Attack of the subclones: accurate detection of mutational heterogeneity

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Attack of the subclones: accurate detection of mutational heterogeneity in bulk DNA from tumours

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Clinical Relevance:

- Advances in mutation detection methods have led to improvements in profiling tumour heterogeneity.
- Accurate mutational profiling is vital for decision making about appropriate targeted treatments.
- Improved identification of tumour subclones may lead to improved therapy responses and/or understanding of therapeutic resistance.

Abstract

Tumours are often polyclonal and therefore heterogenous in their genomic and molecular profiles, which contributes to drug resistance and treatment failure. The methods used to detect these heterogenous differences in tumour samples are critical, but findings have been hindered by methodological inability to detect low frequency subclones in bulk DNA. In this issue, Chang *et al.* have addressed some of these methodological issues.

Introduction

A vital contributor to the lethality of cancer, including drug resistance and treatment failure, is tumour heterogeneity. This includes diversity of genomic, epigenetic, metabolic, immunogenic and metastatic features, which can coexist within a single tumour (intra-tumoural) or between metastatic deposits (inter-tumoural) in the same patient. Individual tumours may be polyclonal and have potential to evolve genomically over time. Methodological confounding factors have previously frustrated the assessment of these features, but technological advances are starting to result in consistent data with meaningful clinical utility. The study by Chang *et al*. in this issue of the Journal of Investigative Dermatology investigates the question of inter-tumoural heterogeneity of the four most common driver mutations in cutaneous melanoma (CM) patients, in *BRAF* (p.V600), *NRAS* (p.Q61) and *TERT* (-

124C>T and -146C>T) and compares different techniques to detect them. *BRAF* and *NRAS* mutations are usually mutually exclusive in CM (Hayward et al., 2017); similarly, the two main *TERT* promoter mutations rarely co-exist (Hayward et al., 2017).

Tumour Heterogeneity

Tumour heterogeneity was recognised as early as 1958 (Foulds, 1958), with the definitions of what constitutes heterogeneity becoming more specific as technological hurdles to identifying different contributing aspects have been overcome. Tumourigenic transformation requires the evasion of stringent cell cycle control mechanisms, which results in the ability to undergo an unusually high number of proliferative cycles. In combination with the acquisition of specific genomic changes, this results in an increased rate of chromosomal instability and increased mutation rates, leading to genetic diversity between cellular populations. The nature of these changes means that clonal evolution is likely 'branching' rather than 'linear' (e.g. as described by Shi *et al*. in CM (Shi et al., 2014)). The evolution of factors might confer a survival advantage, such as the ability for a cell to disseminate beyond the primary site, or development of resistance to treatment, meaning that subclones might exist not only within a single tumour mass but also be shared by primary and secondary tumours. The different factors subject to heterogeneity within tumours have been extensively reviewed, such as by (Marusyk et al., 2012) and (Grzywa et al., 2017).

Heterogeneity of Driver Genes in Cutaneous Melanoma

Specifically focusing on the genetic drivers in CM, Sensi *et al*. was the first to describe the presence of both *BRAF* p.V600E and *NRAS* p.Q61R in the same melanoma, which was mutually exclusive at the single-cell level in 2006 (Sensi et al., 2006). Heterogeneity in *BRAF* p.V600E was subsequently described between primary and metastatic melanomas (Lin et al., 2011, Yancovitz et al., 2012), as well as between metastatic tumours (Yancovitz et al., 2012). Further investigations of the heterogeneity of established driver genes and their associated pathways have been performed, particularly in the context of therapeutic resistance to targeted b-Raf (the protein product of *BRAF*) treatment, with some inconsistency to the results reported. In this issue, Chang *et al*. focus on the main driver hotspot mutations and different methods of detection, in order to provide a systematic assessment of inter-tumoural heterogeneity in CM.

Detection of Mutational Heterogeneity

Heterogeneity can be detected in an individual tumour in a number of different ways. Next generation sequencing of bulk DNA is best performed on fresh-frozen tissue, which can be difficult to obtain, requires specialised bioinformatic skills to analyse and with stringent quality control requirements,

the presence of rare subclones resulting in low allele frequency might be missed. A more accessible method utilises DNA derived from archival tissue (i.e. formalin-fixed, paraffin-embedded (FFPE) tissue). This can result in artificial introduction of genomic changes, but it does allow for sampling from different sections of the same tumour and provides the opportunity to study larger sample sizes due to routine clinical FFPE processing of tumours. The possible sequencing approaches to analyse FFPE tissues also tend to lack sensitivity.

The study by Chang *et al*. in this issue compares methods that are commonly available in standard molecular biology laboratories and that can be used to examine genomic heterogeneity; SNaPshot (Applied Biosystems), Sanger sequencing, mutation specific PCR and droplet digital PCR (BioRad) to examine hotspot variants in *BRAF*, *NRAS* and *TERT*. These techniques examine only a low number of targets, with differing capabilities of detecting low allele frequency within bulk DNA samples. Chang *et al*. examined a total of 271 primary/metastatic CM tumours from FFPE blocks derived from 99 patients using SNaPshot followed by assessment with further techniques in order to examine: a) Concordance of mutational status between the different techniques; b) Ability to detect low allele frequency within bulk DNA; c) Consensus of heterogeneity within individuals between either primary and metastatic tumours, or between metastatic deposits. This study found that for the detection of mutations in *BRAF*/*NRAS*/*TERT* hotspots in bulk DNA, SNaPshot, Sanger sequencing and mutation-specific PCR were 91.5% concordant. Two considerable caveats with the use of these techniques/approaches were however identified. The first was the ability to detect low allele frequency in bulk DNA. Using the more sensitive droplet digital PCR revealed that many samples called as 'wildtype' did in fact contain cells with hotspot mutations. Second, they used uracil DNA glycosylase (UDG) treatment to reduce DNA artefacts introduced by formalin fixation of tumours, which revealed that the number of patients with *TERT* inter-tumour heterogeneity was reduced from 31 to 13. After all these analyses, Chang *et al*. concluded that inter-tumoural heterogeneity was confirmed in 18% of patients examined, which included evidence of polyclonality of the primary tumour and tumour evolution over time. These data are in agreement with those in smaller studies, such as those summarised and combined in the meta-analysis of *BRAF* inter-tumoural heterogeneity by Valachis and Ullenhag (Valachis and Ullenhag, 2017), which found 13.4% of inter-tumoural heterogeneity between primary and metastatic tumours.

Clinical Importance of Accurate Tumour Heterogeneity Identification

The protein product of the *BRAF* gene (b-Raf) activates the mitogen-activated protein kinase (MAPK) signalling cascade, which is involved in cell proliferation, differentiation and development. The *BRAF* p.V600E mutation hyper-activates this pathway leading to uncontrolled cellular proliferation. Given

the ability to therapeutically target the *BRAF* p.V600E mutation, the heterogeneity of *BRAF* has been considerably researched in the context of therapeutic resistance to inhibitors targeting cells containing the p.V600E mutation. These studies have largely focused on investigation of intra- and inter-tumoural mutational status of the key CM drivers and factors involved in the reactivation of the MAPK pathway before and after b-Raf inhibition therapy. The presence of pre-existing distinct cellular clones with distinct main driver mutations likely means an inevitable therapeutic resistance, which will inform further clinical decisions. The ability to identify even low allele frequency driver variants in bulk tumour DNA therefore has significant clinical implications and investigations of the techniques and approaches to identify these mutations are therefore important for the present and near future.

Future Perspective

While Chang *et al*. used standard techniques designed to assess specific candidate driver mutations, the advent of single cell sequencing has revealed the genomic architecture of tumours to an unprecedented level of detail, as first described in CM by Tirosh and co-workers (Tirosh et al., 2016). The cost, availability, analysis pipelines, capacity and resolution of this technique will continue to improve, which will ultimately result in the ability to routinely and accurately detect many aspects of molecular heterogeneity in tumour samples. Importantly, this will include an indication of the proportion of cells containing different features, which will have significant implications in the decision making for selection and timing of treatments.

Figure Legend

Methods that analyse bulk DNA examine mixtures of individual subclones, each of which might not be detectable with the less sensitive methodologies routinely available in laboratories. Furthermore, the presence and frequency of subclones may differ between primary and metastatic tumours. The advent of single cell sequencing allows analysis of whole genomes at the individual cellular level, offering the potential for an unprecedented level of detailed characterisation of subclones that are present within tumours.

Conflict of Interest

None to declare

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