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Review of the Role of Intra-Tumour Heterogeneity in Colorectal Cancer

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Abstract:

Colorectal Cancer (CRC) is a highly heterogeneous disease, with pathologically similar cancers having completely different responses to treatment and patient survival. Intra-tumour heterogeneity (defined as distinct morphological and phenotypic differences) has recently been demonstrated to be an important factor in the development and behaviour of cancer cells, and can be used to determine response to anticancer therapy. There are two main theories to explain the presence of genetic heterogeneity; the stem cell theory, and the clonal expansion theory. These are not exclusive, and both are believed to contribute to the process across different cancer types. Furthermore, recent research has shown that epigenetic factors (the heritable changes in gene expression that are not accompanied by changes in the underlying DNA sequence) can be used to explain the presence of heterogeneity, and may precede the development of genetic alterations. The presence of heterogeneity in CRC has important clinical consequences including questioning the way tumours are diagnosed by single biopsies, it has a role as a biomarker, and finally in the management of the disease. This review aims to summarize the current evidence surrounding heterogeneity in colorectal cancer.

Introduction

Somatic Evolution in Cancer

We have become accustomed to thinking of natural selection as a force for good for the survival of species by eliminating the weak and propagating the fittest. However, when we consider the competition between individual cells in an organism, natural selection can become a liability, as mutations that have individual survival benefits for cells may be detrimental for the whole organism [1]. Cancer is considered a classic example of multilevel somatic evolution, where mutant cells have an advantage by increased proliferation and survival, but at the level of the organism it is usually fatal. Therefore the preference at this level will be for genes and organisation of tissues that suppress cancer [2]. This dichotomy in somatic evolution is not fully understood, although it has been demonstrated that on-going serial cell differentiation in mature tissues can suppress cell level selection and somatic evolution [3].

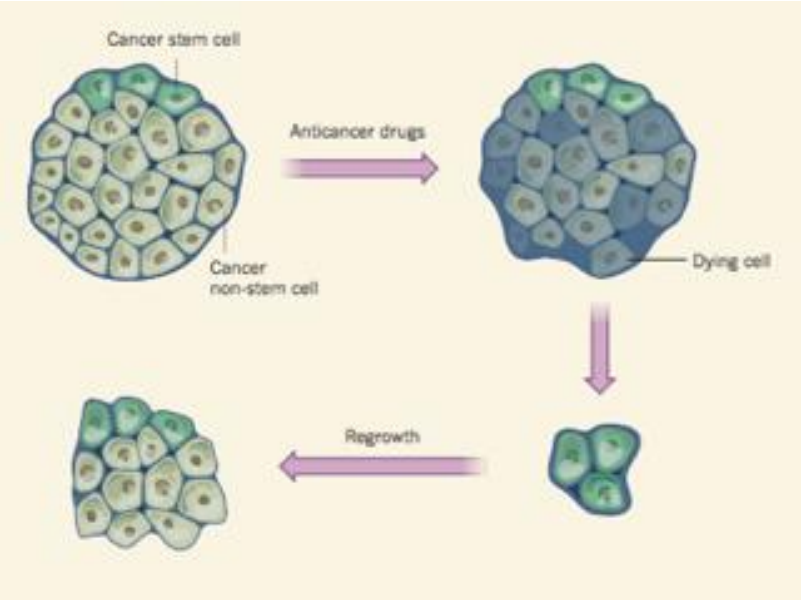
Intra-tumour Heterogeneity

Intra-tumour heterogeneity is defined as the distinct morphological and phenotypic differences within a tumour. This includes cellular morphology, gene expression, proliferation, metabolism, motility and metastatic potential [4]. It has been known since the earliest days of cancer cell biology that phenotypic heterogeneity of cancer cells within a tumour exists, however only very recently have high resolution genome-wide studies confirmed a great amount of heterogeneity within individual cancers and population diversity in mutations involving quantitative trait loci [5,6]. This diversity probably represents a Darwinian, natural selection model of the clonal evolution of cancer biology [7]. There are two models used to explain intra-tumour heterogeneity; the cancer stem cell theory, and the clonal expansion theory. These are not exclusive, and both are believed to contribute to the process in varying levels across different cancer types [8].

The Cancer Stem Cell and Clonal Expansion Theories

The Cancer Stem Cell (CSC) theory originated from the observation of intra-tumour heterogeneity, and the fact that different cells within a tumour behave differently when isolated. CSC are cells within a tumour that have the ability to replicate themselves, as well as giving rise to all cell types found in a tumour of a particular cancer type. Furth and Kahn [10] demonstrated that a single cell from a mouse tumour could initiate tumourigenesis in a recipient mouse, with the resulting cells typically showing further morphologic heterogeneity from the original tumour [9, 10]. In seminal work by Pierce and Wallace [11], it was demonstrated using radiolabelling and auto-radiolabelling techniques that in early stages of tumour growth, DNA labelling was almost exclusively in the non-differentiated cells in a tumour, where as later on, labelling would appear in the well-differentiated areas. This finding supported the theory that the well-differentiated cells derived from CSCs [11]. This CSC theory could also explain the relative resistance of some cancers to single-drug chemotherapy, as the emergence of a genetically variant clonal sub-line, resistant to the treatment may occur. This led to the evidence base for the use of multiple chemotherapeutic agents at once, in order to minimise the chance of a viable CSC surviving, and re-igniting the process (Figure 1) [9].

Figure 1: Heterogeneity leading to anticancer drug resistance. Taken from Cleavers [9].



Around this time, work by Nowell[12] and later by Fearon and Vogelstein [13] lead to a clonal expansion concept of carcinogenesis. This theory, resulting from observations that mutations of oncogenes and tumour suppressor genes cause most cancers, stated that there is sequential selection of more aggressive clonal sub-lines due to acquired genetic variability. Therefore the observed progression of many cancers from a benign start to ever-increasing aggression and instability (e.g. the adenoma carcinoma sequence in colorectal cancer) could be explained.

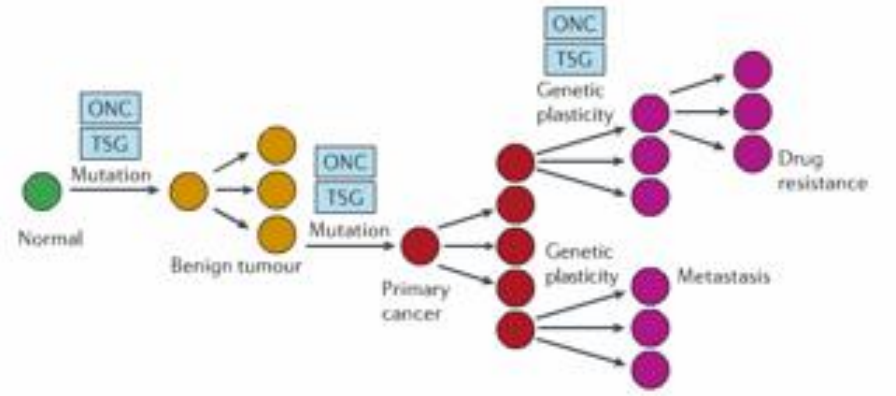
The CSC theory was revived in the late 90s by Bonnet and Dick [14], where it was demonstrated that only the CD34+ / CD38– cells from acute myeloid leukaemia (AML) patients could initiate hematopoietic malignancy in mice. They demonstrated that CSC possessed the ability to self-renew, proliferate and differentiate [15]. Recently, attempts have been made to reconcile the CSC and clonal evolution theories of carcinogenesis. Some cancers, such as chronic myeloid leukaemia (CML) and AML are clearly driven by CSC, however there is still evidence of genomic instability here, and clonal evolution can be seen in action when following immunotherapy, the malignancy becomes tumour-resistant through the emergence of clones that carry mutations in the target areas of this therapy (the BCR-ABL1 fusion gene) [9, 16, 17]. Other cancers such as some CRC develop as a result of serial alterations in oncogenes and tumour suppressor genes (e.g. *APC*, *KRAS* and *TP53*), with the stage of disease roughly relating to the acquisition of these mutations [13].

Epigenetic Progenitor Model

Both genetic and epigenetic processes are thought to be equally important in carcinogenesis [18]. Epigenetic changes are a surrogate marker of clonal genetic changes, as they lead to tumour suppressor gene silencing and oncogene activation [19]. The importance of the relationship between the genetics and epigenetic processes is only now being fully appreciated, and there remains much uncertainty surrounding the sequence of events.

Evidence has shown that epigenetic changes such as CpG island methylation occurs very early on in cancer development, and has been found in normal tissue before a tumour arises. This has led to an epigenetic progenitor model of cancer, where epigenetic alterations affecting tissue-specific differentiation are the predominant mechanism by which epigenetic changes cause cancer [18, 20]. This model involves three steps; the first being epigenetic disruption of progenitor cells, which can lead to a polyclonal expansion of cells that are "neoplasia-ready". This is followed by an initiating mutation step, which was classically considered the first step of tumourigenesis. This step will include mutation of genes such as *APC* or *β-catenin* in the primed cells. Finally a genetic and epigenetic plasticity stage, where there is an environment enabling enhanced ability for both the genetic and epigenetic elements to stably evolve their phenotype (Figure 2) [19].

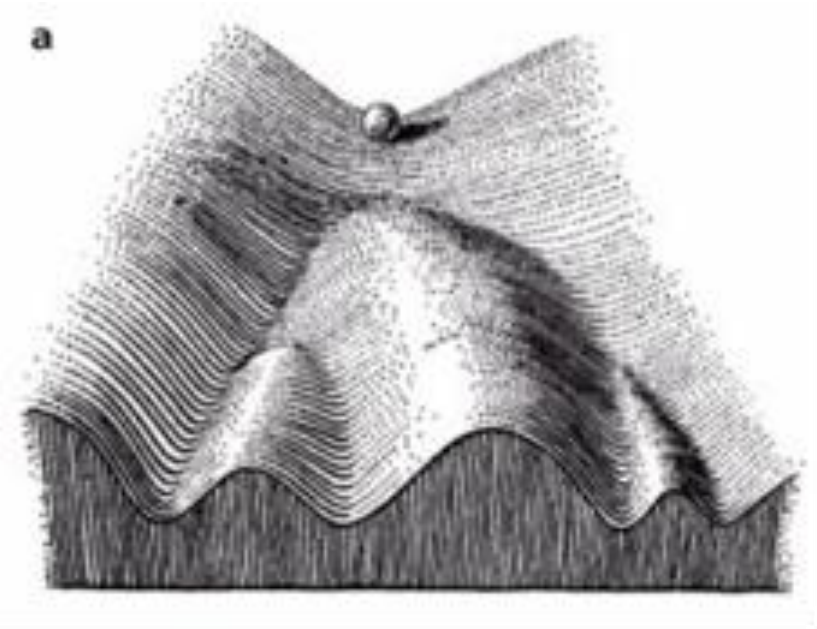
Figure 2: Epigenetic Progenitor model of cancer. Taken from Feinberg [19].



Evidence for this model includes *in vitro* studies demonstrating reversible tumour related growth properties of the progenitor cells, indicating an epigenetic mechanism [21]. One of the first examples of the epigenetic progenitor model in human disease was through a process called Loss of Imprinting (LOI) in the development of pre-neoplastic lesions in the kidney that can lead to Wilms' tumour. In this process, there is CpG hypermethylation in the insulator region immediately upstream of the imprinted *H19* gene on chromosome 11. This results in the transcription factor CTCF failing to bind to the region, and thus a downstream enhancer activates a downstream promoter region of the imprinted insulin-like growth factor 2 *(IGF2)* gene [18]. It has been demonstrated that overexpression of IGF2 can promote tumour growth, or increase the number of target cells that are prone to a tumour initiating event [22].

This epigenetic progenitor model can help explain the highly structured and homogenous genetic and epigenetic nature of normal tissue and how this changes to being highly heterogeneous and disorganised in neoplastic tissue. Waddington [23] initially developed the concept of the epigenetic landscape in 1957 (Figure 3). The original model was designed to explain cell differentiation from the original progenitor stem cell, with the cell depicted as a ball on top of a hillside with valleys of differing depth along the bottom. The height of the valleys represents the relative stability of different states, and the ridges provide 'canalisation' into different states. The same model can be used today as an example of phenotypic stability of a cell in relation to the steepness of the epigenetic environment [6].

Figure 3: Waddington's epigenetic landscape [23].



Changes in the intrinsic (DNA mutations, epigenetic changes) and extrinsic (autocrine, paracrine and endocrine signalling) forces that act on the cell microenvironment can alter the phenotypic stability of the cell (how stable the ball is in the bottom of a valley). It does this by altering the interactions and feedback that exists between expressed genes, in what's termed the gene regulatory network (GRN). Indirect evidence has demonstrated that in the neoplastic environment there are stochastic fluctuations of the GRN (also termed 'stochastic noise'). This enables cells to 'jump' from one valley to the next on the epigenetic landscape, leading to a phenotypic switch [6, 24]. Therefore, the intra-tumour heterogeneity of the microenvironment will lead to phenotypic heterogeneity of the tumour cells, which itself will provide clonal selective pressures within the tumour. This can help explain why tumours alter over time to become metastatic, and can resist cancer therapy [25, 26].

Review

Genetic Heterogeneity

Both the *KRAS* and *BRAF* oncogenes are involved in the MAPK/ERK signalling pathway that regulates cell proliferation, differentiation and apoptosis. The frequencies of these mutations in colorectal cancer have been reported as between 24% and 50% for *KRAS* [27, 28] and between 1% and 13.3% for *BRAF* mutations [28-31]. Extensive intra-tumour heterogeneity of *KRAS*, *TP53* along with other gene mutations have been demonstrated in human prostate, breast, oesophageal and gastrointestinal cancers [32-36]. Significant heterogeneity may also be demonstrated between the primary tumour and their metastases [37, 38].

There is no consensus around what part, or how many samples of a CRC primary tumour should be sampled in order to identify potentially prognostic molecular or histopathological characteristics of a tumour. Baisse et al. [37] performed a study of 15 patients who were treated surgically for advanced primary sporadic colorectal adenocarcinoma (Dukes' C or D) in the late 1990s [37]. They analysed 15-20 areas within the tumour according to the degree of histological differentiation and depth of invasion of the tumour.

In addition, one sample of normal colonic mucosa and lymph node metastases were taken for analysis. The sample location was recorded on a 3-dimentional grid. Samples were tested for gene alterations in *KRAS*, *p53* and LOH in the5qlocus, and18qlocus. They found that 67% of the analysed tumours had tumour heterogeneity in at least 1 gene locus confirming significant tumour heterogeneity in advanced CRC, and recommending a different approach of tumour sampling for prognosis management.

Richman et al. [39] studied 69 primary CRC in 68 patients, and demonstrated that 10.1% patients displayed intra-tumour heterogeneity in *KRAS* codon 12, 13 and *2* or *BRAF* codon 600. Therefore testing DNA from a single block will wrongly assign wild-type status to around 10%. These figures have been replicated by Farber et al.[40]. These papers did not explore the heterogeneity between specific locations in the tumour, as Baisse, Bouzourene [37] had done as the samples had not been mapped at the time of formalin fixation and paraffin embedding (FFPE). Molinari et al. [41] analysed EGFR gene status and protein expression, as well as *KRAS / BRAF* mutations in 38 metastatic CRC. They found *EGFR* gene deregulation in 25 out of 36 primary tumours and 29 out of 36 metastases, *KRAS* mutations in 16 out of 37 cancers and in 15 out of 37 metastases, and *BRAF* mutations in 2 out of 36 cancers and 2 out of 36 metastases. By doing this analysis, they demonstrated that primary colorectal cancer and paired metastasis might exhibit difference with respect to EGFR pathway deregulation mechanisms, which may lead to differing response to treatment.

More recently, Fadhil et al. [42] highlighted that important decisions regarding neoadjuvant treatment are made from a small biopsy sample from the surface of the tumour. This could lead to inappropriate and costly errors in treatment choice. They demonstrated, by comparing molecular markers such as *KRAS*, *BRAF*, *PIK3CA*, *TP53* and MSI, that there was not a significant difference in the markers found in the biopsy sample and the resection sample. This study was on a relatively small patient sample (n=20), and unfortunately they failed to adequately explain what part of the resection sample was tested. This is a significant flaw in this paper, it would be expected that a homogenous result was seen if the same area of the tumour was sampled in the biopsy and resection specimens.

Epigenetic Heterogeneity

The evidence regarding epigenetic heterogeneity in the literature is sparse, with very few studies in the field. One of the reasons for this is that epigenetic alterations can occur in normal, physiological development. For example the methylation status of endometrial cells are related to the menstrual cycle, nutritional status and age of the woman [43]. Epigenetic changes can also occur at different stages of tumourigenesis, further complicating the picture [44]. Therefore deciphering the sequence of events in cancer has proven challenging, and despite the awareness of epigenetic heterogeneity, few examples have been presented to predict the probability of tumorigenicity [45]. Recent research into the heterogeneity of chronic lymphocytic leukaemia (CLL) has provided evidence of highly heterogeneous methylation patterns in the CLL cells compared to normal B cells [46], although this is in non-solid tumour. There was also a proven link between heterogeneous methylation in CLL with aggressive features and a shorter time-to-first-treatment [47].

Heterogeneity of Metastases

Tumours are a result of uncontrolled proliferation of cells. From a benign beginning, they will eventually have the capacity to metastasise, which spreads through the lymphatic and vascular systems; these cells settle down in distant sites and develop metastases. It is particularly important to understand the biology of metastases, as the majority of cancer deaths are a by-product of secondary deposits [48]. Historically, metastases are thought to be the final stage of the clonal succession model of tumour evolution [13]. To support this theory, many studies have demonstrated the close clonal relationships between the primary tumour and its metastases [49-51]. Additionally, gene expression profiles comparing the primary tumour and metastatic site have revealed very similar patterns, which would be unlikely in a polyclonal model [4, 52, 53]. A competing and contradictory theory of a parallel progression of primary and metastatic sites also exists [54]. This states that metastatic spread occurs early in the disease process and therefore greater clonal polymorphism can be observed. To date, unbiased experiments have failed to give a definite answer to which theory is correct [6]. Experiments on some forms of the disease such as pancreatic cancer demonstrated significant genetic divergence between the primary cancer and metastases, despite a common ancestor, whist research on breast cancer has demonstrated much greater genetic similarity, even in metastases that develop months later [55, 56].

Currently, the decision on therapeutic adjuvant therapy is made after examination of the primary tumour specimen [57]. Substantial clonal heterogeneity between primary and metastatic tumours can pose a barrier to targeted therapy, and should be considered, especially in those cancers known to have greater genetic differences between primary and metastatic disease [4]. It is likely that the success of future treatments for metastatic disease will depend on a greater understanding of this process.

Clinical Implications of Intra-Tumour Heterogeneity

The fact that tumours are heterogeneous has various clinical implications. Firstly, solid tumours are biopsied when first discovered. This means small tissue samples are taken from the tumour for diagnostic purposes, and also for selecting the choice of future therapeutic agents in some cases. As the field of oncology advances, and therapies move away from indiscriminate cytotoxic chemotherapy to include a stratified model of treatment dependent on a patient's individual tumour characteristics, the question of whether a biopsy is representative of the whole specimen becomes pertinent. If the molecular features are heterogeneous, then failing to biopsy a certain area may lead to the patient receiving ineffectual treatment, or to deny perfectly effectual medicines based on inadequate biopsy samples. An example of this is the development of targeted biotherapies involving mutations of the *KRAS* and *BRAF* genes [58, 59].

Another clinical implication of intra-tumour heterogeneity is as a biomarker. An example of this can be seen in patients with Barrett's oesophagus, which refers to metaplasia of the lower oesophagus, where normal stratified squamous epithelium with columnar epithelium [60]. The index of clonal diversity (i.e. the extent of intra-tumour heterogeneity) is a strong predictor for malignant progression [61]. Although there is good evidence for this in Barrett's oesophagus, where multiple biopsies are easily obtained and routinely used in clinical practice, no such evidence exists yet for other cancer types. Further trials are needed to demonstrate the role of clonal diversity as a biomarker for disease severity, as heterogeneity within a small sample may be enough to have a predictive value of disease progression [4].

Finally, molecular heterogeneity of a tumour may have implications on the management of disease. Therapeutic agents, be it chemotherapy, immunotherapy or radiotherapy can be thought of as a selection forces that drives natural selection and progression of a tumour [4]. There is evidence that experimental tumours made up of several different clones react differently to cytotoxic drugs compared to monoclonal tumours. This is related to the fact that clonal interactions may potentiate or inhibit the therapeutic action [62, 63]. For example, simple clonal cancers with low levels of genomic instability such as oestrogen receptor-positive breast cancers are associated with a longer duration of response to treatment than genetically more unstable (high-grade) oestrogen receptor-positive cancers [63-65].

Currently, targeted therapy has not become as prominent in the field of cancer treatment as many thought would happen a decade ago. One of the reasons for this is that targeted therapy is based on the most common cell population from the primary tumour, and does not take into account any mutations seen in sub-modal clones in a heterogeneous sample (Sanger sequencing techniques would typically miss a mutation if it were present in less than 20% of cells in a sample) [63, 66, 67]. This may severely limit our ability to predict the response to therapy. As new methods, such as high-sensitivity *KRAS* mutation analysis are developed, there will potentially be a greater role for targeted therapies [63].

Conclusions

It widely recognised that there are founder genetic mutations common to all cells within a tumour from the clonal expansion theory of carcinogenesis [12, 13]. With the recent advances in massively parallel genomic sequencing, which can define the proportion of a tumour with any given mutation, there is now definite evidence of intra-tumour clonal heterogeneity [68]. The driving forces behind this are as yet, unknown, however genomic instability is thought of as a potential mechanism [69]. Much less is known about the role of epigenetics in the carcinogenesis sequence, but in the epigenetic progenitor model, DNA hypermethylation is the root cause of the genomic instability that drives the whole mechanism forward [19]. This suggests a central role of the interplay between genetic and epigenetic factors [18].

Whatever the underlying cause and exact sequence of carcinogenesis, it is becoming increasingly apparent that there are important clinical implications to the presence of intra-tumour heterogeneity. This includes a possible explanation for therapy resistance [62], a need for greater vigilance whist using biopsies to diagnose the disease, and utilizing the presence of heterogeneity as a biomarker [60].

Authors' Contributions

HGJ carried out the literature search and drafted the manuscript. HGJ, DH, JB and GS contributed to the summarization of results and writing of the paper having read and approved the final manuscript.

Authors' Information

HGJ is a trainee surgeon conducting research in the molecular science of rectal cancer at Swansea University. Professors DH and JB are consultant colorectal surgeons at Swansea University and Singleton and Morriston Hospitals. Professor GJ leads a research group in Swansea University School of Medicine investigating carcinogenesis of the gastrointestinal tract.

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