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Cancer Subclones Derived from the Patient's Head and Neck Squamous Cell Carcinoma Tumor Stem Cells for the Screening of Personalized Antitumor Immunotherapy and Chemotherapy

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Abstract

Studying on subclonal evolution of cancer stem cells can help illustrate how the immune system recognizes tumor cells, leading to subclonal treatment by immune-based therapies. Here, we discuss that cancer subclones derived from the patient's head and neck squamous cell carcinoma tumor stem cells can be used for the screening of personalized antitumor immunotherapy and chemotherapy, to maximize benefits and to minimize the adversary effects, toward personalized or precision medicine. We propose a "wait-and-watch" scheme for monitoring a lifetime cancer stem cell subclonal development evolved with local environments to cancer.

Keywords

Tumor stem cells; Subclonal development; Tumor microenvironment; Single-cell; Therapeutics

INTRODUCTION

The US National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) created the Cancer Genome Atlas (TCGA) Project in 2006 with bulk tumor of cellular heterogeneity with one-time point [1]. However, it is essential to track down subclonal evolution of cancer stem cells (CSCs) evolving with treatment, such as Temodar®-driven mutations [2,3]. Indeed, Darwinian pressures arising from systemic therapy, result in the clonal selection of initially rare subclone variants within a tumor. Novel

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DISCLOSURE

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technologies such as single cell circulating tumor cell sequencing would allow real-time monitoring of clonal heterogeneity, including Loss of heterozygosity (LOH) assays at genetic DNA level, at primary and metastatic sites, as well as for improved targeted early intervention, better care, prognosis, tumor subclonal recognition with immunotherapy [4]. This approach is of particular interest in highly lethal cancers like glioblastomas and colorectal adenocarcinomas, which express high intratumoral heterogeneity when a single signaling network is observed [5,6]. Therefore, advances in transcriptome analysis at single-cell resolutions have become increasingly sought out tools to investigate causative signal pathways alterations in cancer, as they can specifically address the shortcomings of studying bulk lysates for heterogeneous tumor biopsies [7]. Single cell stem cells were very tough to extract from heterogeneous populations of tumour, however; recent innovative single-cell sequencing to profile the gene-expression landscapes of more than 20,000 cells in the motor cortex of brain [8], makes it possible for complex tumors to track the subclonal evolution through phylogenetic analysis [9].

Subclonal evolution of cancer as tackled by immunotherapy is recognized by the 2018 Nobel prize in Physiology or Medicine, awarded jointly to James P. Allison and Tasuku Honjo [10] for his discoveries of how the immune system recognizes tumor cells – they have created a change in thinking in subclonal treatment by immune-based therapies. Immunotherapies, which boost the body's own immune system to eliminate cancerous cells, could be an alternative approach to target CSCs [11]. Targeting an interlinked network that connects pluripotency factors and key cell cycle genes of CSCs may improve efficacy [12]. Thus, Immunotherapy using autologous CD19 based modified T-cells (CAR-T cells) for cancer raises hope for cancer patients [13]. A highly competent and educated reservoir of immune cells may exist within the spleen, in which a specialized environment exists for splenic CD11b(+)Gr-1(int)Ly6C(hi) cells, mostly comprising proliferating CCR2(+)-inflammatory monocytes in the marginal zone of the spleen [14]. These cells, closely associated with memory CD8(+) T cells, cross-presenting tumor antigens can result in tipping the balance toward recognition or tolerance but these same cells may also be significantly depleted or completely abrogated during treatment cycles with chemotherapy, leading to lack of tumor-specific immunity [15]. Ability to fight infection and tumor by these immune cells is abolished by chemotherapy due to the destruction of established memory achieved by previous antigen recognition. All these immune approaches need to target CSCs or TICs subclones, therefore; isolation of patient CSCs is essential for the determination of such targeting strategies [7]. We use HNSCC as an example to illustrate such an approach step-by-step.

First, it is essential to define the Head and Neck Squamous Cell Cancer (HNSCC) stem cells by the biomarkers. The aldehyde dehydrogenase (ALDH) is a polymorphic enzyme responsible for the oxidation of aldehydes to carboxylic acids. ALDH can be used in the identification of CSCs [16] in HNSCC. It has been shown that stem cells express elevated levels of ALDH. Chemoresistant cancer cells express high levels of ALDHs, particularly in HNSCC. The ALDH family of enzymes detoxifies both exogenous and endogenous aldehydes. Since many chemotherapeutic agents, such as cisplatin, result in the generation of cytotoxic aldehydes and oxidative stress, we hypothesized that cells expressing elevated levels of ALDH may be more chemoresistant due to their increased detoxifying capacity and

that inhibitors of ALDH may sensitize them to these drugs. In another word, HNSCC-CSCs can be identified by high cell-surface expression of CD44 and high intracellular activity of ALDH, termed ALDHhighCD44high [17]. Microarray analysis of cisplatin-resistant ALDHhighCD44high cells indicates that their signaling pathways have significant implications for the pathobiology of cancer (e.g. TNFα, IFN, IL6/STAT, NF-κB, FGF2) [17]. The activation of ALDH3A1 by a small molecule activator (Alda-89) increased survival of ALDHhigh HNSCC cells treated with cisplatin while treatment with a novel small molecule ALDH inhibitor (Aldi-6) resulted in a marked decrease in cell viability, suggesting a promising strategy [18].

Second, HNSCC cells that are resistant to chemotherapy, lead to tumor recurrent or metastatic that has the poor prognosis with less than 1 year median survival and [17]. Autologous CSCs for the screening of personalized treatment (precision medicine) can be derived from HNSCC pateint specimens. Studies in our lab have revealed that both CSC and its related tumor microenvironment can be used for therapeutic detection before administration to patients. We think that tumor surveillance and response by these patient's autologous CSC screening help determine how patient's CSC react and evolve with therapeutics, i.e., co-evolution of CSCs with therapeutics. Reports from brain tumor stem cells in glioma patients [7] and in primitive neuroectodermal tumor [19,20] with single-cell transcriptome for relapse prognosis [21] and *in situ* hybridization [22] prompted us to realize that patient's HNSCC CSCs (HNCSC) possess a capacity for tumor evolution of therapeutics as such development shows prognostic value for patients. With upregulation of vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) of highly angiogenic phenotypes, tumor endothelial cells (TECs) exhibit higher proliferative and migratory capacity, compared with those of normal endothelial cells (NECs). Such TECs show ALDHhigh cell populations by fluorescence-activated cell sorting (FACS) [23]. Sheng et al. [24] demonstrated that with decreased ALDH activity, the expression levels of stemness-associated markers, CD133+, octamer-binding transcription facto 4 (Oct4) and sex determining region Y box 2 also reduced. They also showed, "an increased number of mice developed tumors in the ALDHhigh group 16 weeks following the injection of 500 cells, whereas tumors appeared at eight weeks in the ALDHlow group". The mice in the ALDHneg group exhibited less tumor formation under these conditions." They conclude that "$ALDH^{high}$ cells had characteristics of self-renewal ability, in a relative resting stage; while the $ALDH^{low}$ cells had characteristics of GCPCs with limited self-renewal ability, but were in a rapid proliferation stage" [24]. Thus, HNCSC cells can be identified with drugs sensitive to ALDH(high)+ cells and isolated with their resistance to fluorouracil (5-FU) *in vitro* and *in vivo*, while tumor endothelial cells (TEC) can be identified with high ALDH activity ($ALDH^{high}$), along with upregulation of stem-related genes such as multidrug resistance 1, CD90, ALP, Oct-4, Platelet-derived growth factor (PDGF)-A [25].

Third, as well documented in glioblastomas [26], medulloblastoma [27], leukemia [28] and germ cell tumors [29]: all of these cancer types evolve with treatment (radiation and chemotherapy) surviving by coming up with new mutations in subclonal evolution, which can be defined by single-cell transcriptome technology (Figure 1). For example, "Although well-tolerated, the efficacy of bevacizumab was somewhat disappointing, possibly due to the high rate of secondary high-grade gliomas in the studied patient cohort and the late use of

bevacizumab in the course of the disease" [30]. Following analysis of tumor specimens, distinct molecular pathogenesis of secondary tumor arising after radiation therapy was determined by cancer genome-scale technology for genomic mutation signatures, particularly, discovered in secondary neoplasms after cranial or craniospinal radiation in high-grade astrocytomas that prognoses for poor clinical outcomes [31]. Surprisingly, this cohort had "a high frequency of TP53 mutations, CDK4 amplification or CDKN2A homozygous deletion and amplification or rearrangements involving receptor tyrosine kinase and Ras-Raf-MAP kinase pathway genes including PDGFRA, MET, BRAF and RRAS2," but, "lacked alterations in IDH1, IDH2, H3F3A, HIST1H3B, HIST1H3C, TERT (including promoter region) and PTEN, which genetically define the major subtypes of diffuse gliomas in children and adults." Such subclonal changes can be tracked down using single-cell RNA sequencing, as shown in 3321 single-cells from six primary H3K27M-glioma and matched models, for the discovery of "oncogenic and developmental programs in H3K27M-glioma at single-cell resolution and across genetic subclones." This subclonal tracking surfaces a therapeutic window [32] on potential therapeutic targets.

To identify a therapeutic window [32] on potential therapeutic targets, we need to define spatiotemporal expression patterns of new biomarkers from HNSCC to significantly improve the efficacy of therapies. As such a new biomarker, known a molecular mechanism, AF4/FMR2 family member 4 (AFF4), the core component of Super elongation complex (SEC), is upregulated dramatically in HNSCC, which is a potential target of therapies for patients with HNSCC [33]. Besides, Disulfiram (DS) has been reported as an inhibitor of ALDH and increasing studies showed it has anti-cancer effects in a copper (Cu)-dependent manner [34]. As "DS/Cu inhibited the expression of stem cell transcription factors NANOG and OCT4, and abolished the clonogenicity of multiple myeloma," we postulate that DS may regulate HNSCC stem cells. Another line of evidence that "HNSCC contains cancer stem cells (CSCs) that have greater radioresistance and capacity to change replication dynamics in response to irradiation compared to non-clonogenic cells [35]," can help characterize the effects of radiotherapy on "CD44+/ALDH+" HNSCC stem cells derived from patients, providing screening for responsible patients, as "CD44+/ALDH+" HNSCC stem cells are of radioresistance. Hyaluronan (HA), an important glycosaminoglycan component of the extracellular matrix (ECM) and its major cell surface receptor, CD44, Nanog/Oct4/Sox2, have been suggested to be important cellular mediators influencing tumor progression and treatment resistance in head and neck cancer [36]. Personalized medicine-based approach can model the patterns of chemoresistance and tumor recurrence using ovarian cancer stem cell spheroids [37]. Gene set enrichment analysis and iPathway analysis identified signaling pathways with major implications to the pathobiology of cancer (e.g. TNFα, IFN, IL6/STAT, NF-κB) that are enriched in cisplatin-resistant $ALDH^{high}CD44^{high}$ cells when compared to control cells. Such pathway analysis establishes the relationship between $CD44^{high}$/$CD133^{high}$/$CD117^{high}$ cancer stem cells phenotypes and Cetuximab and Paclitaxel treatment responses in head and neck cancer cell lines [38].

Fourth, a new concept of "living with cancer subclones" or "co-habit with cancer subclones" [39] sounds odd and against the decades-long dominant trend of "targeted molecular destruction of cancer," however; its focus on modifying the tumor microenvironment [40] gains attention with clinically proven case reports. When the treatment benefits and the side

effects sound odd, can the patients risk their lives for such an intervention? That is particularly promising given the effect of epigenetics [41], immunotherapy [42] and microbiome [43] on cáncer; all lead to a wait-and-watch approach to disease, due to the fact that we can monitor the relapse pathway [44].

CONCLUSION

In Summary, to achieve above benefits for a patient, we need to isolate the patient's CSCs (Which is the REAL CHALLENGE still!), and to determine the mechanism by which subclones of $ALDH^{high}CD44^{high}$ HNCSCs resist to drugs via single-cell transcriptome, as we show with breast cancer, with specified tissue-relevant tumor microenvironment [45]. Measuring survival of HNCSC CSC lines in presence of cisplatin (or other FDA approved drugs) in cellular models for cancer subclonal evolution [39] can help develop therapeutics to drive cancer cells to dormancy, which is a lifetime subclonal evolution process developmentally evolved with local environments to cancer. All the procedures are still ongoing and under way - They need long-term monitoring and confirmation if they indeed work.

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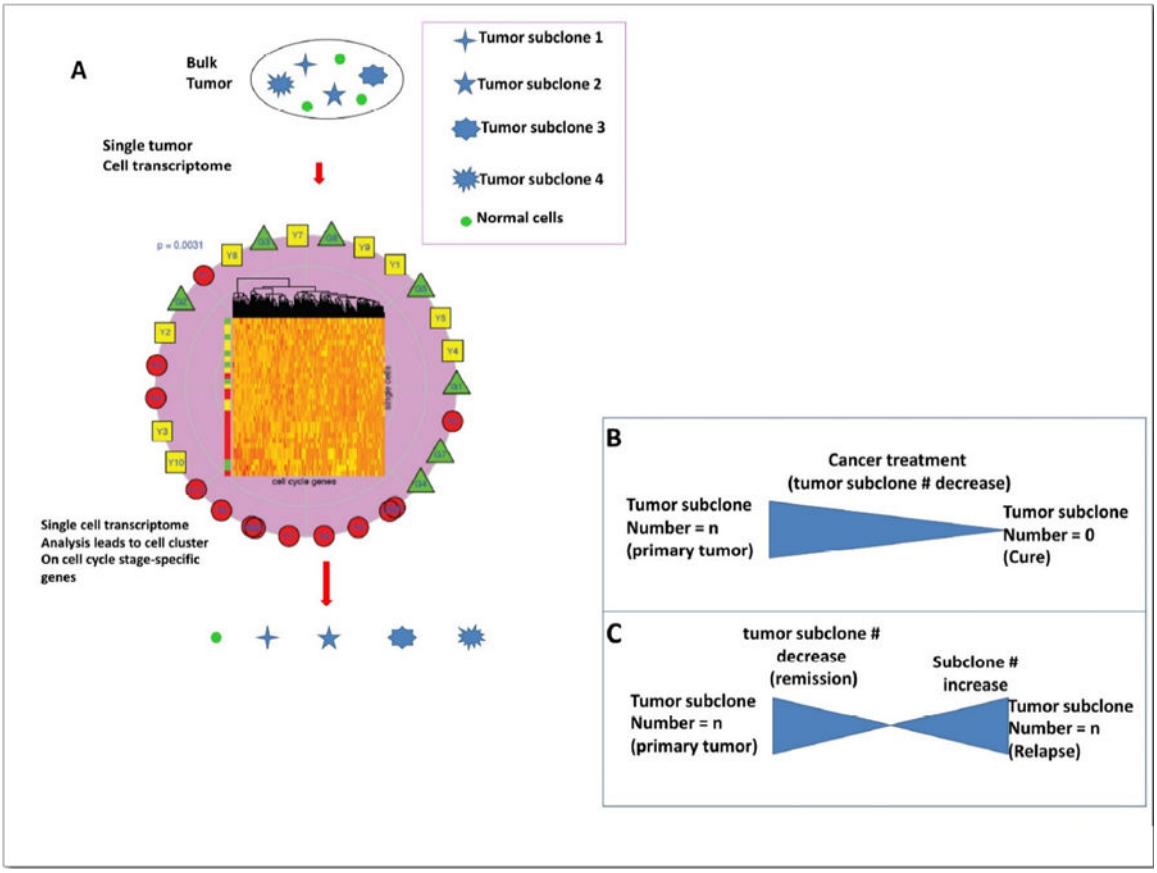

Figure 1.

Sequential perturbations of cell-cycle-phase-specific genes derived from single-cell transcriptomes of patient tumors are applied to treatment. ***A:*** After organizing single-cell transcriptomes by similarity into a sequential order (center-clustering), expression levels of various cell-cycle-phase-specific genes were plotted to visualize the sequential perturbation of individual genes during the cell cycle, a virtual time series. Expression levels were scaled from 0 (undetectable) to 1 (maximum expression). Cell cycle phases were defined and colored. As expected, G0/G1-specific genes had higher expression levels in the G0/G1 phase and an S-specific gene was mainly expressed within the S phase. G2/M-specific genes had high expression levels in G2/M phase and early G0/G1 phase. The sequential expression order suggests that mRNAs of many G2/M-specific genes are not degraded until early in G0/G1 phase after cell division. ***B, C:*** Cancer subclones are defined by single-cell transcriptome-clustered cell cycle gene clustering, which is used to guide treatment. (Adopted from Li, S.C., et al. 2018 [2])