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Review

Molecular testing in oncology: Problems, pitfalls and progress

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ABSTRACT

Recent advances in the understanding of the molecular basis of cancer and the development of molecular diagnostics based on this knowledge have done much to progress the fields of oncology and pathology. Technological developments such as Next Generation Sequencing (NGS) and multiplex assays have made feasible the widespread adoption of molecular diagnostics for clinical use. While these developments and advances carry much promise, there are pitfalls to implementing this testing.

Choosing appropriate biomarkers is a vital first step for clinical use and being able to understand the complex relationship between predictive and prognostic biomarkers is a crucial component of this. Testing for standard of care biomarkers is not straightforward, one must choose carefully between clinical trial assays, assays that analyse the same biological phenomenon or surrogate biomarkers. Sample heterogeneity and population specific difference is assays may skew results and must be controlled for at the assay design stage.

At a technical level, NGS has the potential to revolutionise laboratory practice and approaches to cancer treatment. However, use of this technology requires careful planning and implementation if one is to avoid technical and ethical quagmires. Finally, with FDA regulation of companion diagnostics one may be limited to therapy specific assays.

1. Introduction

If one casts a broad net, molecular diagnostics have featured in the pathologic assessment of cancer since the advent of immunohistochemistry (IHC). The ability of IHC to selectively stain specific protein molecules for subsequent microscopic evaluation merits classification as a molecular technique. Likewise, the ability of in situ hybridisation (ISH) to identify cancer associated abnormalities at the chromosomal or transcriptomic levels similarly falls into this category. Thus, the recent development of mandatory treatment-guiding biomarker assays primarily using PCR based techniques for solid tumours, including lung cancer, marks an addition to the pathology toolset rather than a fresh departure from the morphological roots of pathology.

Traditionally, the role of the pathologist was to diagnose disease and determine prognosis based on the macroscopic and microscopic appearance of tissue. In the setting of cancer diagnosis, the pathologist not only identifies a lesion as malignant, but also suggests whether the lesion may have arisen in situ or is likely to be metastatic. Thus, this information is used for staging and guiding treatment based on the broad classification of the tumour. More recently, identifying the tumour histologic sub-type has shown to be predictive of response to certain types of therapy. A study by Scagliotti et al. [1], showed that in lung cancer histologic sub-type predicted response to one of two cisplatin doublet therapies. Patients with lung adenocarcinoma were shown to have a greater overall survival when prescribed cisplatin/pemetrexed vs. cisplatin/gemcitabine. Conversely, patients with squamous cell histology demonstrated a better overall survival when prescribed cisplatin/gemcitabine vs. cisplatin/pemetrexed.

Advances in our understanding of the molecular basis of cancer have lead to the development of targeted therapies, such as trastuzumab for the treatment of HER-2 overexpressing metastatic breast cancer [2] and imatinib for the treatment of chronic myelogenous leukaemia [3] and gastrointestinal stromal tumours [4]. Both of these early developments demonstrated that a targeted approach could yield significant survival benefits provided the patient's malignancy contained a molecular defect that could be specifically targeted with inhibitor therapy. Thus, targeted therapy has necessitated molecular assays to identify specific aberrations that may indicate or contraindicate a given therapy.

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While molecular testing of tumours is of undeniable benefit, it is not without its pitfalls. This article will explore key areas that are sources of confusion or misinterpretation in molecular testing with a particular focus on predictive and prognostic biomarkers. Laboratory and clinical features that may cause pre-analytical and analytical errors such as sample mix-ups, processing considerations and PCR contamination are also well described in other literature sources [5–7] and are beyond the scope of this article. For the purposes of this review, those sources of uncertainty and confusion that are specific to molecular testing for oncology will be discussed and where possible, potential solutions for these issues will be presented. As the technology and knowledge supporting molecular testing is rapidly evolving, the advantages and challenges of these developments will also be discussed.

2. Not all biomarkers are clinically relevant

It is important to recognise that not all biomarkers are created equal and very few potential biomarkers live up to the standard required for clinical implementation. For predictive markers considered to be companion diagnostics, i.e. those that are used for patient stratification for a clinical trial, the trial itself should provide sufficient evidence for use of the biomarker in a clinical setting. A well known example of this level of evidence is found in the **I**RESSA **P**an-**AS**ia **S**tudy (IPASS) trial [8]. This trial demonstrated that patients with an *EGFR* positive mutation test had a longer progression free survival (PFS) if prescribed gefitinib vs. carboplatin plus paclitaxel. Conversely, patients with a negative EGFR mutation test had shorter PFS when prescribed gefitinib vs. carboplatin plus paclitaxel. The clearly defined nature of the biomarker in this trial and a clear understanding of the underlying biology [9] has lead to its adoption as a marker of treatment suitability for gefitinib [10,11].

Currently, EGFR mutation testing and ALK rearrangement status by break-apart FISH assay are the only two molecular markers considered standard of care for Non-Small Cell Lung Carcinoma (NSCLC) treatment and are the subject of the College of American Pathologists (CAP), International Association for Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP) Guidelines published in 2013 [12]. *EGFR* mutation and *ALK* rearrangement testing are also a feature of the Version 1.2014 National Comprehensive Cancer Network (NCCN) clinical practice guidelines for Non-Small Cell Lung Carcinoma [11]. The 1.2014 version of the NCCN NSCLC guidelines highlight two additional markers for possible implementation in treatment pathways; ERCC1 expression levels as a prognostic marker and predictor of response to platinum based chemotherapies [13–15] and *KRAS* mutation testing as a potential prognostic marker for NSCLC [16,17], although the latter link is not universally supported [18].

To some, the relative paucity of the previously mentioned molecular markers of NSCLC prognosis seems a little surprising, as numerous other biomarkers have been suggested to be important in the management of NSCLC. Frequently, novel technologies can result in biomarkers that show great promise but fail to live up to the standards required for clinical decision-making. As an example, early microarray studies aimed at solving prognostic dilemmas in early stage lung cancer were suggested to provide clinically relevant prognostic information by study authors. However, a review of 16 published microarray studies found that none demonstrated evidence of suitability for clinical use [19]. Established techniques may also be prone to early promise and poor results; a review of IHC antibodies tested for prognostic value in NSCLC failed to identify any single or combined marker that provided sufficient prognostic information to be clinically useful [20].

As a level of uncertainty exists regarding which markers to use in molecular pathology, it is always best to operate with those supported by best practice guidelines. One could easily cite hundreds for articles which promote the utility of a novel marker in lung cancer, or any other cancer for that matter. Most importantly, the chosen markers should be supported by research sufficient to give confidence in the marker's ability to deliver a clinically meaningful result. One need only review the chequered history of ERCC1 testing to realise the importance of prospective trials in establishing the utility of a biomarker. Currently, testing of a broad range of markers is more suited to clinical trials than routine practice. Trials such as the SHIVA trial will aim to assess the clinical utility of emerging markers for targeted therapies [21].

3. Standard-of-care vs. surrogate markers

In pathology laboratories, certain technologies are favoured over others due to their widespread use and availability. A prime example of this is automated immunohistochemistry (IHC), which is available in nearly every histopathology laboratory. Both pathologists and laboratory scientists are familiar with IHC and dialogue around the subject is facilitated by this experience. Within laboratories that do not routinely perform PCR or FISH testing, there is an inclination to favour IHC-based testing. However, this rationale may lead to more problems than solutions as outlined below.

Detection of *ALK* translocations in lung cancer is predictive of response to crizotinib therapy [22]. In a clinical trial that compared crizotinib vs. chemotherapy in advanced *ALK* translocation positive lung cancer, *ALK* break-apart FISH was used to demonstrate the presence of a translocation in the sample and select the patient for inclusion in the trial. Break-apart FISH requires the counting of a set proportion of cells in which a split signal, or split deleted signal i.e. a non proximal 5′ and 3′ *ALK* probe is identified [23]. Analytically, the break-apart FISH assay does not identify specific *ALK* fusions, rather it detects a break in the chromosomal region encoding the ALK tyrosine kinase domain. This assay can detect rarer translocations in which *ALK* is paired with a different fusion partner such as *KIF5B* [24], *KLC1* [25] or others. Thus, the inclusion criteria for clinical trials based upon the break-apart FISH assay is the presence of an *ALK* rearrangement. It is self-evident that assays which seek to act as alternatives to that used in the clinical trial would need to detect the same phenomenon (i.e. an *ALK* gene rearrangement) to remain true to the selection criteria applied in the trial. This is true of chromogenic in situ hybridisation (CISH) which may be considered an equivalent marker to FISH as it is designed to detect the same biological alteration (an *ALK* rearrangement) albeit with a different visualisation mechanism, and has been shown to correlate with the results of FISH assays [26].

As ALK (FISH) is the only marker included in a prospective clinical trial for crizotinib therapy, other assays that show changes in *ALK* are in fact surrogate predictive markers. If one compares the IHC markers to the *ALK* (FISH) assay, it is interesting to note the biological premise for these tests assumes an increase in expression of the protein, or a component of the protein. In the majority of cases, comparison would suggest this is a valid assumption, but this still does not test for the same outcome [26–28]. IHC, on the other hand may detect a potentially treatment relevant increase in ALK protein expression in the absence of a translocation. Nonetheless, it remains to be seen whether such cases occur and what the biological significance is. RT-PCR also detects the presence of the EML4-ALK translocation but is not currently advocated for routine use as it may not identify ALK fusions with rarer fusion partners [29].

A more direct translation from PCR based testing to IHC is seen in the mutation specific antibodies used in detection of mutations in the *BRAF* gene [30]. Markers such as this offer a binary interpretation as staining should be lacking in the absence of the mutation. These antibodies offer the pathologist an opportunity to view the