Deciphering intra-tumor heterogeneity from clonal evolution model, cancer stem cells model or CSCs evolution model

Student: Xiaowen Lu (S1802089) Supervisor: Prof. Frank A. E. Kruyt Date: 2015-Janurary-01 t/m 2015-April-30th

Abstract

One major challenge in effective treatment of cancer is posed by intra-tumor heterogeneity. Understanding the mechanism for the derivation of intra-tumor heterogeneity can provide insight for precise determination of targeted therapeutic treatment and overcome drug resistance. Currently, there are two models that are proposed to explain the origin of phenotypic intra-tumor heterogeneity, i) the clonal evolution model that focuses on heritable origin of heterogeneity such as by genetic mutations and ii) the cancer stem cells (CSCs) model that focuses on non-heritable origin of heterogeneity such as by epigenetic changes, protein stability and microenvironment fluctuation. In this review, I will describe the two models and discuss the underlying concepts, supporting evidences, the limitation of each model and the methods available for the study of each model. Although these two models are often considered as mutually exclusive, recently it has been proposed that these two models can be harmonized into a CSCs evolution model. The methods that can be applied to explore the extent to which intra-tumor heterogeneity can be explained by CSCs evolution model are not yet established. To fill this gap, I propose several new ideas to adapt the existing computational method, such as metabolic network modeling and comprehensive comparative analysis, in order to better explain the intra-tumor heterogeneity and identify relevant therapeutic targets.

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Introduction

Cancer exhibits a wide range of phenotypic intra-tumor heterogeneity on multiple levels, such as cellular morphology, gene expression, metabolism, immunogenicity, motility, proliferation and metastasis potential[1]. Intra-tumor heterogeneity poses a main challenge in the effective treatment of cancer at least in two aspects i) it can misguide the detection of molecular diagnosis biomarkers and even bias the determination of targeted therapeutic treatments, ii) it fuels drug resistance capability of tumor cells.

Such heterogeneity can be attributed to both heritable sources such as genetic variants and non-heritable sources. Currently there are mainly two models that attempt to explain for the widespread intra-tumor heterogeneity, i.e. the clonal evolution model for heritable sources and the cancer stem cells (CSCs) model for non-heritable sources. The clonal evolution model suggests the competition for growth space and resources, involving a sequential acquisition of genetic mutations with growth advantages enabling one or multiple groups of tumor cells (subclones) to become dominant and sweep out the less fitted ones. In this competition process, the co-existence of subclones with different genetic mutants leads to the intratumor heterogeneity. The other model, the CSCs model, proposes that tumors arise from a rare population of cells with stem-cell-like properties, i.e. having infinite selfrenewal capacity and the ability to give rise to differentiated progenitors[2]. Consequently, CSCs can result in the generation of all differentiated cell types within a tumor, and therefore lead to tumor heterogeneity. These two models are fundamentally different mechanisms and have different clinical implications. Understanding which mechanism drives intra-tumor heterogeneity in patient tumors will provide better insight to design more effective treatment strategy.

I will focus this review on the two models that can explain tumor heterogeneity by covering the concepts, provide supporting evidence, discuss the limitation of each model and summarize the methods available for the study of each mechanism. Although these two models are often considered as mutually exclusive, recently it has been proposed that these two models can be harmonized as CSCs evolution model [3]. This model, which overcomes some limitation of both clonal evolution

and CSCs model, bears the potential to better explain the intra-tumor heterogeneity. In order to figure out to what extent CSCs evolution model can explain the intratumor heterogeneity, we proposed several novel computational methods via the adaption of existing computational methods, such as metabolic network modeling and comprehensive analysis.

Intra-tumor Heterogeneity: clonal evolution model as a mechanism

Description of the model

The clonal evolution model was first proposed by Nowell[4], which states that the tumor cells acquire various genetic mutations over time, and the stepwise natural selection for the fittest and most aggressive subclones drives the progression of tumor cells. Such sequential selection is parallel to Darwinian natural selection, where cancer clones are equivalent of asexually reproducing quasi-species. According to the model, the initiation of tumors takes place once the normal cells escape from normal growth control by accumulating multiple mutations, leading to mutated cells with selective growth advantages over the adjacent normal cells and bear the potential to undergo clonal expansion. In the expansion stage, the acquired genetic instability generates tumor subclones with additional novel mutations and if such mutations confer a selective advantage in a certain condition they will allow those new subclones to be the predominant progeny subclones until an even more favorable mutant appears. As such, clonal evolution in tumor cells can result in tumor heterogeneity (Figure 1).



Figure 1 Clonal evolution is driven by acquired novel genetic mutations. The grey eclipses represent normal cells. The different colored eclipses represent subclones with accumulated mutations. At time point t_0 , mutation A initiates the growth of tumor from normal cells. The subclone with mutation A (green eclipses) with growth advantages outcompetes the adjacent normal cells (grey eclipses). Along the time scale, different mutations take place at different time point and drive the tumor clonal evolution in a branched pattern. This results in intra-tumor heterogeneity where tumors are composed of subclones with different mutations and growth properties. (Adapted from Fig.1 in [5])

Supporting evidence for the model and clinical implications

According to the description of this model where tumor subclones acquire novel mutations with selective survival advantage, the most straightforward evidence is that the tumors are found to be composed of one dominant genetic clone together with several genetically distinct subclones. One example is that topological sampling of a renal-cell carcinoma has shown that distinct mutations are detected in different tumor regions, which indicates that multiple subclones develop to different parts of a tumor[6]. Several studies[7-9] of comparing genetic alterations between primary tumor samples and the associated metastatic or relapsed samples have revealed the existence of substantial genetic heterogeneity between primary tumors and metastatic/relapsed samples. More interestingly, these studied found that within

the primary tumor samples, multiple genetically distinct subclones were detected to be co-present with a 'founding' clone that only harbor the common mutations found in all subclones. In these studies, the presence of the subclones with additional mutations are proposed to i) give rise to metastatic or relapsed clones and ii) survive the initial therapeutic treatment. More clinically relevant, the clonal evolution model is also supported by studies where drug resistant sub-clones were observed after antitumor therapies, such as treatment with BRAF inhibitor for melanoma patients with BRAF^{V600}-mutant[10] and with Bcr-Abl tyrosine-kinase inhibitor, e.g., imatinib, for chronic myelogenous leukemia[11]. After treatment subclones evolved with drug-resistance ability in both studies. Two different mechanisms have been proposed to explain how drug resistance subclones emerge, either intrinsic (i.e., mutations present at baseline) or acquired (i.e., development of novel mutations after initial response). As for the intrinsic model, drug resistance can arise when a pre-existing subclone carrying a set of drug resistant related genetic mutations survives the treatment and expands at relapse. One example is that subclones with a secondary mutation in KIT (c-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) are present in gastroinitestinal stromal tumors that bear the potential to resist the therapeutic drugs[12]. According to the other model, the acquired model, the dominant tumor clone evolving into relapse clone involves the gain of novel mutations[10]. Even though it is proposed that BRAF-mutant melanoma obtains drug resistant capacity by acquiring novel mutations, it should be noted that the study did not measure the intra-tumor genetic heterogeneity in the baseline tumor samples. Thus, the so-called acquired resistance can possibly reflect outgrowth of small amount of pre-exiting clones. The determination of which model plays a role in tumor drug resistant requires high-resolution sequencing to identify the existence of subclones that are rare but convey the potential capability to resist drug treatment. Clonal evolution of a tumor can result in both spatial (typically for solid tumors) and temporal heterogeneity. Both levels of heterogeneity are closely relevant to effective tumor treatment. For spatial heterogeneity, one related issue is tumorsampling bias, which can confound the interpretation and validation of biomarkers[6, 13]. In clear cell renal cell carcinomas (ccRCC), except for VHL mutation, it has been demonstrated that around 70% of the driver mutations were subclonal[14], which

indicates that multiple biopsies are required to better identify the clinically relevant mutations. As for temporal heterogeneity, the most relevant issue is emergence of metastasis or replace after treatment. One mechanism of the recurrence of tumor is that treatment can act as a selection pressure to drive tumor progression when preexising subclones possess mutations that are linked with drug-resistant phenotype[15-17]. For instance, in non-small cell lung cancer (NSCLC), it was demonstrated that the presence of MET amplification before treatment is the driving force for the development of drug resistance in patients with an EGFRmutant that are treated with EGFR tyrosine kinase inhibitors. The combined inhibition of EGFR and MET was conceived to be beneficial for patients via preventing the selection of the drug resistance subclones[17]. The other mechanism is that cancer therapy can also generate novel subclonal driver events[18-20]. After treatment with temozolomide in low-grade glioma, multiple de novo mutations were detected in recurrent tumors, such as RB1 and PIK3CA, which are associated with GBM, a high-grade tumor with worse prognosis. These examples indicate that the key step in effective cancer treatment is to trace the clonal evolution history by keeping track of longitudinal analysis of tumors in clinical setting.

In silico depiction of clonal evolution

A tumor's subclonal architecture can be reconstructed from sequencing approaches, which can provide insight into cancer evolution. There are two ways to infer tumor evolutionary history from tumor genomes: i) identifying and comparing subclones from genetic mutations in a single mixed tumor sample and ii) comparing multiple samples in an individual tumor or temporal correlated samples.

When phylogenetic inference is conducted in a single mixed tumor sample, the reconstruction of a phylogeny tree for subclonal evolution is comprised of two step: i) identifying clones and ii) relating the clones to each other. Such schema has been applied to reconstruct the progression in studies of breast tumor and neuroblastoma[21, 22]. Genetic mutations can be ordered during tumor development. Here, the principle of these methods is illustrated in Figure 2a. A single mixed tumor sample is a snapshot of the evolutionary process and usually contains cells from multiple subclones that contain different groups of mutants. The first step

is to identify subclones from the genomic profile of a single mixed sample. Bioinformatics tools[23-25] have been developed to infer the number of cells carrying the mutation (the cellular frequency) from its allelic frequency. For instance, PyClone[23] uses a mixture model to identify clusters of single nucleotide variants (SNVs) with the same frequency and meanwhile it corrects the frequencies for copynumber change and loss of heterozygosity to estimate the fraction of tumor cells carrying these mutations. Thus, clustering mutation frequencies can provide information for population structure of tumor. The second step is to order the clusters in a tree, so that these mutation clusters can be linked to clones. For each node in the tree that represents for a clone in a cancer sample, the clonal genome is given by the mutations that occurred along the path in the tree to this node. There are mainly two approaches to order the clusters into a tree. Firstly, cluster the mutations based on frequencies and then build a tree in an independent second step or, secondly the joint clustering and tree building in an integrated model. The first approach can be implemented by using TrAp[26], which uses frequencies of clustered mutations as input and reconstruct a evolutionary tree with consistent given frequencies by solving a highly constrained matrix inversion. The second approach can be implemented by using two very recently developed tools, PhyloSub[27] and BitPhylogeny[28], which combines clustering and tree reconstuction. Compared with the available tools (i.e., TrAp) for the first approach, the unified methods have three advantages. Firstly, since the clustering and treebuilding steps are not independent, decoupling them can limit the performance of phylogeny reconstructions. For instance, the identified clusters are expected to be one node in the reconstructed tumor phylogeny tree. However, the consecutive clustering and tree-building can lead to a suboptimal tree where one initially established cluster spreads out over different part of the tree [29], which is expected to be avoided by using integrated models. Secondly, TrAp puts a limitation on the number of mutation clusters (up to 25) used for phylogeny reconstruction. With such a limitation, TrAp cannot make use of all genetic mutants to infer phylogeny relationship. This might cause the problem of missing some information embedded in mutations that are left out by TrAp. Thirdly, the combined method, BitPhylogeny, is the only available tool that can be applied to methylation data.

Comparison of multiple samples from an individual tumor or patient can also reveal tumor evolution. Reconstruction of samples collected at different time points, i.e., at different tumor development stage or before and after treatment, is particularly informative to identify the initiation mutant and the order of acquisition of additional genetic mutations at different tumor development stages, which is very relevant to understand the occurrence of treatment resistance and clinical relapse[22]. Provided with genomic profiles from multiple samples, one can use each sample as a node of a phylogenetic tree (Figure 2b). Multiple computational tools[30-32] have been developed to infer evolutionary trajectory among different samples. For instance, MEDICC is a recently published method to infer phylogenetic trees of multiple samples by copy number alternation (CNA) profiles, which calculates the distanced between two genomes by counting the minimal number of changes required to 'translate' one genome to the other one. By applying this method to 177 temporally and spatially distinct high-grade serous ovarian cancer samples from 18 patients a phylogenetic tree was generated to quantify the intratumor heterogeneity and allowed the identification of seven patients with high clonal expansion degree[33]. The authors demonstrated that these patients have significantly shorter survival duration. Interestingly, by reconstructing the evolutionary history of the tumor within each patient a subclone was identified carrying a certain mutant that was associated with chemotherapy resistance.

Multiple phylogenetic methods have been recently developed to automate the modelling of the evolutionary relationship between tumor subclones (Table 1). Most of these methods can be applied to infer evolutionary history from a mixed single sample or multiple samples. The required input for these methods can be either single-nucleotide variation (SNV), copy number alteration (CNA) or both. What needs to be pointed out is that the combination of SNV and CNA can help to determine the order of acquired mutations during tumor development. For instance, if there exists a CNA in a population and the same SNV is found on all copies, one can postulate that the SNV event was before the CNA. On the other hand, if the SNV is only found on one copy, then it can be inferred to happen after the CNA.



Figure 2 Methods to reconstruct evolutionary relationship. (a) Reconstructing phylogeny tree of subclones in a mixed tumor sample. The mixed tumor sample is composed of 4 different suclones, where blue, red, green cells represent tumor cells and grey cells represent normal ones. Mutation profiles are usually measured directly from the mixed tumor sample. To reconstruct the phylogeny relationship of these subclones, the first step is to infer subclone clusters from mutation frequency distribution, where each subclone cluster convey a set of mutants. After identifying subclones within a mixed tumor sample, the next step is to order and link the clusters in the tree. In this example, the leaf nodes are characterized by subclones with different combination of four mutations, A, B, C, and D. The percentage on the tree branches indicate the fraction of cells with a certain set of mutations, e.g., 84.6% of all cells have mutation A, 69.2% additionally have B. Internal node with mutation A and B are fully replaced by its descend nodes with mutation ABC or ABD, which is no longer present in the tumor sample. (b) Reconstruction phylogenetic tree of genomic profiles from multiple samples. Each row corresponds to a measured genomic profile of one

sample, where black cell represents the presence of a mutation. (adapted from Figure 3 and Figure 6a in [34])

Table 1 Computational tools to implement phylogenetic methods for reconstructing evolutionary relationship between subclones (adapted from Table 2 in [34])

Tools	Input Data	Algorithm/Model	Referenced
			(PMID)
PhyloSub	SNV	Tree-stick-breaking process,	24484323
		binomial/MCMC	
PyClone	SNV	Dirichlet Process, beta-	24633410
		binomial/MCMC	
SciClone	SNV	Beta mixture model	24633410
Colmial	SNV	Binomial/EM	25010360
Тгар	SNV	Exhaustive search under	23892400
		constraints	
rec-BTP	SNV	Local search	24932008
ThetA	CNA	Maximum likelihood	23895164
cancerTiming	CNA	Maximum likelihood	24064421
GRAFT	CNV	Patial Maximum likelihood	21994251
MEDICC	CNA	Finite state transducer,	24743184
		Minimum-event distance	
TuMult	CNA	Breakpoint distance	20649963
TITAN	CNA	HMM/EM	25060187
CloneHD	SNV+CNA	HMM, EM, Variational Bayes	24882004
mixClone	SNV+CNA	EM	25707430
BitPhylogeny	SNV+CNV+methylation	Tree-stick-breaking process,	25786108
		Bayesian inference	

*SNV: single-nucleotide variant; CNA: copy number alternation; MCMC: Markov-Chain Monte Carlo; EM: Expectation Maximization; HMM: Hidden Markov Model;

Limitations of the clonal evolution model

First, the performance of the model is constrained by the available mutation information measured from a biopsy that can be mixed with different subclones or even normal tissue. Current developments in single cell sequencing technology provide a potential strategy to overcome this limitation. For instance the mixture of normal tissue in a tumor biopsy can dampen the signal for tumor specific mutation calling. Meanwhile, single cell technology allows one to get access to the sequencing data in a single cell, which is either a tumor or a normal cell, instead of a mixture of both cells. Using such type of data can precisely identify tumor specific mutations. It should be noted that due to the cell-to-cell genetic heterogeneity, some of the identified mutant variants may have no contribution to clonal expansion. This requires the scale-up of the number of sampled individual cells. If large numbers of single cells are analyzed, phylogenetic lineage tree can be constructed to describe their evolutionary relationships and trajectory[35-37].

Secondly, the clonal evolutionary model is mainly focused on genetic heterogeneity, such as heterogeneity revealed on SNV and CNVs. However, this model has not yet considered how other non-genetic variability, such as epigenetic variation, microenvironment variation and functional interactions among clones within tumors, can affect the intra-tumor heterogeneity. For instance, functional cooperation between clones were found to be essential for tumor maintenance in breast cancer[38]. Thus, developing a model which takes into consideration of not only genetic mutations but also different types of non-genetic variability can contribute to better explanation of intra-tumor heterogeneity. The potential solution or methods to achieve a better model is discussed in the next part of the review.

Intra-tumor heterogeneity: cancer stem cells model as a mechanism

Description of the model

The cancer stem cells (CSCs) model proposes that a particular subpopulation of tumor cells with stem cell-like properties, called 'cancer stem cells', drive tumor initiation, progression and recurrence. These cells have similar characteristics of normal stem cells, i.e., the capability to self-renewal infinitely and to differentiate.[2] The differentiated progeny generated by CSCs do not have unlimited self-renewal and differentiation capacity. Such self-renewal and differentiation capabilities lead to the generation of all cell types within a tumor, therefore generating tumor heterogeneity. It should be noted that the CSCs model cannot provide an answer to

the cell of origin for a tumor because CSCs are isolated from end-stage tumors. The precise origin of CSCs is still under debate. They are proposed to originate from normal stem cells that have mutated genes causing loss of the regulation of normal self renewal, or from mutated progenitors that regain the ability to infinite self renewal, or from the de-differentiated cells with activated self renewal related genes (Figure 3) [39].



Figure 3 Cancer stem cells model. Cancer stem cells are proposed to originate either from mutated normal stem cell, mutated progenitor cells or mutated differentiated cells. Cancer stem cells have unlimited self-renewal and differentiation capacities, which can form a clear hierarchy of differentiated cells within a tumor. The co-existence of CSCs and their various differentiated progeny cells results in intra-tumor heterogeneity. (Adapted from Figure 1 in [39])

Supporting Evidence for the model and clinical implications

According to the CSCs model, cancers have a hierarchical organization of tumorigenic and non-tumorigenic cells. The most direct evidence for this model is to purify the tumorigenic population from the mixture with non-tumorigenic cells and to show that only the tumorigenic cells have the capacity to initiate the tumor development. The first experiments indicating the existence of both tumorigenic and nontumorigenic cells within a tumor were performed in an animal model where myeloid leukemia initiating cells were successfully isolated by using the cell surface makers associated with normal hematopoietic stem cells i.e., CD34+/CD38-. These cells were found to be able to initiate leukemia in severe combined immune-deficient (SCID) mouse[40] while other isolated cells, which are CD34+/CD38+ could not. After this discovery, the CSCs were identified for the first time in solid tumors, i.e., breast cancer. A subset of breast cancer cells was isolated by using cell surface makers for normal breast stem cells, CD44+/CD24- and these cells can generate tumors after being xenografted to SCID mouse while CD44-/CD24+ could not[41]. Till now, CSCs have been identified in different types of solid tumors, such as brain[42, 43], colon[44], lung[45], ovary[46, 47], pancreas[48], prostate[49] and melanoma[50].

If CSCs indeed exist in one tumor and their self-renewal capacity stimulates the tumor progression, the clinical parameters, such as survival rate, relapse and metastasis, should be more closely related with tumorigenic cells than nontumorigenic cells. First, the CSCs appear to be more resistant to standard cancer treatment compared to non-tumorigenic cells in different type of cancers, i.e., chronic myeloid leukemia[51], gliomas[52] and breast cancer[53]. Moreover, tumorigenic cells also exhibit differences with the remainder of cells in the capacity of evasion of cell death[54] and metastasis[55]. Collectively, this suggests that an effective cancer therapy requires the selectively depletion of CSCs. Currently, there are two different strategies for targeting CSCs. First, inhibiting the over-activated pathway or protein that controls stemness in CSCs can result in significant reduction of tumor cell growth. Several signaling pathways were found to be essential for maintenance of the capacity of self-renewal, proliferation of normal stem cells. The dysfunction of these pathways may lead to the generation of CSCs, which offers new strategies for cancer treatment. Particularly, some of the signaling pathways are characterized to be responsible for the formation of CSCs, such as Hedgehog, Notch and Wnt/beta-catenin pathways[56, 57]. For instance, blocking over-activated Notch pathway in glioblastoma by gamma-secretast inhibitors can effectively reduce neurosphere growth in vitro and reduce tumor growth in vivo[58]. Since these signaling pathways are also active in normal stem cells, inhibition agents of these pathways can not only targets the CSCs but also the normal stem cells. The main challenge for targeting the signaling pathways is to modify the inhibition agent or

use drug combination to improve the specificity of treatment. Moreover, targeting the cell surface markers can also provides useful methods to inhibit tumor growth. For instance, applying an antibody directed against CD44 can inhibit the growth of xenotransplanted acute myeloid leukemia (AML)[59]. The second approach is to stimulate the differentiation of CSCs so that it can restrain the capability of selfrenewal. The most well-known example is using all-trans-retinoic acid to enhance the tumor differentiation in the treatment of acute promyelocytic leukemia[60]. Due to the clinical need for better treatment, future research should make effort to understand what genetic or molecular differences lead to the functional differences between the tumorigenic population and non-tumorigenic cell population. In addition, due to shared properties between CSCs and normal stem cells it is important to study to what extent CSCs differ from normal stem cells to minimize the harmful impact of the treatment on normal stem cells.

Techniques for the study of CSCs model

CSCs are mostly identified and enriched via the approaches for normal stem cells identification. The most common scenario in CSCs identification is as follows. First, one or multiple cell surface markers, which are often well established in normal stem cells, are examined for differential expression in one tumor sample. Based on heterogeneous expression profiles of the markers, CSC-enriched populations are sorted out of the remainder of the cancer cells and then transplanted into immunodeficient mice by limiting dilution assay[61] to assess its tumor initiation capacity[62].

Although the xenograft limiting dilution method is considered as the 'golden standard' for identifying human CSCs, this method still has some caveats. First, xenografts can only capture a snapshot of the state of CSCs when a tumor sample is collected. The empirical validation of the stability of the CSCs is still not available. Instead, some studies have indicated that the cancer cells can fluctuate between CSC and non-CSC states[63-66]. For instance, H3K4 demethylase JARID1B is identified to be differentially expressed in human melanoma cells, where JARID1B-cells cycle faster than the JARID1B+ ones[64]. However, the researchers found that JARID1B-can arise from JARID1B+ cells and vice versa. This indicates that one subpopulation

of cancer cells can have temporal heterogeneity which is required to maintain the development of the tumor. Given such observations, the plasticity of CSCs should be taken into consideration when xenograft experiment at a fixed intrinsic state is applied to represent the CSC status of a tumor from which cancer treatment is determined. The second caveat is the immune-compromised mice used in the method. The immunodeficiency facilitates the transplantation of the human cells to the mice. However, such system lacks the elements that are considered to be substantial for the growth of tumors[67, 68]. Thirdly, the method depends on the specificity of the markers. However, CSC cell surface markers remain largely unknown for most tumor types, especially for solid cancers. Even though several cellsurface markers have been proposed to identify CSCs in some solid tumors, such as in breast[41], brain[42, 43] and colon[69] tumors, some markers are selected based on the observation that they show heterogeneous expression patterns in a tumor instead of direct evidence for their functional linkage with stem cells. This can lead to the scenario the cells identified by these markers are simply a subclone with growth advantages instead of CSCs. Such markers can lead to conflicting results. One example is CD133+, which is among others used to identify CSCs in gliomas[52]. This result was contested by the experiment in rat that CD133- cells can be tumorigenic and give rise to CD133+ glioma cells[70]. Moreover, the currently CSCs xenograft model does not take consideration of the possibility that more than one type of CSCs may exist within one tumor sample. Different CSCs may co-exist in sample tumor consisting genetically different subclones. An alternative method to identify CSCs in solid tumor is sphere-forming assays[71]. Cells from tumors, usually solid tumors, which are able to grow in suspension in non-adherent culture condition and form a 3-D sphere-shaped structure, are identified to be CSCs. This strategy has been applied to multiple solid tumors, such as brain tumor[42, 72], breast tumor[73] and melanoma[74], and provided evidence for the presence of CSCs in these tumors. One caveat of this approach is that this type of assays requires small amounts of cells to be plated. However, a tumor can be viewed as a complex social system[75], which requires interaction between different tumor cells and normal cells in the tumor environment. Without the stimulus provided via the interaction with surrounding

cells or environment, a CSC does not form a sphere. As such, this can lead to the under-estimation of the number of CSCs.

Limitations of the CSCs model

The CSCs model argues that tumor stem cells undergo epigenetic modification, which is similar to the differentiation process of normal stem cells, to form a hierarchical lineage with phenotypically various progeny that have limited proliferation capacity. According to the model, tumor cells are viewed as a genetically homogenous population and attribute the phenotypic heterogeneity mostly to epigenetic variation. Thus, a major deficiency of this model is that it ignores the existence of the genetic distinct subclones. One tumor might be composed of multiple genetically different subclones, which differ in proliferation potentials. Moreover, these subclones can possess different cell-surface makers. Thus, the fractionation of CSCs out of non-CSCs can be simply the segregation of subclones with high proliferation capability with subclones with low proliferation capability. In this context, it is necessary to test the tumor initiating capability of CSCs in a genetically identical subclone. Additionally, it is suggested to carry out genetic analysis in xenografts and compare its mutation profile with that of the primary tumor to figure out whether novel genetic mutations emerged that may bear selection advantage and promote the expansion of the tumor. Such analysis can shed light on whether CSCs also undergo evolution procedure.

CSCs evolution model: a combination of CSCs and clonal evolution model

Hypothesis for CSCs evolution model

In the previous paragraphs, we have discussed two different models that can describe the origin of phenotypic intra-tumor heterogeneity observed at different levels, ranging from cellular morphology to metastasis potential. The clonal evolution model, which focuses on tracing the heritable source i.e., genetic mutants, of intra-tumor heterogeneity, hypothesizes that subclones with specific genetic

mutations will have growth advantage that will promote clonal expansion. On the other hand, the CSCs model, which explains the non-heritable sources of intra-tumor heterogeneity, proposes that the CSCs in the tumor have infinite self-renewal and differentiation capacity, which is analogous to normal stem cells. According to this model, the tumors are organized into a hierarchy of tumorigenic and non-tumorigenic progeny. Both models can explain the observed intra-tumor heterogeneity to some extent. However, as mentioned above both have some limitations and fail to explain the heterogeneity completely.

Even though these two models are fundamentally different, they are not mutually exclusive; instead, they can be unified to complement for each other's limitation and better explain the intra-tumor heterogeneity. For the clonal evolution model, the main issue is that even cells within a genetically homogenous subclone can still exhibit differences in functions, such as cell longevity, proliferation capacity and sensitivity for chemotherapy[76]. One possible explanation is that there are CSCs in such genetically homogenous subclones, which can result in a hierarchal structure of cells with functional heterogeneity. As for the CSCs model, the cancer stem cells are thought to be non-static entities; instead they can evolve. Studies that combined cancer genetic analysis and functional xeno-engraftment have revealed that subclonal genetic diversity exists among functionally defined tumorigenic cells, i.e., CSCs[77, 78]. The genetic variation detected in tumorigenic cells mirrors subclonal patterns, which supports the evolution of CSCs. Moreover, these different genetic mutations that identify subclones within the tumorigenic cells can lead to functional heterogeneity including aggressiveness of xenografting repopulation[77].

Taken together, I propose CSCs evolution model as a unification of CSCs and clonal evolution models. In this model, the emergence of a set of genetic identical CSCs give rise to a tumor that consists of a hierarchy of a minority of CSCs (i.e., tumorigenic cells) and a large proportion of more differentiated non-tumorigenic cells. Progressing with the time of tumor growth, the initial set of CSCs accumulates growth/self-renewal advantageous genetic mutations. These genetic mutants lead to the emergence of a new subset of CSCs that bear growth advantages and can outcompete the initial CSCs set. As such, these CSCs can expand in subclones, which

start and drive the clonal evolution in a tumor. In other words, clonal evolution occurs within the CSC compartment of tumors (Figure 4).



Figure 4 CSCs evolution model. A set of genetically identified CSCs (red CSC) initiates the growth of a tumor that is composed of a hierarchy of CSCs and differentiated non-tumorigenic cells. Along the time of the tumor progression, genetic mutations that convey growth or self-renewal advantages are accumulated in the initial CSCs, which give rise to a novel set of CSCs (green CSC). This triggers the tumor to undergo clonal evolution. The newly emerged CSCs bear growth advantage and have the potential to outcompete the initial CSCs.

Potential research techniques and methods

It remains largely unknown what are the most suitable techniques and methods that can be applied in the study of the CSCs evolution model. Here, by revisiting and integrating the available experimental and computation methods, I come up with several potential ways that can be applied to reveal insight of the CSCs evolution model.

Comparative analysis

Based on the description of the CSCs evolution model, the following straightforward questions need to be solved first; i) whether there exist genetically distinct CSCs

among different genetic homogenous subclones; ii) if yes, what are the genetic and epigenetic differences among these CSCs and do they exhibit functional variations such as proliferation capacity; iii). Cancer types where the existence of CSCs has been confirmed are likely to follow the CSCs evolution model. However, it should be noted that perhaps not all cancers follow the CSCs model[79]. It is necessary to figure out in what cancer types the CSCs evolution model plays a role. To answer these questions, the first substantial step is to successfully identify and isolate the CSCs in each genetically defined subclone. To achieve this, two steps of experiments are required. The first step is to identify genetic homogenous subclones. According to the examples listed in the previous section[77, 78], this can be achieved by applying xenograft assays after the use of genomic analysis to classify the subclones based on the genomic mutation profiles. The next step is to identify and isolate the CSCs in each subclone, which can be realized by using specific surface makers. Another way to study the diversity of CSCs in subclones is starting from single cells. In a recent study[80], researchers established 4 subclones from 4 different single cells derived from tissue from one glioblastoma patient. Differences in morphology, the self-renewal and proliferation capacities among these 4 subclones were observed. Comparing the subclones identified via genetic analysis showed that these subclones derived from single cells can sustain the genetic homogeneity within a subclone to the most extent, which ensure the identification of CSCs specific to only one genetic subclone.

Current technical developments in single cell level DNA sequencing[81], RNA sequencing[82] and epigenome profiling[83] make it possible to generate genetic data for CSCs that are low frequency in cancers. Provided with this rich data resource, we can carry out all type of comparative analysis. For instance, we can compare the RNA sequencing measured from different CSCs and identify the most differentially expressed gene sets. A simple gene ontology (GO) term analysis on these differentially expressed genes can reveal which cellular components, molecular functions or biological processes are enriched in these differentially expressed genes. This can potentially pinpoint the signaling, regulatory metabolic pathways whose activation or suppression is the underlying reason for the observed functional difference among these CSCs from subclones. Moreover, comparative analysis of

DNA or RNA sequencing data can also reveal difference of mutations in cancer genes, i.e., oncogenes or tumor suppressor genes, among CSCs from subclones. The drug inhibitor information is already available for some of the cancer genes, such as gefitinib and erlotinib hydrochloride as inhibitors for epidermal growth factor receptor (EGFR)[84] and nilotinib as an inhibitor for Bcr-Abl tyrosine kinase[85]. Together with the knowledge of effective drug inhibitors, knowing which cancer genes promote the growth of different subclones is very valuable in providing instruction for designing drug combinations to inhibit the growth of all subclones within a tumor.

The previously described comparative analysis can provide an explanation for intratumor heterogeneity on one dimension, i.e., the difference between the evolving CSCs. If the data for CSCs and its differentiated cell population from a genetic homogenous subclone are available, one can explain intra-tumor heterogeneity from another dimension, i.e., heterogeneity attributed to non-heritable sources such as variation on epigenetic level. In the study of stem cells, it has already been shown that transcription regulatory networks play a key role in the programming of differentiation and dedifferentiation[86, 87]. For instance, comparing the epigenetic landscapes of CSCs against the differentiated progeny can identify the transcriptional factors (TFs) whose activation or dysfunction can manipulate the programming between tumorigenic and non-tumorigenic cells[88]. The identification of such TFs can shed light on therapeutic targets that are essential for the dedifferentiation capacity.

Metabolic network modeling

Some studies have already shown that genetic variations in tumors could lead to variation in metabolism, such as difference in serine metabolism dependence[89] or TCA cycle function[90]. Such cases indicate the potential to use modeling and simulation of cancer cells to figure out the extent to which enzymes, metabolites or pathways exhibit heterogeneity i) between the evolving CSCs identified in genetically defined clones or ii) between CSCs and their differentiated progeny. This can provide informative molecular mechanisms underlying the observed phenotypic heterogeneity.

Here, I will discuss about using metabolic network modeling to explore how the CSCs evolution model can explain intra-tumor heterogeneity. In metabolic network modeling, metabolic reactions are represented as a stoichiometric matrix (S) of size m*n (m rows and n columns), where each row represents a metabolite, each column is a reaction and each element is the stoichiometry coefficient of the metabolite in that reaction. Constraint-based modeling, a systems biology approach, is applied to simulate the basic phenotypes, such as cell growth (i.e., represented by the biomass equation) or the production of certain metabolites. There are two main types of constraints imposed on the network: mass balance (stoichiometric) and capacity boundaries. The former constraint is represented as equations that balance the inputs and outputs of each reaction and the second constraints is represented as inequalities that put capacity constraints on the each reaction flux. These two types of constraints define the solution space for the simulated phenotypes, i.e. one can find the optimal flux distribute for all the reaction in the solution space that can lead to the maximal cell growth. (see [91] for more detail).

The key step in metabolic network modeling is to reconstruct a genome-scale metabolic network that is most representative for a specific cancer cell, or a cancer cell line. Recently, several studies have successfully reconstructed constraint-based metabolic networks for individual cell lines, tissues or patients. Here, I present a summary of the available methods to reconstruct the cancer cell/cell-line/tissue specific metabolic networks (Table 2). The basic idea underlying the reconstruct is to use different types of omics data to infer active reactions in certain cancer cells. For instance, gene expression or protein abundance profiles of a sample can be converted to a reaction activity according to the gene-protein-reaction(GPR) association. The reactions that are not supported by the data, such as non-active reactions, can be removed from the networks. After obtaining the metabolic network, which can reflect the biological status of a certain cell line, one can genome-widely predict the phenotypic response, such as cell growth or producing certain metabolite, following gene knockouts/knockdowns[92], or simulate the effects of drug applications[93].

Table 2 Algorithms for the reconstruction of a tumor cell, cell line or tissue specificmetabolic network

Algorithm	Reconstructed model	Integrated data	Purpose of the model
MBA: Model Building Algorithm[94]	General cancer model [21694718]	Transcriptomic data from 60 tumor cell lines	Assessing metabolic drug targets
INIT: Intergrative Network Inference for Tissues	Tissue specific model for both cancer and health samples[22615553]	Immunohistochemical staining data, metabolomics data and transcriptomic data	Identifying reporter metabolites associated with cancer
mCADRE: Metabolic Context-specificity Assessed by Deterministic Reaction Evaluation	26 tumors and cell lines[23234303]	Transcriptomic data	Identifying cancer- specific pathways
iMAT: integrative Metabolic Analysis[95]	HepG2 cell line specific model[96]	Transcriptomic and proteomic data	Indetifying pathway via which p53 functions as a tumor suppressor
MPA: Metabolic Phenotype Analysis[97]	392 breast tumor samples models	Transcriptomic data	Predicting cell growth, metabolite biomarkers

When metabolic network modeling is applied to study the CSCs evolution model to better explain the intra-tumor heterogeneity, one can reconstruct a specific metabolic network for each set of CSCs identified in a genetically defined subclone by integrating their transcriptomic, proteomic, or epigenomic data. After the model reconstruction of each set of CSCs, first, it is possible to simulate the growth rate difference among these sets in the presence of certain selection pressures, such as shortage of nutrition in the growth median or the application of drug inhibition. A set of CSCs with maximal growth rate can be predicted to convey selection advantages. It might outcompete the other subclones and needs specific drug treatment to inhibit its growth to achieve effective tumor therapy. Secondly, one can also reveal the difference of enzyme, metabolites phenotype or pathway activity among all the sets of CSCs in a certain growth medium. It should be noted that these selection pressures could be easily simulated in metabolic network modeling by constraining the flux of transporter reactions for growth nutrients or the flux of the reactions whose enzymes are inhibited by applied drugs. Thirdly, current advances focusing on simulating the cell-cell interactions[98, 99] make it possible to consider cancer development as a result of a community of various cells. This is particularly interesting to simulate the tumorigenic cells grow together with non-tumorigenic cells and figure out the mutual interaction between them.

Cancer can be considered as a phenotypic representation of a dysfunctional biological network involving multiple cellular processes such as transcription regulatory, translation and signaling networks in addition to metabolism. It is still in demand of breakthrough in mathematic modeling so that we can systematically reconstruct the other types of networks. Future work should be focused on developing methods[100, 101] to bridge the various types of networks into a whole picture to better understand the interplay between different cellular processes. This is particularly important to simulate the effect of microenvironment change in the determination of cell fate, where molecular changes in the microenvironment can trigger the activation of specific signaling pathways, which can provoke the response on transcriptional regulatory networks. Consequently, protein/enzyme activity can be affected due to the transcription of its encoding gene is regulated by the transcription network.

Taken together, the integration of different types of omics to networks will enable us to reconstruct a biologically representative network. It can trace and track the dynamic genetic and environment change and estimate the consequence of the variations. Most importantly, it can shed light on identifying novel biomolecules targets for cancer treatment and the determination of effective therapeutic strategies such as drug combination.

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