Review Article

Technologies and methods used for the detection, enrichment and characterization of cancer stem cells

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ABSTRACT

Cancer stem cells (CSCs) represent a subclass of tumour cells with the ability for self-renewal, production of differentiated progeny, prolonged survival, resistance to damaging therapeutic agents, and anchorage-independent survival, which together make this population effectively equipped to metastasize, invade and colonize secondary tissues in the face of therapeutic intervention. In recent years, investigators have increasingly focused on the characterization of CSCs to better understand the mechanisms that govern malignant disease progression in an effort to develop more effective, targeted therapeutic agents. The primary obstacle to the study of CSCs, however, is their rarity. Thus, the study of CSCs requires the use of sensitive and efficient technologies for their enrichment and detection. This review discusses technologies and methods that have been adapted and used to isolate and characterize CSCs to date, as well as new potential directions for the enhanced enrichment and detection of CSCs. While the technologies used for CSC enrichment and detection have been useful thus far for their characterization, each approach is not without limitations. Future studies of CSCs will depend on the enhanced sensitivity and specificity of currently available technologies, and the development of novel technologies for increased detection and enrichment of CSCs.

Natl Med J India 2010;23:346–50

INTRODUCTION

A growing body of evidence suggests the existence of cancer stem cells (CSCs), a rare but critical subpopulation of cells within a tumour that share characteristics similar to normal stem cells. Among these characteristics are the ability for self-renewal, production of differentiated progeny, prolonged survival, resistance to damaging therapeutic agents and anchorage-independent survival, which collectively provide a tumour an effective mechanism to proliferate, migrate and metastasize, despite intervention with traditional cytotoxic and targeted therapeutic modalities.[1] Emerging first from the observation that only a minority of cancer cells are proliferative in a given heterogeneous population, CSCs have since been identified in a number of malignancies, including breast,[2] prostate,[3] colon[4] and brain,[5] as well as haematopoietic malignancies such as acute myeloid leukaemia (AML)[6] and multiple myeloma.[7] While the existence of CSCs continues to gain acceptance in the oncology research community, it remains unclear whether tumours arise as a result of CSCs transformed from normal stem cells, or from differentiated cells that became malignant and experienced a de-differentiative event to assume a stem cell-like phenotype.[1] The former of these two paradigms suggests that tumours arise from true stem cells, and mechanisms to support this concept have been reported.[8] The latter paradigm suggests that tumorigenesis occurs as a result of malignant cells with genetic alterations that permit increased proliferation and invasiveness, as well as have a capacity for self-renewal. In solid organ tumours, the occurrence of an epithelial-to-mesenchymal transition (EMT) has been reported, where epithelial tumour cells undergo a set of genetic changes to detach from their primary sites and assume a more motile, invasive, mesenchymal cell-like phenotype. The EMT is typically characterized by loss of e-cadherin expression, increased expression of vimentin, secretion of matrix metalloproteinase enzymes, and upregulation of various EMT-inducing transcription factors such as twist, snail, slug, Zeb-1, Zeb-2, and others.[9] Interestingly, the self-renewal mechanisms mediated by EMT during cancer metastasis share similarity with mechanisms that are used by adult stem cells when actively involved in tissue reconstruction, and a stem cell-like phenotype can be generated when transformed mammary epithelial cells undergo induction by EMT *in vitro*.[10,11] Whether derived from normal stem and/or progenitor cells, or from de-differentiated malignant cells, sufficient evidence supports the concept that the characteristics possessed by CSCs endow this population with increased invasive and metastatic capacity. Thus, a better understanding of the mechanisms involving the origins of CSCs, as well as the mechanisms that allow CSCs to migrate, metastasize and colonize distant organs, is required. This could have a profound impact on improved patient management and reducing resistance to therapy. The primary challenge to the study of CSCs, however, is their rare occurrence. In pancreatic cancer, for example, CSCs are reported to represent 0.2%–0.8% of the total pancreatic tumour cells.[12] Thus, efficient and sensitive assays must be used to identify and investigate the CSC population. The current review discusses the technologies that have been used to isolate and characterize CSCs to date as well as new potential directions for the enhanced enrichment and detection of CSCs.

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METHODOLOGIES FOR ENRICHMENT AND DETECTION OF CSCs

Our past, current and future investigation of the role of CSCs in malignant disease initiation and progression is directly dependent on their successful identification and characterization. Many efforts to advance our understanding of CSCs, however, have been limited due to their paucity. Nonetheless, a variety of methods to study CSCs have been devised using existing technologies (Fig. 1). The most predominant of these methods has been the use of flow cytometry. Using fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS), the CSC population can be specifically quantified, isolated and analysed by expression of cell surface markers derived from the characterization of normal stem cells (Table I). Flow cytometry is available for use in many tumour types, including haematopoietic malignancies as well as solid tumours. Bonnet and Dick were the first to report evidence of CSCs using FACS, demonstrating that CD34+/CD38– cells were the predominant leukaemic-initiating cells in mouse models, and that this population possesses differentiative, proliferative and self-renewal capacities consistent with leukaemic stem cells.[6] Additionally, Clarke and colleagues found that only a minority of breast cancer cells grown in immunocompromised mice had the ability to form new tumours.[2] These tumorigenic (i.e. tumour-initiating) cancer cells had stem cell-like phenotype, and could be isolated and separated from non-tumorigenic cancer cell populations by FACS using CD44+/CD24– cell surface markers.[2] In pancreatic cancer, tumorigenic cancer cells exhibiting CD44+/CD24+/ESA+ surface marker expression were isolated by FACS and when implanted into immunocompromised mice, demonstrated enhanced tumorigenic potential compared to non-tumorigenic cancer cells in a manner similar to breast cancer cell models.[12]

Acquisition of side populations (SP) based on the exclusion of Hoechst 33342 dye is yet another method used to enrich CSCs employing FACS. Isolation of CSCs by acquisition of SP is based on the principle that adult stem cells have high expression of proteins in the ATP binding cassette (ABC) transporter family responsible for efflux of foreign materials from the cell, and thus cancer cells with a stem cell-like phenotype that have similarly increased expression of this marker will eject the Hoechst 33342 and be found in the SP.[13] Employing this technique, Hung and colleagues demonstrated the ability to enrich CSCs in human lung cancer cell lines, as well as in lung tumour xenografts from NOD/SCID (non-obese diabetic severe combined immunodeficiency) animal models.[14] Using the SP method, Bleau *et al*. showed evidence that the PTEN/PI3K/Akt signalling axis regulates ABC transporter activity in glioma CSCs.[5]

While FACS has been widely used in the isolation and enrichment of CSCs, this method is not without its limitations. From a functional standpoint, the use of flow cytometry requires that a sufficient number of total cells be analysed to obtain statistically significant numbers of rare CSCs.[15] Discriminating between true labelling and non-specific staining is not possible as it is in microscopic interrogation of specimens, where chromagen aggregates and other staining artifacts can be dismissed by the pathologist.[15] Additionally, recent data call into question some of the markers used to isolate and identify CSCs by FACS. Radovanovic and colleagues showed, for example, that when glioma cells were segregated based on CD133 positivity, there was no significant difference in expression of 'stemness' genes and long-term self-renewal capacity between CD133+ and CD133– populations.[16] And while SP isolated from FACS-separated

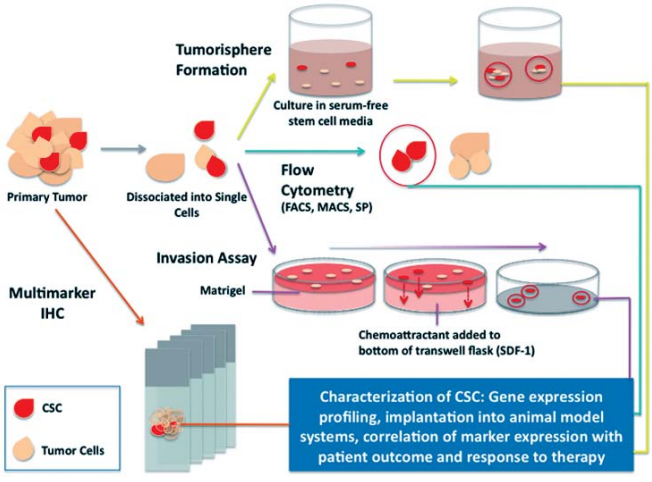

FIG 1. Methods used to detect and enrich cancer stem cells (CSCs) include (clockwise) tumorisphere formation, flow cytometry, invasion assay systems, and multimarker immunohistochemistry (IHC). Primary malignant tissues (i.e. resected tumour, biopsy, pleural effusion) are taken from the patient and dissociated into single cell suspensions. These cell preparations can be placed into culture for *tumorisphere formation*, where tumour cells with stem-like character grown in non-adherent conditions, incubated in serum-free stem cell media will form tumorispheres in suspension. Cell preparations can also be processed by *flow cytometry*, where they can be sorted into tumorigenic (red) and non-tumorigenic populations (tan) based on surface marker expression. Cell preparations can additionally be placed into *invasion assay systems*, where CSCs layered onto matrigel will adhere and migrate towards a chemoattractant added to the lower chamber of a transwell culture system. Spheroids (from tumorisphere formation), cells expressing stem-like character (flow cytometry), or cells that have migrated through matrigel (invasion assay systems) are collected for further characterization (i.e. gene expression profiling, implantation into animal model systems, proteomic assays). CSCs can be identified in primary archival tissues that have been formalin-fixed by *multimarker IHC*. While fixation of primary tissues makes the performance of functional assays unavailable, multimarker IHC allows for the detection of CSC populations for retrospective study, and additionally permits the histological localization and morphological characterization of CSCs.

TABLE I. Cencer stem cell (CSC) markers, malignancies and multimarker profiles

CSC marker	Malignancy	CSC multimarker profile	Malignancy
CD24	Breast, pancreatic	CD34+/CD38–	AML
CD29	Breast (mouse)	CD44+/CD24–	Breast
CD34	AML	CD24+/CD29+	Breast (mouse)
CD38	AML	CD133+/ALDH-1+	Liver
CD44	Breast, prostate, pancreas, colon and rectum	ESA+/CD44+/ CD156+	Colon and rectum
		ESA+/CD44+/CD24+	Pancreas
CD133	Brain, colon and rectum, liver, prostate		
CD166	Colon and rectum		
EpCAM (ESA)	Pancreas, liver, colon and rectum, ?prostate, ?breast		
ALDH-1	Breast, liver		

adrenocortical carcinoma cells showed expression patterns consistent with a less differentiated phenotype, there was no significant difference between the proliferative capacities of SP *v*. non-SP groups, and both groups displayed the ability to give rise to their own phenotype as well as the opposite group's phenotype.[17] Similar results from SP assays have been reported in other malignancies as well. Confounding issues with the use of FACS and SP-based methods have recently been reviewed by Welte and colleagues, where they found that 2 independent research groups who used the same reagents and sorting technique to characterize the tumorigenic capacity of CD133+ cells arrived at opposing conclusions.[18] Further, Welte *et al*. reported several studies from other investigators that found no significant difference in tumour incidence or latency periods of ABC transporter-positive and -negative populations *in vivo*.[18]

Immunohistochemistry (IHC) techniques have been successfully applied for the clinical diagnoses and prognoses of cancer for many years. The application of IHC towards the identification of CSCs has the potential to overcome some of the barriers presented by the use of FACS. IHC protocols are relatively simple, reproducible, standardized, inexpensive and widely available for use without the requirement of large, technically complex equipment. The use of IHC also provides the opportunity to identify CSCs from various archival tissues (e.g. formalin-fixed paraffin embedded, frozen tissues, etc.) allowing for retrospective investigation. Further, microscopic evaluation of tissue architecture, localization of marker staining, and intensity of staining using IHC can be used to better delineate truly positive CSCs as compared with FACS. Using IHC, our group reported the first evidence of CSCs within bone marrow of early breast cancer patients, finding that the median prevalence of CK+/CD44+/CD24– cells in the bone marrow was 65%, compared with <10% in primary tumours.[19]

Recent technological advances have significantly improved the sensitivity and specificity of secondary detection systems, allowing for performance of multimarker IHC. Such advances could be particularly beneficial for the detection of CSCs by IHC, as the ability to interrogate multiple markers simultaneously in the same tissue increases specificity and characterization of detected CSCs. In particular, the development of quantum dots (QDs), nanometer-sized semiconductor particles covalently linked to a variety of biorecognition probes, has helped to revolutionize the fields of molecular and cellular biology. The strong intensity of fluorescence and photostability of QDs, as well as broad excitation profiles and narrow emission spectra are key optical and electrical properties that, relative to organic fluorophores, make QDs best equipped for use in multimarker IHC. QDs were first used as probes for *in vivo* targeting and imaging,[20] but their use as secondary conjugates in IHC/IF (immunofluorescence) assays has increased in recent years. The use of QDs as secondary conjugates in IHC/IF has been enhanced further by the integration of multispectral imaging technologies onto standard fluorescent microscopy platforms. Systems such as the Nuance (CRI Inc., Boston MA) are an especially beneficial tool in this application for their ability to (i) enhance signal to background fluorescence by unmixing of spectra and (ii) quantify emissions of narrowly specified wavelengths. Loda and colleagues successfully performed CD44/CD24/Ki67 QD multimarker IHC in formalin-fixed paraffin embedded primary breast carcinoma tissues, finding significant differences between CD44+ and CD44– patient groups.[21]

Wang *et al*. have developed a particularly elegant IHC assay, where up to 4 markers can be simultaneously assessed.[22] A cocktail of 2 primary antibodies from 2 different species (e.g. mouse and rabbit) are incubated with the tissue. This is followed by QD secondary antibody conjugates for each respective primary species. The tissue is then re-blocked, and a second round of two primary antibodies (mouse and rabbit) are added, followed by QD secondary antibody conjugates added to bind the second round of primary antibody labelling. Because there are 4 markers in total with just 2 species being interrogated, there is potential for non-specific crosstalk between unbound or unblocked primary antibody from the first round of staining with QD secondary antibody from the second round of staining. To assess for this occurrence, Wang *et al*. introduced a control slide where no primary antibody was added during the second round of staining. Using multispectral imaging, the amount of non-specific interaction between the QD secondary antibodies of the second round and primary antibodies of the first round can be quantified, and subtracted from the amount of true interaction between the primary antibody from the second round and secondary antibody from the second round.

While the use of IHC to identify CSCs can be beneficial for many reasons (as described above), it does not allow for functional characterization of CSCs. In fact, some of the functional characteristics used to define CSCs can also be used to isolate them. Segregation of CSCs using serum-free stem cell medium can be achieved, where CSCs will be enriched through tumorisphere formation. Using pleural effusions of metastatic breast cancer samples, Burchell and colleagues showed for the first time that mammosphere culture of pleural effusion enriches for cells with a CSC phenotype, and that the CSC population isolated from those mammospheres are capable of inducing tumours in immunocompromised mice.[23] Based on the increased invasive capacity of CSCs, Yu and Bian have proposed a novel strategy for CSC enrichment.[13] Transwell culture cabins are coated with matrigel, and cell suspensions are seeded onto the top chamber in serum-free stem cell culture media. After a brief incubation, poorly adherent cells are washed away, while the more adherent CSC population remains bound to the matrigel. Fresh serum-free stem cell culture media is then added, and the bottom chamber of the transwell culture cabin is filled with serum-free stem cell culture media with chemoattractants added (i.e. SDF-1). After 24–48 hours of incubation at 37 °C, the less invasive cells at the top of the matrigel layer are carefully removed, while the more invasive CSCs that have migrated to the bottom of the matrigel surface are harvested for subsequent analysis. As a proof of principle to validate this method, Yu and Bian attempted to isolate CSCs from the U87 glioma cell line. A fairly high tumorisphere formation rate was observed from migrating cells collected from the bottom portion of the transwell culture system, 41.02%, in contrast to cells that were poorly adherent or cells that adhered to the matrigel but failed to migrate through the matrigel, where only 0.79% of cells had the ability to form tumorispheres.[13] While this method has yet to be attempted in cell suspensions from clinical samples (i.e. resected tumours or pleural effusions), the preliminary results indicate that this may be a promising new methodology for CSC enrichment.

CIRCULATING TUMOUR CELL (CTC) CAPTURE: POTENTIAL FOR CSC ENRICHMENT?

Examining the haematogenous compartment for evidence of metastasis has increased significantly within the oncology research community in recent years. In addition to acquiring mechanisms for intravasation into blood vessels, tumours require an adequate

blood supply for survival and initiate programmes for angiogenesis, providing them direct access to the circulatory system and opportunity to disseminate tumour cells into the bloodstream. The circulatory system also provides an ideal compartment for repeated sampling, effectively serving as a liquid biopsy with a potential to monitor metastasis in real time. This sharply contrasts with other compartments where tumour cells may have disseminated such as the bone marrow, which is more invasive and difficult to acquire than a blood draw, and is not amenable to repeated sampling. As a result, CTCs in the blood have emerged, of late, with outstanding predictive and prognostic capacity in a number of malignancies including breast, prostate and colorectal cancer.[24–29] While the enumeration of CTCs has been the primary means to collect clinically useful data, the characterization of CTCs may improve the prognostic and predictive capacity. Because CTCs represent the subpopulation of tumour cells with the highest invasive and motile capacity which have broken free from their primary site, it is fair to hypothesize that there would be an enriched population of CSCs among CTCs. It is reasonable then to speculate that most CTCs have undergone a set of genetic alterations causing them to de-differentiate to assume a stem cell-like phenotype, and CTCs with stem cell-like phenotype are endowed with an increased capacity for anchorage-free survival, escape from anticancer therapy, and are ultimately the population responsible for seeding secondary metastatic sites (Fig. 2).

However, as CTCs are rare events occurring as few as 1 CTC/ml of whole blood, the primary barrier to the expanded study of CTCs has been their sensitive and efficient detection and isolation. Currently available technologies for isolation of CTCs include density gradient centrifugation, reverse transcriptase-polymerase chain reaction (RT-PCR)-based detection and affinity-based CTC capture. All these methods have limitations. Density gradient centrifugation has poor sensitivity, with CTC recovery as low as 10%–65%.[30,31] RT-PCR-based detection, using markers specific for epithelial and malignant cells, has been used recently to evaluate the presence of CSC transcripts in CTCs. In 226 blood samples of patients with metastatic breast cancer, ALDH-1 expression and EMT marker expression was observed in 62% and 69% of CTC+ patients, respectively.[32] In patients whose disease progressed in the face of palliative chemo-, antibody-, or hormonal therapy, ALDH-1 expression was observed in 62% and 44% of patients, respectively, compared with just 10% and 5% in patients who responded to therapy.[32] Despite these promising data, the RT-PCR technique suffers from issues with marker specificity due to basal expression in normal, nucleated blood cells, and provides no way to enumerate or to do any post-isolation morphological evaluation of CTCs.

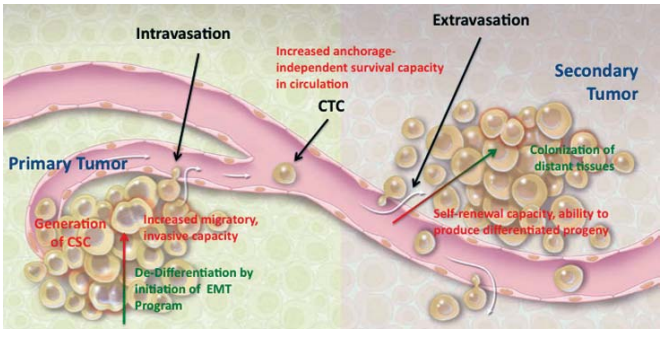

FIG 2. Epithelial-to-mesenchymal transition (EMT) to cancer stem cell (CSC) model of metastases—The importance of circulating tumour cells (CTC) in enrichment of CSCs. EMT in primary tissues bestows cells in transit, CTC, with stem cell-like character and all the necessary abilities to leave their site of origin, survive in circulation, invade other tissues, and colonize secondary tumours. Thus, the isolation of CTC could help to enrich CSCs for further characterization. (Cartoon adapted from Chiang and Massague, *N Engl J Med* 2008;**359:**2814–23)

Affinity-based methods, which primarily use EpCAM to enrich CTCs from normal blood cells, currently represent the 'gold standard' in CTC isolation, but to do variable expression levels of EpCAM between tumour types, may not be sufficiently sensitive. While EpCAM expression is reported in some tumour cells displaying CSC phenotype, such as pancreatic cancer and hepatocellular carcinoma,[12,33] it is unclear whether EpCAM expression is retained in tumour cells with the CSC phenotype in other malignancies. Perhaps the most severe limitation of these technologies, with respect to the current review, is their inability for subsequent characterization of CTCs (i.e. establishing primary cultures, interrogating markers of interest, etc.), which does not allow for the investigation of CSC subpopulations among CTCs.

To traverse these barriers, our group has developed a novel membrane microfilter device to selectively capture CTCs by exploiting the size differences between tumour cells and normal blood cells.[34] Essentially, the device is composed of an acrylic housing cassette, which holds a 6 mm × 6 mm, 10 mm-thick microfilter fabricated from parylene. Each microfilter contains ~40 000 evenly distributed, uniform 8 mm pores. Whole blood samples are drawn from patients by standard phlebotomy practice, diluted 1:1 in PBS, undergo a brief fixation in 1% formalin for 10 minutes, and are then mechanically flowed through the microfilter, which allows the majority of smaller, non-tumour normal blood cells to pass while retaining the larger CTCs for subsequent analysis. In model systems and clinical blood samples, the microfilter device showed superior sensitivity of CTC capture compared with the CellSearch platform, currently the only US FDA-approved technology available for CTC capture and enumeration.[35] We have also demonstrated the ability to perform molecular characterization of captured CTCs in model systems including FISH, multimarker IHC/IF and laser capture microdissection for subsequent nucleic acid analysis (unpublished data). With the additional access to isolated CTCs provided by the microfilter device, such molecular characterization techniques can be integrated so that the presence of CSCs in circulation can be investigated. Addressing the limitation of currently available technologies for CTC capture, we have also developed a microfilter device capable of isolating viable CTCs from blood. We have demonstrated the ability to capture viable tumour cells from blood samples in model systems, where cells can be captured and placed into culture systems for successful passage and propagation (unpublished data). Additionally, the microfilter device is small and portable, which potentiates its use in point-of-care diagnostics. Currently, our novel CTC capture technology is being used in clinical trials to evaluate the potential for monitoring therapeutic efficacy in metastatic cancer patients, and provides an exciting opportunity to investigate and further characterize CTCs for the presence of CSC subpopulations.

PERSPECTIVES

Improvement in the management of patients with cancer is the ultimate objective sought by investigators attempting to expand our understanding of CSCs and the mechanisms that drive malignant disease progression. One of the most critical factors driving an increased understanding of CSCs is the technology

available for their isolation and characterization. While the limitations of currently available CSC isolation technologies have been discussed in this review, their employment has already begun to show potential for translational impact. Lander and colleagues have identified a prospective inhibitor, salinomycin, by high-throughput compound screening, which shows an ability to selectively eliminate CSCs in breast cancer model systems.[11] Another approach being explored in the direct targeting of CSCs is differentiation therapy, where CSCs are forced towards the reversal of maturation arrest, permitting CSCs to terminally differentiate and thus be more susceptible to currently available chemo- and radiotherapeutic agents.[35] Yet a third approach being explored towards CSCs eradication is their targeting through T-cell activation. Mine *et al*. have recently demonstrated that PBMC activated by natural immunogenic peptides Notch-1 and Numb-1 successfully eliminate CD44+/CD24– breast cancer cells in model systems.[36] While efforts to improve therapeutic efficacy through CSC targeting is under way, all novel agents currently being tested suffer from the inability to differentiate between normal adult stem cells and CSCs. Because the markers used to identify CSCs have been derived from expression characteristics of normal adult stem cells, this limitation provides the possibility of non-specific elimination of non-tumour stem cells vital to the survival and maintenance of normal tissues while attempting to target CSCs. Thus, further defining the characteristics of CSCs as they differ from normal adult stem cells remains to be accomplished.

In conclusion, the use and modification of currently available technologies and molecular biology techniques have yielded beneficial data in understanding the role of CSCs in driving the initiation and progression of malignant disease. Future efforts to improve the sensitivity and efficiency of methods used in CSC detection and isolation will further help to elucidate their role in cancer development and ultimately lead to more effective patient management.

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