Tumour heterogeneity: principles and practical consequences

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Abstract Two major reasons compel us to study tumour heterogeneity: firstly, it represents the basis of acquired therapy resistance, and secondly, it may be one of the major sources of the low level of reproducibility in clinical cancer research. The present review focuses on the heterogeneity of neoplastic disease, both within the primary tumour and between primary tumour and metastases. We discuss different levels of heterogeneity and the current understanding of the phenomenon, as well as imminent developments relevant for clinical research and diagnostic pathology. It is necessary to develop new tools to study heterogeneity and new biomarkers for heterogeneity. Established and new in situ methods will be very useful. In future studies, not only clonal heterogeneity needs to be addressed but also non-clonal phenotypic heterogeneity which might be important for therapy resistance. We also review heterogeneity established in major tumour types, in order to explore potential similarities that might help to define new strategies for targeted therapy.

Keywords Tumour heterogeneity · Phenotypic · Clonal · Epigenetic · Molecular · Functional plasticity · Intratumour · Intertumour · Spatial · Temporal

Introduction

In principle, a biospecimen or a group of biospecimens of any disease can be defined as homogeneous by applying an arbitrary classification system based on a priori-defined criteria: All cells in a single specimen or all specimens in a group are equal. In the biomedical field, this is essentially done for two reasons: to define clinically meaningful patient groups with similar prognosis and/or therapy response and to facilitate research on mechanisms of disease. *In fine*, this is an attempt to overcome the heterogeneity inherent to biological systems. The framework of disease classification has developed along historical lines, heavily influenced by pathology as its primary custodian: mostly organ based, from macroscopy to microscopy, the latter providing histological characteristics and since the final decades of the twentieth century further subdivided by immunohistochemical markers. That is not the end of the story: the tremendous impact of molecular biology on medicine has added a new layer of complexity and in an increasing number of cases provides an alternative window on disease classification (e.g. 'what is wrong in the cell at a molecular level' rather than 'what does it look like').

While the concept of heterogeneity can be applied to a wide range of cohort characteristics, such as populations defined by ethnicity or common geographic ancestry (e.g. Caucasian/African/Asian etc.), social ancestry (e.g. Ashkenazy Jews) or exposure to harmful agents in an occupational or habitual context (e.g. asbestosis, tobacco smoke), this is beyond the scope of this review. We will focus on tumour heterogeneity, between and within patients, in the latter case both in the primary tumour and between the primary tumour and its metastases. There are two major reasons to study tumour heterogeneity. The first is that it represents the basis of acquired resistance to therapy [1, 2]. The second is that it limits reproducibility of clinical research and precision in diagnostic evaluation of tumours [3]. In this rapidly developing field of tumour 'subtypes', we will address the different levels of heterogeneity, its biological background, current understanding of the phenomenon and its evolving impact on biomedical research (clinical and translational) and on diagnostic pathology.

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Concepts of tumour heterogeneity

For practical purposes, we will make a distinction between phenotypic and clonal genomic heterogeneity of tumours.

Phenotypic heterogeneity

In the context of diagnostic pathology, the term 'tumour heterogeneity' emerged from macroscopic and microscopic observation. The heterogeneity in histological appearance of different tumours (intertumour heterogeneity) as well as of different areas in a single tumour (tumour heterogeneity) is of uncontested clinical relevance. This is exemplified by histopathological classification of tumours according to morphological patterns, the very basis for clinically useful stratification. Since the advent of molecular pathology, intertumour heterogeneity has been extensively investigated at the RNA level by transcriptional profiling. This has allowed the definition of molecular subtyping for several types of cancer, which does not necessarily completely overlap with prevailing histological classifications.

Quite often, a characteristic diagnostic pattern (e.g. mucinous differentiation) only occurs in part of the tumour. This is considered tumour phenotypic heterogeneity. Phenotypic heterogeneity is biologically relevant because heterogeneous tumours often behave differently from homogenous tumours, and this can be of prognostic significance [4]. Conceptually, poorly differentiated tumour components are thought to determine the biological behaviour of the whole lesion, a notion recently challenged by the observation of clonal tumour progression from phenotypically better differentiated areas in lung adenocarcinomas [5]. Different areas of a tumour can have different functional properties, for example, the invasion front as compared to the tumour centre. As sampling of a tumour for molecular analysis is often performed indiscriminately, this might compromise the validity and the reproducibility of the results, depending on the questions asked [6].

Microscopic observation has taught us that the histological characteristics of a tumour can change over time. Phenotypic heterogeneity can be but is not always the result of heterogeneity at the level of the genome. Cancer cells often have the capacity to differentiate, which results in phenotypic heterogeneity. Tumour cells tend to adapt to their microenvironment, which can affect their morphology and behaviour [7]. As a consequence, phenotypic heterogeneity can represent divergent states of differentiation (which can be reflected, e.g. in patterns of mRNA expression or of protein expressed), which has also been called phenotypic functional plasticity. Examples are epithelial mesenchymal transition or stem cell-like capacity of tumour cells (known as 'stemness') which is associated with tumour progression. Occasionally, the phenotype of tumour cells changes along with specific alterations in their genome. In this situation, phenotypic and clonal heterogeneity coincide [8]. A recent report in melanoma has documented phenotypic heterogeneity in association with acquired resistance to therapy [9]. Autocrine mechanisms and paracrine interaction with factors emitted by tumour stromal cells might be responsible for this phenomenon.

A somewhat esoteric form of phenotypic heterogeneity is so-called stochastic plasticity, which conceptually is the result of the stochastic nature of biochemical processes within cells (transcription, translation, posttranslational modification of proteins, availability of chaperone proteins, to name but a few), which implies that no two cells are exactly identical [10, 11]. This is the most likely explanation for intercellular variability in immunoreactivity, which pathologists regularly are confronted with in performing marker studies for diagnostic or research purposes.

One implication of phenotypic heterogeneity is that it might be involved in tumour progression. Parting on the principle that alterations in the genome and in the epigenome have been shown to constitute major factors responsible for tumour progression, it is unknown what the relative contribution of these very different mechanisms is to this crucial tumour characteristic [7, 12, 13]. Subclones in a tumour with differences in phenotype can synergize, which might favour tumour progression, or antagonize which might inhibit progression [12]. Even quantitatively minor subclones can drive tumour progression in interaction with the microenvironment in what has been called 'non-cell autonomous expansion' [13]. Functional constraints associated with phenotype might modulate tumour progression [7].

Genomic heterogeneity

Next-generation sequencing of tumour cell populations as well as of single cells has provided evidence that differences in genome alterations exist between individual cells in a tumour. This is most striking in tumours characterized by mismatch repair deficiency, which hypermutate because errors of replication are no longer corrected. This is the mechanism responsible for familial colorectal cancer in the context of Lynch syndrome. Once a mutation has conferred growth advantage to a cancer cell, that cell might grow out into a new clone. This is one of the basic paradigms underpinning cancer progression. We will call differences in genome abnormalities between subclones in a tumour clonal heterogeneity. Which clones will emerge as a result of genomic instability is subject to selection pressure, in which metabolic conditions (e.g. hypoxia), microenvironmental factors in topographically confined niches and therapeutic interventions play a role [1, 14, 15]. Expansion of clones may be different between a primary tumour and its metastases due to clonal selection during the metastatic process [16], which accounts for variations between them. To what extent non-clonal evolution such as phenotypic plasticity, induced by differences between the microenvironment in different metastatic sites are involved, is unknown [6].

Clonal heterogeneity encompasses mutational and non-mutational epigenetic mechanisms, such as gene promoter methylation, both transmissible to progeny cells. The role of epigenetic events in tumour heterogeneity has been less investigated. Epigenetics includes patterns of expression of short and long non-coding RNAs, which may be involved in non-clonal phenotypic plasticity. Clonal mutational heterogeneity, on the contrary, has recently drawn much attention, as it provides a convincing explanation for tumour progression and of the emergence of secondary resistance to targeted therapy.

Resistant subclones are suspected to be one of the most important mechanisms leading to partial response to treatment and failure of targeted therapies [1, 2]. Minor subclones, already present but below the limit of detection and equipped with yet unknown resistance mechanisms, can outgrow the remaining tumour under the selection pressure imposed by therapy. Alternatively, a resistant clone can develop due to a newly acquired mutation. For such events to be addressed adequately, two requirements have to be met by molecular diagnostics: the assay needs to be sufficiently sensitive to allow identification of minor, low-level tumour subclones, and sampling should be regularly repeated ('longitudinal'), because the emerging subclone that eventually determines outcome may not be detectable in a single biopsy or even in multiple pretreatment biopsies. Biomarkers released from primary and metastatic sites might be heterogeneous, which could have important implications for the choice of treatment [17]. As key biological attributes of metastases may significantly affect disease outcome, the view that treatment decisions should not be based solely on the features of the primary tumour is rapidly gaining ground [18]. We have recently shown in breast cancer that the clinical significance of a biomarker detected in primary tumour tissue changes when the same biomarker is detected in lymph node metastases [12]. For a long time, the prevailing hypothesis was that metastases developed from a single cell or subclone [19], but recently, also polyclonal seeding from one metastatic site to another has been described [20]. Consequently, optimal clinically relevant biomarkers should reflect intratumour molecular heterogeneity, as, for example, genomic instability of the tumour [21].

Statistical extrapolations of sequencing data indicate that at the time of diagnosis, many tumours already harbour (minor) subclones with mutations that confer resistance to targeted therapy [22]. This fits well with the clinical observation that almost all targeted monotherapies invariably fail. Recently, colorectal cancer patients were reported of which some metastases shrunk under anti-epidermal growth factor receptor (EGFR) therapy while others did not. When therapy was suspended, the original dominant sensitive tumour clone detected in peripheral blood before acquired resistance was reactivated and re-expanded [23]. Such remarkable plasticity of tumour heterogeneity has led to so-called adaptive therapy, intended to control but not necessarily eliminate the prevalent cell clone [24]. As an alternative, combinations of targeted drugs are being developed against multiple specific driver mutations [25]. The different types of intratumour heterogeneity are summarized in Table 1.

Genomic heterogeneity as a microevolutionary process

DNA alterations include mutational changes (e.g. point mutations, deletions, insertions, translocations, inversions) or quantitative variations in gene copy number. Patterns of mutational changes in tumours are commonly addressed using the conceptual framework of Darwinian phylogenesis [16, 26] with the notion of a phylogenetic tree. In analogy, somatic alterations common to all cells in a tumour are called 'truncal mutations', presumably responsible for oncogenic transformation of the single cell that started the tumour. Subsequent somatic mutations give rise to multiple 'clonal branch mutations' which ultimately ramify into 'subclonal mutations'. In a graphic representation of this concept, trunk and branch lengths can be plotted proportional to the number of mutations acquired on the corresponding trunk or branch, in order to visualize proximity or distance of evolutionary junctions. In the context of tumour treatment, the ideal molecular targets would be truncal mutations as all cells in a tumour have them in common. Low-level frequency subclones would be tiny, barely detectable branches that fork from the trunk or one of the (sub) branches (Table 2).

Tumours are composed of very dynamic cell populations in which these 'microevolutionary' processes are ongoing. This implies that heterogeneous subclones will continue to emerge, and their development over time defines the second basic dimension of tumour heterogeneity: 'temporal' heterogeneity [62], 'spatial' heterogeneity being the first as discussed in a previous paragraph (Fig. 1).

Table 1 Types of intratumour heterogeneity

Types of intratumour heterogeneity	Examples	Methods
Microscopic tissue heterogeneity	Histology (histotype, tissue reaction, differentiation, tissue composition, …) Different functional areas (e.g. centre and borders of tumour)	Microdissection
Molecular heterogeneiy-clonal type	Genetic evolution Epigenetic evolution	NGS, FISH, Single cell Seq.
Molecular heterogeneity-non-clonal type	Phenotypic functional plasticity (EMT, stemness,…) related to different functional areas and stromal interaction Stochastic plasticity (single cell)	Single cell RNA seq., gene expression profiling by microarrays, in situ methods, IHC, proteomics

Table 2 Summary of the publications per tumour type, excluding reviews

Cancer type	Type of heterogeneity	Method[a]	Genes	References
Breast cancer	Primary/local recurrence	IHC, FISH	ER, PR, HER2, Ki67,	[27–31]
	Intermetastatic	NGS	K8, K5/6	[32]
	Primary/metastasis/cfDNA	ddPCR	PTEN ESR1	[33]
Colon cancer	Primary/metastasis	Sanger	KRAS	[34–36]
	Primary/metastasis	PCR-RFLP	KRAS	[37]
	Primary/metastasis	Sanger	KRAS, BRAF	[38]
	Primary/metastasis	N.A.	KRAS	[39]
	Primary/metastasis	PCR-SSCP	KRAS	[40]
	Primary/metastasis	NGS	Multigenes	[41]
	Primary/metastasis/CTC	NGS	Multigenes	[42]
Skin melanoma	Intratumoural	IHC	SSX	[43]
	Intratumoural	RT-PCR	MAGE, NY-ESO, SSX	[44]
	Intratumoural	Sanger	BRAF NRAS	[45]
	Primary/metastasis/ intermetastatic	Sanger, IHC, RT-PCR	BRAF, NRAS, MEK1, MEK2 AKT1	[46]
Prostate cancer	Intratumoural	NGS	TMPRSS2-ETS, PTEN	[47, 48]
	Intermetastatic	FISH	TMPRSS2-ETS	[49]
	Intermetastatic	Array CGH	Multigenes	[50]
	Intermetastatic	Methylation	Multigenes	[51]
	Primary/metastasis	NGS	Multigenes	[20, 52]
Renal cell carcinoma	Intratumoural	M-Seq	Multigenes	[53]
	Intertumour	miRNA and RNA sequencing, DNA methylation array, SNP arrays, exome sequencing, RPA	Multigenes	[54]
	Intratumour	SNP arrays (copy number alteration)	Multiregion	[55]
	Primary/metastasis	Whole-exome multiregion sequencing, SNP array, mRNA expression profiling	Multigenes	[26]
Lung cancer	Intratumoural	Sanger and mutant-enriched PCR	EGFR	[56]
	Primary/metastasis	Sanger and Scorpion ARMS	EGFR	[57]
	Primary/metastasis	Sanger	EGFR, KRAS	[58]
	Intratumoural, primary/ metastasis, primary/recurrence	Sanger, Cycleave PCR	EGFR	[59] [60]
	Primary/metastasis	Sanger	EGFR	[61]
	Intratumoural, primary /metastasis, temporal	PNA-LNA PCR clamp method and Cycleave PCR WES	EGFR	[14]
	Intratumoural		Whole exome	

FISH fluorescence in situ hybridization, *IHC* immunohistochemistry, *ddPCR* droplet digital PCR, *PCR-RFLP* PCR restriction fragment length polymorphism, *N.A.* not available, *NGS* next-generation sequencing, *PCR-SSCP* PCR-single-strand conformation polymorphism, *RT-PCR* reverse transcription-PCR, *WES* whole-exome sequencing, *PNA-LNA PCR clamp method* peptide nucleic acid-locked nucleic acid PCR clamp method, *Scorpion ARMS* Scorpion amplification refractory mutation system-PCR assay, *M-Seq* multigene exome sequencing, *RPA* reverse phase protein array

Liquid biopsy

The term '*liquid biopsy*' was originally introduced for circulating tumour cells (CTCs), recovered from (liquid) peripheral blood. Methods to analyse liquid biopsies had to be sufficiently sensitive to detect a single tumour cell among thousands of normal haematopoietic cells [63]. It became subsequently clear that a liquid biopsy does contain not only CTCs but also cell-free nucleic acids and exosomes. Analysis of these components provides surrogate or complementary biomarkers, which overcome the limitations of invasive tissue biopsies. While circulating tumour DNA (ctDNA) can be released into the bloodstream not only from tissue in the tumour but also from lysed CTCs, ctDNA might contain DNA from tumour cell clones that have not

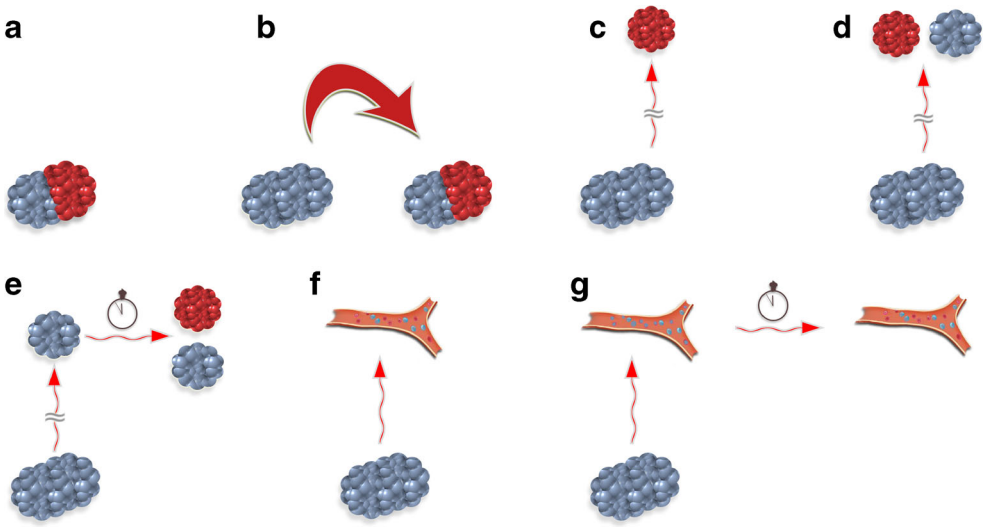

Fig. 1 Spatial heterogeneity within the primary tumour compartment (**a**) and between separate tissue compartments (**b**). Heterogeneity between primary tumour and metastasis (**c**). Intermetastatic heterogeneity (**d**). Temporal heterogeneity between metastases (**e**). Heterogeneity between tissue (primary or metastasis) and the soluble compartment (**f**) and temporal heterogeneity evident in the soluble compartment (**g**)

released CTCs, as ctDNA can be found in peripheral blood in the absence of detectable CTCs [64]. In a range of different solid malignancies, ctDNA has been detected and levels have been shown to go up with increasing disease stage [64].

Liquid biopsies are significantly improving our knowledge on the dynamics of and mechanisms involved in dissemination of malignancies. Molecular analysis of CTCs and ctDNA has contributed to our understanding of the biology behind cancer recurrence and acquired mechanisms of resistance and how to deal with these clinically. Aggressive tumour clones can be detected by the presence of CTCs and ctDNA in blood during follow-up and may be related to treatment outcomes [2], indicating that therapy itself may represent the selection pressure factor for clonal evolution [1]. Today, the relevance of liquid biopsies for clinical practise and its relation to tumour heterogeneity are still uncertain. It is evident that CTCs and ctDNA can come from different and heterogeneous metastatic sites; therefore, sensitivity and reproducibility in detecting tumour clonal range still have to be proven [65, 66].

Methods to detect intertumour and tumour heterogeneity

Methods to detect phenotypic heterogeneity

Phenotypic heterogeneity has been studied first and foremost by microscopy, as has been discussed in the "Introduction" section. In terms of molecules involved, it can be studied at RNA or protein level. In situ methods, even if non-high throughput and usually non-quantitative, are preferred in formalin-fixed paraffin-embedded (FFPE) tissues because results can be projected into morphology, e.g. in terms of tumour area (centre, invasion front), tumour differentiation (lineage, grade), cell type (tumour, stromal) and intracellular level (nucleus, plasma membrane, cytoplasm).

RNA expression, both of coding and non-coding RNAs, can be assessed by extractive procedures as well as by in situ methods. For FFPE tissues, a range of methods is available from real-time quantitative RT-PCR [67] to microarrays [68, 69] and in situ hybridization (ISH). The sensitivity of the latter has been optimized by the introduction of signal amplification as well as the RNAscope technique (Advanced Cell Diagnostics Inc., Hayward, CA) [70–72], which allows investigation of multiple transcripts in the same tissue section.

Whole-transcript sequencing by next-generation sequencing (NGS) (RNA sequencing or as it is customarily called RNA-seq) can be performed on RNA extracted from FFPE [73, 74]. As stated above, the main disadvantage of extractive methods is the absence of parallel morphological information, for which the only solution would be RNA-seq of single cells extracted from fresh tissues by cell sorting [75].

Phenotypic heterogeneity at the protein level has mainly been investigated in FFPE tissues by targeted assays, using antibodies in immunohistochemical assays or after extraction by reverse phase protein array technique [76–78]. Other valuable methods to study phenotypic heterogeneity, even in FFPE specimens, are liquid chromatography tandem mass spectrometry (LC-MS) [79, 80], matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (MALDI imaging) [81–83] and multiplexed ion beam imaging [84]. The last two methods are performed in situ with preservation of morphological characteristics.

Methods to detect genomic heterogeneity

NGS is now the method of choice to analyse the clonal architecture of cancer [85], as it has the capacity to detect 1–5 % of mutated alleles against a wild-type background [86]. Whole-

exome (WES) and whole-genome sequencing (WGS) are ideally suited to detect clonal heterogeneity at maximum resolution, because they allow examination of the entire coding genome (WES) or even the entire genome (WGS) [87]. For practical and economic reasons, most clinical genotyping to date utilizes 'hotspot' genotyping [88] or targeted sequencing panels [89–91] of clinically relevant genes in FFPE tissues. In addition to sequencing (which includes WES and shallow WGS [92–94]), oligonucleotide microarray-based comparative genomic hybridization (array-CGH) can be used to map genome-wide copy number variations in FFPE tissues [95]. This technology can also be used for CTCs or cell-free DNA (cfDNA).

Methods to detect epigenetic heterogeneity

Detection of DNA methylation at individual loci and within gene promoter sequences has been performed in FFPE tissue specimens using conversion of non-methylated cytosines by bisulfite treatment into uracyl residues. This leaves methylated cytosines (5Cm) intact; the difference will then be picked up in a sequencing reaction [96]. Methylation-specific multiple ligation-dependent probe amplification (MS-MLPA) allows simultaneous assessment of promoter methylation of multiple genes in a single experiment [97], which eliminates the bisulfite conversion step [96]. Global DNA methylation can be assessed with array-based methods, such as infinium methylation [98], or by WGS [99, 100]. Chromatin-protein interactions can also be studied in FFPE specimens by combining chromatin immunoprecipitation with massive DNA sequencing (ChIP-seq) [101, 102].

Methods applied to liquid biopsies

Liquid biopsy performed at different time points during patient follow-up conceptually provides an ideal way to address spatial and temporal heterogeneity during tumour evolution. In terms of methodology, this has materialized through advances in the field of CTC isolation and ctDNA analysis. As CTCs constitute a very minor population among nucleated blood cells, an enrichment step is needed before they can be isolated. The enrichment step is a potential limitation, as it is usually done using antibodies against preselected markers which might introduce a selection 'bias'. In addition, CTC content in peripheral blood may be so low that a large volume of blood would have to be taken to arrive at a sufficient number of cells to analyse [103]. There are several methods to isolate CTCs, but the only FDA-approved technology is the CellSearch® (Veridex, Raritan, NJ), which is based on initial enrichment of cells expressing the epithelial cell adhesion molecule (EpCAM), followed by immunofluorescence staining targeting an epithelial marker (cytokeratin (CK) 8, 18, 19) and a leucocyte marker (CD45) and DAPI for nuclear staining [103]. A recent development is the DEPArray (Silicon Biosystems, Bologna, Italy), a dielectrophoresis-based platform which can effectively process a relatively small number of cells. The device analyses and sorts single, viable, rare cells using an image-based selection process, followed by entrapment of cells inside dielectrophoretic cages, with the possibility to simultaneously enrich for different cell populations [104, 105]. Selected cells in specific cages can then be moved by software-controlled modulation of electrical fields and ultimately recovered for downstream molecular analyses [104, 105].

After isolation, CTCs can then be used for genotypic and phenotypic characterization using one specific or a few molecular markers, such as human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR) and oestrogen receptor (ER) for characterization of breast cancer CTCs [64]. Furthermore, CTCs can be potentially used for high-throughput molecular characterization such as CGH, NGS and even for WES as recently reviewed by Ignatiadis and Dawson [103].

Heterogeneity in different tumour types

Breast cancer

Breast cancer is a heterogeneous disease consisting of different tumour types, both histologically and molecularly (i.e. triple negative, HER2-enriched, luminal A and luminal B). Using mathematical modelling, Wang et al. showed that triple-negative tumour cells have a higher mutation rate than ER-positive tumour cells [106]. However, tumour heterogeneity of breast cancer has been found at morphological as well as molecular level. Of the four predictive biomarkers used in clinical practise for breast cancer (ER, PR, Ki67 and HER2), expression of PR [107] and Ki67 [108] is highly heterogeneous, and in order to obtain reliable data, adequate sampling is crucial, both for clinical applications as well as for research using tissue microarrays [109]. Furthermore, discordance in tumour biomarker status in different areas of a primary tumour and between a primary tumour and its metastases has been described in studies exploring new biomarkers in lymph node [27–29] as well as distant metastases [30, 31]. Recent findings indicate that, already at initial diagnosis, primary breast cancer is composed of multiple clones and that the repertoire of somatic genomic alterations in metastatic lesions may differ from that of the primary tumour. By whole-genome shotgun sequencing (WGS) of paired primary and metastatic metachronous tumours from a single breast cancer patient, Shah et al. found several non-synonymous mutations in metastases that had not been identified in the primary tumour diagnosed 9 years earlier [110]. Intratumour heterogeneity is likely amplified by selection pressure exerted by therapy, as has been shown for *PTEN* loss [32] and ESR1 mutations as detected in biopsies from metastatic sites and liquid biopsies

(cfDNA). Such a mechanism might drive development of secondary resistance to hormone therapy [33].

Recently, intratumour genomic and phenotypic heterogeneity was studied during metastatic progression in breast cancer through analysis of cellular genotypes and phenotypes at the single-cell level. These studies showed that distant metastases are the most diverse, both genetically and phenotypically, with probable implications in frequent therapy resistance of advanced stage disease [111]. Given the complexity of the biology of breast cancer in terms of heterogeneity and mechanisms involved in progression, analysis of CTCs or cfDNA in liquid biopsies might help to monitor patients during treatment and in parallel shed some light on poorly understood aspects of the metastatic process [112, 113]. A practical proposal for markers with known heterogeneity (HER-2, Ki67, PR) is to sample at least three different areas of the tumour. This is particularly relevant for large lesions in a neo-adjuvant setting, when it is done on core biopsies before surgery therapy planning.

Colorectal cancer

Intratumour phenotypic (histological) heterogeneity is a well-known feature of colorectal cancer (CRC). Multigene, WGS and WES mutation analysis has shown heterogeneity in an increasing number of genes, but these are mostly private rather than driver mutations. Clonal intratumour heterogeneity of driver mutations in oncogenes (including *KRAS*, *NRAS*, *BRAF* and *PIK3CA*) or tumour suppressor genes (e.g. TP53) is less frequent.

In a clinical context, gene alterations are only relevant if they have an impact on treatment choice. Currently, this encompasses alterations predictive for response to anti-EGFR treatment, including (according to the current National Comprehensive Cancer Network practise guideline 2. 2016) *KRAS*, *NRAS* and *BRAF*. The predictive value of *PIK3CA* mutations for response to anti-EGFR therapy needs further evaluation [114]. According to the presently available data, the pattern of mutations of these genes is largely homogenous, both in the primary tumour and when primary and recurrent disease is compared. An increasing number of reports describe CRCs with discordant *KRAS* mutation status between primary and metastases, but this remains a small proportion (about 5 %) of cases [115]. Complete concordance [37, 38, 40] even after anti-EGFR therapy [39] or low-level discordance has been reported in other publications [34–36]. Of note, in a meta-analysis, Han et al. [40] observed a markedly higher (18.7 %) discordance rate between primary tumour and lymph node metastases. Occasionally, discordance between a primary and its metastatic tumours appears to be related to morphologically distinct areas in the primary tumour (e.g. mucinous vs. non-mucinous), with a clonal metastasis reflecting morphology and mutation pattern of the corresponding area in the primary tumour [8].

A recent UK-practise guideline [116] states that *K/NRAS* testing for practical purposes can be performed on primary or on metastatic tissue, with a preference for metastatic tissue if available. This seems justified given relatively rare mutational heterogeneity of colorectal cancer. As long as mutation testing is limited to *N/KRAS* and *BRAF*, this guideline therefore is acceptable.

With the advent of multigene sequencing, mutational heterogeneity in genes other than *RAS* has become apparent, even though driver mutations have remained highly concordant, including oncogenes 'beyond *RAS*' [41]. (Neo)adjuvant chemotherapy may increase mutational homogeneity through selection of subclones with driver mutations rendering them non-responsive [41], but more studies are needed to confirm this hypothesis. Liquid biopsy approaches have allowed studies of mutations in cfDNA and of copy number variations in CTC, through which evolution of the tumour genome can be followed over time [42, 117]. This is an extension of studies on tissue, regarding concordance of mutation status between primary and metastastatic/recurrent tumour. Analysis of cfDNA might also be a more sensitive approach to detect genome heterogeneity in the primary tumour. Mutations 'private' to cfDNA, which must have been already present in the primary tumour as small subclones, have been described [42, 118]. The emergence of various *KRAS*, *NRAS* and *BRAF* mutations over time in a patient has been reported as an example of 'temporal heterogeneity' in the course of anti-EGFR therapy. Resistant cells consistently displayed activation of mitogen-activated protein kinase (MEK) and extracellular signal-regulated kinase (ERK), which persisted after EGFR blockade [119]. In summary, before contemplating mutational heterogeneity in CRC, clinical and histopathological information needs to be taken into account and technical artefacts need to be excluded [3].

Skin melanoma

In the past two decades, a variety of gene abnormalities has been identified in melanoma. An expanding panel of recurrent driver mutations has emerged, which appears to activate specific oncogenes or inactivate tumour suppressor genes. A subdivision of melanoma into four clinical subgroups of disease has been proposed: melanoma arising from non-chronically sun-damaged skin, melanoma arising from chronically sun-damaged skin, melanoma arising from mucosal surfaces and melanoma arising from acral surfaces. These are characterized by unique combinations of genome-wide aberrations in DNA copy number and oncogenic alterations [120]. Recently, four genomic subtypes have been distinguished in cutaneous melanoma: *BRAF*, *RAS (N/H/K)*, *NF1* and triple wild type [121]. Interestingly, melanomas with a high number of infiltrating lymphocytes, which is associated with more favourable prognosis, do not all have the same distinct genomic subtype [121], presumably due to the tumour microenvironment and the emergence of tumour-specific neo-antigens.

Intratumour heterogeneity in skin melanomas is striking when immunohistochemical staining patterns for melanoma antigens (i.e. SSX, NY-ESO-1, MAGE-A1) are considered, as they have appeared mostly heterogeneous with a focal expression pattern [43, 44]. Laser capture microdissection (LCM) of nodular melanomas of single patients has shown different mutations of *BRAF* or *NRAS* in different areas of the tumour, which provides evidence for the existence of different subclonal cell populations [45]. The spectacular successes obtained with treatment by *BRAF* inhibitors but with rapid regrowth of the tumour is a striking example of intratumour heterogeneity as it relates to acquired resistance to targeted treatment. Emerging evidence indicates that different tumour localizations in a single patient can simultaneously develop different molecular mechanisms of resistance, e.g. *NRAS* mutations (Q61R or Q61K) in one metastasis and *BRAF* amplifications or *MEK1* mutations in another [122]. Shi et al. studied temporal and spatial heterogeneity by WES, analysing nine different cutaneous metastases of a single patient treated with dabrafenib. They found a broad spectrum of different genomic alterations, including alternative splicing and amplification of *BRAF*, *KRAS* mutation (G12C) and *PTEN* deletion, resulting in 20 % of patients having at least two different mechanisms of resistance [123]. The common denominator of resistance mechanisms appears to be restoration of ERK signalling [46], which includes *NRAS* and *MEK1* mutation, as well as overexpression of BRAF or activation of alternative kinases, including platelet-derived growth factor receptor-β (PDGFRβ) and insulin-like growth factor 1 receptor (IGF1R) [124]. Resistance to BRAF inhibitors could also follow phenotypic alterations of melanoma cells due to rewiring of oncogenic signalling pathways, such as MAPK restoration at the level of RAF through increased expression and subsequent dimerization of CRAF [122]. The Wnt signalling pathway has been shown to effectively modulate phenotypic plasticity of tumour cells, when activated by a hypoxic microenvironment [125].

The emerging pattern is that melanoma in not only different patients but also different metastases in a single patient and even different subclones within a single tumour localization can develop different resistance mechanisms, genomic and/or phenotypic and unpredictable a priori [126].

Prostate carcinoma

Prostate carcinomas show very particular heterogeneous morphology that has been recognized for a long time. This is used in a clinical context as Gleason grading, which comprises the separate evaluation of the two dominant morphologically different growth patterns in the tumour. The sum of these represents the most important prognostic factor in prostate cancer. Advanced stage prostate carcinomas often show several different histological growth patterns. An interesting question is how these growth patterns, perceived in a two-dimensional tissue section, translate into three-dimensional tissue architecture. One assumption is that the specific (fibromuscular) stroma of the prostate grows in 'bridging' structures which dissect the tumour epithelial cell clusters. The study of microdissected samples of tumour from areas with different Gleason grade has indeed shown that, in spite of morphological heterogeneity, they share the same clonal genome abnormalities such as the break points in the characteristic *TMPRSS2-ERG* translocation [47, 48]. In contrast, *PTEN* gene deletions tend to be heterogeneous and emerge in branches of the 'phylogenetic tree' notably in advanced prostate cancer, even within a single tumour node [127].

In studies of the genome of metastases of prostate cancer, monoclonality in terms of the presence and type of *ETS* gene fusion has been reported [49, 50], as well as similar patterns of gene promoter methylation [51]. However, some studies of individual patients found evidence of polyclonal seeding from one prostate cancer metastasis to another [20]. Patterns of tumour spread can be complex also in their clinical consequences. Haffner et al. described a case of prostate cancer in which the cause of death was related to a minor low-grade subclone of the primary tumour, rather than the high-grade dominant subclone [52]. This might have been due to selection pressure exerted by therapy. Such observations call for further studies on prognostic and predictive biomarkers, notably those driving subclones, along with quantitative metrics of heterogeneity [21].

Renal cell carcinoma

Phenotypic (histological) tumour heterogeneity is striking in renal carcinoma, as reflected in an increasing number of morphological subtypes, and this is also true at least for some oncogenic driver mutations [53]. In addition, genomic driver aberrations tend to be different from one case to another (intertumour heterogeneity) [54]. How many subclones might be detected depends to a large extent on the sensitivity of the method used to detect mutations [21]. Gerlinger et al. [26] performed multiregion exome sequencing of clear cell renal cell carcinoma (ccRCC) and constructed phylogenetic trees by segregating genome aberrations into 'truncal', 'shared' as well as 'private' mutations in tumour subclones, including in metastases. They found that in every site, a mutation in the von Hippel-Lindau (*VHL*) tumour suppressor gene forms the trunk of the phylogenetic tree. All ccRCC phylogenetic trees analysed thus far have shown a branched rather than a linear evolutionary pattern. One study using multiregion copy number profiling reported that subclones within a single ccRCC more closely resembled subclones in other ccRCCs than its own subclones [55]. Other studies on single patients, using multiregion exome sequencing, have shown that there might be more similarities between mutational patterns in different metastases than between the primary tumour and its metastases [26]. Such observations have clinical consequences, as subclones with different targetable driver mutations might require complex multidrug approaches. Multiregion sequencing of the primary tumour, as well as longitudinal follow-

up of the patient with liquid biopsies to analyse cfDNA, may be required to obtain a comprehensive picture of mutational patterns in evolving tumours.

Lung cancer

Pulmonary carcinomas, notably those caused by chronic carcinogen exposure (cigarette smoking), exhibit among the highest mutational burden in cancer, and yet a limited array of driver mutations appears to be involved. This has been studied in detail in non-small cell lung cancer (NSCLC) and has resulted in a remarkable success of molecular prediction of therapy response. *EGFR* mutation status predicts sensitivity/resistance to the EGFR tyrosine kinase inhibitors erlotinib and gefitinib, while *ALK*, *RET* and *ROS1* translocations predict response to crizotinib and ceritinib. The most common resistance event is *EGFR* mutation (T790M), which has been detected in minor subclones in primary NSCLC [56, 128] which will emerge as resistant in tumours under EGFR-TKI therapy [128]. Additionally, intratumour heterogeneous *EGFR* and *KRAS* mutation status has been reported in primary tumours and metastases in about 25 % of NSCLC [57, 58]. This has been challenged in single studies reporting spatial (intratumour as well as primary vs. metastatic) and temporal homogeneity in NSCLC [59, 60]. Interestingly, a recent analysis [61] of patients with EGFR-TKI-resistant NSCLC due to an *EGFR* T790M mutation revealed not only spatial intratumour heterogeneity in primary and metastatic lesions but also high temporal plasticity of T709M status in the primary (evaluated by serial biopsies). Tumours with acquired mutations conferring resistance after TKI treatment reverted to the wild-type (therapy sensitive) state after TKI withdrawal, which in some patients even changed back to the mutated state once TKI therapy was re-initiated. The most detailed study of genome heterogeneity concerns a series of 11 NSCLC investigated by whole-exome multiregion sequencing [14], which revealed intratumour heterogeneity in all tumours. Of all mutations detected, 60 % were truncal as they were homogeneously present in the whole tumour. Even more importantly, almost all (20 out of 21) currently known recurrent cancer-associated gene mutations were truncal. This homogeneity was also found for copy number alterations, all of which were homogeneously present.

Conclusions

The major issues in tumour heterogeneity today are to recognize drivers related to tumour evolution and to establish the relationship between subclonal evolution at the molecular level and therapy resistance. Tumour heterogeneity must be examined more closely both in terms of topography (multiple samples to detect low-level subclones, discordant metastatic clones including in lymph node metastases) and temporally by repeated liquid biopsies during patient follow-up. The emerging practical importance of liquid biopsies underscored is its recent European Medicine Agency (EMA) registration for companion diagnostic purposes. Furthermore, a crucial goal is to unravel that the role of the microenvironment is in spatial phenotypic heterogeneity. Factors involved in tumour progression are not only intrinsic genomic instability but also tumour cell-microenvironment interactions and selection pressure exerted by therapeutic intervention, including radiotherapy and targeted or cytotoxic chemotherapy. Old and new in situ methods of analysis will be used, and the results will have to be scrutinized in terms of clinical significance in multivariable computational models. The final goal is to obtain reliable biomarkers that reflect clinically relevant heterogeneity. This will allow identifying new targets for combinatorial therapies as well as better definition of the risk for therapy resistance. Standardization of molecular analysis, using sampling protocols that take tumour heterogeneity into account, is necessary to obtain reproducible results important for diagnostic practise and for translational and clinical research.

Compliance with ethical standards This review was complied without the direct involvement of human participants and/or animals, but only by the analysis of published studies. Therefore, for this type of study, formal consent was not required.

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