Tumour heterogeneity: Does it matter?

Abstract

Introduction: It has long been recognised that tumours are composed of a mosaic of cells and numerous methods have been developed to detect tumour heterogeneity, including *in situ* hybridisation, multi-regional sampling, cytological assays, and whole genome and single cell sequencing. Using these methods, heterogeneity has been observed at the genetic, epigenetic, and phenotypic level in numerous cancers. With the advent of deep sequencing technology, we now appreciate a greater complexity of distinct genotypes and phenotypes that drive the biological behaviour of cancer. Despite decades of progress in detecting tumour heterogeneity, the question remains: to what extent does it matter?

Areas covered: This review explores the evidence for and against the importance of tumour heterogeneity in three main areas: prognostication, development of targeted therapeutics and tumour resistance; summarising current understanding before evaluating ongoing experimental and clinical developments.

Expert opinion: Theoretical understanding and *in vitro* detection of intratumour heterogeneity promises much but is yet to translate into meaningful clinical benefit. However, the recent emergence of a host of technological innovations and upcoming clinical trials may soon change the landscape of this field.

Keywords: Cancer stem cell, drug resistance, liquid biopsy, tumour evolution, tumour heterogeneity

1. Introduction

Tumour heterogeneity can be described in terms of intertumour and intratumour heterogeneity **(Figure 1)** [1]**.** Intertumour heterogeneity is variation between tumours from different patients (interpatient) or from different tumours within the same patient. Intertumour heterogeneity can be related to host factors, such as tumour microenvironment, somatic mutations occurring within the tumour of each individual patient, and germline mutations [2].

Intratumour heterogeneity reflects the variation of tumour cells within a single tumour and can be defined in terms of cellular morphology, cell surface markers, gene expression, epigenetic profiles, motility, and metabolism.

2. Models of tumour heterogeneity

The two main models explaining how heterogeneity arises are the stochastic or clonal evolution model and the cancer stem cell (CSC) model **(Figure 2)** [3]**.** The stochastic model proposed by Peter Nowell in 1976 [4] states that tumours arise from a single mutated cell, which acquires additional mutations over time. Each of these individual mutations gives rise to subpopulations of tumour cells. According to basic principles of Darwinian evolution, these subpopulations may possess survival advantages and become dominant owing to different capacities for proliferation, invasion, and migration. The clones that possess an advantage expand, while the clones with less fitness become extinct. Importantly, tumour microenvironment may differ in space and time. For instance, in regions of relative hypoxia, clones that are suited to survive under these environmental conditions thrive. Together, these factors create heterogeneity spatially and temporally.

Virchow and Cohnheim were the first to suggest the idea of CSCs which they believed to arise from “activation of dormant embryonic tissue” [5]. The CSC model holds that a subset of cells are tumourigenic. These cells have the ability to self-renew and sustain the CSC population or to differentiate into heterogeneous cell types. In this model CSCs generate heterogeneity by creating a differentiation hierarchy composed of cells of distinct subtypes. This model predicts that CSCs can be isolated from the tumour bulk based on intrinsic properties and that since they sustain tumour growth, their demise is essential to effective therapy. This model was first supported by a xenograph model from acute myeloid leukaemia (AML) whereby a CSC population of CD34+/CD38- cells was isolated from patients and then transplanted into immunocompromised mice to recapitulate many aspects of human AML [6]. Similar xenograph approaches have demonstrated CSC populations in a variety of cancer types including colon [7], pancreas [8], liver [9], breast [10] and glioma [11]. However, this approach has been criticised because of the artificial immunocompromised environment into which the cells are transplanted. The definition of CSC and the cell surface markers used to identify them continue to be a matter of intense debate [12]. While the stochastic model and the CSC model ascribe a different origin to tumour heterogeneity, they are not mutually exclusive and a combination model has also been proposed [3] whereby hierarchical organisation can be driven by a dominant clone and it can also be modified due to genetic and epigenetic events that impair cell differentiation [13]. Recently, a few new models have been proposed to

explain clonal evolution including the “Big Bang” and neutral evolution models, which hold that the rate of tumour evolution is dynamic with periods of intense mutational bursts and relative stasis [14,15]. This view contrasts with the traditional model that clonal evolution arises gradually. While this model was initially observed in colorectal tumours, emerging evidence suggests neutral evolution may be common [15].

3. Prognostic indicator

Cancer prognosis is traditionally determined by the presence or absence of certain histological features within a tumour and the determination of stage and grade, which are ‘artificial’ features of the inherent biology of the tumour. In some cancers, molecular markers are a mainstay of prognosis. For instance, oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) status for breast cancer [16] and KRAS mutations for colorectal cancer [17] are biomarkers that determine treatment options. In clinical practice these determinants are often based on a single biopsy taken at a specific point in time, which may or may not be representative of the entire tumour or capture the changing behaviour of the tumour in response to therapies. For pathologists, tumour heterogeneity poses a challenge as they decide which samples of the entire tumour are taken for histological analysis and which are discarded. For small tumours (≤3 cm in diameter) often the entire tumour can be analysed, while in larger tumours sampling the entire tumour is not cost-effective or indeed time effective. In deciding which samples to include in the analysis, pathologists follow international guidelines [18,19] with the aim of obtaining accurate representation of the entire tumour.

To address these issues, the pathologist must move beyond histopathological characterisation of tumours. New methods are emerging to capture multi-level tumour heterogeneity including combining bright-field dual-colour chromogenic and silver in situ hybridisation assays along with image-guided computational workflow (ISHProfiler), which has been tested in several types of solid tumours. For instance, Zhong and colleagues used this technique to provide information on tissue morphology and copy number variation at a single-cell resolution and then used statistical analysis to quantify heterogeneity of a variety of genes across several cancer types [20]. One of the alterations they analysed was PTEN deletion, which is known to be heterogeneous, subclonal, and associated with tumour progression. An ISHProfiler was used to generate signal colour maps of each tissue core whereby PTEN was illustrated using coloured squares. The tissues were then classified visually into two groups: homogeneous or heterogeneous; three subclasses: homogeneous deletion and non-deletion, and heterogeneous; and six further subgroups: homozygous deletion, hemizygous deletion, cellular homogeneity, cellular heterogeneity with either homogeneous or heterogeneous genetic status, and intra-tumour heterogeneity. Importantly, this technique allows the integration of genome sequencing with the spatial and morphological context and allows tumour heterogeneity to be quantified. Additionally, while genome sequencing often fails to detect minor subclones and the presence of mutations in the same or different cells, this can be achieved using ISHProfiler. Moreover, it can be performed on large cohorts unlike single cell sequencing.

Additionally, spatial transcriptomics, which captures positional information of RNA sequencing data in an intact tissue section, may lead to far more precise tumour characterisation that incorporates heterogeneity. For instance, Stahl and colleagues developed a method for determining the spatial distribution of RNA transcripts by annealing fixed cancer tissue directly to barcoded reverse transcriptase primers. Then, reverse transcription is performed followed by sequencing and computational reconstruction for multiple genes [21]. This group also applied spatial transcriptomics to obtain spatially resolved unbiased high-throughput gene expression data in bulk and combined this with histological staining in mammalian tissue [22]. These techniques incorporate positional information with RNA sequencing, remove the need for laborious single-cell isolation and sequencing, while allowing a greater characterisation of tumour heterogeneity.

Several studies support the idea that intratumoural heterogeneity matters because it provides a barrier to reliable prognosis. Gerlinger and colleagues characterised intratumour heterogeneity at the genetic level in renal cell carcinoma [23]. They sampled spatially distinct regions of a single renal tumour (**Figure 3**). Of all the somatic mutations detected using multi-regional sequencing, 63-69% were not detectable across all regions of the tumour. Specifically, loss of function mutations in the tumour suppressor genes PTEN, SETD2, and KDM5C were spatially separated within a tumour. They concluded that different regions contain either favourable or unfavourable prognostic signatures, which can give an inaccurate prognostic picture if sampled individually. Assigning tumour subtype and grade is challenging for pathologists in the face of marked morphological heterogeneity, such as is found in renal cell carcinoma collision tumours which are composed of multiple tumour subtypes [24]. When differing tumour grades are present in a single sample, the highest grade is typically assigned [25], which is not reflective of the tumour biology and may be misleading. For instance, in a study using whole-genome sequencing to track the evolution of the lethal clone in a patient who died of prostate cancer, the clone arose from a relatively low-grade focus and not a higher-grade primary cancer [26]. These studies highlight the importance of multiregional and longitudinal sampling and challenge a feature-based approach to prognostication.

Another study supporting the role of multiregional sampling is a retrospective study where the heterogeneity of the foci in multifocal invasive breast carcinoma was related to shorter survival [27]. Five individual foci were sampled from 574 patients and heterogeneity in tumour grade was found in 5.5% of cases. Between the foci examined in a single patient, the molecular phenotype differed in 10-12.7% of patients. Patients who had more heterogeneous phenotypes had a shorter survival. This study concluded that sampling additional tumour foci has an impact on therapeutic decisions, as it did for 8 out of 574 patients in this study. Arguments against multifocal sampling are based on the finding that a large majority of multifocal carcinomas were homogenous with respect to molecular phenotype and that actionable therapeutic consequences are only possible in a small group of patients. The benefit to a small group of patients must be weighed against associated risk of complications and additional costs associated with multifocal sampling.

Whether the degree of intratumour heterogeneity is a prognostic indicator itself remains actively debated. In support of this idea, a more diverse tumour may be more likely to generate a clone that is resistant to cancer therapy or have a greater metastatic potential and

therefore the patient may have a worse prognosis. If this is the case, adjuvant or earlier treatment may be justified to increase the chance of successful treatment with a smaller volume of disease. As an example, patients with intratumoural heterogeneity of HER2 expression and gene amplification are reported to have poorer survival than patients without HER2 heterogeneity, according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines [28]. However, conflicting evidence from another study demonstrated that the ratio of tumour cells with HER2 expression/centromere on chromosome 17 more than 2.2 was not correlated with poorer prognosis [29]. Nevertheless, ASCO/CAP guidelines recommend that tumours equivocal for HER2 should be further assessed by FISH in two or more representative fields of the invasive tumour [30] even though there are few large studies examining the clinical implications of intratumoural HER2 heterogeneity on prognosis. The role of intratumoural HER2 heterogeneity in assessing response to targeted treatment is addressed in the section that follows on targeted therapeutics.

Beyond breast cancer, the role of heterogeneity as a prognostic indicator continues to be an area of debate. Other studies have challenged the multiregional sampling approach and the robustness of heterogeneity as an independent prognostic indicator. Rini and colleagues demonstrated that despite substantial heterogeneity in clear cell renal carcinoma, a 16-gene signature could predict prognosis [31]. The gene signature was developed using a recurrence score algorithm based on the expression of 732 genes and clinical outcomes in 942 patients with stages I-III clear cell renal cell carcinoma. Of these genes, 516 were correlated with recurrence-free interval and 11 were selected as the most indicative of prognosis and combined with five reference genes to yield a 16-gene signature. The majority (77%) of the somatic mutations were homogenous and there was very low intratumoural heterogeneity in the 16 genes that comprised their recurrence score. However, the specific molecular features that have prognostic value has not reached a consensus and is likely highly dependent on the type of tumour.

Adding further support to the conclusion that heterogeneity itself is not a robust prognostic indicator, Andor and colleagues asked whether measures of genomic instability and genetic intratumour heterogeneity could predict overall and progression-free survival (PFS) in 1,165 cancers from 12 tumour types using univariate Cox models for each cancer type [32]. Interestingly, intratumour heterogeneity was not a linear prognostic biomarker and a reduced risk was observed for the most highly diverse tumours. While an increased risk of mortality was associated with an increase in the number of clones, this was only observed for up to four clones. Additional diversity beyond four clones did not increase mortality. Other studies have confirmed these findings showing that in ovarian, non-small cell lung cancers (NSCLC), gastric, and ER-negative breast cancers, intermediate levels of copy number variation is associated with the worst survival [33]. An upper limit of tolerable genomic instability may explain the non-linear relationship between the level of heterogeneity and survival. Additionally, there may be a range of heterogeneity that drives a proliferative advantage but beyond these limits heterogeneity may impede clonal growth.

4. Targeted therapeutics

Targeted therapy aims to exploit a tumour's reliance on a key survival pathway. Over the past several decades, this approach has significantly improved patient outcomes in a range of solid tumour types, including breast [34], colon cancer [35], NSCLC [36,37], chronic myeloid leukaemia (CML) [38], gastrointestinal stromal tumours (GISTs) [39], and renal cell carcinoma [40]. However, it is apparent that targeted therapies do not lead to improved survival for all patients and the clinical benefit is often of limited duration in patients who improve initially [41,42]. One explanation is that little progress has been made to incorporate information about metastatic or intratumour heterogeneity. For instance, patient management for breast cancer relies on aspects of *inter*tumour heterogeneity such as ER/PR/HER2 status of the primary tumour alone, but metastatic sites are hardly ever biopsied for phenotypic characterisation and *intra*tumour heterogeneity is rarely considered. Because of this, HER2 targeted therapy without chemotherapy is rarely sufficient to eradicate HER2-positive cancer cells when there is significant intratumour heterogeneity. In a single-arm phase II study enrolling HER2-positive breast cancer patients, HER2 intratumour heterogeneity was assessed by histopathological evaluation of two core biopsies from different geographical areas [43]. This metric was found to be a robust predictor of pathological complete response to a dual-HER2 targeted therapy regimen consisting of trastuzumab emtansine (T-DM1) and pertuzumab.

The relative effectiveness of imatinib in CML versus solid tumours is another example of how heterogeneity determines the success of targeted therapy. While imatinib is highly successful in CML with a low level of intratumoural heterogeneity and with reliance on a single driver mutation, it is less effective in solid tumours with increased heterogeneity and complex clonality [38,44]. One of the major challenges in delivering effective targeted therapies lies in obtaining an accurate view of the genomic landscape of a tumour to select an appropriate therapeutic regimen [45].

Tissue biopsies pose a major barrier to the relevance of tumour heterogeneity for the development of targeted treatments as the time required to yield actionable results is often not feasible. Currently, the gold standard for tumour genome profiling is paired tumour and normal tissue from biopsy. Sample processing required for histopathological assessment often leaves insufficient material for sequencing [46]. In a study involving 381 patients with NSCLC with biomarker testing, 29% of cases had insufficient tissue [47]. In NSCLC, in which genetics-based approaches for treatment selection has been established, next-generation sequencing and analysis of the tumour sample is not often obtained in time to influence clinical decision-making. In a retrospective study of 300 patients with NSCLC with biomarker testing, 80% of patients did not have biomarker information including EGFR and ALK results upon their first oncology appointment and empiric chemotherapy was started instead of targeted therapy [48].

An emerging solution to this challenge that may demonstrate intratumour heterogeneity "matters" in terms of guiding treatment in a time efficient manner are "liquid biopsies" based on circulating cell free DNA (cfDNA) or circulating tumour DNA (ctDNA), and circulating tumour cells (CTCs). While the mechanism of ctDNA release from tumour cells has not been

fully elucidated, most studies suggest apoptosis or necrosis of tumour cells as its main source [49]. These methods yield results more quickly than standard biopsies [50] and could potentially alleviate the challenges of spatial heterogeneity [51]. Given its short half-life, ctDNA may also be more reflective of the real-time tumour genome than other sources [52,53]. A meta-analysis of 16 studies examining the diagnostic and prognostic value of ctDNA in gastric cancer in terms of tumour size, stage, and *H. pylori* infection demonstrated a sensitivity of 0.62 (95% CI=0.59-0.65) and a specificity of 0.94 (95% CI= 0.89-0.98) [54]. Overall survival and disease-free survival were associated with the presence of ctDNA. Limitations include the introduction of potential bias due to the lack of well-established ctDNA targets and thus preferential selection of positive findings. While the specificity was high, the low sensitivity is a significant limitation. Also, the vast majority of studies included were from one geographical region, which may limit the applicability of these findings. A study examining ctDNA to monitor treatment response demonstrated that a decrease in cfDNA concentration and mutation allele frequency are associated with response to treatment with poly ADP-ribose polymerase (PARP) inhibition in prostate cancer [55]. Similarly, a case study from a patient with metastatic breast cancer followed over 3 years, ctDNA provided real-time information during treatment that correlated with disease progression and also with the tumour lesion imaging [51]. While these results need to be validated in larger cohorts, across different types of cancer, and with more consistent methodology, “liquid biopsies” hold the possibility for assessing prognosis in real-time.

Liquid biopsies have also been used to monitor the impact of individual oncogenic alterations on lesion-specific responses in colorectal cancer [56]. Through serial ctDNA monitoring, this study demonstrated that mutant MEK1 levels declined with treatment, but that another mutation, $KRAS^{Q61H}$, increased during therapy. $KRAS^{Q61H}$ was also found on a non-responding metastasis, revealing that monitoring of ctDNA can expose heterogeneous resistance mechanisms arising within individual lesions in the same patient.

Similarly, another study used ctDNA monitoring to identify mutations associated with resistance to targeted HER2 therapy in colorectal cancer as part of the HERACLES trial and follow up postmortem studies [57]. ctDNA was used to monitor the evolution of individual metastases during treatment and identify spatially resolved determinants of resistance. Currently, almost all clinical trials evaluating the efficacy of new anticancer drugs are based on RECIST criteria, which are based only on data from cytotoxic therapies and are not suited to evaluate the impact of heterogeneity. Thus, this study paves the way for an improved metric to evaluate therapeutic efficacy.

Recently, the US Food and Drug Administration approved use of cfDNA analysis for EGFR mutation analysis for NSCLC, which is the first blood-based companion test to determine which patients are candidates for the drug erlotinib. Despite showing promise as a biomarker they are not widely used in clinical practice and fresh tumour biopsies remain the gold standard.

General limitations of liquid biopsies include dilution by non-tumour DNA, which can complicate their interpretation [58]. Also, validation is lacking in terms of its sensitivity and specificity as a representation of clonal distribution and evolutionary pattern of a tumour. Finally, more research is required to interpret “liquid biopsy” findings due to the baseline

mutation rate in healthy individuals and the large percentage of mutations that do not result in a disease phenotype [59].

Ongoing clinical trials are examining the utility of "liquid biopsies" to monitor response to treatment in real-time. One such study is DETECT III, a multi-centre randomised phase III trial with 120 participants with the aim of addressing tumour resistance due to heterogeneity. This study, which ends in 2020, compares HER2-targeted therapy, lapatinib, combined with standard therapy to standard therapy alone in patients with HER2-negative breast cancer who have HER2-positive CTCs in their blood. The primary outcome measure is CTC clearance rate defined as the proportion of patients with at least one CTC detected in 7.5 mL of peripheral blood drawn before treatment that have no CTCs in the blood after treatment. The intervention tests whether heterogeneity detected through CTCs should guide treatment decisions when tumour resistance is evident. This study will help elucidate the utility of "liquid biopsies" in monitoring treatment response and resistance.

5. Drug resistance

Despite major advances in cancer therapies, including targeted therapies and check point inhibitors, most metastatic tumours remain fatal due to their remarkable capacity to evolve drug resistance [60,61]. Tumour heterogeneity is likely the reason why individual cancer cells have different responses to the treatment. Heterogeneous tumours are composed of a variety of subclones that can expand under selection pressures, such as chemotherapy (**Figure 4**) . The expansion of treatment-resistant clones during the course of cancer therapy has been shown in colorectal cancer [61], melanoma [60], NSCLC [62,63], CML [64], glioma [65]. For instance, first and second generation EGFR tyrosine kinase inhibitors (TKI) are currently first-line management for NSCLC with activating mutations in the kinase domain of the EGFR gene [66]. However, resistance commonly develops. In 50% or more of the patients who develop disease progression, the EGFR T790M point mutation is found [63]. Thus, osimertinib, a third generation EGFR TKI was developed to target both the EGFR-TKI-sensitising and the EGFR T790M resistance mutation [67]. In a double-blind, phase III trial of 556 patients with previously untreated EGFR mutation-positive NSCLC, the median progression-free survival of those treated with osimertinib was longer than standard EGFR-TKIs (18.9 versus 10.2 months) [67]. Thus, sequencing targeted therapies to address anticipated treatment-resistant clones can be a successful strategy. To further complicate this approach however, recent studies also show that different metastases within the same patient have heterogeneous resistance mechanisms [56]. Additionally, the mutational damage to tumour DNA caused by chemotherapy may be propagating new drug-resistant subclones and lead to more aggressive disease [65,68].

The underestimation of tumour heterogeneity in terms of resistant mechanisms may explain the ultimate failure of most cancer treatments. The current approach relies on the principle of a maximum tolerated dose, which is the maximum dose of administered drug that results in an acceptable level of toxicity and side effects. By administering the highest possible dose in the shortest time, the rationale is to prevent resistance before it occurs. However, this assumes a homogenous population and that resistant populations are not present prior to therapy. Emerging evidence demonstrates that tumour cells can possess multiple and

heterogeneous resistant mechanisms prior to therapy [69,70]. Therefore, this strategy may simply promote the success of resistant clones and lead to their proliferation.

Combination chemotherapy using agents with overlapping drug toxicity and different mechanisms of action has been widely used with the aim of minimizing therapeutic resistance [71]. However, this approach is complicated by cross-resistance whereby development of resistance to one drug can lead to resistance to another drug [72]. Other approaches have been proposed to address the issues of tumour resistance and heterogeneity by manipulating the tumour population via its evolutionary response to treatment. Gatenby and colleagues proposed an "adaptive therapy model" whereby long-term therapeutic strategies can be developed to anticipate drug resistance before it occurs [73]. This approach was applied to two human breast cancer cell lines, MDA-MB-231/luc, a metastatic triple-negative line and MCF7, an ER-positive and less aggressive human breast cancer cell line, which were orthotopically implanted in the mouse mammary fat pat and treated with paclitaxel using adaptive therapy algorithms linked to tumour response [74]. Two adaptive therapy algorithms were assessed: dose modulation and treatment skipping. For dose modulation, doses were adjusted based on the tumour size measured by MRI twice per week. The dose was reduced by 50% if the tumour volume decreased by 20% or more. The drug dose was increased by 50% in the tumour increased in size by 20% or more. The dose was repeated if the tumour size was within 20% of the previous volume. The treatment-skipping algorithm began with a moderate dose and then the subsequent treatment was based on the percent of tumour growth rate measured twice per week. MRI was used to measure tumour size and guide the therapeutic approach. Animals treated with the "adaptive therapy" approach had increased PFS and their tumours were of a smaller size at the end of the study compared to controls treated with maximum dose density. Limitations of this study include: a small cohort of animals (n = 4 or 5 per treatment group), only two cell lines, and application to only one type of cancer.

Given the emerging evidence of the importance of heterogeneity and clonal evolution in driving tumour resistance, several clinical trials have attempted to use these Darwinian principles to address tumour resistance. Tracking non-small cell lung cancer evolution through therapy trial (TRACERx) [75,76] is an ongoing multi-regional observational prospective cohort study of 842 patients with primary NSCLC, which aims to characterise tumour heterogeneity trajectories in space and time through longitudinal tumour sampling and sequencing (**Figure 5**). The primary outcome measure is to characterise the relationship between intratumour heterogeneity and clinical outcome after surgery and adjuvant therapy after 5-years of follow-up. Study limitations include the lack of sequencing for the entire tumour, which may not capture a complete picture of tumour heterogeneity. Also, the study is not randomised and thus is mainly assessing the feasibility of collecting samples for patients and observing trends over time. Results from the first 100 patients have been recently reported [75] and it was found that most targetable driver mutations (including EGFR, BRAF, and MET) were almost exclusively clonal and arise early, which explains the uniform and robust treatment response seen when these mutations are targeted [74]. However, 75% carried late subclonal driver mutations, which were only apparent through a multiregional whole-exome sequencing approach. Also, an increased level of copy number aberrations was correlated with worse prognosis. Treatment response follow up data on these patients is pending and thus the role of these subclonal driver mutations is currently unknown.

Another clinical trial seeks to address tumour resistance by target early emerging clones identified by "liquid biopsy." Re-challenge with panitumumab driven by RAS dynamic of resistance (CHRONOS) is a phase II trial assessing the efficacy and safety of challenging panitumumab RAS-extended wild type metastatic colorectal cancer patients with resistance to EGFR targeted therapy as confirmed by ctDNA [77]. Patients' ctDNA is monitored during treatment for the presence of extended-RAS mutations and a re-challenge is timed according to levels of mutant RAS ctDNA. In preclinical models the RAS-axis mutant cones decay when cetuximab is withdrawn, suggesting that tumour cells can regain sensitivity to further treatment. Additionally, ctDNA levels of mutant RAS has been shown to correspond to patients that benefited from re-challenge treatment [78]. This trial demonstrates that the use of "liquid biopsy" can be used to track the clonal evolution of tumour cells and may be a strategy to address resistance to targeted therapies in colorectal cancer.

Another ongoing clinical trial, Posthumous Evaluation of Advanced Cancer Environment (PEACE) Study, is a multi-centre prospective observational study that aims to integrate deep whole-exome sequencing data from primary tumours after surgical resection with sequencing data from metastatic sites from post-mortem samples in patients who have died from advanced cancer. Through longitudinal sampling from diagnosis to death, this study hopes to capture the events leading to metastasis and identify specific lethal subclones. By combining data from TRACERX and PEACE studies [79,80], it is hoped that the evolutionary trajectory of tumours can be characterised and inform methods by which disease progression can be monitored and predicted.

8. Expert opinion

Since the first description of tumour heterogeneity by Muller and Virchow in 1833, we now possess improved abilities to detect tumour heterogeneity and an enriched understanding of its importance. The genomic instability that gives rise to this heterogeneity is now considered a hallmark of malignancy [81]. While the morphological perspective on tumour heterogeneity dominated for many decades, more recently other methods have been developed to detect and characterise tumour heterogeneity. While *inter*tumoural heterogeneity has formed the basis of current prognostic indicators and targeted therapeutics for select cancer types, *intra*tumour and metastatic heterogeneity is largely ignored in patient management. An improved understanding of intratumour heterogeneity has the potential to revolutionise prognostication, combat drug resistance, improve the efficacy of targeted therapies, and ultimately lead to better survival outcomes.

However, despite our improved ability to detect the complexity of tumour heterogeneity, much work is required to incorporate this information into actionable diagnostic, prognostic, or therapeutic strategies. An enhanced understanding of heterogeneity and more advanced methods to detect it, may not translate into clinical utility. For instance, while increased information about tumour heterogeneity can be gained by performing multiple biopsies from the same patient, it is challenging, increases the risk of side effects, and is costly. Furthermore, the utility of this approach clinically has yet to be validated in large studies and may not be warrant the risks and costs. At the moment, this approach may

be better used to predict the risk of recurrent disease and inform the use of preventative medicines rather than treatment.

One of the major barriers to delivering effective targeted therapies lies in obtaining an accurate view of the genomic landscape of a tumour to select an appropriate therapeutic regimen. Thus, it is essential to move beyond histopathological characterisation of tumours and incorporate new methods to more precisely capture tumour heterogeneity. New emerging methods include combining bright-field dual-colour chromogenic and silver in situ hybridisation assays and spatial transcriptomics. These techniques are likely to change the metrics by which we evaluate therapeutic response to anticancer therapy and may replace RECIST metrics in future clinical trials. Additionally, advances in "liquid biopsy" and single-cell whole genome sequencing may lead to genetic mapping of entire tumours at the level of individual cells and faster biomarker data that could advance current approaches to cancer treatment.

Several important clinical trials are currently underway that will be important for gathering evidence for the role of intratumour heterogeneity in clinical outcomes. Clinical trial strategies to assess the importance of intratumour heterogeneity are challenging, as they require repeated investigations in each patient and collaboration of clinicians and scientists. More trials are required that employ a randomised design and a large number of study participants. Ideally, intratumour heterogeneity could be assessed and monitored during disease progression for each patient using sequencing technologies. Monitoring clonal dynamics and response to treatment could be helpful in characterising the genetic changes underlying resistance and inform therapy adjustments.

Overall, the increased understanding and enhanced tools to detect heterogeneity has the potential to improve diagnostics, prognostics, and especially the personalisation of anticancer treatment. For example, it may lead to overcoming treatment resistance and guide treatment switching for an individual patient in real-time. However, there are several key barriers preventing the adoption of these new concepts and techniques that must be overcome. These barriers including scientific uncertainty. For instance, the ongoing debate around the importance of heterogeneity as a prognostic marker and uncertainty about the utility of the "liquid biopsy." Additionally, expense in terms of both cost and time hinders the integration of our improved understanding of tumour heterogeneity into routine care. The recent emergence of a host of technological innovations and data from upcoming clinical trials may imminently change the landscape of this field.

Article highlights

- Much progress has been made in our ability to detect tumour heterogeneity and current methods now include *in situ* hybridisation, multi-regional sampling, cytological assays, and whole genome and single cell sequencing.
- Two major explanations have been proposed to explain the origin of tumour heterogeneity including the clonal evolution and the cancer stem cell model.
- Heterogeneity can provide a barrier to reliable prognosis due to biopsy sampling bias.

- Whether the degree of heterogeneity is a prognostic indicator remains actively debated and is likely highly tumour-type dependent.
- While *inter*tumour heterogeneity has been an important consideration in the selection of targeted therapeutics, *intra*tumour and metastatic heterogeneity is rarely considered.
- "Liquid biopsies" based on circulating cell free DNA, circulating tumour DNA, and circulating tumour cells provide a new approach to alleviate the challenges of spatial heterogeneity.
- Underestimation of heterogeneity of tumour resistance mechanisms may explain the failure of most cancer treatments. To address this, an "adaptive therapy model" to anticipate drug resistance has been employed successfully in a mouse model study.
- Several ongoing clinical trials using longitudinal tumour sampling and sequencing in space and time will be important for assessing whether treatment approaches that capture tumour heterogeneity in real-time will be beneficial for patient outcomes.

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Annotations

* Of interest
** Of considerable interest

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