How "precise" is precision medicine in hematology?

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Massive parallel sequencing, the foundation of next generation sequencing (NGS), allows us to sequence the entire exome (the coding sequences of the genome) of leukemia or lymphoma cells, and can be combined with RNA-Seq to evaluate the transcriptome. Using these techniques, one can search for mutations, indels, gene fusions, copy number alterations, alternative splicing, and gene expression profiles from a blood sample from a person with leukemia. Importantly, these analyses can be performed in just a few days and at modest cost. Given these advances, we might expect data from NGS to be quickly integrated into therapy decisions to effect so-called precision or *personalized medicine*.[1,2] In fact, cancer treatment was envisioned as one of the most promising applications of PM. In particular, haematological neoplasias, and especially leukemias, were seen as the most direct candidates, given the accessibility of neoplastic cells. Interestingly, and surprisingly, this is not yet widely used, although it continues to generate interest and debates.[3] In contrast, our current situation is similar to having many or most pieces of a puzzle, but limited ability to put them in the right place to complete the picture. Several important limitations and challenges associated with using these data to precisely treat persons with leukemia or lymphoma have emerged and are summarized below along with possible solutions. We do not deal here with germline mutations; only with those somatic.

Distinguishing the wheat from the chaff

Many mutations identified by NGS are present before a cell is transformed and are unrelated to leukemia development. These mutations are termed *passenger* mutations. *Passenger* mutations are distinguished from *driver* mutations which cause leukemia transformation. *Passenger* mutations increase over time and are more frequent in older persons.[4,5] They are also present in persons without leukemia or any blood disorder.[6] One way to distinguish *passenger* from *driver* mutations is to consider *drivers* as only those recurrently identified in a substantial proportion of persons with leukemia or clonal hematopoiesis,[7,8] and not age-adjusted normals. However, this approach risks ignoring infrequent mutations (black holes), which may be important in a specific person's leukemia. In an attempt to identify mutations which may be *drivers*, we developed OncoScore (revised version submitted for publication in Scientific Reports; available as Bioconductor software at: *https://bioconductor.org/packages/release/bioc/html/OncoScore.html* and as a web tool at: *http://www.galseq.com/oncoscore.html*). OncoScore is dynamic software which tracks medical literature in real time, and suggests a numerical score of the probability a mutation is a *driver* mutation. We tested the possible clinical value of OncoScore in a cohort of 23 persons with Chronic Myeloid Leukemia at diagnosis and found it was better correlated with sustained response to tyrosine kinase-inhibitors than the total number of mutations or Sokal score.[9] Further work and analysis will be needed to ascertain the real value of this software.

If the impact of a specific mutation on transformation is unknown, it is also possible to try to predict its impact on the encoded protein function with software such as PolyPhen, DAVID or PROVEAN.[10-13] These software tools help to indicate whether the observed mutation is likely to cause perturbation in the protein function. Alternatively, animal models could be used to characterize the functional significance of a particular mutation; however, these models require a substantial amount of time, and thus are frequently incompatible with the dynamics and time frames allowed in clinical medicine.

All these tools are imperfect and require improvement and complementation with additional decision making instruments. However, they represent a first step in the direction of differentiating *driver* and *passenger* mutations.

Determining mutation hierarchy

Once mutations in a persons' leukemia cells are identified, we must then add a further dimension: the temporal order in which mutations are acquired. Data from diseases such as MPN-associated myelofibrosis indicated different sequences of mutation acquisition results in different phenotypes despite a similar genotype.[14] Reconstruction of the order of acquiring mutations is also important in other settings. Some leukemias first acquire important *driver* mutations (*e.g.*, *BCR/ABL1*, *NPM/ALK*, *PML/RARalpha*) with substantial tranforming ability such that additional mutations are dispensable. In other diseases, such as myelodysplastic syndrome (MDS), the first mutations are only weakly transforming, and additional genetic alterations are needed for the fully transformed phenotype.

Targeting the earliest *driver* mutation(s) holds the greatest therapeutic promise when they carry a relevant transforming potential.[15,16] In this setting (CML, APL, ALK+ lymphomas), PM can change disease prognosis.[17] Alternatively, therapies targeting several different *driver* mutations are a potential therapeutic strategy when the transforming potential of the initial mutation is low. Here, the available evidence for a benefit to patients is more limited, although some promising data are emerging.[18] Knowing the mutation hierarchy could also reveal why the same type of mutation, such as *ALK* containing fusion genes, have different therapeutic implications in different cancers; in lymphoma *versus* lung cancer, for example, the same drug (crizotinib) obtains quite different therapeutic responses. Hierarchical variant reconstruction is possible but requires sequencing many individual leukemia colonies or sequential studies. In addition, this is presently feasible for myeloid neoplasms, but less so for other cancers. Alternative strategies such as single cell exome/RNA-Seq analysis are being developed to facilitate reconstruction of clonal hierarchy and to eliminate the need to

sequence colonies arising from single cells. Statistical methods to infer the order of acquisition of multiple mutations in a cancer were recently suggested by Papaemmanuil *et al.*[19] and Caravagna *et al.*[20] These methods are important as they show that cancer progression follows a defined trajectory, not a random pattern. However they are valid for groups of patients, but cannot assess the order of development of mutations inside a single leukemia.[14]

All these considerations are valid when the therapeutic strategy is "functional" targeting, *e.g.*, blocking the enzymatic activity of the product of a mutated gene such as a tyrosine kinase. Paradoxically, *passenger* mutations could represent ideal targets for immune therapy since they are present in all cancer cells, before the *driver* mutation.[21]

Signal transduction pathways

An ideal initial *driver* mutation is one which carries most of the leukemogenic activity and which can be directly targeted. *BCR/ABL1* in CML is an example. However, most leukemias and lymphomas are more complex, with a median of >10 mutations/cases and several sub-clones at the time of diagnosis.[22] Several software packages, for example DAVID,[12] are designed to address this complexity using inputs such as lists of mutated genes or Differentially Expressed Genes (DEG) derived from RNA-Seq analyses. The output can identify the pathway(s) used by the leukemia or lymphoma which could be targeted. Clearly, therapeutic specificity is reduced with this approach as targeting is focused on a pathway used by many normal functions, not solely by the *driver* mutation. Nonetheless, this approach holds promise when direct targeting is not yet available or feasible.

Which NGS strategy is best?

Whole genome or exome NGS is attractive, but targeted sequencing is gaining favor.[19] The strategy is to sequence specific genes (or mutations) identified as recurrent in a specific tumor type and which are *actionable*, *i.e.*, can be directly targeted with current drugs. Advantages of this approach are: (1) lower cost; (2) higher coverage (mean number of times each nucleotide of the target region is sequenced), which decreases the risk that some targeted loci are insufficiently covered for reliable variant-calling (despite improvements in NGS, it is still common to find parts of targeted genes insufficiently covered); and (3) less complex bioinformatics. Using this approach, one can interrogate <1,000 instead of >13,000 genes. The obvious potential drawback is missing *non-conventional* mutations (*black holes*). However, the use of panel-based sequencing is, in some regards, contrary to the original goal of NGS-based PM, namely, characterizing the universe of genetic abnormalities in an individual cancer. Panel-based sequencing is, instead, a *standardized* approach to PM, which can be unable to reconstruct mutation hierarchy in a person with leukemia or cancer.[23] It is difficult to foresee which strategy, comprehensive or panel-based, will be most useful in the future.

In medio stat virtus

Virtue, as is often the case, likely lies between these alternative strategies. Presently, use of pre-defined panels is probably clinically the most feasible one, and will allow physicians to familiarize themselves with the complexities of NGS without being overwhelmed by a mass of *omics* data. However, in the near future, complete unbiased NGS will, in our opinion, predominate. This evolution may result from improvements in sequencing technology and reduced cost, thus solving, for example, the *black holes* problem. New, user-friendly bioinformatics tools are likely to be developed, and physicians and researchers will become more familiar with them; new communication skills will also be needed to convey this complex information to patients and their families. Importantly, new clinical trial designs are needed to test the clinical relevance of data from NGS (see below).

A new type of clinical trial is needed

Proving clinical benefit from any therapy intervention requires rigorous methodology, best exemplified by randomized clinical trials. NGS, however, makes it increasingly difficult to identify homogeneous cohorts of persons for study using this trial design. Consequently, new types of clinical trials are needed which preserve the value and rigorous approach of the controlled study, but also take into consideration the NGS-based molecular profile of the tumor.[18,24-26] A possible solution could reside in trials in which the strategy of using PM data or not to inform treatment decisions is evaluated in a controlled way, rather than the single therapeutic intervention.

Conclusions

NGS has increased our understanding of leukemia/lymphoma development, and must be translated into better therapy. Consequently, NGS will likely change the way physicians treat lymphomas and leukemias.[27] In this scenario, panel-based sequencing will be a bridging-technology to whole-exome or even whole-genome sequencing and RNA-Seq. We are entering an exciting era of PM in general, and leukemia therapy specifically. However, we must also solve the important logistical problems that the use of NGS will inevitably cause. We must find new ways to evaluate therapy strategies, and *off-label* use of approved drugs must be streamlined and simplified. Finally, we must develop new types of clinical studies and new ways to render the complexity of PM information understandable and useful to patients.

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