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A Review of Tumour Initiating Cells in Veterinary Oncology and Potential Implications in Canine Transmissible Venereal Tumours.

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Review Article

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ABSTRACT

The canine transmissible venereal tumour (CTVT) is a transplantable neoplasia considered an allograft. However, information about the origin and carcinogenesis process is scarcely known. Currently, some neoplasms are believed to arise from tumour-initiating cells (TIC's) classically described in human myeloid leukemia. TVT intrinsic characteristics provide evidence of a possible TIC's participation in carcinogenesis process of this malignancy. In this review, we highlight CTVT as a cancer stem cell model and describe the four main TIC markers commonly used to enrich tumour initiating cells in canine and human tumours.

CANINE VENEREAL TRANSMISSIBLE TUMOUR (CTVT)

The CTVT, one of the oldest somatic tumours, has been widely inquired to determine source, mode of transmission, and factors associated to spontaneous regression [1]. Other potential tumours include transplantable reticulum cell sarcoma in Syrian hamsters (Mesocricetusauratus), and hemocitical leukemia in clam (Mya arenaria) [2]. In humans, there are no descriptions of naturally transplantable tumours. Nonetheless, transmission may occur in specific situations such as child birth, transplantation, and surgical procedures. In any case, a certain degree of immunosuppression is needed to successful transplantation [3].

Initially described in London, 1810 [4], and later by Novinski, 1826, CTVT is now considered a transplantable allografts. The aneuploid karyotype, and some specific chromosomic features as the insertion of LINE-1 sequence near the C-MYC gene, in all CTVT's distinct geographic regions, propose a common origin. This supports the theory of continuous cell transplantation to the generations [5, 6].

Nowadays, the origin of TVT is estimated thousands of years ago in ancestral old wolves of East Asia. Along the natural history of the disease, genetic instability would have generated cumulative character of mutations, followed by genetic stabilization after subsequent passages [6].

CTVT has been detected in most continents, except Antarctica. It is commonly reported in tropical and subtropical regions,

including the southern region of the United States, Central and South America, Southeast Europe, Ireland, China, Middle East, Africa, Bahamas, Japan, India and Brazil [7, 8].

Strakova and Murchison [4] conducted a historical and present analysis of CTVT global distribution that determined mean prevalence rates between 1-10 % in most countries of South and Central America, Asia and Africa. Canada, Czech Republic, Finland, New Zealand, Switzerland, Sweden and the United Kingdom were declared free of the disease. Geographical variations in parts of Europe and the United States have also been reported. Developed countries with stable economies have lower prevalence.

Likewise, through a historical analysis in London and Thailand, Strakova and Murchison [4] found significant reductions in the prevalence after applying public policy liability, and sterilization campaigns, which eradicated the illness at present. Similarly, strict quarantine policies for imported dogs keep New Zealand free of such disease. Likewise, according to the authors, poor healthiness and the quantity of stray dogs are also associated with higher rates.

In Brazil, a regional epidemiological study conducted in the Municipality of Botucatu, from 1994 to 2003, showed a CTVT prevalence of 17.1 % in 5798 cytopathology examinations in dogs, all performed by the Cytology Department, Faculty of Veterinary Medicine and Animal Science, UNESP, Campus Botucatu. The annual incidence ranged from 11.8 % to 24.1 % during the same period [8].

Data relating to breed predisposition, sex or age are contradictory, depending mainly on the region or country under study. In general, the disease is more prevalent in sexually mature animals, commonly between 2 and 8 years of age 7 with no breedand sexual predisposition [4, 7].

TVT mode of transmission is quite peculiar. The success of neoplastic cell transplantation depends on many factors such as the presence of the continuity of solutions in epithelial barriers of the receptor tissue, the immune status of the host, and the tumour cell viability. Coitus matches the most common way of transplantation by means of cell exfoliation and implantation to the genital mucosa. However, social behaviors, including licks, scratches, bites and the act of sniffing, as well as delivery and maternal habits, are actively involved in the transmission, at least to a lesser extent [7].

CTVT neoplastic cells, after transplantation, must overcome antitumour defense mechanisms by recognizing the major histocompatibility complex of class II (MHC II) in tumour cells for T lymphocytes and NK of the host. The loss of expression of class II molecules, and CHM conversion of the tumour microenvironment for local immunosuppression state, are the main immune evasion strategies. More recently, acquired genetic modification has contributed to the resistance [4].

TVT has three evolutionary phases: progressive (4-6 months), static, and regressive [7, 9]. In each, a significant variation in the mitotic and apoptotic index, number of intratumoural lymphocytes and mast cells, vascular microvessel, and in stromal characteristics can be present [9]. However, cutoffs regarding the variables mentioned are nonexistent. In addition, the exact definition of stage depends on the clinical follow-up.

This neoplasia often displays a vegetative formation, as a polypoid multilobular, friable, with a white, or red surface, bleeding and ulcerating, measuring up to 15 cm. In females, it frequently locates in the vestibule or vagina. In males, the bulb of the glans, pars longaglandis, and the glans penis are the most affected areas. The extragenital sites such as skin, oral cavity, conjunctival mucous membranes, lymph nodes, spleen, ovaries, breast, perianal region, liver, lung, skeletal muscle and testes may also be invaded, even in the absence of genital lesions [7,8]. The anatomical site reflects clinical signs usually associated with the compression effect or tissue invasion at the proximities. Although uncommon, metastasis in inguinal lymph nodes, external iliac, mesenteric, kidney, spleen, liver, eye, tonsils, brain, pituitary, skin, bone and peritoneum, are also reported [7].

The diagnostic methods for TVT include cytology, histopathology, immunohistochemistry, cyto-genetics and polymerase chain reaction [7].

The fine-needle aspiration (FNA) is the method with the highest specificity and sensitivity for diagnosis. In general, the cell population is represented by abundant round cells, measuring between 14 µM to 30 µM, and characterized by individualized cytoplasm with distinct contours, slightly basophil containing multiple scattered light vacuoles and round. The nuclei are eccentric or central with regular borders, aggregated chromatin, and distinct nucleoli. Mitotic figures are common [10]. The cytologic subtypes are categorized into plasmacytoid, lynphocytoid, and mixed, based on the quantity and similarity of oncocytes with lymphocytes and plasma cells. Flórez et al. [11] describe the lynphocytoid type as composed of over 60 % rounded cells, with a fine granular appearance cytoplasm, containing discrete quantities of clear vacuoles. Their nuclei are round, tending to be centralized, with evident heterochromatin, and 1 or 2 distinct nucleoli. The plasmacytoid type consists of more than 60 % of oval cells with abundant cytoplasm, and a lower nuclei: cytomplasmratio. Its cytoplasm has a larger amount of light vacuoles and eccentric nuclei. The mixed one presents plasmacytoid and lynphocytoid cells. However, none of their population exceeds 59 % of the total cells.

The cytomorphological classification is of prognostic importance [12]. The plasmacytoid are usually associated with higher frequency in extragenital sites, metastasis, nuclear abnormalities (e.g. amount of micro-nuclei.), nuclear lobulations, macrokaryosis, and high nucleolar area, number of nucleoli, and nuclear and nucleolar diameter [13]. Besides, they showed higher growth fraction

(Ki-67 index), P-glycoprotein expression, DNA damage in the comet assay, and lower response to chemotherapy [12,14-16].

The common histological features fit venereal tumour into the spectrum of round cell tumours. The CTVT is described as sheets and neoplastic cell nests distributed in diffuse pattern, and supported by trabeculae of fibrovascular stromal tissue. Cells are large, round, with large and vesicular nuclei, mostly with a unique and prominent central nucleolus. The cytoplasm is granular, eosinophilic, vacuolated, with distinct edges. Variable infiltration by lymphocytes, plasma cells and macrophages are usually seen, depending on the clinical phase [17].

CTVT cells exhibit immunoreactivity to vimentin, lysozyme, α-1 antitrypsin, and specific antigen macrophages (ACM-1), and non-reactivity for cytokeratin, S100, CD3, and α-actin of smooth muscle [7,17-19]. Some cases demonstrate positive immunophenotype for NSE (Neuron Specific Enolase), desmin, IgG, IgM and light chains of immunoglobulin [17,18]. Moreover, these cells are negative for Melan A, CD117, CD79, CD20, PAX-5 and E-cadherin. The expression of the above-mentioned markers allows to hypothesizing histiocytic origin of CTVT [17].

The polymerase chain reaction may also be used to diagnose CTVT. Some researches detected a rearrangement of the C-MYC gene by a sequence insertion called long interspreadnuclear element (LINE-1). This genetic event is present in all VCTT, regardless geographic region [6, 7].

CTVT and tumour initiating cells (TIC's)

Recently, O'Neill [20] through a concise review approached CTVT to another related disease called Facial Disease Tasmanian Devil (Sarcophilusharrisii), or DFTD, from the perspective of the carcinogenesis model related to tumour initiating cells. The author enumerates common characteristics to TIC's and normal stem cells, including unlimited self-renewal, asymmetric division, and gene expression associated to potential stem, further than addressing the observed potential for allotransplantation and xenotransplantation in this malignancy.

The potentiality of TVT with an extended self-renewal is supported by the natural history of the disease, and confirmed by experimental studies of transplantation in mice, coyotes, jackals, foxes and domestic dogs [20].

The murine experimental models based on arranged serially xenotransplantations in CTVT also support the hypothesis of self-renewing capability of these cells [5,21,22]. Mice with a severe combined immunodeficiency and non-obese diabetic specimens (SCID/NOD), inoculated with cell suspension from the primary tumour, primary cell cultures, or second-passage cells of murine xenografts, developed tumours within weeks [5,22,23]. Here, it is interesting to note that mice inoculated with suspensions of inactivated tumour cells did not develop cancer, leaving out the possibility of viral participation [22].

The cell quantity required to consider a new tumour in dogs and murine models is already known [7]. Experimental models of allotransplantation were reproduced successfully in dogs by subcutaneous inoculation, until 1×10^8, of original or cultivated tumour cells [24]. However, only 13 % of transplanted cells survived and contributed to tumourigenesis in other experiments [21].

Koike et al. [25] during a serial allotransplantation in puppies managed to keep histological features and karyotypes of original tumours for 12 years, which comprised 50 passages. Karlson et al. [26] also maintained CTVT by 40 generations of dogs for 17 years, without histopathological changes. Most studies used cell populations "non-enriched" *in vitro* for any TIC [20], which may suggest high presence of this cell type in such neoplasia. Indeed, it is highly likely that less differentiated and homogeneous tumours contain a high number of tumour initiating cells [27].

Gene expression related to profile tumour initiator was described in Tasmanian devils [20]. Performing a transcriptomic study on DFDT samples, a cancer analogous to CTVT, Murchison et al. demonstrated overexpression of SOX-2, nestin, OCT6, CD44 and CD133, which introduces molecular evidences as a potential initiator. However, gene expression reflecting any profile of the total tumour population not necessarily supports the premise of only tumour subpopulations would carry TIC phenotype [20]. Without overestimating Murchison´s findings, such outcomes advise to carry out detailed studies on CTVT [20].

Other indications not mentioned by O'Neill regarding TIC's perspective in CTVT are described below.

The chemoresistance, an intrinsic characteristic of TIC's, is manifested by the protein expression of the ABC family related to ATP-dependent transporters. The carriers are classified into seven subfamilies from A to G. The ABCB1, or P-glycoprotein MDR-1 (multi-drug resistance protein 1) is commonly overexpressed in tumour stem cells [28]. Gaspar et al. [16] demonstrated greater immunoexpression of P-glycoprotein in plasmacytoid subtypes and metastases from CTVT. Flórez et al. [29] showed variable MDR-1 gene expression in primary cell culturesof CTVT samples and highlighted significant differences among treated and untreated cell culture with vincristin, suggesting a modulating effect of drug on gene expression.

Through a CTVT *ex vivo* study, Bassani-Silva et al. [30] determined immunoexpression of survivin in > 95 % of oncocytes, and low percentages (0 % to 6.5 %) of positive tumour cells for cleaved caspase-3. Survivin and caspase-3 are proteins participating actively in anti-apoptotic mechanisms of Wnt/β-catenin pathway, which are commonly, overexpressed in cancer stem cells [31].

Tumour initiating cells

The clonal evolution model of cancer was the main oncological research topic in the last century. It postulates equal prob-

ability of any tumour cell to acquire genetic and/or epigenetic changes, which would confer growth advantages, self-renewal and generation of upcoming malignancies. In the same period, the model of tumour initiating cells was set on the unicity of tumour cell subpopulation to present a specific capacity for self-renewal, multi-lineage differentiation, and tumourigenesis.

Progenitor and differentiated cells derived from stem parental population would lose such capabilities, being considered as non-tumourigenic [32]. At the end, neoplasias would fit in a model of hierarchical evolution of the disease. However, both models are not mutually exclusive. In fact, they could manifest concomitantly in different stages of carcinogenesis [33].

At this point, a brief review of terminology used in basic biology of normal stem cells becomes necessary. Stem cells are undifferentiated units having the potential of multi-lineage differentiation determined by genetic and epigenetic programs, and unlimited self-renewal. Self-renewal occurs by symmetrical or asymmetrical division [34]. The main feature, especially of the tumour initiating cells, is the asymmetric division property responsible for expanding the population of original cells, and for originating progenitor cells with limited potential of differentiation and self-renewal [35,36]. In contrast, the symmetric division creates two daughter cells containing the same phenotypic profile of the parent cell.

The stem cell potentiality is another important term, which can be explained through embryogenesis. Embryo totipotent cells are able to produce all organism cells, and extraembryonic tissues. Pluripotent cells from the embryoblast originate all body tissues. Omnipotent and multipotent cells exist in the embryo and the adult. In adults, the multipotent cells have multi-lineage differentiation capacity (e.g. multipotent hematopoietic stem cell). Finally, unipotent cells differentiate into only one line (e.g. omnipotent stem cells of the intestinal crypts) [35].

Stem cells can be further classified as the distribution and location on embryonic stem cells, fetal, adult, amniotic, umbilical, epidermal, neural and tumour [35].

Currently, the source of tumour initiating cells is under discussion. Normal stem cells would be the first candidates for malignant transformation, as can be seen in patients with chronic myeloid leukemia (CML), and acute lymphoblastic leukemia (ALL). CML is characterized by the presence of specific chromosomal translocations, such as t (9:22) or Philadelphia chromosome, in their hematopoietic stem cells $CD34^+ CD38^- CD19^-$ [37].

Another potential target is the multi-potent progenitor cells. Chromosomal translocations and aberrant gene expression have been described in $CD34^+ CD38^- CD19^-$ cells in patients with ALL [37]. The evolution of patients undergoing CML to the blast crisis phase involves the activation of self-renewal programs in progenitor cells. Expressed in cellular terms, different stages and conditions also have different origins [38].

The re-differentiation or dedifferentiation of distinctive somatic cells to more primitive states with the acquisition again of self-renewal is another model explaining the origin of cancer stem cells [39]. Somatic cell must be reprogrammed to gain unlimited proliferation and asymmetric division capabilities [36].

The scientific community uses the definitions of tumour cell and initiating neoplastic stem cell interchangeably. However, Kreso and Dick [40] distinguish both malignant populations based mainly on tumourigenic capacity and prospective isolation. In short, TIC's would not be subject to isolation because the concurrence of initiator and non-initiator transitory stages at the same cell derived from reversible cellular plasticity [34,41]. Finally, the authors reserve the term in cases of the lack of mapped surface markers, and consequently, of prospective isolation.

Since 2006, the American Association for Cancer Research (AACR) contemplates tumour stem cell as such a unit with prolonged self-renewal and ability to multi-lineage differentiation [42].

The expression "tumour initiating cell" covers indeed tumour stem cells, or cells capable of unlimited self-renewal and differentiation. Progenitor cells are those failing to maintain their long-term potential of self-renewal, but still having tumourigenic potential [34].

In 1997, Bonnet and Dick described the first evidence of TIC by investigating patients with AML. $CD34^+/CD38^-$ cells were isolated from these patients and xenotransplanted in SCID / NOD mice, resulting in complete reconstitution of the phenotypic spectrum in AML. The authors named this tumourigenic subpopulation as leukemic cells. Beyond described properties, these aberrant units disclosed high potential for self-renewal, proliferation and differentiation, including the capability of prospective selection, regardless of the primary tumour phenotype [43]. Moreover, studies showed leukemic cells belonging to negative subpopulations for lineage markers (Lin-) [38].

After the discovery of TIC´s in hematopoietic malignancies, Al-Hajj et al. in 2003 reported a low amount of $CD44^+ CD24^-$ / baixo cells in human breast tumours. They are also capable of cancer initiation, imitation of the primary neoplastic characteristics in SCID / NOD mice, mammosphere formation, and high activity of the enzyme aldehyde dehydrogenase (ALDH) [44]. In subsequent years, replicating Al-Hajj's work helped to describe human solid tumours, including lung, ovarian, brain, and melanomas [45].

In veterinary medicine, candidates for TIC's in solid tumours have been already studied in dogs and cats. Table 1 summarizes main markers and published works.

Tumour initiating cell markers

The "enrichment", isolation and/or identification of TIC's is not simple. Currently, several methods contribute to this process. Namely, they are as follows: The expression of "specific" surface markers, fluorescence or magnetic separation (MACS and FACS), SP cell analysis, measurement of ALDH levels, immunocytochemistry, immunohistochemistry, microfiltration, multicellular tumour spheroid model (MCTS), clonogenic assay, characterization of chemoresistance, tumourigenicity in animal models, gene expression, and transcription of factors by RT-qPCR or RT-PCR [31, 67].

Despite of technique diversity, intrinsic limitations and potential biases are also present (ISLAM et al. [31]). However, it is noteworthy that the evidence of tumour initiator potential must meet the minimum criteria of prolonged self-renewal and differentiation capacity [34].

Evaluating self-renewal capacity by xenotransplantation testing and/or non-adherent spheroid formation in cell culture is taken as the main standard to characterize TIC's [31,34]. The finding of the initiator profile usually has misinterpretations due to generalization and extrapolation of outcomes. Despite TIC's unification by intrinsic characteristics, a considerable phenotypic variation exits, which depends on their niche. This provides certain heterogeneity [33,42].

Various identified surface antigens are used to detect TIC's by flow cytometry. However, a wide expression of markers not implies the existence of any particular initiator phenotype [42]. Some technical issues have influence on surface molecule expression, including the use of enzymes (e.g. Collagenase, hyaluronidase) to prepare suspensions, and on the loss of cell viability [68]. Further, the amount of starter cells can be under, or overestimated, especially *in vitro* systems [33]. Moreover, the non-exclusion of cells such as leukocytes, fibroblasts, endothelial cells and necrotic debris from analysis may ultimately underestimate their tumourigenic potentiality [69]. Therefore, beyond the surface marking, functional assays such as the ALDH activity and SP cells are to be implemented [67].

Among the most applied markers cited by the literature are CD44, CD34, CD90 (Thy-1), CD133 (prominin-1), ABCG5, CD24, EpCAM, CD105 (Endoglin), CD117 (c-kit), ALDH, CD38 CD26, CD29, CD166, CD15, nestin, CD13, ABCG2, CD20, CD271, c-met, CXCR4 / CD184, CD98, Trop-2, CK5, 063, BMI-1, OCT-4, and OCT3/4 [31,70]. The first four are commonly found in almost all studies.

The CD44 antigen, a family member of the cartilage link protein, is a transmembrane glycoprotein of adhesion composed of seven extracellular domains, a transmembrane domain, and a cytoplasmic domain. The size of the extracellular domains varies according to the insertion of exons cracking products. The structure of the polymer is responsible for binding to collagen, laminin, glycosaminoglycans, fibronectin and hyaluronic acid, and cellular receptors, including L-selectin and E-selectin [71].

CD44 protein is classified in standard, or hematopoietic, (CD44s), and variable isoform (CD44v). Variable isoforms (v1 to v10, and combinations) are expressed on hematopoietic and epithelial cells during embryogenesis, leukemias and carcinomas [71,72]. The variable isoform CD44v6 is commonly associated with breast and colorectal tumours. Other less common isoforms were also studied, including CD44E, and expressed in epithelial cells. R2 and CD44R1 isoforms are categorized in mononuclear leukocytes and granulocytes [73].

Variable isoforms have a number of functions. They are as follows: organization of the synthesized hyaluronic acid molecules (although there is no evidence of hyaluronic acid production by leukemia initiating cells), local concentration of glycosaminoglycans, metalloproteinases, cytokines and chemokines, TIC's adhesion to the stem cell niche, endothelial migration, quiescence mediated by the ligand-receptor interaction, resistance to stress generated by low oxygen saturation, regulation of drug resistance genes, potential malignant cell invasion, and activation of apoptosis resistance of routes including blocking caspase-3 [71,72].

The contribution of CD44 protein is partially related to interactions with the extracellular niche (ECN). ECN is defined as specific locations composed of epithelial cells, mesenchymal cells, and extracellular matrix molecules responsible for regulating adhesion, retention, implantation, mobilization, quiescence, activation, differentiation, and division of stem cells [71]. Hyaluronic acid is the main molecule in ECN, and the main binder of CD44 protein.

Therefore, much of receptor functions are linked to interactions with its primary ligand [72]. The extracellular matrix (ECM) of CTVT undergoes quantitative and qualitative changes during periods of progression and regression. Mukaratirwa et al.[74] observed a significant increase in the hyaluronan membrane and cytoplasm of oncocytes during the progressive phase. This suggests a molecule involvement in masking antigens and histocompatibility complex beyond an anti-apoptotic protective effect.

CD44 was originally detailed in mammary tumours, and squamous cells of head and neck [67]. Because of its consistent distribution in other cancer types, besides the differences between the tumourigenic potential of $CD44^+$ and CD44- cells, the protein has been considered a common marker for TIC's [75]. Researchers had focused on the correlation between isoforms and specific types of TIC's. As an example, isoform CD44v6 was associated with increased aggression in human non-Hodgkin lymphomas [73].

CD34 trans-membrane phospho-glycoprotein has 115 kDa of molecular weight. In 1984, it was firstly identified in hematopoietic stem cells. The protein has a glycosylated extracellular domain, a single helix, and a cytoplasmic membrane end. Located on lymphocytes surface, L-selectin (CD62L) is the most commonly studied ligand. The ligand-receptor interaction induces endothelial cell adhesion and migration [76]. The protein still participates actively in the increase of cell proliferation and differentiation

block, the migration of hematopoietic cells and cellular morphogenesis. It is characterized almost exclusively by endothelial and hematopoietic stem cells [77].

CD34 glycoprotein has been identified and associated with "enriched" population of hematopoietic stem cells. Recent evidence suggests that $CD34^+$ cells hold potential of transdifferentiation. Such cells would be capable of differentiating into cardiomyocytes, hepatocytes, and respiratory epithelium. $CD34^+$ progenitor cells were disclosed in cornea, salivary glands, striated muscle, intestines, and skin. Specific descriptions were given for each location [76].

The evidences point not only to take CD34 glycoprotein as a marker of hematopoietic progenitor cells, but also as a potential marker of undifferentiated cells [76]. The satellite muscle cells, corneal keratocytes, fibrocytes and mesenchymal stromal cells are accepted as tissue-specific progenitor cells. While maintaining the functional state of progenitor cells, during *in vitro* and *in vivo* researches, these biological units subexpressed CD34 protein when compromised to more advanced differentiation states [76].

The revealing of CD34 antigen as a marker TIC's occurred during experiments with SCID and NOD/SCID mice, even in the 90s. The hypothesis of leukemic initiating cells took into account the phenotypic characteristics and normal hierarchical organization of the hematopoietic system. According to several authors, primary transplanted cells of AML human patients into mice initiated and sustained tumour growth. The cells with $CD34^+CD38^-$ profile, or leukemic starters, were isolated and purified prospectively. Consequently, such units acquired the capability of initiating leukemia in mice, regardless of cellular heterogeneity of the primary tumour [43].

Other markers associated to CD34 also enabled to detect phenotypically similar populations in solid tumours [78]. These biological conglomerations are a fraction of hematopoietic stem cells $CD34^+$ cells, which have $CD133^+$ protein co-expression with high expansion potential *in vitro*, and the ability of differentiation in other cell types [79].

Unveiled 40 years ago during a research on leukemia cells in mice, the Thy-1, or CD90, is a membrane glycoprotein with a molecular weight between 25 and 37 Kda. Initially, it was labeled as a differentiating marker predominantly expressed on thymocytes and brain cells of mice [80]. Recent studies confirm CD90 protein distribution on the surface of thymocytes, lymphocytes, fibroblasts, endothelial, neuronal cells, hematopoietic and mesenchymal stem cells, and ovarian follicular cells, even in cancer [81]. The protein is inserted into the cell membrane by glycophosphatidyl inositol anchor molecules (GPI), without trans-membrane domain. In 1994, the First International Canine Leukocyte Antigen Workshop determined Thy-1 expression in thymocytes, nodal and peripheral T lymphocytes, B lymphocytes, monocytes, granulocytes, neural and kidney cells and mesenchymal stem cells [82].

Thy-1 protein is linked to immune or non-immune roles. The immunological function has to do primarily with T lymphocyte activation, interactions with antigen presenting cells, and maturation of thymocytes. Deficiency of Thy-1 induces defects in T lineage, including flaws in the maturation and proliferative response. The most important non-immune functions are apoptosis, adhesion and cell migration, tumour suppression, cicatrization, fibrosis, proliferation and neuronal differentiation [80]. Stimulation of $CD90^+$ stem cells by cytokines induce its entry into the cell cycle, whereas the $CD90^-$ remains in a state of quiescence. In addition, some studies propose an association between protein expressions and the tumourigenic ability [83, 84].

$CD34^+CD90^+$ cells from bone marrow, umbilical cord, and peripheral blood generate long-term cultures and differentiated cells originate from diverse lineages. In fact, they are able to generate myeloid erythroid (ME) and B lymphoid lineages with greater efficiency when compared to $CD90^-$ cells [79]. Furthermore, some authors indicate that only Thy-1^+ cells enter to the S/G2/M phases by stimulation [79].

CD133, or prominina-1 (AC133 or PROM-1), is a highly conserved gene of the animal kingdom. It encodes a membrane protein composed of 865 amino acids with 5 trans-membrane domains, and two extracellular loops glycosylated in human species. The prominina-1 has an extensive dissemination mainly in adult epithelial cells of glandular tissue. $CD133^+$ cells stay in the bile and pancreatic ducts, the salivary ducts and secretory cells, lacrimal glands, sweat, cervical and endometrial uterine, even on the bottom of pits and gastric crypts, both duodenal and colonic. CD133 also appears in epithelial cells of the proximal tubules and Bowman's capsule [85].

CD133 antigen was firstly noted when obtaining specific monoclonal antibody, or AC133 epitope, coming from $CD34^+$ hematopoietic stem cells of adult bone marrow, and fetal liver cells. The co-expression in $CD34^+$ hematopoietic cells allowed the recognition of CD133 antigen as a stem cell marker [85].

Presently, CD133 protein function has not been fully elucidated. Although used for the identification of neuronal, hepatic, renal and prostate stem-cells / progenitors, findings of CD133 positive subpopulations not necessarily represents TIC's presence. The latter is because the protein expression also occurs in differentiated adult cells [86].

More importantly, the dependent epitope of AC133 human glycosylation is part, not synonym, of the CD133 antigen. The prominin-1 recognized by the 13A4 murine monoclonal antibody is homologous to A133 in humans [87]. In addition, the AC133 expression is restricted to less differentiated cells. In addition, the AC133 expression is restricted to less differentiated cells, even by verifying the loss of protein as a stimulus for differentiation [85,88].

As part of the stem cells, $CD133^+$ normal cells are able to reconstruct differentiated tissues *in vivo* (e.g. prostate epithelium),

and regenerate them (e.g. in the Duchenne muscular dystrophy -DMD- in mouse) [87]. Likewise, prominin-1 has been linked to TIC subpopulations in several types of human, murine and canine cancer, including glioblastomas, medulloblastoma, prostate, colonic, ovarian, laryngeal, pancreatic, hepatocellular, and lung neoplasias, even melanomas [85,86].

Among the most outstanding functional characteristics related to prominin-1 expression are a higher potential tumourigenic *in vivo*, resistance to apoptosis, metastasis capacity, cell metabolism, chemotherapy and radiation resistance, expression of genes and factors associated to maintaining an initiator tumour status, tumour sphere formation, ALDH expression and efflux ability of DNA colorants in assays of "side population".

The multi-lineage differentiation implied by the reconstitution of post-transplant heterogeneous tumours is also another significant feature [85,86,88]. The expression or suppression of prominin-1 depends on extrinsic and intrinsic factors to the tissue microenvironment, including hypoxia, concentration of cytokines (TGF-β), microRNA profiling, and epigenetic regulation mechanisms [89].

More recently, several studies indicate tumourigenic ability of CD133- glioma cells, beyond the reacquisition capability of $CD133^{+}$ phenotype *in vivo* (CHEN et al.). In dogs, similar results have been reported in CD34 melanoma cell lines [61].

Future directions

Currently, there are no studies regarding a cancer stem cell phenotype for CTVT. The sorting of potential subpopulation of TIC's based on above described surface markers, side population analysis, and ALDH activity should be employed as the first step toward CTVT characterization to shape a cancer stem cell model. Furthermore, sphere tumour formation, clonogenic assays, *in vivo* tumourigenicity, multipotency evaluation, self-renewal ability, and a differentiating gene expression would help to delineate a hierarchic model tumour. A comparison between fresh samples and cell lines from the same tumour would be also important to verify differences between both systems, since *in vitro* systems may not be representative of the primary patient condition.

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All authors developed the concept for the review and contributed to writing and editing of the manuscript.

CONFLICTS of INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

The following abbreviations are used in this manuscript:

TIC: tumour initiating cell

CTVT: canine transmissible venereal tumour

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