chronic myeloid leukaemia (CML), but also in a considerable minority of patients with chronic phase CML.

One of the first reports of TKI resistance concerns the development of kinase domain mutations, which remains the most commonly encountered and best understood mechanism of resistance. Kinase domain mutations lead to changes in the amino acid sequence and the structure of the BCR-ABL protein. TKI binding in the altered structure may either be impaired or abrogated altogether. Imatinib, a first generation TKI, is potentially susceptible to the widest number of kinase domain mutations. To improve outcomes in imatinib resistant cases, second generation TKIs such as nilotinib, dasatinib and bosutinib were subsequently developed. These agents are more potent BCR-ABL inhibitors, with efficacy in certain imatinib resistant mutations. However, all TKIs were ineffective against the T315I gatekeeper mutation, until the rationally designed third generation TKI ponatinib became available. The most potent of all BCR-ABL inhibitors, ponatinib has demonstrated efficacy against all KD mutations commonly encountered in the clinic, and remains the only effective TKI against the T315I mutation [1].

It was hoped that ponatinib would have been the last weapon needed against kinase domain mutations. However, CML also developed a defence in response: compound mutations, which denote more than one KD mutation existing on the same BCR-ABL1 molecule. The resultant changes in the conformation of the BCR-ABL protein from more than one amino acid exchange can disrupt TKI binding in multiple locations, conferring resistance beyond that seen with singleton KD mutations.

The role of compound mutations as a resistance mechanism of relevance, particularly to ponatinib, is well established. Initial reports of treatment resistance, supported by comprehensive functional data and crystallographic models, have subsequently been confirmed by clinical experience [2]. The T315I/E255K compound mutation, in particular, is resistant to all TKIs available clinically. However, there is ongoing debate on the frequency of compound mutations, and by extension, whether it is common enough to be of clinical relevance. Of central importance is the lack of standardised technology to detect compound mutations, and the inability to distinguish compound mutations from polyclonal mutations, especially when mutant allele frequency is low. (Polyclonal mutations denotes a situation where different mutations occur on separate *BCR-ABL1* alleles, with no BCR-ABL protein having more than one amino acid exchange.)

For instance, whilst Sanger Sequencing is the standard of care for mutation detection, it has a low sensitivity and can only detect mutations with allele frequency of > 10-15%. Furthermore, Sanger sequencing is semi-quantitative, and cannot reliably distinguish whether mutations are compound versus polyclonal when the sum of all mutations add up to < 100% (Fig. 1A). More sensitive mutation detection techniques, such as the Agena MassARRAY® System, has demonstrated sensitivity of detecting low level mutants to ˜0.1% [3]. However, such assays are also poorly suited to discerning whether multiple mutations are compound versus polyclonal.

A number of studies have demonstrated the potential of assays using Next Generation Sequencing to improve sensitivity of detecting kinase domain mutations, and report whether mutations are in polyclonal versus compound configuration. NGS, with its unprecedented potential to sequence efficiently and relatively cheaply on a per molecular basis, has the potential to be the ideal method for sensitive mutation sequencing. Its ability to allow visualisation of results at the molecular level read by read, would also potentially de-convolute configuration of low level mutations, differentiating between compound versus polyclonal. Results from initial studies suggest compound mutations may be much more common than first thought. Complex and diverse clonal architecture with concurrent co-existence of many different sub-clones may lie underneath what is seen with Sanger sequencing, with up to 13 different mutated sub-clones co-existing in one reported case [4–6]. In many patients, the same mutations were found both as components of compound mutations, and as individual mutations in the same individual, suggesting that the same nucleotide substitution occurred independently multiple times within an individual patient, with both selection and deselection occurring in phylogeny of unlikely complexity. Such results imply that ponatinib would be ineffective in a substantial proportion of patients. In retrospect, however, early reports overestimated the abundance and complexity of sub-clonal mutation architecture, and the number of patients who fail multiple TKIs because of highly resistant multiple compound mutations remain low. This is particularly the case in CP-CML [7]. Indeed, under experimental conditions, artefactual compound mutations may be derived as a product of PCR recombination artefacts, and is likely responsible for many cases of compound mutations observed with early NGS techniques (Fig. 1B). Parker et al demonstrated this by mixing plasmids with known mutations together prior to PCR pre-amplification and cloning, which resulted in colonies with compound mutations that were not present in the starting plasmids. In contrast, the same plasmids, when PCR-pre-amplified in separate reactions prior to being mixed, then cloned, results in polyclonal colonies each with only the mutations in the starting plasmids [8].

Based on experience from early experiments, NGS based mutation detection methods continues to be refined and mature. For instance, application of molecular barcodes to each cDNA template prior to PCR-

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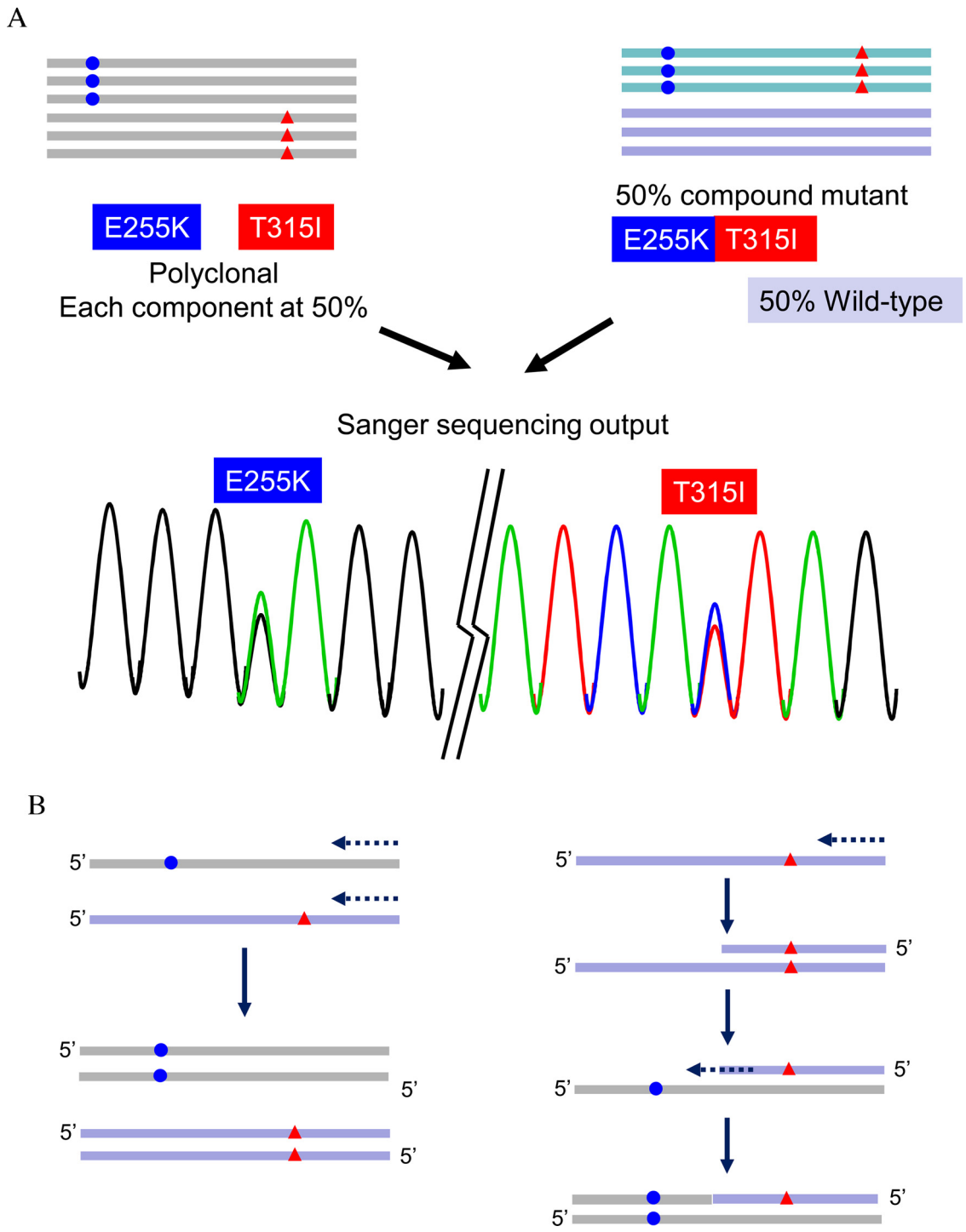

Fig. 1. A) Sanger sequencing results reflect the average signal from all molecules within a sample mixture, and is unable to differentiate polyclonal mutations from compound mutations. In these simulated sequencing chromatograms, the E255K (circle) and the T315I (triangle) point mutants are present in different configurations on different BCR-ABL molecules. The mutated base present at 50% on a wild-type background in both instances lead to similar read outs. B) In normal PCR reactions, extension from one primer continues to cover all regions of interest. The resultant amplicons are identical copies of the original templates (left panel). One possible mechanism of PCR recombination artefact results from a disruption to the extension process. The premature termination results in a shortened amplicon, which can itself act as a primer. When a number of different templates are present as in a PCR reaction, these shortened amplicons bearing one point mutation can anneal to templates bearing a different point mutation. The result is an artefactual amplicon with compound mutations (right panel). Only one strand is illustrated here to simplify the schematics.

amplification may avoid artefactual compound mutations that results from PCR recombination. Improvements in bioinformatic techniques not only result in more accurate variant calling, but can also allow for discernment of mutation configuration. With further development and validation, NGS based methods has the potential to change the current paradigm of mutation testing in CML. Such techniques will also be invaluable in other diseases where small molecular inhibitors are susceptible to resistance through compound mutations, such as EGFR inhibitors in lung cancer [9].

Although NGS based assays represent a significant advance in

diagnostics, these techniques require specialised equipment and expertise, and may not be applicable in all instances. In a paper published in the January of Leukemia Research, a group of Korean researchers demonstrated a method to sensitively identify mutations at low allele frequency, with information on whether they are compound versus polyclonal. Starting with a patient's white cells, they reverse transcribed RNA, then pre-amplified the cDNA using primers targeting the *BCR-ABL1* region. The templates are then incorporated into plasmids singularly before cloning, followed by direct sequencing on the colonies that grew. They laboriously and diligently studied 218 peripheral blood samples from 45 CML patients with multiple mutations, performing subcloning and sequencing. Whilst Sanger Sequencing detected 19 mutations in 45 samples, subcloning and subsequent Sanger sequencing of colonies detected 556 mutations from subcloning sequencing of 900 colonies, revealing complex architecture involving minor, low frequency clones, many in compound configurations. The researchers were also able to perform experiments in subsequent samples from the same patients. Sequencing 4357 colonies in 218 post baseline samples, 57.5% had compound mutations, and most had 2 or 3 point mutations on the same allele, with one clone having up to 7 component mutations.

This methodical and well performed study demonstrated that subcloning and sequencing can reliably increase the sensitivity of Sanger sequencing, and provide information re mutation configuration. However, it may not be without limitations. As a cohort study, the authors are unable to ascertain the true incidence of compound mutations without an accurate denominator. Furthermore, the lower limit of detection for mutant allele frequency, and a false positive detection rate, has not been established. In addition, the current design is not entirely free from the prospect of PCR recombinant artefacts, which may be introduced in the initial pre-amplification step. The magnitude of this problem is uncertain, as most mutations detected at baseline eventually expanded in patients who received therapy that in retrospect would have been inappropriate. Indeed, minor clones, such as T315I present at 5% allele frequency, could be demonstrated in historical, pre-baseline samples from patients who subsequently went on to develop treatment resistance mediated by T315I (Kang et al, Supplementary Table 7). Additionally, the data supporting most of the mutations detected in compound configuration is credible. Finally, given the laborious nature of the work flow, such a method is unlikely to see wide spread adoption in a clinical setting, and may remain largely a research tool. Notwithstanding these potential deficiencies, this report demonstrates the usefulness of cloning as a technique – increasing the sensitivity of low frequency mutant detection and allowing detection of compound mutations – without the requirement of a sophisticated and expensive NGS platform with a bioinformatics team. It also allows population dynamics to be assessed, which may lead to clues uncovering other aspects of disease biology.

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