Intratumoural Heterogeneity as a Major Challenge for Cancer Modelling and Successful Treatment

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Abstract: Cancer heterogeneity refers to the fact that cancer cells are characterised by different genomic/transcriptomic/proteomic compositions, which often confer behavioural properties, such as enhanced drug resistance, survival and propensity to form metastasis. Modern methodology, such as single-cell barcoding, now allows a deeper look into this mechanic, and therefore a possibility to derive more efficient, new treatments. However, cell culture approaches are commonly used to evaluate those novel approaches, and the ability to faithfully model cancer heterogeneity is still in its infancy.

Keywords: Cancer heterogeneity, cell culture, resistance, recurrence, evolution

Introduction

It is common in biological research to assume that the difference between cells of a certain type, e.g.: endothelial cells, epithelial cells or even skin cancer cells, is negligible small. However, in many physiologically healthy tissues it has been demonstrated that differences in the transcriptome or proteome of cells of the same nature (e.g.: skeletal muscle stem cells) are potent enough to allow those cells to undergo different tasks (e.g. some muscle stem cells divide and replenish the niche, while others differentiate to develop/regenerate skeletal muscle fibres). In other words, the decision which cell performs what task within a tissue is not necessarily stochastic, but influenced by many factors that eventually alter the cells' transcriptomic/proteomic landscape, and therefore changes their response to their environment. This is called cellular heterogeneity. In a tumour, where increased genomic instability creates a more permissive environment for the development of pro-survival heterogeneity, this difference can be linked to increased therapy evasion, relapse and recurrence.

190 years of tumour heterogeneity research

One gram of tumour mass (1 cm^3) is composed of $1 \cdot 10^8$ to $1 \cdot 10^9$ cells [1,2] and cancer cell development is highly dynamic. Intra-tumoural heterogeneity was first mentioned in 1833, when Muller and Virchov [3] noted different cellular morphologies in cells derived from the same tumour. Since then, different phenotypic displays were associated with various behavioural consequences [4], such as a different rate of mitosis, and finally, in 1976, Peter Nowell proposed the theory of clonal cell evolution [5]. Indeed, the importance of research into tumour heterogeneity has gained immense momentum in the last few years (Figure 1), as modern tools allow researchers to efficiently investigate the subtle differences between individual cells.

The article was awarded free publication and an honorarium of $300.00.

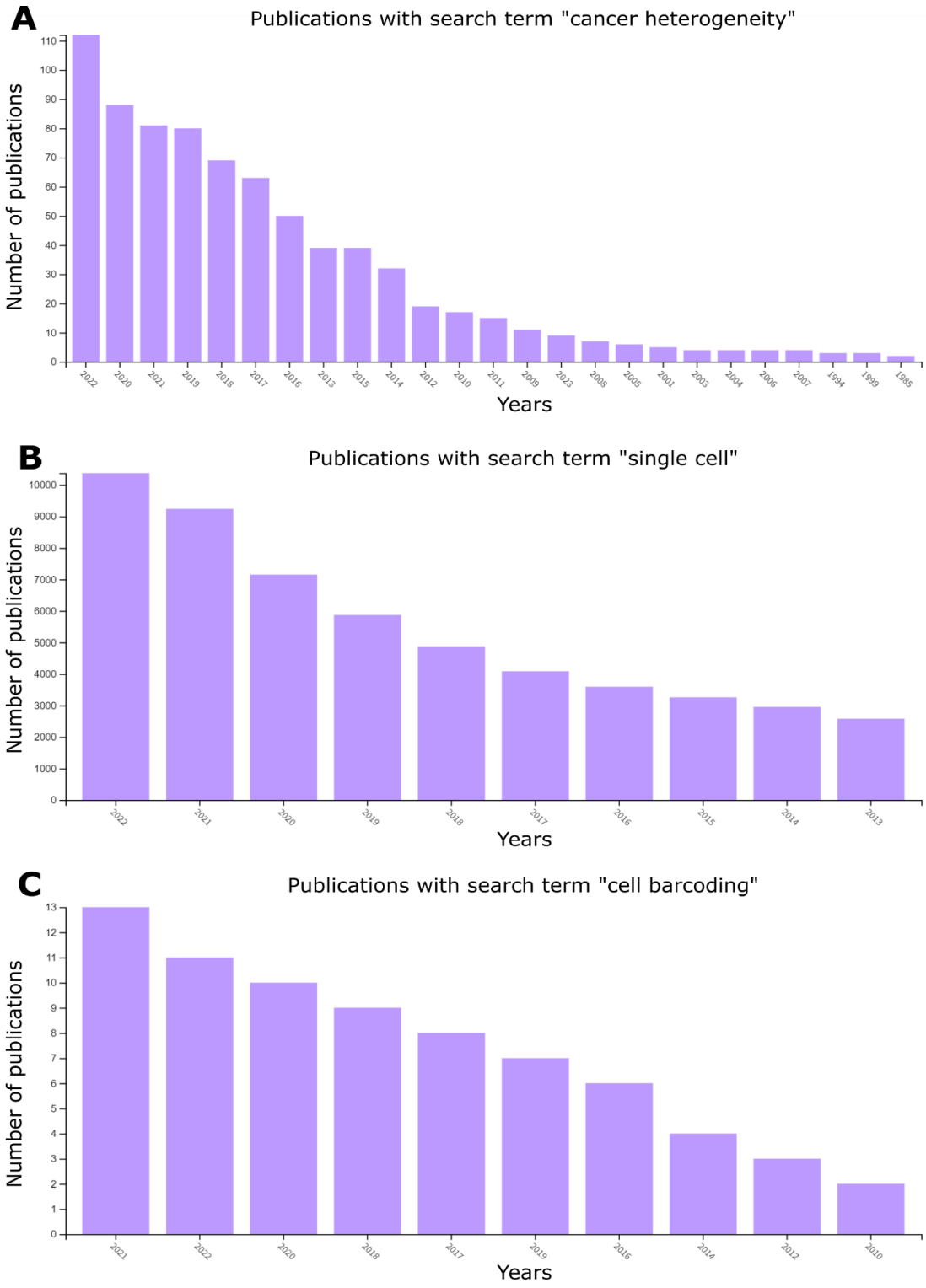

Figure 1: (A) Publications shown by Web of Science when searching for "cancer heterogeneity" in the category topic (accessed 05.03.2023) demonstrating the growing research interest. This research is aided by advanced methodology that allows for example investigation at the single cell level (B – search term "single cell" in the topic") and even longitudinally (C – search term "cell barcoding").

Heterogeneity occurs in different forms

Today, we distinguish between inter-tumoural (between patients) and intra-tumoural heterogeneity (within one tumour). Both are major obstacles in the efficient treatment of any cancer (Figure 2 A,B). Inter-tumoural heterogeneity is mostly attributed to patient specific factors, such as germline variations or environmental factors [6]. Intra-tumoural heterogeneity can be further divided into spatial heterogeneity, e.g. a non-uniform distribution of cancer cells with a distinct molecular signature within and across primary and/or secondary tumour sites and temporal heterogeneity, e.g. cells adopting a changing molecular signature over time (Figure 2 C,D). Genetic, epigenetic, phenotypic and transcriptomic alterations are all considered to contribute to this acquired heterogeneity [6].

Temporal heterogeneity (Figure 2 D) is one of the driving forces of cancer cells transforming from benign to malignant, as cells sequentially acquire alterations that increase their proliferation, evasion and suppression of cell death signals, increase induction of angiogenesis and activation of molecular events that trigger tissue invasion and metastasis [7] (Figure 3 B – top). However, due to their unstable nature, cancer cells do not end evolving after metastasising, instead they often acquire an even more heterogeneous phenotype as the disease progresses [8]. This can lead to the generation of distinct sub-populations that exhibit different sensitivities to common treatments (Figure 3 A,B). Additionally, cancer cell evolution is not always a stochastic event but does occur in response to challenges in their environment. Administrating of any therapy exerts evolutionary pressure on the present cells, which propagates the development of tumour cells that are, and continue to be, resistant to the performed intervention. Consequently, these cells are much harder to target, and this difficulty increases with every relapse. Intra-tumoural heterogeneity is therefore thought to be a strong driver of cancer evolution and subsequently drug resistance [9,10].

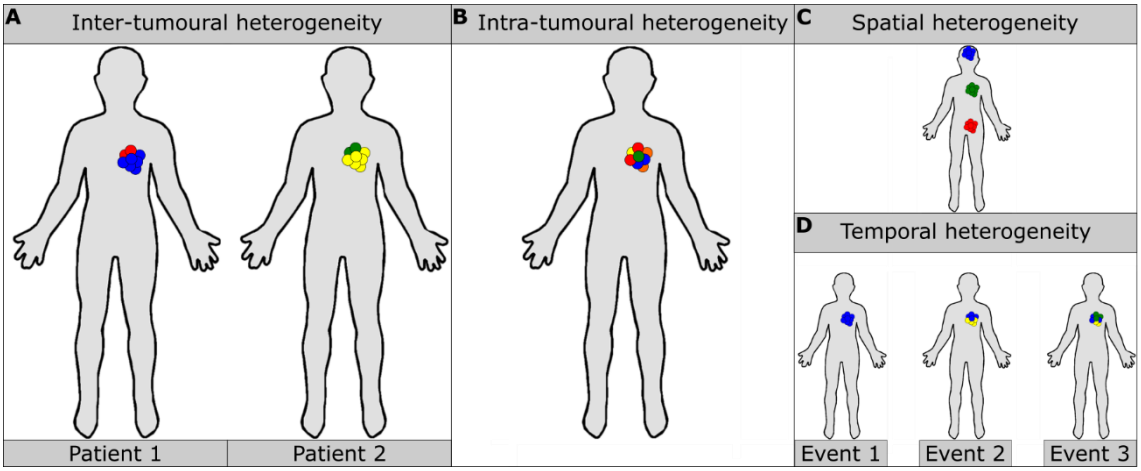

Figure 2: Tumour heterogeneity occurs in different types. (A) Inter-tumoural heterogeneity describes the difference of the same type of cancer (e.g.: lung cancer) in different patients. (B) Intra-tumoural heterogeneity refers to the difference between cells within a tumour or within a primary and secondary site in the same patient. It is further divided into (C) spatial heterogeneity which describes the difference of cancer cells between tumour sites or within one tumour and (D) temporal heterogeneity which describes cancer cell evolution over time. Different colours represent different cell populations.

Genomic instability drives tumour heterogeneity

A strong driver of intra-tumoural heterogeneity is genomic instability, which can result from exposure to exogenous mutagens (smoking / UV irradiation) or from compromised endogenous processes, such as inefficient DNA mismatch repair, or response to oxidative stress (Reviewed in 3, 4). Genomic instability refers to the increased tendency of genome alterations occurring during cell division. Normally such events would be detected by DNA damage checkpoints, mitotic checkpoints or the DNA repair machinery, but all of those processes can be altered/compromised in cancer, which allows cells to accumulate genome alterations, predisposing it for malignant transformation [13]. Chromosomal instability, defined as consistent gains and losses of

either whole or large parts of chromosomes, is known to contribute to genomic instability in many cancers [14].

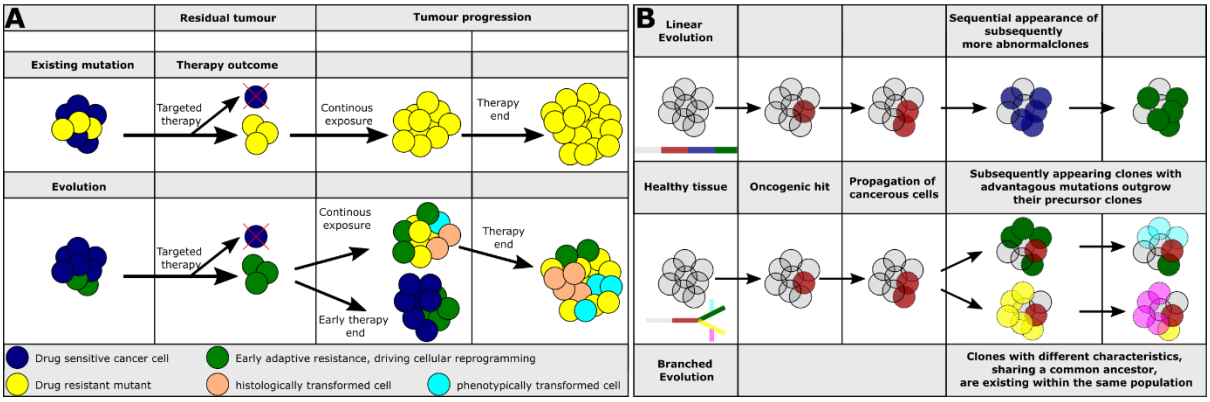

Figure 3: (A) Top: therapy resistance is conferred through an existing mutation, which allows cells with it to grow during and after treatment, causing relapse. Bottom: cancer cell evolution confers resistance. During therapy, adaptive cells are reprogrammed to resist therapy, often leading to the recurrence of a heterogeneous tumour. (B) The two major paths of tumour evolution are either linear (top) or branched (bottom). In linear evolution, an oncogenic hit causes transformation of a cell, which subsequently expands, and the population then acquires a beneficial mutation. This mutations confers improved survival onto the cells, causing the cells to outgrow the precursor clone. This population then acquires the next positive mutation for their survival, again outgrowing the precursor, ultimately generating a homogenous, population with more and more abnormalities. In the branched model, mutations are not acquired subsequently, but simultaneously, which leads to a heterogeneous population with various sub-clones, that all share a common ancestor. Different colours represent different cell populations.

Tumours most likely require more than just genomic instability to develop clinically relevant heterogeneity, but it is still considered to be at the centre of clonal diversity. The original clonal evolution model, proposed in 1976, suggests that initiation of tumour growth is based on a stochastically arising event that causes a healthy cell to undergo malignant transformation. This is followed by subsequent acquisition of genetic diversity through genomic instability (Figure 3 B - top). The arising subpopulations are then subjected to evolutionary selection, causing the emergence of populations with an increasingly abnormal molecular signature [5]. Tumour phylogeny can elucidate if clonal diversity is generated through linear branching (clones being direct descendants from each other), collateral branching (different clones harbouring distinct private mutations), or a combination of both (Figure 3B). A study investigating the Darwinian evolution of clones within childhood cancers (24 neuroblastomas, 24 Wilms tumour, 8 rhabdomyosarcoma) found that collateral branching (sometimes in combination with linear branching) appears to be the typical model for the analysed childhood cancers. Additionally, high risk types with less favourable prognosis were associated with more branchpoints, longer branches and a general higher risk of relapse [15]. This might partially explain why the low risk subtypes are more successfully treated, as a lower genomic instability indicated by linear branching would be less likely to generate therapy resistant subclones fast enough to evade eradication. If relapse after treatment occurs, it is generally within 1 – 2 years after treatment, and resistance is thought to be acquired by mechanisms including activation of compensation/bypassing signalling cascades [16], acquisition of mutations [10] and cell fate changes [17]. Evidence also suggests that these are not necessarily changes occurring in response to treatment, but can be already present in a small population of cells pre-treatment [18,19].

Intra-tumoural heterogeneity can be observed on the microscopic and transcriptomic level, with evidence of a correlation between cancer cell morphology and distinct gene expression emerging as of late. Cell morphology (Figure 4) can be linked to physiological processes, such as cell matrix interaction, drug responsiveness [26], [27] aging [28], cell cycle progression [29], metastatic potential [30] and gene expression. For example, certain genes, such as the tumour suppressor PTEN, can reduce the

morphological heterogeneity of human and mouse metastatic myeloma cells [27].

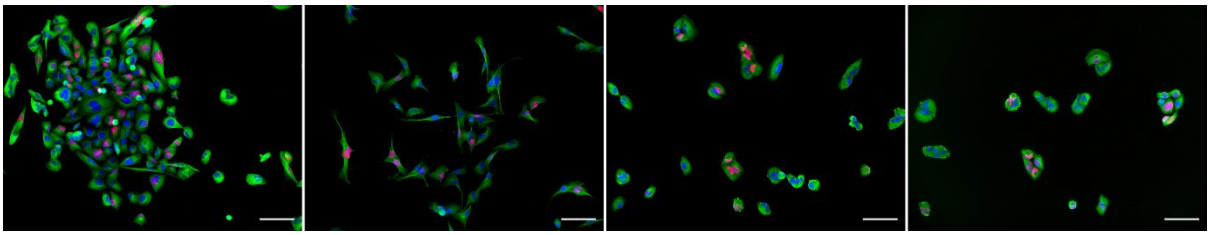

Figure 4: Examples of different morphologies observed in sub-cloned rhabdomyosarcoma cells

Accurate *in vitro* modelling of cancer heterogeneity is paramount for therapy development

Lung cancer is one of the more aggressive cancer types, and the associated survival rate declines with time after diagnosis. 40% of patients survive for at least one year after diagnosis, however that number drops to 15% for the first 5 years post diagnosis and to 10% for ten years post diagnosis [20]. Tumour heterogeneity, which tends to be particularly high in aggressive hard to treat cancers, is one of the factors playing into this, because it allows the tumour cells to develop a resistance to the previously used treatment (Figure 3 A - top). Studying their evolution and the emerging heterogeneity is paramount, so that the healthcare field can develop new, effective drugs against cells that have become resistant to gold standard methodology. As such, it is important to develop models in preclinical research that allow faithful study of this phenomenon in a cell population that is as close to the patient tumour as possible.

Even though clonal heterogeneity is such a well-described phenomenon in a multitude of cancers, observing the same in tumour-derived cell lines has been reported far less. This is problematic, because *in vitro* cell culture is the predominantly used tool for screening potential new therapies and drugs, and them not faithfully reflecting the *in vivo* situation adds further complication to an already challenging field of study. The importance of this problem is illustrated by the example of a human colon carcinoma line, where two distinct subclones differed in morphology and ability to grow anchorage independent *in vitro*, and generated histologically distinct tumours after separate xenografting, with different therapy responses [21].

Furthering the challenge, *in vitro* cell culture can already be equated to exerting evolutionary pressure on a mixture of cells, because the cells that are best adjusted to develop under these precise conditions (e.g.: 5% CO_2, 37°C, 2D culture, plastic surface, specific media) will outgrow the rest, and eventually heterogeneity of the original cell pool will be lost. Media composition, which often vary between laboratories, is another parameter that will favour one cell type over another [22]. Clonal variation between existing cell lines has been demonstrated previously, with MCF7, an estrogen receptor^{+ve} breast cancer cell line, from 27 different laboratories. Only 35% shared coding non-synonymous single nucleotide variants, insertions and deletions between them. Interestingly, the acquisition of heterogeneic phenotypes seemed to be predominantly based on continuous time in culture; five biological replicates of MCF7 cells, grown in different culture media for the same timeframe, generated the same subclone. Additionally, genetic heterogeneity could be re-established after cloning single cell derived clones, suggesting that it can be ongoing genetic instability, rather than stochastic events, that underlie the generation of genetically different subclones [23]. Similar conclusions were reached with the lung adenocarcinoma cell line A549 [23] and cervical cancer HeLa [24] cells.

While this is concerning regarding reproducibility of results between different laboratories using different clones of the same cell lines, it is an interesting phenomenon for the identification and study of clonal heterogeneity in cancer cell lines. It suggests that even after long term *in vitro* culture, when separated by single cell growth, these clonal derived cell lines can still undergo the same genetic instability events that cause them to generate a heterogeneous subpopulation *in vivo*.

The appearance of heterogeneity in cell culture is important for testing new drugs for cancer treatments. For example, 321 compounds were tested in the MCF7 clonally derived lines, with the response

varying strongly. 55 compounds decreased proliferation in at least one line by > 50%, but only 48 of them also decreased proliferation by < 20 % in at least one other sub-clone, which essentially means that most drugs only effectively kill one subclone, as another subclone, with a different composition of cells proved more resistant. As a consequence, it might be more worthwhile testing the response of cancer cell to a drug on individual clones of one cell line instead of using different cell lines [25].

Conclusion

Considering the importance that intra tumoural heterogeneity plays in disease progression and drug response, it is imperative that pre-clinical research into drug efficiency takes into account that not all cancer cells are equal – even when derived from within the same tumour site.

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Conflict of Interest

The author declares no conflict of interest.