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Will cancer stem cells provide more effective biomarkers?

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1. ABSTRACT

Cancer stem cells (CSCs) represent a rare subpopulation of tumor cells responsible for tumor initiation and eventual patient relapse. Strong preclinical evidence points to the importance of this population for therapeutic intervention and currently there are several anti-CSC therapies under development. While we begin to advance these therapies into the clinic, there is a need to develop biomarkers to measure both patient response and prognosis. This review will discuss the experimental evidence for the role of CSCs in patient outcomes as well as the need, challenges and the hope that CSC biomarkers may provide.

2. INTRODUCTION

Human tumors are comprised of a variety of different cell types. These include endothelial cells, pericytes, infiltrating immune cells and cancer-associated fibroblasts, in addition to the tumor cells themselves (1). Even within the tumor cell population there is significant heterogeneity at both the genomic level and phenotypic levels. This heterogeneity has long presented a problem for cancer drug development, due to the differential sensitivity of cells within the tumors. This also presents important considerations for developing biomarkers as changes in biomarkers may reflect changes in the composition of the tumor rather than frank response of the tumor to therapy.

Over the last decade a significant amount of research has demonstrated that among the different cell types present within a tumor, is a cell type referred to as a cancer stem cell (CSCs). CSCs, found in both liquid and solid tumors (2,3), are unique not only in their phenotype but also in their role in the tumor. They are distinctive in their ability to initiate tumorigenesis and have been shown to be resistant to many therapies, including common chemotherapies and radiotherapy. Therefore they often persist in patients following treatment and are posited to lead to eventual relapse of the patient (4). Currently there is a significant effort within the research community to further understand and ultimately develop therapeutics aimed at eradicating this tumor subpopulation. In addition to the need for new therapeutics there is a need to redesign our clinical trials for anti-CSC monitoring. Effective therapies are often determined by RECIST, response evaluation criteria in solid tumors, which was established in 2000 (5). RECIST provides a standard for measuring decreases in abject tumor burden in both adults and pediatric cancers in clinical trials. While this is often an effective endpoint for evaluation of new therapies, anti-CSC therapies which only target the CSC population are unlikely to lead to reduction of the tumor burden owing to the fact that CSCs represent only a relatively small proportion of the tumors. Rather, anti-CSC therapies are anticipated to have the greatest effect on tumor progression and relapse with more suitable end points being progression-free or overall survival. While these endpoints are the most relevant to the patient, since reduction in tumor burden does not effectively predict a better overall outcome for the patient, they do require larger and lengthier trials. Therefore, surrogate biomarkers of response are need to provide an effective way to measure the ability of a given therapy to reduce the CSC frequency in early and smaller trials and build confidence in the efficacy of the therapy.

This review will explore our current understanding of cancer stem cell biology with an emphasis on how this may translate to patient outcome. It will also outline critical areas for biomarker development and the challenges faced.

3. CSC BIOLOGY

CSCs were first identified by Bonnet *et al.* in acute myeloid leukemia (2). Since then CSCs have been identified in nearly all solid malignancies (4). While CSCs are often identified with different cell surface markers depending on the malignancy from which they were isolated, many similar underlying biological attributes have been uniformly ascribed to them. Paramount among all of these is the ability to self-renew which leads to seeding the tumor both during its initial outgrowth but also during relapse (6-8). Self-renewal leads to both the maintenance of the CSC reservoir through symmetrical division but is also responsible for fueling the more differentiated cells through asymmetrical division (9). The process of self-renewal in stem cells, both normal and malignant, is not subjected to the controls of senescence so that the reservoir of CSCs is a long-lived population capable of persisting for years. This property has been speculated to allow the acquisition of mutations culminating in the initial tumorigenic event. Coupled with this property is an increased innate resistance to chemotherapeutics. CSCs have been reported to have both increased expression of drug efflux pumps as well as to be relatively quiescent leading to the ability to escape the effect of cytotoxics (4). These properties, coupled with the long-lived nature of CSCs, allow the CSCs to repopulate a tumor after chemotherapeutics debulk the tumor initially and may lead to the gain of further genetic mutations that allow for the escape of the tumor and mediate drug resistance. Indeed, there have been numerous reports both from preclinical cell line and xenograft models as well as from clinical samples demonstrating that the CSC population is expanded after therapeutic intervention (10).

3.1. Intrinsic resistance

The definition of a CSC can be defined as "a subclass of neoplastic stem cells that propagate malignant clones indefinitely and produce an overt cancer" (11). This operational definition speaks to the beginnings of the neoplastic process but also has important implications to the growth and survival of tumor cells. Several elegant lineage tracing experiments published in 2012 showed the origination of tumor growth from the CSC population but also showed that these cells were able to survive chemotherapy and repopulate the tumor once chemotherapy was halted (6-8).

CSCs have been reported to be resistant to several chemotherapies and some targeted therapies (10). The reasons behind this inherent resistance have been reported to be the result of several different mechanisms: (a) increased ability to efflux drugs, (b) increased ability to repair DNA, (c) general quiescence of CSCs. Like normal hematopoietic stem cells (12), several groups have shown that CSCs can be defined by their ability to efflux the vital dye Hoechst 33342, the so-called side population (SP). Further, it has been shown that the SP can harbor cells with increased tumorigenic potential (13). Two different members of the ABC transporter family, P-glycoprotein (MDR1) and breast cancer resistance gene (ABCG2), can efflux Hoechst dye affording resistance to different therapies (14,15). Indeed, CSCs become more sensitive to chemotherapy in the laboratory when a general ABC transporter inhibitor, verapamil, is added to cell cultures suggesting that drug efflux is one way in which the CSCs escape therapeutic intervention (16,17). Therefore, combinations of CSC targeting drugs with inhibitors of drug transporters could be a viable option for therapy in the clinic.

Other studies have highlighted that CSCs have an increased ability to repair DNA affording them yet another mechanism to escape chemotherapeutic intervention. While the mechanism of action of chemotherapeutics varies, several, including platin-based drugs and doxorubicin, rely on DNA damage to induce apoptosis of cells. CSCs from several malignancies have increased DNA repair activities, which have been reviewed elsewhere (18,19). In addition to increased ability to repair DNA damage, CSCs have also been shown to more efficiently use the DNA checkpoint pathways resulting in greater integrity of the repair system. Following either single strand breaks or double strand breaks Chk1 and Chk2 kinases, respectively, are activated to induce cell cycle delay so that efficient repair of the DNA can take place (20). Pre-clinical studies using the checkpoint inhibitors, AZD7762 and KU55933, resulted in increased sensitivity to chemotherapy in NSCLC and to radiation in breast cancer CSCs (20,21). These studies highlight the possibility of combinatorial therapies that may be capable of eliminating CSCs from tumors.

Yet another mechanism employed by CSCs to escape therapy includes relative quiescence. Since most chemotherapies and radiotherapy kills actively dividing cells, cells that are slow cycling show greater resistance. CSCs of a wide variety of malignancies show a relative low level of proliferation in comparison to other cells present in the tumor. Demonstration of the slow cycling nature of CSCs has been accomplished through the use of labels that are lost through divisions such that long-term label retention is the result of slow cell proliferation. Using the dye PKH26 which is incorporated into the lipid bilayer of cells and is diluted out under subsequent divisions, Richichi *et al* (22) demonstrated that the long term label-retaining cells found in glioblastoma were able to self-renew *in vitro* and were tumorigenic *in vivo*. Similar studies have also been shown to mark CSCs of colorectal (23),

ovarian (24), pancreatic (25) glioblastoma (26) and a gastric cell line (27). Likewise, these studies also showed an increased chemotherapeutic resistance of the long-term label retaining cells.

All of these studies highlight that CSCs are more resistant to chemotherapy and radiotherapy thereby making it essential that oncologists and researchers consider new therapeutic strategies to eliminate these cells and develop new biomarker strategies specifically designed to assess the extent of CSCs remaining after therapy.

3.2. Prevalence and patient survival

In addition to showing that CSCs are resistant to conventional therapies pre-clinically, the prevalence of CSCs in a multitude of malignancies has been linked to poor patient prognoses in the clinic. In breast cancer both the CD44+CD24-/lo and ALDH+ population have been shown to mark cells with increased tumorigenic potential (2,28). Likewise, both of these populations are also correlated with a poor prognosis (28-30). In glioblastoma, several studies have linked the high prevalence of the glioblastoma CSC surface marker CD133 with a worse clinical outcome (31,32). In colorectal cancer, the prevalence of CSCs not only predicted a poor prognosis, but also predicted a poor response rate to the conventional chemotherapy FOLFOX (33).

Other studies have determined expression patterns of proteins and genes associated with CSC self-renewal and pluripotency as an alternative to the use of surface markers. For example, high expression of nestin was linked to poor prognosis in glioblastoma (31,34). Eppert *et al* also showed that a gene expression signature associated with leukemic CSCs could predict overall survival in acute myelogenous leukemia (34).

These studies suggest that the prevalence of CSCs in a tumor may shape the therapeutic strategy employed among patients and further highlight the need for reliable diagnostic biomarkers for CSCs.

3.3. Role in Metastasis

Since CSCs are thought to be the initiators of primary tumor formation, it stands to reason that CSCs are also responsible for seeding metastatic lesions (35,36). Further, within the circulating tumor cells there should be a subpopulation of circulating CSCs (circCSCs). Initially this was shown in breast cancer patients, where approximately 35% of circulating tumor cells were shown to express the CSC surface markers $CD44^{+}CD24^{-/lo}$ (37). Since then, several studies have found a considerable fraction of circCSCs in the patients of lung, colon and hepatocellular cancer (38-40). Recently, experimental evidence has shown that these circCSCs are able to establish tumors in mice (42-44). It has also been demonstrated that when circCSCs, but not other circulating tumor cells isolated from breast cancer patients are injected into the tail vein of mice, they are able to establish tumors at other sites suggesting that circCSCs are primarily responsible for metastatic spread (41). Similarly, in hepatocellular carcinoma CD90+CXCR4+ cells isolated from the blood of patients resulted in animal models that had distant metastasis (40).

The clinical significance of circCSCs is just beginning to be explored. It is hampered, in part, by the limitations of finding rare cells in the blood (45). To circumvent this problem, most methods rely on the enrichment of tumor cells with EpCAM. Enrichment of tumor cells is a required step during analysis due to the high numbers of lymphocytes and red blood cells in comparison to the tumor cells. However, loss of EpCAM by cells undergoing epithelial mesenchymal transition, necessary for metastatic spread, concerns several investigators (46). Newer methods of detection are being explored as alternatives to EpCAM enrichment and may help to characterize the different populations of tumor cells, including circCSCs (47). These new methods include negative selection strategies, addition of other markers to enrich for CTCs, and physical separation methods that rely on size since tumor cells are larger than lymphocytes and red blood cells (48). Once reliable methods can be established then the relevance of the number of circCSCs to both the extent of metastasis and clinical outcome can be established. In addition, if a correlation between modulation of circCSCs and tumor CSCs in response to therapy can be established, circCSCs may serve as a biomarker for not only metastasis but patient response to anti-CSC therapies. This would provide a minimally-invasive approach to monitor such effects, making it possible in settings where serial biopsies are not feasible.

4. BIOMARKER OPPORTUNITIES

The link of CSCs with both patient prognosis and metastasis implies that an understanding of how CSCs are modulated following therapy could prove to be a useful biomarker to predict the clinical course of patients. Therapies that decrease the overall prevalence of CSCs may thus portend a favorable outcome. Measurement of CSCs may either be through direct sampling of the tumor or could potentially be accomplished through circulating factors, such as circulating CSCs or soluble factors. These will be discussed in more detail below.

4.1. Tumor biomarkers

Quantitation of the CSC population within tumors would be the most direct method of demonstrating a correlation between CSC prevalence with clinical course. However this approach is challenging due to many factors. First the prevalence of CSCs within a tumor is generally low, requiring a large population of cells to be counted in order to ensure an accurate reading. This can

be difficult in small samples, such as fine needle aspirates and core needle biopsies which may also induce a sampling bias depending on where the needle is placed within the tumor. The second difficulty of this approach is the lack of knowledge of the most relevant marker to rely on for accurate CSC quantitation. CSC heterogeneity, even within a single patient is just being explored, and a thorough understanding of the hierarchical organization of tumors is still forth coming (49). This limitation may be circumvented by using functional assays of CSCs, such as sphere formation. This approach was employed by Schott AF *et al* to assess the anti-CSC effects of a gamma secretase inhibitor, MK-0752, in a phase I study of breast cancer patients (50).

Another approach to measure anti-CSC effects in the clinical setting is to measure CSC gene signature responses. In acute myeloid leukemia, a leukemic stem cell signature portended a poorer prognosis in patients with this signature versus those who did not (35). While this approach was successful in predicting outcome, monitoring a patient's response to therapy with this method may be more difficult. The ability to detect the signature of a relatively small population of cells, such as CSCs, can often be masked by the other, more abundant, cells present within the tumor.

The tumoral monitoring of CSCs post-therapy is necessary to establish direct clinical evidence that targeting CSCs improves both progression free and overall survival. Such monitoring may also help to define a threshold of suppression that is necessary to achieve clinically meaningful results. While the field is advancing anti-CSC therapies into the clinic it will be necessary to establish methods to achieve this goal.

4.2. Circulating cancer stem cells

While measurements of CSC populations within the tumor will be important in understanding how therapies work in shaping cellular dynamics, more minimally-invasive techniques to assess therapeutic effectiveness will improve patient care. One promising area of research is circulating tumor cells (CTCs). While a relationship for the number of CTCs and patient outcome has been established for several years, subset analysis of the CTCs is just beginning in many solid malignancies. In head and neck cancer, CTCs were analyzed for the expression of the CSC marker CD133 in addition to N-cadherin, cytokeratin and CD45 (51). The authors found a statistically significant difference in survival for patients with detectable N-cadherin expressing cells after resection. In colorectal cancer, Katoh *et al.* detected the CSC marker CD44v9 in CTCs of patients (52). In their study, stage III and IV patients with the detectable CSC marker CD44v9 in CTCs had a poorer prognosis in comparison to patients without detectable CD44v9.

While there is clearly significant further work to be conducted, these studies collectively point to the possible utility of measuring cicrCSCs and the potential that it may have in predicting the propensity for metastatic spread as well as for clinical prognoses. Further exploration in combination with measuring direct tumor effects will allow for the decision on the usefulness of this approach to measure response to therapy or predict clinical course.

4.3. Circulating tumor DNA and other soluble factors

In addition to CTCs, other soluble factors are being explored as potential biomarkers of disease, with Circulating tumor DNA (ctDNA) being one exciting new area. CtDNA levels have been shown to correlate with tumor burden in several solid malignancies, including CRC (53) and breast cancer (54). In both of these studies, samples taken from the same patients during disease progression showed the superior sensitivity of ctDNA as compared to traditional biomarkers (CEA for CRC and CA15-3 for breast cancer) to predict overall tumor burden. In fact, in the analysis of Dawson *et al* it was found that ctDNA was a better indicator of tumor burden than the level of CTCs.

While these studies highlight the utility of ctDNA for determining tumor burden, changes in the CSC population per se are not likely to lead to dramatic changes in tumor burden. Therefore, when thinking of the use of ctDNA for CSC therapy, the greatest utility may be as a biomarker of relapse and allow an earlier measurement for progression-free survival. However, optimizations in this approach are still required due to the relatively low sensitivity, lack of broad applicability and cost (55). Despite new techniques designed to address some of these concerns, further improvements to methods will also be required when the tumor burden is very low or for early stage disease (56).

Other soluble factors, such as cleaved proteins and secreted factors have been used as biomarkers for cancer monitoring. This may be possible for the monitoring of CSCs, however this requires the identification of a factor unique to the CSC and not also made by the other tumor cells. At the moment, such soluble biomarkers displaying unique expression profiles are not yet identified.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Pre-clinically the role of CSCs in tumor development, maintenance and eventual regrowth following therapy is now firmly established. The next wave of research should be focused on the clinical relevance of this population and the translation of preclinical learnings to the clinic. Indeed, several companies are pursuing therapies directed at this population (57). While anti-CSC therapies enter the clinic, biomarker studies to firmly

establish the CSC theory with clinical disease course will become essential.

In addition, given the rarity of the CSC population, tumor shrinkage and standard criteria to measure patient response will not apply. Therefore, demonstration of the efficacy of a novel anti-CSC therapy will need to rely on a surrogate biomarker. Initially this will require demonstration of CSC reduction within the tumor itself but could evolve rapidly to include soluble factors including circCSCs themselves, if a correlation with peripheral and tumoral suppression can be established.

Ultimately, if a connection between prevalence of CSCs and overall patient outcome is firmly established in the clinic, then CSC-specific biomarkers will provide the greatest value to both patients and the clinicians who treat them.

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