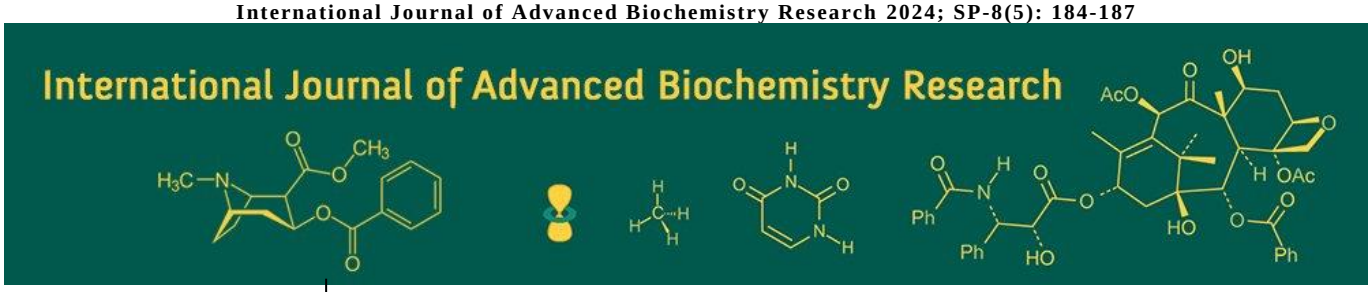

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Cancer stem cell and epithelial mesenchymal transition in chemoresistance of canine solid tumours

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Abstract
There is increasing evidence for the presence of cancer stem cells in several solid tumors, and these cancer stem cells have a potential role in tumor initiation, aggression, and recurrence. Solid tumors occur frequently in dogs, where such tumors exhibit complexity when examined histologically. These tumors are composed not only of proliferative luminal epithelial cells, but also of myoepithelial cells and /or mesenchymal cells with cartilage and osseous tissues in a solitary mass. The origin of the complexed histogenesis remains speculative, but cancer stem cells (CSCs) are likely involved. Canine CSCs are relatively resistant to the cytotoxic effects of common chemotherapeutic drugs and ionizing radiation, indicating that failure of clinical therapy to eradicate canine mammary cancer may be due to the survival of CSCs. The epithelia to mesenchymal transition (EMT) has been associated with cancer invasion, metastasis, and the acquisition of stem cell characteristics. Present investigation shows that canine CSCs predominantly express mesenchymal markers and are more invasive than parental cells, indicating that these cells can be induced to undergo EMT by transforming growth factor-β (TGF) and that these cells have an increased ability to form tumor sphere. CSCs possess self-renewing capacity, differentiation potential, high tumorigenicity in immunodeficient mice, and resistance to chemotherapy and radiation. Analysis of the characteristics of CSCs may contribute to the elucidation of the histogenesis underlying canine solid tumors, formulation of novel CSC-targeted therapeutic strategies, and development of biomarkers for early diagnostics and prognostic applications.

Keywords: Solid tumor, tumor growth factor, cancer stem cell, glioblastoma, cluster of differentiation (CD)

Introduction
Solid cancer consists of heterogeneous cells that contain a sub population of tumor cells with stem cell properties, including self-renewal capacity, differentiation potential, tumorigenicity in immunodeficient mice, and resistance to chemotherapy and radiation [1, 2]. Such tumor cells, termed cancer stem cells (CSCs) or tumor-initiation cells (TICs), are generated either from mutational events in normal tissue stem cells or from the acquisition of stem cell properties by differentiated cells; these tumor cells exist at the apex of a hierarchy of cancer tissues [3, 4, 5] in various cancers such as skin, liver, and glioblastoma, mammary carcinoma. CSCs are organized as tree-like hierarchies [6, 7]. CSCs have the capacity to self-renew, recurrence, to initiate and maintain the tumor, can produce heterogeneous lineages of cancer cells to compose the bulk of the tumor and is the primary cause of metastasis [8, 9, 10]. The cancer stem cell model therefore proposes that tumor development is akin to abnormal organogenesis [8]. In addition, CSCs are resistance to many current cancer treatments, including chemo- and radiation therapy. Therefore, conventional therapies, while killing the bulk of the tumor cells, ultimately fail because they do not eliminate the CSC population, which survives to regenerate the tumor [11]. Further understanding the properties and mechanisms of CSCs is essential in the development of effective-anti-cancer therapies. In humans CSCs were first identified in acute myeloid leukemia [12] and more recently in melanomas [13], glioblastomas [14] and epithelial cancers [15]. In the canine model, we were the first to identify CSCs of a canine osteosarcoma cell line [16] and have subsequently isolated CSCs from a range of canine solid tumors including glioma, hemangiosarcoma and squamous cell carcinoma. Recent evidence has suggested that tumor progression metastasis is dependent upon aberrant activation of epithelial to mesenchymal transition (EMT) in

cancer cells, resulting in the acquisition of invasive and metastatic properties [17]. Classically, EMT is an evolutionarily conserved developmental pathway involved in tissue morphogenesis, organ fibrosis and wound healing [18]. The hallmark of EMT is the loss of cell surface E-cadherin, which is associated with disassembly of adherent junctions, acquired motility and expression of mesenchymal markers including Vimentin and Fibronectin [19]. The EMT program is regulated by multiple transcription factors, including Twist, Snail and members of the zinc finger and homeodomains (zhf) family [20]. It is now known that EMT activation is also associated with the maintenance of stem cell properties, and in vitro it has been that emergence of CSCs occurs as a result of EMT [20].

Properties of Cancer stem cells

CSC share similar properties and presence of surface marker with the normal stem cells present in adult mammalian tissue. CSC and stem cells have many similar characteristics like as self-renewal, indefinite self-replication, asymmetric cell division, generating a large number of differentiated cells and expressing specific surface marker and regulatory molecules [21]. Though, both stem cell and CSC exist for a prolonged period of time but the major difference between their functional properties is that normal stem cells always function under control whereas the division and differentiation of CSC are out of control leading to large number of tumor cells with diversified cell population. In a nutshell, CSC is defined by four distinct properties (a)self-renewal—like stem cells, the CSCs sub population in a culture plate are transplanted through multiple generation (b)differentiation— CSCs have the capacity to differentiate into bulk population of daughter tumor cells (CSCs and non-tumor cells (non-CSCs) indicating its pluripotency. (c) tumorigenicity—a small proportion or subpopulation of CSCs have tumorigenic potential when transplanted into SCID/nude mice or animals and (d) CSCs in addition to common surface marker like stem cells, express specific surface markers by which these are separated from non-stem cells [22].

Tumor microenvironment and characterisation of CSCs

Inside a tumor there are diversified population of cells starting from normal fibroblast, cancer associated fibroblast (CAF), normal stem cells, T cell, endothelial cells, cancer progenitor cell, cancer stem cells (CSCs), cells in the epithelial mesenchymal transition (EMT), extracellular matrix (ECM). Since, CSC and stem cells share many common properties, the isolation and identification of CSC can be done with several *in vitro* assays such as sphere forming assays [23] , Hoechst dye exclusion (SP cells) [24], detection of enzymatic activity of aldehyde dehydrogenase1 (ALDH1) [25], detection of surface markers [24], signalling pathway identification [26], serial colony-forming unit assays (replating assays) [27], label retention assays [1], and migration assays [28]. These *in vitro* assays are used to isolate stem cells in a tumor but in addition serial transplantation in animal models is required to identify the particular CSCs [1]. Still advanced and optimised methods are currently used to isolate and identify the CSCs. Those are Flow cytometry analysis, fluorescent associated cell sorting (FACS) analysis, polymerase chain reaction (PCR) analysis, immunofluorescence staining analysis which uses cell surface specific markers for CSCs such as surface cell-adhesion molecules (e.g., CD133, CD24, hyaluronic acid (HA) receptor CD44), cytoprotective enzymes (such as aldehyde dehydrogenase, ALDH), transcription factors or stem cell pluripotency marker (e.g., OCT-4, SOX-2), and drug efflux pumps (e.g., ATP-binding cassette (ABC) drug transporters and multidrug resistance transporter1, MDR1) [24]. Among these CD133, CD24 and CD44 surface marker are commonly used. In canine mammary carcinoma, sphere forming assay are used to identify CSCs as free floating spheres are characteristic of the solid tumors in vitro and they are rich in stem cells and progenitors [29]. Currently, the isolation of CSCs in head and neck cancer (HNSCC) involves (1) detection of side-population phenotypes by Hoechst 33342 exclusion, (2) sphere-formation assays, (3) assessment of aldehyde dehydrogenase (ALDH) activity, and (4) identification of CSC-specific cell surface markers CD44, CD133, and ALDH through FACS and immunohistochemistry [30].

Immune evasion and cause of metastasis

The two main reasons for progression, spreading and metastasis of cancer are through CSCs and epithelial mesenchymal transition (EMT). While CSCs initiates tumorigenesis, the EMT helps in formation of new CSCs. EMT is a complex cellular event in which epithelial cells lose their morphology or phenotype by shedding cell-to-cell adhesion molecules, such as desmosomes, tight and gap junctions, lose apical-basal polarity and attain frontal-rear polarity and acquire a mesenchymal phenotype that is associated with high motility and invasive properties [31]. Depending upon the process of transformation EMT is classified into Type I, Type II and Type III EMT. Type I is associated with embryogenesis and organogenesis, Type II with wound healing, tissue regeneration and organ fibrosis whereas Type III is mainly associated with tumor progression [32]. Cells undergoing EMT are identified through loss of epithelial marker such as E-cadherin, EpCAM and cytokeratins and gain of mesenchymal markers like Fibronectin, Vimentin and N cadherin. The process invol ves activation of nuclear transcription factors (ZEB1, SNAIL, SLUG, TWIST, KLF8, and E47), signal transduction pathways (*HOX* transcription factors, the Wnt-βcatenin, Notch and Sonic Hedgehog (SHh) pathways, and the Polycomb gene *Bmi-1*, and TGF-β signaling) [33] and extracellular matrix (ECM). In Pancreatic ductal adenocarcinoma (PDAC), one of the lethal malignancies nestin plays a major role in tumor initiation and maintenance [34]. Both CSC and EMT are responsible for tumor formation, invasion, metastasis and chemoresistance associated with ovarian cancers [35].

Conclusion

As these cells play a major role in cancer initiation and progression, the isolation of these cells is of major interest for the development therapeutic approaches aiming at directed targeting of these cells. In recent years, targeted immunotherapeutic drugs have been developed as for treatment of malignancy and lengthening overall survival times (OST) in canine patients.

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