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Tumour heterogeneity in the clinic

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Recent therapeutic advances in oncology have been driven by the identification of tumour genotype variations between patients, called interpatient heterogeneity, that predict the response of patients to targeted treatments. Subpopulations of cancer cells with unique genomes in the same patient may exist across different geographical regions of a tumour or evolve over time, called intratumour heterogeneity. Sequencing technologies can be used to characterize intratumour heterogeneity at diagnosis, monitor clonal dynamics during treatment and identify the emergence of clinical resistance during disease progression. Genetic interpatient and intratumour heterogeneity can pose challenges for the design of clinical trials that use these data.

There is great promise that knowledge of the biological drivers of cancer will lead to personalized cancer treatment. Oncologists increasingly use molecular characterization of a sample of primary or metastatic tumour to guide their selection of treatments for an individual patient. However, they usually rely on a limited sample of cancer tissue that cannot represent heterogeneity between and within patients.

Cancer genomics studies, including large-scale collaborative sequencing projects such as The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC), have catalogued genetic interpatient tumour heterogeneity for cancers of the same histological subtype. Non-genetic phenotypic and functional heterogeneity is also well recognized (see the Review by Meacham and Morrison on page 328), as is heterogeneity of the tumour microenvironment (see the Review by Junttila and de Sauvage on page 346). Comprehensive characterization of multiple tumour specimens obtained from the same patient illustrates that remarkable intratumour heterogeneity might exist between geographical regions in the same tumour (spatial heterogeneity), as well as between the primary tumour and a subsequent local or distant recurrence in the same patient (temporal heterogeneity). Inter- and intratumour heterogeneity pose a challenge to personalized cancer medicine because a single needle biopsy or surgical excision is unlikely to accurately capture the complete genomic landscape of a patient's cancer. Genomic characterization of cell-free circulating tumour DNA (ctDNA) or circulating tumour cells (CTCs) may offer an opportunity to assess clonal dynamics throughout the course of a patient's illness and identify drivers of therapeutic resistance. Here, we review the clinical implications of interpatient and intratumour heterogeneity for cancer diagnosis, making a prognosis, treatment selection and resistance. We discuss how clinical trials that are restricted to molecular subtypes of cancer could incorporate studies of tumour heterogeneity so that we can better understand the clinical impact of heterogeneity on therapeutic effectiveness and the emergence of treatment resistance.

Current models for diagnosis and treatment

Modern cancer treatment is based on accurate tissue diagnosis of samples obtained from needle biopsy or surgical excision. Cancerous tissues are analysed under a light microscope to evaluate histopathology, and immunostaining and selected molecular tests are used to establish a specific cancer diagnosis. Treatment is based on the

anatomical location and tissue of origin of the primary tumour when cancer is localized to an organ site or when cancer has metastasized and the primary site can be identified by imaging or pathological examination. When solid tumours recur after treatment for localized disease or progress after systemic treatment for metastatic disease, taking another biopsy to guide treatment decisions is not routine¹. Instead, further systemic treatment of patients with progressive metastatic disease is typically based on the identification of predictive biomarkers in archived primary specimens, which may no longer represent the current disease such as *BRAF* mutation in melanoma, *HER2* (also known as *ERBB2*) amplification or overexpression in breast cancer, *KRAS* mutation in colorectal cancer and *EGFR* mutation in non-small-cell lung cancer^{2–6}.

Intratumour heterogeneity and clonal evolution

The current approach to molecular biomarker testing to inform cancer treatment focuses on interpatient tumour heterogeneity. However, there is a growing recognition that intratumour heterogeneity within the same patient is clinically relevant because the status of predictive biomarkers that are used for making clinical decisions may evolve during tumour progression, in particular metastatic dissemination of the primary tumour to a distant organ or for established metastatic disease under the selection pressure of treatment. Nowell's theory of clonal evolution states that cancers arise from a single cell of origin, develop genomic instability during replication and then undergo enrichment for the most aggressive clones through the processes of metastasis and the eradication of sensitive clones with cancer treatment⁷ (see the Reviews by Burrell *et al.* on page 338 and Klein on page 365). For example, discordance between oestrogen receptor (ER) expression in a primary breast cancer and subsequent distant metastases that may appear many years after completion of primary treatment is observed in 7–25% of patients^{8–11} (Table 1). Change in ER status may have important implications for treatment because patients with tumours that lack ER expression do not benefit from treatment with endocrine therapy such as tamoxifen or aromatase inhibitors¹². Loss of ER or *HER2* expression in breast cancer during metastasis is associated with a poorer outcome^{13,14}. Although data from other tumour types are more limited, discordance of prognostic or predictive biomarker testing results between the primary tumour and metastases has been reported in other settings^{15–24} (Table 1).

Before metastases are clinically apparent, clonal heterogeneity can

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Table 1 | Selected single parameter biomarker tests that are routinely used to inform clinical decision-making for advanced solid tumours, and reported frequencies of discordance between primary tumours and metastases

Tumour type	Biomarker	Prognostic or predictive biomarker	Evidence of discordance
Oligodendroglioma	1p and 19q co-deletion <i>MGMT</i> promoter methylation	Prognostic/predictive Prognostic/predictive	Not applicable
Medullary thyroid	<i>RET</i> mutation	Prognostic ¹⁰²	Unknown
Breast	ER expression	Prognostic/predictive	7–25% ^{8,11,14}
	PR expression	Prognostic	16–49% ^{8,11,12,14}
	<i>HER2</i> amplification	Prognostic/predictive	3–24% ^{13,24}
Lung	<i>EGFR</i> mutation	Prognostic/predictive	0–38% ^{103,104}
	<i>EML4-ALK</i> translocation	Prognostic/predictive	1–2% ^{18,105}
Gastric	<i>HER2</i> amplification	Prognostic ¹⁰⁶ /predictive ¹⁰⁷	1–3% ^{20,21}
Colorectal	<i>KRAS</i> mutation	Predictive	0–10% ^{22,108}
Melanoma	<i>BRAF</i> mutation	Prognostic/predictive	4–25% ¹⁰⁹
Gastrointestinal stromal	<i>KIT</i> mutation	Predictive	Acquired mutations evolve during tyrosine kinase inhibitor treatment ^{110,111}
	<i>PDGFRA</i> mutation	Predictive	

ER, oestrogen receptor; PR, progesterone receptor

be identified within the primary tumour²⁵. For example, complex patterns of *HER2* gene amplification detected by fluorescence *in situ* hybridization are seen in breast²⁶ and gastro-oesophageal cancers²⁷. Similar patterns of regional intratumour heterogeneity have been observed with mutation testing in other tumour types, including *KRAS* in colorectal cancer, *BRAF* in melanoma and *EGFR* in non-small-cell lung cancer^{23,28–31}. Intratumour heterogeneity may account for resistance despite the matching of targeted treatment to the mutation, such as trastuzumab for *HER2* amplified breast cancer³², *EGFR* monoclonal antibody treatment for *KRAS*-wildtype-colorectal cancer³³ and *EGFR* tyrosine kinase inhibitor treatment for *EGFR* mutant non-small-cell lung cancer³⁴, through the selection of clonal subpopulations with mutations that confer treatment resistance.

Strategies to measure intratumour heterogeneity

Recognition of intratumour heterogeneity to inform treatment decisions requires test methods that can be applied to clinical tumour samples. Genome-scale technologies provide an unbiased characterization of clonal heterogeneity within tumours beyond a specific genetic locus or a set of loci (see the Review by Burrell *et al.* on page 338). Studies with karyotype analysis and comparative genomic hybridization allow for detection of clonal subpopulations within the same tumour that can be differentiated on the basis of DNA content and chromosomal imbalances³⁵. Newer techniques such as single nucleotide polymorphism arrays provide greater resolution and can identify smaller-scale allelic imbalances in specific genetic loci. Next-generation sequencing technology allows for the systematic enumeration of single nucleotide mutations and the identification of rare clonal subpopulations that are present in a small fraction of tumour cells. Sequencing studies of normal tissue, early pre-malignant precursors and malignant lesions derived from the same patient have been performed in secondary acute myeloid leukaemia derived from myelodysplastic syndrome³⁶ and invasive breast cancer with adjacent pre-invasive neoplasia³⁷. Clonal lineage has been reconstructed with the identification of antecedent founding clones in a pre-malignant precursor from which malignant disease evolved with the outgrowth of subclones with additional genomic alterations³⁷.

In metastatic disease, recent studies have characterized the emergence of treatment-resistant subclones that were present at a minor frequency in the primary tumour^{38–44}. This raises the tantalizing possibility that the model of cancer diagnosis and treatment in the future could involve characterization of subpopulations within the primary tumour⁴⁵, monitoring of clonal dynamics during treatment and eradication of treatment-emergent clones. Clinical sequencing using

less invasive sampling methods such as cytology specimens, CTC analysis and ctDNA would greatly facilitate this approach^{42,43,46–52}. A recent study⁵² demonstrated that ctDNA detected using targeted gene sequencing for *PIK3CA* and *TP53* mutations was associated with survival in patients with metastatic breast cancer. Levels of ctDNA were more closely correlated with response to treatment than CTCs or levels of the circulating cancer antigen CA15-3 detected in serum⁵³. A further study involving serial ctDNA exome sequencing of six patients with advanced solid tumours demonstrated an increased representation of certain mutant alleles with the emergence of treatment resistance⁵⁴.

Challenges of clinical assessment

Beyond initial proof-of-concept studies, larger clinical efforts are required to evaluate whether in-depth genomic characterization and serial monitoring of clonal dynamics leads to better patient care. The falling cost of next-generation sequencing has made high-coverage DNA sequencing of clinically relevant cancer genes accessible at the point of care^{55,56}. Genomic assessment of interpatient and intratumour heterogeneity in the clinical environment⁵⁷ has several practical challenges.

Surgical resections of primary tumours or metastatic lesions provide large volumes of tumour tissue that are required for assessment of regional heterogeneity and clonal diversity. Tumour specimens are routinely formalin fixed and paraffin embedded (FFPE) after surgical excision to preserve histology. Although tumour nucleic acids can degrade with formalin fixation and this can limit researchers' ability to perform genome-scale analyses, particularly for RNA sequencing, advances in technology mean that the analysis is becoming more feasible. In addition, deciphering the precise spatial orientation of stored FFPE tumour blocks using the routine clinical annotation that is included in surgical pathology reports to reconstruct intratumour heterogeneity can be difficult. Serial characterization of metastatic lesions through core needle biopsy could be used to identify clonal evolution, but sampling bias may occur because only a limited geographical region of a tumour is analysed. ctDNA is more amenable to serial sampling and presumably represents cancer genomes from multiple metastatic sites. However, ctDNA analysis is in its infancy and is not yet routinely established in the clinical environment. Furthermore, whether there are important mutations that are unique to non-circulating populations of tumour cells is not yet known.

In the United States, clinical laboratories that test human specimens for the purpose of providing information on diagnosis, prevention or treatment of disease to the supervising physician must adhere to Clinical Laboratory Improvement Amendments (CLIA) standards and be accredited by the College of American Pathologists (CAP) for reimbursement⁵⁸, and similar regulatory standards exist in

other countries. Genome-scale sequencing was previously outside the purview of a clinical laboratory owing to the cost of massively parallel sequencing platforms, high-performance computing capacity and the sophisticated bioinformatics expertise that was required for sequence alignment and mutation calling. The recent development of bench-top next-generation sequencing instruments that offer high coverage ($\geq 250 \times$ read depth) of a large targeted panel of clinically relevant cancer genes is well suited to the work flow of a clinical laboratory^{59–61}. The Next Generation Sequencing Standardization of Clinical Testing (Nex-StoCT) workgroup recommends that all clinically actionable mutations should be confirmed by independent analysis using an alternative method before reporting to the treating clinician⁶². This poses a problem when high-coverage next-generation sequencing identifies a low-frequency mutation that cannot be confirmed by Sanger or PCR sequencing owing to the limitations of sensitivity of direct sequencing methods.

Mutation verification can delay the reporting of results to the oncologist if multiple clinically actionable variants are detected by next-generation sequencing. Patients with metastatic cancer and their oncologists may not be willing to wait for these results before initiating a new treatment⁶³. Deciding which mutation or mutations are clinically relevant, prioritizing mutations for treatment matching when multiple mutations are detected and developing a framework to report results to clinicians that can be easily interpreted are complex tasks. Few mutations have been validated with a high level of evidence for the prediction of treatment response⁶⁴. Specific mutations may

have different clinical implications depending on a cancer's tissue of origin, such as *BRAF*(V600E) mutation in patients with melanoma or colorectal cancer^{65,66} and their response to vemurafenib monotherapy. For mutations in tumour-specific contexts for which there are no clinical studies available, preclinical drug sensitivity encyclopaedias can be mined to infer potential clinical relevance^{67,68}. However, there are concerns about validating predictive genomic biomarkers across cell-line screening data sets⁶⁹ and the lack of reproducibility of pre-clinical experiments⁷⁰.

Trial designs that assess tumour heterogeneity

Despite the challenges associated with genomic assessment in the clinical environment, molecular characterization — from genotyping to targeted genome sequencing — through the use of stored FFPE samples or serially procured fresh tumour biopsies is increasingly used to complement histopathological diagnosis. Clinical-trial design frameworks for cancer diagnostics and therapeutics must be developed to efficiently and dynamically incorporate such genomic data and assess the value of matching profiled patients to specific interventions or targeted therapies.

There are several premises on which clinical-trial design frameworks in the cancer genome era are based. First, genetic aberrations exist in human malignancies with a subset that are present in different cancer types at variable frequencies. Aberrations with functional relevance that lead to cancer initiation, growth and metastasis are the targets of greatest clinical interest because they could potentially be used for diagnosis, prognosis and predicting response to therapy.

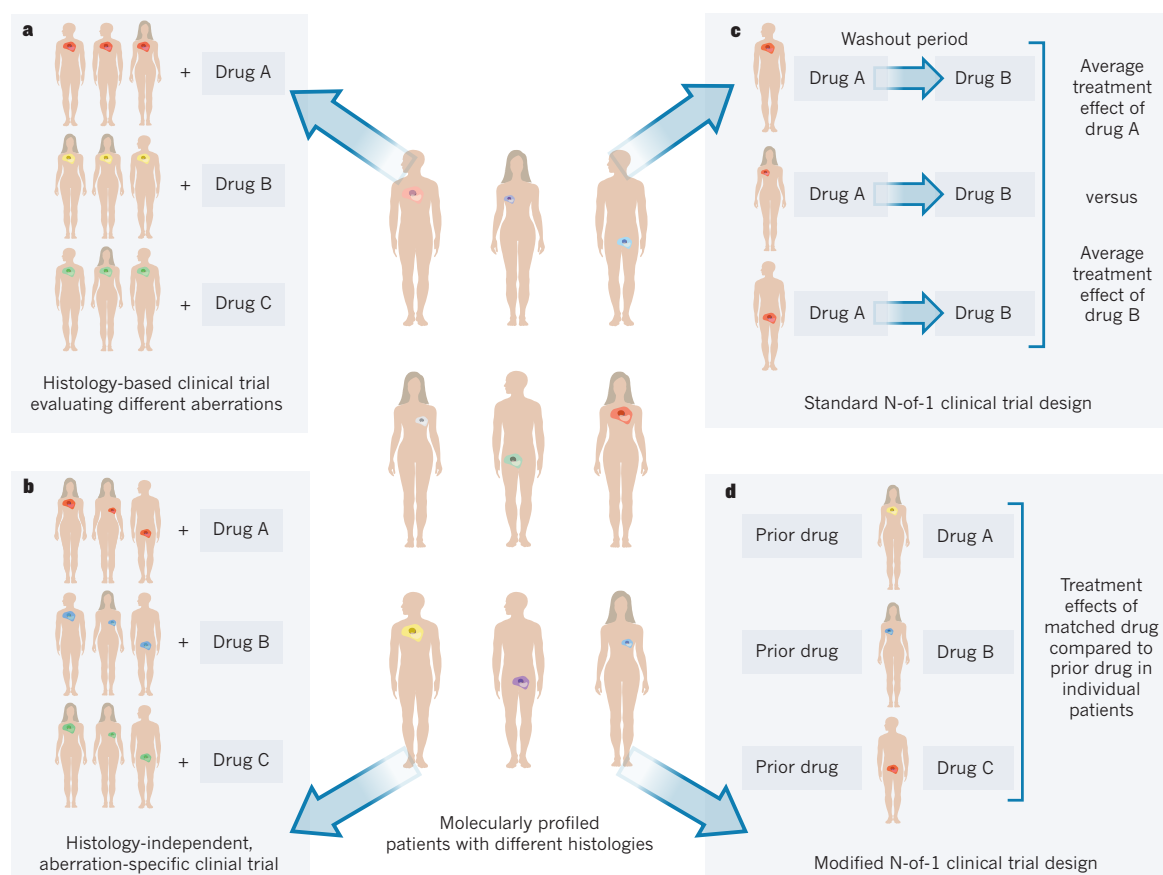


Figure 1 | Clinical-trial design frameworks. In a population of molecularly profiled patients who have tumours of different histologies (shown by position of tumour) and molecular aberrations (shown as different colours), the framework for a clinical trial can take a number of forms. **a**, Histology-based clinical trials evaluate different molecular aberrations by enrolling patients with the same tumour histology but who harbour different aberrations, and match groups of patients to different drugs. **b**, Histology-independent, aberration-specific clinical trials, or ‘basket’ trials, enrol patients with different tumour histologies but who

harbour the same or related molecular aberrations, and match drugs to the aberration specific or related groups. **c**, Standard N-of-1 trials randomly assign patients to different drugs in different sequential orders, with washout periods between drugs to minimize crossover effects. At completion, the individual effect of each drug and the average effects of each drug across individuals can be analysed. **d**, Modified N-of-1 trials use each patient as his or her own control and compare the treatment effect of the current matched drug with that of the most recent earlier drug.

Table 2 | Selected worldwide large-scale clinical molecular profiling programmes by institution or consortium

Trial or programme name	Platforms or techniques	Genes and mutations	Cancer types	Tumour sample
Cancer Research UK, London				
Stratified Medicine Programme ¹¹²	PCR FISH	9 genes 3 genes	Melanoma, NSCLC, CRC and breast, prostate and ovarian cancer	Archival
Dana-Farber Cancer Institute, Boston, Massachusetts				
PROFILE ¹¹³	Sequenom	OncoMap: 41 genes, 471 mutations	All solid tumours	Archival
Curie Institute, Paris; French National Cancer Institute				
SHIVA (NCT01771458)	Ion Torrent PGM CytoScan HD	AmpliSeq: 46 genes 29 genes	All solid tumours	Fresh biopsy
Gustave Roussy Institute, France (non-paediatric trials)				
MOSCATO ⁷⁵ (NCT01566019)	aCGH PCR	NA 96 mutations	Solid tumour phase I patients	Fresh Biopsy
SAFIRO1 (NCT01414933)	aCGH PCR	NA 2 genes	Breast cancer	Fresh Biopsy
MSN	PCR FISH	Seqcan: 30 genes 5 genes	Melanoma, SCLC and NSCLC	Fresh Biopsy
Massachusetts General Hospital, Boston				
NS ¹¹⁴	SNAPshot	14 genes, >50 mutations	NSCLC, CRC, melanoma and breast cancer	Archival
MD Anderson Cancer Center, Houston, Texas				
T9 Program ¹¹⁵	Sequenom	>40 genes	All solid tumours	Archival
IMPACT ⁷³ (NCT00851032)	PCR FISH	10 genes 1 gene	All solid tumours	Archival
Clearing House protocol ¹¹⁶	PCR Illumina NS, Ion Torrent NS NS	~100 genes T200: 200 genes Whole genome	All solid tumours	Archival or fresh biopsy
Memorial Sloan-Kettering Cancer Center, New York				
IMPACT (NCT01775072)	Illumina HiSeq Sequenom or MiSeq	275 genes (Research assays) NS (Clinical assays)	All solid tumours	Archival
Netherlands				
Centre for Personalized Cancer Treatment ¹¹⁷	Ion Torrent PGM 5500xl SOLiD	~150 genes >2,000 genes	Solid tumours	Fresh biopsy
Norwegian Cancer Genomics Consortium				
Nationwide programme ¹¹⁸	NS	Whole exome	9 tumour types, both solid and haematopoietic	Archival or fresh biopsy
Princess Margaret Cancer Centre, Toronto, Canada				
IMPACT ⁶⁰ (NCT01505400)	MiSeq Sequenom	TSACP: 48 genes, >700 mutations. Customized panel: 23 genes, 279 mutations	Selected solid tumours	Archival
Vall d'Hebron Institute of Oncology, Barcelona, Spain				
NS ^{72, 119}	Sequenom Illumina GAIx	OncoCarta, 19 genes, 238 mutations NS	Breast cancer, solid tumour phase I patients	Archival
Vanderbilt-Ingram Cancer Center, Nashville, Tennessee				
PCMI ¹²⁰	SNAPshot	6–8 genes and >40 mutations	Melanoma, NSCLC, CRC and breast cancer	Archival
WIN Consortium				
WINTHER ⁸³ (NCT01856296)	NGS CNV CGH	NS NS NA	Solid tumours	Fresh biopsy (tumour and matched normal)

aCGH, array comparative genomic hybridization; CGH, comparative genomic hybridization; CNV, copy number variation; CRC, colorectal cancer; FISH, fluorescence *in-situ* hybridization; GAIx, genome analyzer Iix; NA, not applicable; NGS, next-generation sequencing; NS, not stipulated; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction; PCMI, personalized cancer medicine initiative; PGM, personal genome machine; SCLC, small-cell lung cancer; TSACP, TruSeq amplicon Cancer Panel.

Second, there are specific interventions or tolerable medicinal agents that may effectively modulate such targets. Last, intratumour heterogeneity and clonal evolution occur and there are feasible technologies to measure these phenomena in the clinical setting. The reliable quantification of both spatial and temporal variations in

the molecular landscape of cancers would enable the development of therapeutic strategies to interrogate them. Although, currently, most approved targeted therapies and clinical trials focus on inter-patient heterogeneity, considering intratumour heterogeneity will increasingly become important in the future.

Trial designs for interpatient heterogeneity

Clinical-trial design frameworks that focus on interpatient tumour heterogeneity are possible, assuming that detailed genomic characterization is feasible⁷¹ (Fig. 1).

Longitudinal cohort with nested trials

One framework currently used by many large cancer institutions and national cancer cooperative groups is to prospectively profile a large number of patients to establish a longitudinal cohort with clinical annotation such as demographics, histopathological diagnosis, earlier therapies and outcome (Table 2). Thus far, most clinical molecular profiling programmes worldwide have focused on the genomic characterization of limited but presumably representative specimens obtained at a single time point, typically in patients with metastatic disease who are suitable for systemic therapy. It is logical that a current sample would more accurately reflect the patient's current disease than an archived sample, although it is unknown whether a small current specimen (for example, from a needle biopsy) is preferable to a larger historical sample (for example, from surgical resection). In instances in which archived tumour tissue is too scant to yield sufficient DNA, or has been exhausted owing to serial evaluations of single markers, then a fresh tumour biopsy would be necessary for genomic profiling. Although there is great enthusiasm for molecular characterization of tumour samples, the clinical use of this approach is still unproven. Some clinical trials of targeted drugs limit enrolment to patients with specific molecular perturbations; however, the effectiveness of such drugs is usually unconfirmed. Nonetheless, the coupling of a molecular characterization strategy with a drug development programme has been widely embraced, despite disparate results from different retrospective series and the lack of definitive supportive data^{72,73}. In this context, patients with specific molecular aberrations are often 'opportunistically' enrolled into clinical trials of matching targeted agents. This framework is attractive to those running large programmes who have access to a robust panel of early phase clinical trials that test different molecular targets^{60,63,73–75}. The panel of clinical trials can be 'nested' or embedded as distinct research activities under the auspices of an overarching platform of molecular profiling and target–drug matching.

Histology-based design

Other frameworks involve the evaluation of the target–agent matching strategy in large, prospectively conducted clinical trials (Table 2). For instance, histology-based and biomarker-integrated multicentre clinical trials aim to assess a variety of targeted agents matched to specific molecular profiles within a single tumour type (Fig. 1a). The FOCUS 4 trial supported by the UK Medical Research Council, for example, will enrol patients with advanced colorectal cancer who have responsive or stable disease after 16 weeks of chemotherapy⁷⁶. On molecular profiling, patients with tumours that harbour commonly mutated oncogenes such as *KRAS*, *BRAF* or *PIK3CA* will be given targeted agents or a placebo. Other histology-based clinical trials include the US-based BATTLE-2 trial (NCT01248247) in lung cancer and the I-SPY 2 trial in breast cancer (NCT01042379)^{77–79} (Table 3).

Histology-agnostic, aberration-specific design

An alternative framework employs a histology-agnostic, aberration-specific design in which patients whose tumours harbour identical or related molecular profiles are treated in the same 'basket' with the same therapeutic regimen (Fig. 1b). An example is the inclusion of different tumour types that harbour *PIK3CA* mutations or amplifications into a basket trial that evaluates a PI(3)K α -isoform specific inhibitor (NCT01219699). This strategy may be adapted to increase enrolment of patients with tumour types that demonstrate early signals of antitumour activity while excluding those who lack preliminary response. Although this framework will not directly lead to regulatory approval, given its exploratory nature, it does provide a platform to determine the differences in functionality of the same molecular alteration across multiple cancer types.

N-of-1 clinical trial design

The N-of-1 clinical-trial design framework has been pursued for non-oncology diseases, most frequently in neuropsychiatric, pulmonary and musculoskeletal conditions^{80,81}. In their standard context, N-of-1 trials involve individual patients who are typically blinded and randomly assigned to different treatment regimens or to a placebo in different sequential orders, with washout periods, in which patients receive no treatment, between regimen alterations to minimize crossover effects (Fig. 1c). There are limitations to the application of this framework in oncology. For instance, the switch from one regimen to another may occur before there is sufficient time for antitumour activity to be manifested, such that there may be an underestimation of therapeutic efficacy while increasing the risk of inducing drug resistance. Modified N-of-1 designs have been used to investigate the value of individualized therapy. The concept of using each individual patient as his or her own control, for example, to assess the growth modulation index by comparing the time to progression or progression-free survival (PFS) on a current regimen with that attained on the most recent prior treatment, represents such a modification of the N-of-1 design⁸² (Fig. 1d). This framework may become increasingly relevant for subsets of patients with rare molecular alterations, for which large randomized trials may never be feasible. The WINTHER trial⁸³ (NCT01856296), led by the Worldwide Innovative Networking (WIN) Consortium, is an example of a modified N-of-1 design that is using a variety of advanced profiling technologies to comprehensively characterize oncogenic events in 200 patients with different cancers. The trial compares patients' PFS on therapy guided by profiling results with that achieved on the regimen immediately preceding trial enrolment. However, the validity of this approach is unknown, given the uncertain correlation in PFS between sequential inactive therapies⁸².

Trial designs for intratumour heterogeneity

Establishing clinical-trial design frameworks in the context of intrapatient tumour heterogeneity and clonal evolution is challenging because dimensions of both space and time must be incorporated to reflect the dynamic nature of tumour biological characteristics within individuals.

Geographical heterogeneity

The execution of the aforementioned frameworks is typically based on molecular profiling of tumour specimens obtained from one geographical location. These samples, in addition to other biorepositories such as tissue banks and autopsy programmes, provide a means to build knowledge bases that help us to gain insight into complex molecular events such as intratumour heterogeneity^{40,84,85}. One such initiative to build this type of knowledge base is the REACT study (NCT01505400). The aim of this study is to genomically evaluate all archived tumour samples from a cohort of molecularly profiled patients to assess heterogeneity and clonal evolution.

To prospectively assess geographical or spatial heterogeneity, profiling of multiregional tumour samples would be indicated. Although this is feasible (but rarely performed in surgical resections), it is impractical and potentially risky to take biopsies from multiple deep-seated metastatic lesions in every patient to examine the genotypes of different tumour cell clones. If tumour biopsies using fine-gauge needles (23-gauge or smaller) could yield sufficient quantities of tumour nucleic acids for molecular profiling, these would be an attractive alternative to large-bore needles owing to the lower risk of procedure-related complications. An ongoing prospective study called MATCH (NCT01703585) evaluating the quality and quantity of DNA obtained using different sizes of biopsy needles, could determine whether the use of fine-gauge needles is feasible for targeted sequencing. In addition, there are prospective and retrospective tumour-specific programmes that explore heterogeneity and evolution in relation to drug therapy (Table 3). The PREDICT programme for patients with renal cell cancer who are treated with neoadjuvant everolimus or sunitinib is an example of an explicitly designed study to evaluate heterogeneity in the primary tumour

Table 3 | Prospective and retrospective programmes that evaluate tumour heterogeneity by institution or consortium

Trial or programme	Platforms or techniques	Coverage and depth	Cancer types	Fresh tumour acquisition	Additional specimen collection	Drugs	Additional procedures	Type of heterogeneity
Dana-Farber Cancer Institute, Boston, Massachusetts; Broad Institute, Cambridge, Massachusetts; and Brigham and Women's Hospital, Boston								
CanSeq ¹²¹	Sequenom NS	OncoMap Whole exome, depth NS	NSCLC, CRC, MBC and prostate cancer	Biopsy at set time point depending on tumour type	No	No	No	Interpatient
Heinrich Heine University, Düsseldorf								
DETECT III ¹⁰¹ (NCT01619111)	NS	NS	HER-2 ⁺ MBC with HER2 ⁺ CTCs	No	CTCs (CellSearch)	SOC chemotherapy or endocrine therapy +/- lapatinib	No	Intratumour
Massachusetts General Hospital, Boston								
Biopsies of Cancer Patients for Tumor Molecular Characterization (NCT01061944)	NS	Genes NS, depth NS	All solid tumours	Biopsy of metastasis (SOC)	NS	No	No	Interpatient
Mayo Clinic, Scottsdale, Arizona								
BEAUTY ¹²²	NS	Whole genome, depth NS	Non-metastatic breast cancer	Biopsy of primary pre- and post-neoadjuvant chemotherapy. Primary resection	NS	Paclitaxel +/- trastuzumab AC or FEC	Xenografts	Intratumour Interpatient
MRC Clinical Trials Unit, London								
FOCUS 4 (ref. 76)	PCR assays	3 genes	CRC	Diagnostic, on treatment and PD biopsy (lesion NS)	No	5 treatment arms	No	Interpatient
MD Anderson Cancer Center, Houston, Texas								
BATTLE-2 (NCT01248247)	PCR FISH	11 biomarkers	NSCLC, PD on chemotherapy	Biopsy (lesion NS)	NS	Erlotinib, MK2206, AZD6224, sorafenib	No	Interpatient
BATTLE-Front line (NCT01263782)			NSCLC, treatment naive					Interpatient
National Cancer Institute, Bethesda, MD								
I-SPY 2 (NCT01042379)	TargetPrint HER2, Mamma-print	71 genes	Stage 3 breast cancer	Pre-specified serial primary biopsies. Primary resection	Blood	Experimental drugs with SOC chemotherapy	Breast MRI	Interpatient
MPACT ¹²³	NS	22 genes for treatment, 80kb sequenced, 383 amplicons with ≥80% covered >450x	All solid tumours	Biopsy of metastasis	NS	MEK, mTOR, PARP, WEE1 inhibitors	No	Interpatient
MATCH ¹²⁴	NS	Genes and depth NS	All solid tumours and lymphoma. PD on 1 SOC treatment	Pretreatment and PD biopsy (lesion NS)	NS	Multiple targeted therapies on clinical trials	RNA-Seq	Intratumour Interpatient
PREDICT Consortium								
E-PREDICT S-PREDICT (ISRCTN 22979604)	GAllx HiSeq	Whole exome, transcriptome, average depth 30x	Renal cell cancer	Biopsy of primary and metastasis. Nephrectomy	NS	Everolimus or sunitinib	Functional RNA interference	Intratumour Interpatient
Princess Margaret Cancer Centre, Toronto, Canada								
MATCH (NCT01703585)	MiSeq	48 genes, 212 amplicons average depth ~1,000x	CRC, MBC, gynaecological cancers	Serial biopsies of metastases at study start and on PD	Blood: CTCs, ctDNA. Archival tumour	No	No	Intratumour Interpatient
	Sequenom	23 genes, 279 hotspots						
University College London								
TRACERx (NCT01888601)	NS	Whole genome, whole exome, depth NS	NSCLC	Biopsy of primary and metastasis. Primary resection	Blood: ctDNA	SOC chemotherapy	Functional imaging	Intratumour Interpatient

AC, adriamycin and cyclophosphamide; CRC, colorectal cancer; CTCs, circulating tumour cells; ctDNA, circulating tumour DNA; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; FISH, fluorescence *in situ* hybridization; GAllx, genome analyzer Iix; kb, kilobases; MBC, metastatic breast cancer; MRI, magnetic resonance imaging; NS, not stipulated; NSCLC, non-small-cell lung cancer; PCR, polymerase chain reaction; PD, progressive disease; SOC, standard of care.

Table 4 | The different clinical-trial design frameworks and tumour-sampling strategies that can be used to evaluate intratumour heterogeneity and clonal evolution from pre-malignancy to the development of resistant metastases, using breast cancer as an example.

Ductal carcinoma <i>in situ</i>	Localized cancer	Micrometastases	Macrometastases	Resistant disease
Clinical evaluation				
Surveillance of pre-malignancy to malignancy	Forming a prognosis of metastatic potential	Monitoring response to adjuvant therapy	Targeting treatment to match driver clones	Targeting treatment to match resistant clones
Evaluation strategies				
Multiregional sampling, if feasible	Multiregional sampling Monitoring using CTCs or ctDNA	Monitoring using CTCs or ctDNA	Multiregional sampling Molecular imaging Serial sampling CTCs or ctDNA	Multiregional sampling Serial sampling for CTCs or ctDNA Molecular imaging
Clinical-trial design frameworks				
Longitudinal cohort	Longitudinal cohort Histology-based design	Longitudinal cohort Histology-based design	Histology-based design Histology-agnostic basket design	Histology-based design Histology-agnostic basket design N-of-1 design

CTC, circulating tumour cell; ctDNA, circulating tumour DNA.

through multiregional sampling^{86,87}. Ultimately, the development of non-invasive visualization techniques, such as molecular imaging using radionuclide-based methods that can quantify the expression of tumoral targets with high sensitivity and specificity, would be ideal⁸⁸.

Temporal heterogeneity

Serial tumour sampling, especially at crucial time points in the disease course such as the development of metastatic disease or progression after initial response to systemic therapy, may reveal the emergence of dominant clones. This type of dynamic examination of clonal evolution is being conducted by programmes such as PREDICT^{86,87}. Until less invasive techniques such as characterization of CTCs or ctDNA are validated to yield sufficient sensitivity and specificity to be representative of clonal distribution and evolutionary pattern, fresh tumour biopsies will probably be used to monitor these events, although limited biopsies may also not reflect the full genomic landscape^{89,90}. Sensitivity of detection of somatic mutations is related to their frequencies in the analysed segments of cancer-related genes, and can be increased by using new techniques such as amplification and deep sequencing of selected genomic regions. ctDNA has already been used as a tracking tool for distinct existent clones⁵¹, as well as an early predictor of treatment response or resistance^{42,43,52}. Optimization of these methods to transition them from research to diagnostic laboratories would enable their applications in clinical trials and eventually in routine cancer care. Advances in molecular imaging that would make longitudinal surveillance possible would be desirable, although it is uncertain whether imaging can ever provide resolution at the level of target expression in tumour cells and be able to reflect changes in the clonal milieu.

Trial designs

The evaluation of geographical and temporal variations in tumour molecular profiles is complex. Their integration into the aforementioned clinical-trial design frameworks that focus on interpatient tumour heterogeneity is possible but would necessitate that the frameworks become dynamic models that consider changes across space and time within individuals. By using these frameworks, geographical heterogeneity and clonal evolution in tumour samples can be prospectively measured, but must first be correlated with clinical outcome to determine whether they portend a prognostic and/or predictive role. Interventions to modulate these phenomena would only be planned if they are demonstrated to have an important link to clinical outcome.

An example of intratumour heterogeneity in the clinic

Given the complexity of intratumour heterogeneity and clonal evolution, it is impossible to provide approaches that are universally applicable. As such, in reality, it is expected that adaptations of clinical trial designs for individuals will be tailored to the unique features of specific malignancies. By using breast cancer as a example, practical

applications of these frameworks for prognosis and therapy (Table 4) are discussed below.

Pre-malignancy to malignancy

The establishment of a longitudinal cohort would enable long-term follow-up of patients with pre-malignant lesions, such as ductal carcinoma *in situ* (DCIS), for whom the disease might progress to invasive breast cancer. Retrospective analyses of cases with synchronous DCIS and invasive ductal carcinoma have shown that this progression is associated with the appearance of subclones that harbour specific genetic aberrations, such as amplifications of *MYC*, *CCND1* and *FGFR1* (refs 91–93). The prospective quantification of geographical and temporal heterogeneity can be achieved by multiregional sampling of DCIS in surgical specimens, and by serial sampling in cases of DCIS recurrence. The identification of biomarkers of progression that may predict the transition from pre-malignancy to malignancy would be relevant. A comparison of surveillance strategies with or without molecular assessment of such biomarkers in different geographical locations and in serially collected samples of pre-malignant lesions can be undertaken to validate their prognostic role.

Metastatic potential of localized cancer

Both the longitudinal cohort strategy and the histology-based design to evaluate multiple aberrations would be reasonable frameworks to consider for metastatic potential of localized cancer. Comprehensive molecular portraits of the four main primary breast cancer subtypes (luminal A, luminal B, basal-like and HER2-enriched) have recently been published⁹⁴. Multiregional sampling and molecular profiling of primary tumour and regional lymph nodes can be carried out in patients who have undergone curative resections. In addition, depending on the sensitivity of detection, CTCs can be enumerated and profiled, and ctDNA can be extracted and analysed for the presence of somatic genomic alterations. Patients can then be monitored prospectively to determine if the detection of specific biomarkers in multiple locations within the primary surgical specimen or in the circulatory system can help to identify those tumours with biologically aggressive behaviour beyond the prognosis given by standard clinicopathological factors.

Monitoring for early micrometastases

After definitive local therapy and systemic adjuvant therapy, serial enumeration of CTCs or prospective sequential profiling of ctDNA can be performed, either as a longitudinal cohort or in a histology-based design to evaluate different molecular aberrations⁵¹. Single-cell exome sequencing to detect single nucleotide mutations is being developed^{95–97}, such that molecular characterization using captured CTCs could eventually be possible⁹⁸. These samples can be used as a tracking tool for distinct existent clones that can be assessed to monitor response to adjuvant therapy and to predict disease relapse.

Targeting oncogenic driver clones

In patients who develop macroscopic metastases from breast cancer, current systemic therapy consists mainly of hormonal therapy, cytotoxic chemotherapy and a limited number of targeted agents, such as HER2 inhibitors for HER2-positive tumours, or mTOR inhibitors in hormone-receptor-positive tumours⁹⁹. At present, other than HER2-targeting, selecting treatment based on a molecular profile is not proven to be superior to standard algorithms in metastatic breast cancer. As such, the design of therapeutic clinical trials that are either histology-based or histology-agnostic to evaluate the benefit of target–drug matching compared with conventional approaches, would be considered investigational. Exploring the impact of intratumour heterogeneity in a therapeutic context adds a further layer of complexity. Even if current technologies such as minimally invasive multiregional sampling of metastases or molecular imaging are able to identify functional tumour subpopulations that are geographically distinct, the design of clinical trials to interrogate these subpopulations is challenging. For instance, if two potentially important clones, one with *PIK3CA* mutations and the other with *FGFR1* amplification coexist, then hypothetical therapeutic possibilities can include either concurrent combination or sequential treatment with PI(3)K and FGFR1 inhibitors (ideally distinguished using carefully designed randomized trials). The accessibility to approved or experimental agents in such scenarios may be limited. Furthermore, the most optimal approach to combine or sequence two or more agents to yield sufficient biological target modulation with tolerable toxicity is often undefined and requires dose-finding studies. Finally, even if appropriate drug combination strategies are determined and can effectively suppress clonal evolution, thus ameliorating or delaying the onset of resistance, a previously undetected or new driver clone may ultimately arise. In contrast to the uncertainty of ‘drugging’ intratumour heterogeneity successfully, the use of CTCs or ctDNA as early biomarkers of treatment response of metastatic breast cancer seems to be more readily tangible⁵².

Emergence of resistant clones

Intratumour heterogeneity is a key factor that may lead to primary drug resistance because the extent of genomic assessment and molecular characterization determines our ability to identify potentially important subclones¹⁰⁰. In patients who have clearly responded to treatment but in whom disease subsequently progresses, a repeat tumour biopsy to detect the expansion of pre-existent resistant subclones or the emergence of newly acquired resistant clones may be highly informative. An important caveat is that clonal population size and architecture cannot be assessed through biopsy sampling of a single metastatic site. If a change in genotype is observed when another biopsy is taken at the onset of progression after systemic treatment, this may be due to either clonal evolution or as a result of an earlier false negative due to sampling bias. To circumvent such limitations of tumour biopsies, characterization of CTC or ctDNA in plasma could be an attractive alternative if they are demonstrated to be more reflective of the global molecular status. Furthermore, these circulating ‘liquid tumours’ may also precede radiological evidence of tumour growth^{42,43,54}. These strategies to identify and tackle primary or acquired resistance can be integrated into clinical trials using histology-based or histology-agnostic frameworks. For instance, DETECT III (NCT01619111)¹⁰¹ is a multicentre, histology-based, randomized phase III study that compares lapatinib (as a HER2-targeted therapy) combined with standard therapy with standard therapy alone in patients with HER2-negative breast cancer who have had HER2-positive CTCs detected in their blood. When the sample size is small, an N-of-1 trial design may be used to sequentially assess, in the same patient, the effects of different agents that may have antitumour activity against the resistant clones. It would seem logical to interrogate an emerging resistant clone as early as possible, using the combination or sequential therapeutic strategies previously described, although the timing for pharmacological counteraction of clonal evolution may also require full assessment through well-conceived clinical trials.

Future directions

The occurrence of intratumour heterogeneity and clonal evolution in cancers, resulting in malignant growth, invasion, metastasis and resistance acquisition has long been recognized. The availability of molecular profiling technologies such as next-generation sequencing coupled with advances in bioinformatics has enabled these previously elusive phenomena to be assayed in the clinical setting. The challenges ahead are immense, and include the reliable and accurate elucidation of geographical and temporal variations in patient samples and the subsequent correlation with both prognosis and treatment response. Current efforts are focused on gathering evidence to support the idea that intratumour heterogeneity substantially affects disease outcome, although the relationship is probably context dependent. Clinical trial strategies to interrogate intratumour heterogeneity are challenging, and for researchers to gain a deeper understanding into these molecular complexities would require not only the active participation of patients who are willing to undergo repeated investigations, but also the collaborative engagement of clinicians and scientists. Without a full understanding of the spectrum of a patient’s mutations, we may risk expending large resources on the development of fundamentally flawed approaches to biomarker-directed therapeutics.

The knowledge that significant intratumour heterogeneity is present in most patients has important implications for predictive biomarker development in the context of early clinical trials. First, quantitative biomarkers (for example, RNA expression) may be misleading, as they are based on the average expression across a heterogeneous tumour. Second, sequencing approaches may be misleading, unless careful attention is paid to detecting minor clones of clinical significance. Last, phenotypic and functional heterogeneity that results from events other than genomic alterations, for instance due to epigenetic alterations or plasticity, is likely to have an important effect on treatment response (see Review by Meacham and Morrison on page 328). Although we do not yet have the knowledge base to successfully individualize treatment by accounting for both interpatient and inpatient heterogeneity, we believe that the delivery of comprehensive personalized cancer medicine will eventually be possible. ■

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