Understanding the Controversy in Cancer Stem Cell and Circulating Tumor Cell

Saman Milanizdeh¹, Mahsa Khanyaghma¹, Mahdi Mirahmadi²

ABSTRACT

New evidences have been raised suggesting that, cancers, as well as normal tissues, might also be hierarchically organized. Thus a minor fraction of tumor cells, endowed with stem cell-like features, and so termed cancer stem cells (CSCs), have been known as responsible for tumor initiation and maintenance. Another cell group which has caused controversies and circulates freely in the peripheral blood of cancer encountered patients is circulating tumor cells (CTCs). They defined as tumor cells that either primary sites or metastases are culprit of them. Characteristics and detection methods of CTCs and CSCs and their therapeutic application have been explored in this review with focus on their similarities and differences.

Keywords: Cancer Stem Cell (CSCs); Circulating Tumor Cells (CTCs); Epithelial-Mesenchymal Transition (EMT)

Despite tremendous progress in cancer researches, it is still debated which is the cell of origin and which cells propagate the tumor after initiation (1). It is assumed that cancer originating cells are different from the tumor propagating cells after its inception (2). Moreover, it was revealed that a rare subpopulation of undifferentiated cells may be responsible for cancer initiation, maintenance and metastasis. These cells are so called cancer stem cells (CSCs). Identification and characterization of CSCs resulted in challenges about the traditional “stochastic model” of tumor development, which assumes that each cancer cell is tumorigenic. Limitless proliferation potential, angiogenesis, multipotency, self-renewal, presence in hypoxic niches, the upregulation of DNA damage response. The increase in drug efflux and immune evasive features are the characterizations of CSCs (3). Interestingly, expression of DNA repair mechanisms, drug transporters and detoxifying enzymes by CSCs can lead to resistance to traditional agents, and thus are known as responsible for tumor relapse (4). In a solid tumor, total cells consist of only 1% CSCs despite cancer progression.

References

Although, their frequency appears to be highly variable (5). The clonal theory of cancer initiation is the base for CSC hypothesis (6). CSCs have been identified in many types of cancers, including colorectal, breast, ovarian, pancreatic, prostate, head and neck, and melanoma (7). There are various ideas about CSCs ancestors including progenitor cells, differentiated cells, or even cells from outside the tumor (1). To be more specific, normal stem cells may be transformed to CSCs by means of two different mechanisms: De-differentiation of differentiated cells which gives them stem cell properties and cells with new mutations that acquire self-renewal capacity to form CSCs (8).

**CSC markers**

Cluster of differentiation (CD) molecules located on the surface of cells are mostly used to characterize CSCs which can be isolated by FACS. These markers are putative stemness markers including CD133, CD44, CD166 (9). Nevertheless, there are still debates on phenotypic characterization of some CSCs.

**CD133**

Human CD133 which belongs to Prominin family is involved in postnatal tissue regeneration, angiogenesis and inflammation. CD133 has recently been identified as a major potential among the several other cancer stem cell markers. Subpopulation of CD133 positive cancer cells had been demonstrated self-renewal and highly tumorigenic potential (10). Moreover, these cells were highly enriched in tumor initiating cancer cells. These cells were resistant to chemotherapy and able to retain spheroid cultures as stem cells (11). There are several findings suggesting CD133 as a potential diagnostic and prognostic marker which occurs at early stages and contribute to tumorigenesis via intracellular signal transduction (12). Initial works had been identified CD133 as a reliable CSC marker in primary human cancer (13), however, it has been demonstrated by a following study in both mouse and human that CD133 is detectable in majority of tumor cells and is not restricted to rare cell subsets (14). A research performed by Ricci-Vitiani et al (15) revealed that CD133+ cells can rise to visible tumors in immunodeficient mice. However, another study showed the ability of CD133- cells tumorigenecity and give rise to controversies (16).

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**References**

CD44

CD44 is a transmembrane protein that plays roles in lymph node homing, lymphocyte activation and cell adhesion and allows cell-cell and cell-ECM (extracellular matrix) interactions through the binding to hyaluronic acid (HA). It is also involved in the activation of the tyrosine kinase receptor c-Met (17). CD44v isoforms are mainly expressed by cancer cells and it has been proposed as CSC marker of several solid tumors, including breast, pancreas, head and neck, non-small cell lung, hepatocellular and colon cancers (18).

It has recently been found that CD44 is a robust marker of tumorigenic CRC cells with stem cell-like properties and not CD133 (19). As an example, the putative breast cancer stem cell phenotype was identified as CD44+ and CD24-/low (20) and independently associated with aldehyde dehydrogenase activity (21). CSC properties were detected by cells which express CD44 on their surface including formation of a sphere by a single cell and a xenograft tumor that resembles the original lesion (22). In a research that conducted by Patel et al., significant increase in CD166 expression in adenomatous glands and an age-dependent increase in CD44s and CD166 expression, had been found correlating further with the number of polyps (23). Like CD133 there is still debate about the efficiency of this marker. Early studies demonstrated that CD44 expression is related to metastatic increment; however, another research showed that down-regulation of CD44 is associated with the presence of vascular invasion, more advanced tumor stage, infiltrating tumor border and lymph node involvement (24).

CD166

Mesenchymal CD166, also known as activated leukocyte cell adhesion molecule (ALCAM), is a stem cell marker which has been reported as a co-CSCs marker with EpCAM and CD44. The EpCAM high /CD44+/CD166+ phenotype reported to be alternatively used as CSC marker instead of CD133 (18).

References

In recent studies expression of membranous CD166 was associated with shortened survival (23). It has been revealed that loss of CD166 is associated with an increase in tumor size, lymph node metastasis, tumor infiltration and a shorter overall survival. On the other hand decrease in CD166 expression had correlated with unfavorable clinical outcome and tumor progression (4). CD166 together with CD44 seems to be involved in cell-matrix and cell-cell adhesion; as a result its decrease may be associated with higher metastatic potential of tumors.

Other Markers:
CD29 or Integrin beta-1 is an extracellular receptor for cell migration, proliferation, survival, differentiation and death signals (25). It acts as an adhesion molecule in cell-collagen interactions; thus, it's down regulation may affect differentiation (26). Moreover, CD29 was shown to be increased in metastatic cancers(27). CD24 is a mucin-like adhesion molecule which seems to regulate cell adhesion and signaling. These evidences suggest that this molecule might play a role in the process of cancer metastasis. (28). Furthermore it may be related to some carcinogenic factors such as degree of differentiation (29). A group of antigens which are present in germ cells, trophoblasts, and cancer cells are cancer/ testis antigens (CTAs) (30). These antigens are highly immunogenic and regulate self-renewal and metastases. It is demonstrated that they may be crucial for stemness so can be used as a CSC marker (31).

Tumor microenvironment and cytokine loops have demonstrated priority in maintenance of CSCs and tumor development ability (32). Regardless of which method we use, detection of CSCs is dependent on microenvironment and bone marrow reconstitution as well as on the timing of post-transplant analyses. In a recent study it has been revealed that normal tumor cells can convert to CSCs within the tumor depending on environmental stimulators (33).

References
In another study, it has been demonstrated that myeloma stem cells have stronger proliferative capacity in presence of myeloma patient's stromal cells than presence of normal control bone marrow stromal cells (34).

**CSC assays:**
The foundation of CSC theory was reached through in vivo experiments of xenotransplantation of human tumor cells into immunodeficient mice and using an in vitro sphere-forming assay (36). This method allows CSC detection on a single-cell level. Other assays which recently have been used for CSC detection includes: the colony assay, the sphere assay, the side population (SP) assay by Hoechst labeling, (37) staining for CSC surface antigens (such as ABCG2 and other markers), aldehyde dehydrogenase (ALDH) activity assay, (38) and label-retaining cell assay using PKH (Paul Karl Horan) dyes. To generate an overview about CSC assays, some of them will explain briefly:

**Microsphere Assay (Neurosphere assay):** assesses forming sphere-shaped cell aggregates by the ability of neural, breast, heart pancreas and prostate stem cells to grow in serum-free medium in nonadherent conditions (36). However, sphere assays may not detect quiescent CSC.

**SP Assay:** It uses the ability of P-glycoprotein to actively transport some dyes, such as Hoechst or Rhodamine, out of the cells to assess CSCs. SP asssay is very dependent on stain and culture conditions and beside easy procedure it needs strict attention (39).

**Surface antigens staining:** There are different surface antigens like CTAs or to be more specific ABCG2 protein which might be used for CSC surface antigen staining (40).

**ALDH activity assay:** Although aldehyde dehydrogenase activity is affected by chemotherapy, High levels of ALDH activity have recently been manufactured for characterization, isolation and study of CSCs in leukemia, prostate and cervical cancer (38).

**PKH Label-Retaining Cell Assay:** As an example, PKH26 labeling method has been analyzed cells asymmetric division to identify CSCs (41).

References
Therapeutic application:
CSC model hypothesized that to eradicate disease; CSCs have to be completely eliminated. However, CSCs have been reported to be relatively resistant to standard anticancer therapies (42). As a result, new therapeutic procedures have been developed recently to fight against cancer including re expression of tumor suppressor genes by epigenetic manipulations (such as histone deacetylase inhibitors and DNA methyltransferase inhibitors) or immunotherapy targeting CSCs (such as generation of cytotoxic lymphocytes against chronic lymphocytic leukemia and Hodgkin lymphoma stem cells) (43).

Disseminated Tumor Cells (DTC):
Disseminated tumor cells (DTCs) are considered as micrometastases. They can remain in a dormant state for many years before giving rise to macrometastases (44). DTCs can be self-seeding or cross-seeding resulting to the primary tumor or aggressive metastatic variants (45). Moreover, it has been confirmed that DTCs has a putative stem cell phenotype (46). In a research conducted on DTCs, putative breast cancer stem cell phenotype has been characterized in majority of cells (46). It is demonstrated that stem cell markers express in DTCs or in derivative cell lines (47).

Circulating Tumor cells (CTCs):
Cells that can be shed from a primary tumor and circulate through the blood stream are known as circulating tumor cells (CTCs). To be more specific, an epithelial cell in cancer patient's blood stream with cytokeratin expression and intact nucleus without a detectable CD45 marker is a CTC (47). CTC detection is a tempting idea as it may lead to early prognosis and treatment. Moreover, serial minimally invasive real-time liquid biopsy makes it an ideal approach for cancer investigation. CTCs have been detected in almost all cancers, Including colon, breast, prostate, lung, ovary, pancreas, liver, gastric esophageal, renal, bladder, thyroid, nasopharyngeal and melanoma. They are extremely rare in patients without malignancy and can be detected in bone marrow (48).

References

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before the development of metastases (48). Moreover, Gerber et al (49) demonstrated that CTCs may be responsible for tumor relapse. TNM classification for staging patients resulted in insufficient information for treatment and adjuvant therapy (50), consequently a small proportion of CTCs that have self-renewal potential can be applicable for metastasis and therapy response detection (51). It has been revealed that in a nude mouse model more than 1000 CTCs are required to produce metastatic lesions (52). CTCs have physical and genetical properties which make them suitable for metastases including increased cell elasticity and genetic exchanges between tumors (53). It is assumed that by increasing tissue hypoxia and compete for resources, neovascularization and lymphangiogenesis happens to generate CTCs and spread them to blood vessels (54). However, CTCs May show different characteristic than those of the cells of metastatic tumors (55). In recent studies, appearance of amplification of a specific androgen receptor suggested that at least a subset of CTCs are tumor derived (56). Molecular analysis of CTCs were demonstrated some HER2-positive CTCs in breast cancer patients whose primary tumor was HER2 negative (57) as well as some HER2-negative CTCs emerging in HER2-positive breast cancers subjected to anti-HER2 therapy (58). CTCs had some similarities in copy number changes to the primary tumor and additional different copy number changes. Differences in profile between CTCs and primary tumors in breast and colorectal cancer has been demonstrated (59) which can be due to missing of small metastatic subclone in primary tumor or gaining additional genomic characteristics (60).

References
It has been observed that up to 1 million cells enter the circulation daily per gram of tumor tissue (61). However, metastatic inefficiency leads to elimination of most tumor cells and eliminate metastasis (62).

CTC Markers and Assays:

CTC markers were commonly chosen for their potential as markers of stemness, drug resistance and apoptotic resistance. In a recent study, increased expression of ALDH1, CD44, CEA, CD133, MRP5 and survivin gene markers were determined 33%, 11%, 100%, 7%, 48% and 55%, respectively (63). Based on physical and biological properties of CTCs their enrichment includes variety of methods to distinguish them from hematopoietic cells (64). Moreover, worse progression-free survival has been associated with upregulation of survivin, ALDH1 and MRP5. However, CD133 has been identified as a marker correlating with recurrence (65). Other CTC markers include CD26, EGFR and myeloid leukaemia cell differentiation protein Mel-1. Proliferative index Ki67 was also measured in breast cancer for detection of CTCs biological activity (66). Stemness features of CTCs has been characterized from metastatic breast cancer patients (67) and has been reported to be specified for CD44+/CD24− or ALDH1 high/CD24− subpopulation (68). A recent work demonstrated that a small subset of CTCs which express epithelial cell adhesion molecule (EpCAM), CD44 and CD47 is indeed capable of inducing metastasis in luminal breast cancer patients (52). As these cells are rare in peripheral blood highly sensitive and specific methods required for their assay. Although fluorescence-activated cell sorting (FACS) is the widely used method nowadays by negative selection of EpCAM/CD45 or positive selection of tumor specific markers (69), different CTC detection approaches have been developed which have high sensitivity including quantitative RT-PCR assays, automated microscopic systems, filtration devices (for size selection) and CTC microchips (70), detection of physical cell properties (by dielectrophoretic field flow fraction) (71), or microfluidics (by the use of label-free biochips) (72) and next-generation sequencing (for characterisation of CTC genome) (73).
Among all techniques, CellSearch system, based on EpCAM marker, is used as the FDA-approved technology for CTC detection (74). However, there are encouraging results published about CTC detection in cancer patients using other systems like Maintrac®. These methods are only some samples of CTC detection and there are a lot of researches still going on about their association between CTC's molecular characterization and cancer.

**Therapy, prognostic and predictive utility**

Although epithelial CTCs were first described well over 100 years ago (75), only recently CTC enumeration has been shown to be clinically useful as a prognostic biomarker in epithelial malignancies including breast (76), colon (77), and prostate cancer (78). There is evidence about detection of <3CTCs/7.5 ml in cancer patient's blood and expectation for chemotherapy response(79). 25–50% of patients with stage II-III CRC relapses as a consequence of undetected spread of malignant cells (80). It was shown that the number of CTCs in the blood is related to biological futures including tumor vascularity or invasiveness and have prognostic value but is not correlated with tumor mass measurements. (81). Moreover, it has been demonstrated that number of CTCs are reduced after effective chemotherapy, targeted kinase inhibition, or hormonal therapy (82).

**References**

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It has been confirmed that CTCs in cancer patients with metastatic disease is associated with free survival, overall survival and worse progression (83). In another study the number of CTCs (above 5 CTCs per 7.5 ml of blood) demonstrated the same results (83). However, the rate of decline of CTCs after surgical resection remains to be determined. A putative drug resistant profile of CTCs independent of tumor type and the stage of the disease was found suggesting individualization chemotherapy and can be superior or additive to conventional imaging methods (84). Cancer early diagnosis is a tempting characterization of CTCs which can be reached by possibility of easily and reliable detection of them.

**Epithelial-mesenchymal transition (EMT):**
Factors that determine tumor growth, survival, angiogenesis, and invasion regulate the metastatic potential of a tumor. In the epithelial-mesenchymal transition (EMT), epithelial cells lose their phenotype and gain invasive properties and become more resistant to apoptosis and transform to mesenchymal stem cells (85). Although it is known to occur in embryonic development, some epithelial cancer cells may undergo EMT to be able to enter peripheral circulation. It has been suggested that EMT might be related to the acquisition of stem cell properties, invasion and metastasis (86). However it is not clearly proved that EMT is crucial for invasion (87). Increase in therapy resistance and cellular migration has been revealed to be associated with EMT and stem-like properties of the cancer cells (88). In this process tumor cells lose expression of some epithelial marker proteins including E-cadherin (89), EpCAM (90), and cytokeratin (91), whereas mesenchymal markers such as vimentin and N-cadherin (92) and growth factors including c-MET(93), TGF-β (94), Wnt (95) and FOXC1 (96) are up-regulated.

**References**
For example, a transcription factor that induces EMT, TWIST1, is expressed in bone marrow cells of breast cancer patients and is known for distant metastasis and local tumor progression (97). Whether CTCs first undergo EMT and then shed to blood or whether tumors that have eroded into a blood vessel release CTCs and then they undergo EMT is still a mystery. It has been reported that CTCs with expression of mesenchymal markers demonstrated worse prognosis than the expression of cytokeratins alone (98).

**Detection Difficulties:**
Detection of CSCs in solid tumor tissues needs invasive procedures (tumor tissue resection) and can't be applicable for all cancer types. Besides, the exact functionality of CD markers that used to identify CSCs is not clear. It needs molecular assays on CSCs to determine their application; nevertheless, it is hampered by rarity of this type of cells in cancer patients. Some detection approaches like SP assay uses toxic materials which needs more attention. Moreover, there is doubt about specificity and sensitivity of applied methods. There are other defects in CSC assays such as insufficient knowledge about some CSC mechanisms like sphere formation. If we can understand exact mechanism new doors for CSC detection may be opened. There is several matter of complication in CTC detection methods as there isn't a standard procedure for their enrichment and detection. As an example, sample volume is suggested to be more than 30 ml which allows for more frequent CTC detection and characterization (99). To be more specific, Poisson distribution leads to selection of more than 7.5 ml blood for CTC detection. Another problem is the wide range of methods that have been used for CTC detection and needs for validation and quality control of them. Nevertheless, FDA-approved CellSearch technique has recently gets a lot of considerations. There are drawbacks for affinity-based enrichment methods that CTCs can only be detected in the blood samples of approximately 50% of patients with metastatic cancers.

**References:**
Evaluation in the management of patients with cancer still needs more consideration and effort (100). DTC metastasis in the different cancer types such as breast cancer may take long time to be clinically detectable and the mechanism of this cancer dormancy is largely unknown (101). By undergoing EMT transition, tumor cells may escape detection by conventional methods (102). As an example, vimentin is expressed in both carcinoma cells that have undergone EMT and blood cells. However, collagen adhesion matrix (CAM) assay is a useful method to overcome this deficiency which uses a functional cell separation procedure based on their invasive properties (103). Routine use of EpCAM antibody to study EMT is another defect that prevents CTC detection and leads to pre selection for cells that have retained significant expression of epithelial markers. As an example, vimentin is expressed in both carcinoma cells that have undergone EMT and blood cells. However, collagen adhesion matrix (CAM) assay is a useful method to overcome this deficiency which uses a functional cell separation procedure based on their invasive properties (103). Routine use of EpCAM antibody to study EMT is another defect that prevents CTC detection and leads to pre selection for cells that have retained significant expression of epithelial markers.

Discussion

Response to custom treatment methods will be narrow in different cancer types such as hereditary colorectal cancers (104) due to their deficiencies in certain genes that rules for definite products (105) (i.e. Adenomatous polyposis coli (APC) and mismatch repair (MMR) genes for this cancer) (106). As the evidences come up with new researches in CSCs and CTCs, contribution of these cells in colorectal cancer occurrence and relations between these cells are crucial for prognosis and selecting appropriate treatment methods. Moreover, a recent work has shown that different metastatic sites harbor different genomic aberrations (107) and biopsy of one or two accessible metastases may not be representative. Minimal residual disease (MRD) that remains in the patient during or after treatment detection and molecular analysis of them is a crucial step to prevent the progression of DTCs/micrometastases to overt metastases. It has been suggested that DTCs can replenish the pool of CTC and they can be detected for a long period of time after primary tumor resection.

References

CTCs have long been known to be the reason of metastasis from the primary tumor and may be beneficial for insight into the process of metastasis in human cancers. An important step to use CTCs as a biomarker is standardization of the various techniques of CTC detection for their assay. Other improvements are optimization of markers used to detect and time point with regards to surgical resection. As an example, a large scale clinical validation of CTCs resulted in inclusion of CTCs in a tumor-node-metastasis (TNM) cancer staging in breast cancer as cMYC(i+) in 2010 (108). Obtaining CTC levels can be used for measuring response to therapy or treatment break (77) and detection of chemotherapeutic agents for therapy (109). Prediction of the site of mutation can be developed by determining molecular changes and efforts for new mutation detection methods. These approaches may have a diagnostic or screening potential if they are used with single cell sequencing (110).

Because mouse models of tumors often metastasize late, they need to be validated by observational studies in human cancers. It has been proposed that the cells responsible for initiating overt metastases might also be stem cells disseminated from the primary tumor into the circulation to be precursor for metastasis formation (70). CTCs molecular characterization may demonstrate their cellular origin which can be from primary or metastatic tumor cellular masses. Certain evidence suggests that CTCs might be identified partly as cancer stem cells because of similarities such as increased resistance to chemotherapy and decreased proliferation during circulation. Moreover, both CSCs and CTCs demonstrate EMT futures as well as expression of EMT proteins. Determining CD molecules expression as markers for CTCs and CSCs status may guide for cancer treatment. Although it may lack sufficient specificity by expression of CD133 or CD44 in non-malignant colonic epithelial cells (51).

**Conclusion**

CSCs seemed to have high clinical output; however, it did not reveal completely reliable results. We are still in our very first steps to understand their behavior which needs more time for researchers to prove their ability to cancer prognosis and treatment. Future perspective of cancer may be highly dependent to CSC characterization. Identifying specific CD markers and physical properties of CSCs can be beneficial to reach this goal. Although FDA approved Cell search system for CTC detection is available, more accurate procedures to estimate CTCs in blood of cancer encountered patients is developing. Phenotypic characterization is one of CTCs detection methods; nevertheless, CSCs detection by phenotypic characterization still needs more efforts. There are some similarities between CTCs and CSCs; however, the same origination for these cells can't be proved completely. More studies about their properties need to be done and by new approaches for their identification, in near future, they may be used as new diagnostic and prognostic factors.

**References**