Immunophenotypic and molecular profile of cancer stem-cell markers in ex vivo canine transmissible venereal tumour (CTVT)

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1 INTRODUCTION

Canine transmissible venereal tumour (CTVT) is considered a transplantable tumour allograft composed of somatic cells. Continuing efforts have been made to determine its origin, phenotype and factors associated with transmission and biologic behaviour (Baez-Ortega et al., 2019; Murchison et al., 2014).

Cancer stem cells (CSCs) are considered unique subpopulations inside the heterogeneous cell population of a tumour, which are solely responsible for tumour initiation, metastasis (Baccelli & Trumpp, 2012) and recurrence post-treatment (Baccelli & Trumpp, 2012; Glumac & LeBeau, 2018; Krause et al., 2014). It has been proposed that CTVT might exemplify the cancer stem-cell (CSC) process given that it possesses long-term self-renewal capacity, potential for serial transplantation, expression of specific stem cell genes and an undifferentiated phenotype, which are all hallmarks of CSCs (Clarke et al., 2006; Liu et al., 2012; O’Neill, 2011). Despite this hypothesis, the presence and origin of stem cells in CTVT remain unverified.

The majority of CSC studies use in vitro systems to characterise and enrich the sample for CSC subpopulations. However, these systems can alter gene and protein expression, which can interfere with CSC proportions (Hill, 2006). Therefore, it may be preferable to study CSC
subpopulations in fresh tumour biopsy samples rather than cell lines (Guth et al., 2014).

Various methodologies have been used to identify CSCs in solid tumours (Tirino et al., 2013). The most common one is sorting cells by surface expression of the classical haematopoietic stem cell (HSC) markers CD44, CD133, CD90 and CD34, both in veterinary and human medicine (Calloni et al., 2013). All of these proteins have several functions in stem cell biology.

CD34 plays a role in cell adhesion and differentiation, being expressed in primitive human stem cells (HSC) of bone marrow, in endothelial cells (Calloni et al., 2013; Kim & Ryu, 2017), and in cancer stem cells (CSCs). CD34 has been associated with endothelial cells/endothelial progenitors, angiogenesis and vasculogenesis (Kapoor et al., 2019). CD44 is a cell surface adhesion receptor identified in HSC and tumour-initiating cells from leukaemias, mammary and colonic tumours (Zöller, 2015), epithelial cells (Teye et al., 2016), leukocytes (Wang et al., 2001), tumour cells (Yan et al., 2015) and vascular endothelial cells (Olofsson et al., 2014). CD44 and its isoforms play important roles in a variety of physiological and pathological settings (Yu & Stamenkovic, 2000) and has a relationship with tumour progression and vasculogenesis (Matou-Nasri et al., 2009; Savani et al., 2001).

CD133 (also known as AC133 and prominin-1) is the most frequently used cell surface antigen to detect and isolate CSCs from various solid tumours (Kim & Ryu, 2017). CD133 was initially discovered as HSC (Yin et al., 1997), and its biological function has been linked to stem cell fate decisions and emerges as an important physiological regulator of stem cell maintenance and expansion (Calloni et al., 2013). The physiological functions of CD133 in normal biology and in the progression of cancer remain elusive (Glumac & LeBeau, 2018) and its involvement in membrane organisation is suggested (Su et al., 2015).

Although the function of the CD90 protein has not yet been completely elucidated, it has been implicated to have a significant role in cellular adhesion and migration, regulating cell–cell and cell–matrix interactions (Rege & Hagood, 2006); the protein is an indicator of cell cycle status and an HSC marker (Calloni et al., 2013). Besides its expression in certain normal cell types, CD90 has been described as a CSC marker in various malignancies, such as hepatocellular carcinoma, oesophageal cancer, glioma, lung and breast cancers (Yan et al., 2013) and insulinoma (Buishand et al., 2016). Despite this, there is no universal CSC marker, so the assembly of markers into panels is required (Greve et al., 2012).

We evaluated the immunophenotype of CTVT in relation to the classical CSC markers CD44, CD133, CD34 and CD90 in order to search for initial evidence of CSC subpopulations in this tumour.

## METHODS

Thirty-eight CTVT samples were prospectively selected between January 2014 and January 2015. Patients were chosen according to the following criteria: cytological diagnosis of TVT regardless of location, mass size, age, breed or sex, and no chemotherapy in the previous 6 months (Table 1). At least three tumour fragments measuring 1 × 1 × 1 cm each were obtained by incisional biopsy. Areas of ulceration, haemorrhage or inflammation were avoided. Data on follow-up were obtained from the hospital’s electronic database. Most patients were lost to follow-up, thus precluding statistical comparisons in relation to days for complete clinical remission.

Cytological samples for morphological assessment were acquired by imprinting of biopsy cut surfaces onto glass slides. Air-dried smears were stained using a rapid Romanovsky staining kit (Laborclin, PR, Brazil). Smears were scanned at 200× magnification in order to find the most representative fields. Neoplastic cells were classified as plasmacytoid or lymphocytoid (Flórez et al., 2012). Cytological subtypes were classified according to the percentage of each cell type in relation to the total number of neoplastic cells; plasmacytoid tumours had >59% plasmacytoid cells, lymphocytoid tumours >59% lymphocytoid cells and mixed tumours <59% of each cell type. All photomicrographs were obtained via a software-equipped microscope (AxioVs40 version 4.8.2.0 in an Axio Lab. A1 microscope, Carl Zeiss, Jena, Germany) and analysed in the Image J digital image-processing program (freeware).

Flow cytometry was performed on cells harvested by smooth scraping of the surface of incisional biopsy samples with a surgical blade, which were then transferred to microtubes containing 4% paraformaldehyde for 30 min at 4°C. Next, samples were centrifuged at 172 × g for 10 min, washed three times in phosphate-buffered saline (PBS) solution and stored in PBS at 4°C until analysis.

Specimens were adjusted to a density of 1 × 10 (Glumac & LeBeau, 2018) cells/ml, and incubated with Fs block (BD Bioscience, San Jose, CA) for 30 min at 4°C. Samples were then washed once with PBS and incubated for 30 min at 4°C, protected from light, with previously titrated primary antibodies (Table 2) and isotype-matched immunoglobulins as negative controls. Next, samples were washed three times with PBS and analysed in a flow cytometer (FACSCalibur, BD Bioscience, San Jose, CA); CTVT cells were gated according to their large size and high complexity. At least 25,000 events were acquired for analysis. A pool of non-stained CTVT cells was used to calibrate data according to their large size and high complexity (Figure 1). CD45<sup>−</sup> cells (intra-tumoural leukocytes) were gated and excluded from subsequent analysis. All experiments were conducted independently and in duplicate. Data were represented as an average value of both experiments. All antibodies showed cross-reactivity with canine tissues as provided on the specification sheet. Anti-CD45 antibody was added to remove contamination by differentiated haematopoietic cells and leukocytes. Post-acquisition analysis was performed using FlowJo software (TreeStar, Ashland, OR). CD45<sup>−</sup> cells were analysed for CD44, CD133, CD90 and CD34 fluorescence. Values were reported as percentage of positive cells. Positive controls included normal canine haematopoietic cells obtained from the clinical routine of the teaching veterinary school at the same university.

Tumour fragments were fixed in 10% formaldehyde for 24–48 h prior to routine paraffin embedding, sectioning and mounting of 4 µm sections on positively charged slides (Amitel, São Paulo, SP, Brazil). Tissue sections were deparaffinised in xylene and rehydrated in graded ethanol and water. Sections were then immersed in epitope retrieval
| Case | Sex | Age (in years) | Breed          | Location         | Outcome (in days) |
|------|-----|---------------|----------------|------------------|------------------|
| 1    | F   | 3             | Mixed          | Skin             | CCR (345)*       |
| 2    | M   | 3             | Mixed          | Genital          | L/F              |
| 3    | F   | 8             | Weimaraner     | Genital          | L/F              |
| 4    | F   | 8             | Mixed          | Genital          | L/F              |
| 5    | M   | 4             | Mixed          | Genital          | L/F              |
| 6    | M   | 7             | Mixed          | Genital          | L/F              |
| 7    | F   | 3             | Mixed          | Genital          | CCR (49)         |
| 8    | F   | 8             | Mixed          | Genital          | CCR (35)         |
| 9    | F   | 10            | Pitbull        | Genital          | L/F              |
| 10   | M   | 9             | Mixed          | Genital          | L/F              |
| 11   | M   | 3             | Mixed          | Skin             | L/F              |
| 12   | M   | 5             | Mixed          | Genital          | L/F              |
| 13   | F   | 7             | Mixed          | Genital          | L/F              |
| 14   | M   | 4             | Mixed          | Genital          | CCR (69)         |
| 15   | F   | 4             | Pitbull        | Genital          | CCR (116)        |
| 16   | F   | 6             | Mixed          | Genital          | CCR (34)         |
| 17   | F   | 1             | Mixed          | Genital          | CCR (247)        |
| 18   | M   | 4             | Mixed          | Genital          | L/F              |
| 19   | M   | 8             | Mixed          | Genital and skin*| CCR (63)         |
| 20   | F   | 8             | Mixed          | Eyelid conjunctiva | L/F            |
| 21   | M   | 6             | Mixed          | Genital          | L/F              |
| 22   | F   | 4             | Mixed          | Genital          | L/F              |
| 23   | M   | 3             | Mixed          | Genital          | L/F              |
| 24   | F   | 2             | Mixed          | Genital          | CCR (35)         |
| 25   | F   | 2             | Mixed          | Genital          | CCR (39)         |
| 26   | F   | 12            | Mixed          | Genital          | CCR (79)         |
| 27   | M   | 8             | Pincher        | Oral and nasal   | CCR (37)         |
| 28   | M   | 10            | Belgian Shepherd | Genital          | L/F              |
| 29   | F   | 5             | Pitbull        | Genital          | CCR (99)         |
| 30   | F   | 7             | Dachshund      | Genital          | CCR (80)         |
| 31   | F   | 3             | Mixed          | Genital          | L/F              |
| 32   | F   | 6             | Mixed          | Genital          | L/F              |
| 33   | M   | 8             | Mixed          | Genital          | CCR (36)         |
| 34   | F   | 4             | Mixed          | Genital          | CCR (49)         |
| 35   | F   | 2             | Mixed          | Genital          | L/F              |
| 36   | M   | 6             | Standard Poodle | Genital          | L/F              |
| 37   | F   | 8             | Pitbull        | Genital          | L/F              |
| 38   | M   | 2             | Mixed          | Genital          | L/F              |

CCR, complete clinical remission; L/F, lost to follow-up.

Note: Complete clinical remission was defined when no tumour cells were detected by cytology and no gross lesions were observed.

*Only skin samples were collected.
### TABLE 2 Antibodies, sources and antigen specificities used for flow cytometry

| Antibody | Clone | Source and isotype | Antigen specificity | Manufacturer | Fluorochrome |
|----------|-------|-------------------|--------------------|--------------|--------------|
| CD44     | IM7   | Rat IgG2b, k      | Canine CD44        | BD Biosciences | PerCP-Cy5.5  |
| CD133    | 13A4  | Rat IgG1k         | Canine CD133       | eBioscience   | PerCP-eFluor 710 |
| CD45     | YKIX716.13 | Rat IgG2b, k   | Canine CD45        | Serotec       | PE           |
| CD90     | YKIX337.217 | Rat IgG2b, k   | Canine CD90        | eBioscience   | APC          |
| CD34     | 1H6   | Mouse IgG1        | Canine CD34        | Santa Cruz    | FITC         |

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP-CY5.5, peridinin chlorophyll.

### FIGURE 1
Representative flow cytometry images to illustrate gating strategy and immunophenotyping analysis of canine transmissible venereal tumour cells. (a) Gating of the tumour cells according to their size (x axis, FSC-H) and complexity (y axis, SSC-H). (b) Gating of CD45⁻ cells, excluding CD45⁺ leukocytes for the stem cell marker analysis (red line = PE isotype control; blue line = stained sample). (c) Frequency analysis of CD34⁺ cells (red line = unstained control; blue line = stained sample). (d) Frequency analysis of CD133⁺ cells (red line = unstained control; blue line = stained sample). (e) Frequency analysis of CD44⁺ cells (red line = unstained control; blue line = stained sample). (f) Frequency analysis of CD90⁺ cells (red line = APC isotype control; blue line = stained sample). APC, allophycocyanin; CD, cluster of differentiation; FSC-H, forward scatter height; SSC-H, side scatter height.

Solution (citrate buffer pH 6.0) for 30 s at 125°C and then for 30 s at 90°C in a pressure chamber (Pascal, Dako Cytomation, Glostrup, Denmark). Sections were cooled to room temperature for 20 min and then placed in a water bath for 5 min. Subsequently, the sections were immersed in 10% hydrogen peroxide for 30 min in a dark chamber at room temperature in order to inhibit endogenous peroxidase activity. Next, slides were incubated with primary antibodies CD44 and CD34 (Table 3) overnight at 4°C in a humidified chamber. Immunohistochemical reactions were detected using a polymer link detection system (Superpicture, Thermo Fisher Scientific, Carlsbad, CA) according to the manufacturer’s recommendations.

CD34 labelling was performed by incubating sections with polyclonal rabbit anti-goat biotinylated immunoglobulins (Dako Cytomation, Glostrup, Denmark) in a 1:400 dilution for 30 min; next, slides were incubated with HRP/streptavidin complex (Novolink, Novacastra Laboratories, Newcastle, England). Immunohistochemical reactions were visualised by incubating the samples with 3,3′-diaminobenzidine (DAB) chromogen for 3 min. All sections were counterstained with...
TABLE 3 Antibodies, sources and antigen specificities used for immunohistochemistry

| Antibody | Clone | Source and isotype | Antigen specificity | Manufacturer | Dilution |
|----------|-------|-------------------|-------------------|--------------|---------|
| CD44     | IM7   | Rat IgG<sub>2b,k</sub> | Canine CD44      | BD Biosciences | 1:50    |
| CD34     | C18   | Goat IgG          | Canine CD34      | Santa Cruz   | 1:2000  |

Harris haematoxylin for 2 min, placed in a water bath for 5 min and mounted with permanent mounting medium. For negative controls, primary antibodies were replaced by antibody diluent (Novocastra, Leica Biosystems, Wetzlar, Germany). All immunohistochemistry procedures were performed manually.

CD44 immunolabelling scores were calculated by multiplying the percentage of positive cells (score 0 to +4) and staining intensity (score 0 to +3) in five microscopic fields (400×) with the highest immunostaining regions (‘hotspots’). Percentages of positive cells were scored as 0 (no positive cells), 1+ (< 10% positive cells), 2+ (10–50% positive cells), 3+ (51–80% positive cells) or 4+ (> 80% positive cells). Membrane staining intensity was assigned as 1+ (mild), 2+ (moderate) or 3+ (marked). A total score of 0–12 was assigned (Huang et al., 2015). Epithelial cells from mucous membrane or epidermis from the tissue around the CTVT were used as internal controls for CD44 immunostaining.

The RT-rtPCR technique was performed following the MIQE guidelines (Bustin et al., 2009). Total RNA was extracted from CTVT tissue using an RNAspin Mini RNA Isolation Kit (GE HealthCare, San Francisco, CA) according to the manufacturer’s guidelines. Purity and quantification of isolated RNA were measured spectrophotometrically (Nanodrop spectrophotometer, Thermo Scientific, Wilmington, DE). Total RNA was reverse transcribed into cDNA using random primers. Primer and probe assays for RT-rtPCR were purchased (Applied Biosystems, Foster City, CA) and those for CD44 (assay Cf02693346_m1) and CD34 (Cf02673965_g1) were included. The 18S rRNA (assay 4352930E) and GADPH (assay Cf04419463_gH) were used as reference genes to normalise the results. Each sample was analysed in duplicate and negative controls were enrolled; the efficiency was verified and established at 95–105%. The rtPCR analyses were carried out using a thermocycler (LightCycler® 96, Roche Diagnostic, Mannheim, Germany). Analyses of relative gene expression data were performed according to the 2<sup>−ΔΔCq</sup> method (Livak & Schmittgen, 2001).

All data were assessed for normality by the Shapiro–Wilk test. All variables are expressed as median and interquartile ranges. Statistical differences between cytological groups were determined using the Student’s t-test or Mann–Whitney U test. p Values ≤0.05 were considered significant. The statistical analysis was performed with the aid of SPSS 23.

3 | RESULTS

Genital tumours were present in 33 of the 38 (87%) dogs. Only one case had concomitant genital and extra-genital tumours. Most patients were lost to follow-up, thus precluding statistical comparisons in relation to days for complete clinical remission.

FIGURE 2 Cytological sample of a canine transmissible venereal tumour showing round neoplastic cells with characteristic clear cytoplasmic vacuoles. Plasmacytoid and lymphocytoid cells are highlighted by white and black arrows, respectively. Mixed subtype. Romanovsky stain, 400×

TABLE 4 Expression levels of CD44, CD34, CD133 and CD90 in flow cytometry and RT-rtPCR for 38 canine transmissible venereal tumours

| Marker | Flow cytometry (%) | RT-rtPCR (in units) |
|--------|-------------------|---------------------|
| CD44   | 98.95 (91.92–99.65) | 216.35 (140.1–396.37) |
| CD34   | 0.026 (0.0006–0.077) | 0.31 (0.20–0.66) |
| CD133  | 30.15 (11–61.17) | NP |
| CD90   | 1.78 (0.17–4.25) | NP |

Note: The values are presented as median and interquartile range (Q1–Q3). Flow cytometry results are expressed as percentage of positive cells. NP, not performed.

Cytological analysis of 38 CTVT showed 22 (58%) plasmacytoid and 16 (42%) mixed types (Figure 2). No predominantly lymphocytoid subtypes were noted. In addition to tumour cells, variable numbers of inflammatory cells were observed, including small lymphocytes, neutrophils and macrophages. No eosinophils or mast cells were detected.

There were no significant differences between cytological groups and CSC markers (Table 4). CTVT samples exhibited different mRNA levels for CD44 and CD34 (Table 4). There were no significant differences in CD44 and CD34 mRNA levels between cytological groups.

CD34 immunostaining was negative in all neoplastic and stromal cells. Only endothelial cells displayed strong, diffuse and granular staining restricted to the cell membrane (Figure 3a). CD44 immunostaining was restricted to the tumour cell membrane in all samples (Figure 3b).
FIGURE 3  (a) Neoplastic cells from a canine transmissible venereal tumour were negative for CD34. Only endothelial cells in intratumoural vessels were positive (arrow). (b) Neoplastic cells from a canine transmissible venereal tumour displayed CD44 membranous staining. DAB chromogen, Harris haematoxylin counterstain, 200×

Minimum and maximum CD44 scores were 1 and 12, respectively (median = 3; Q1 = 2; Q3 = 6) (Table 5). There were no significant differences between cytological groups and the CD44 immunohistochemical score. CD133 expression had a median of 30% and CD90 had low expression.

### DISCUSSION

The expression of the commonly used CSC markers CD44, CD133, CD90 and CD34 were assessed for neoplasms, similarly to other tumours tested (Blacking et al., 2012; Buishand et al., 2016; Bustin et al., 2009; Calloni et al., 2013; Flórez et al., 2012; Greve et al., 2012; Huang et al., 2015; Kapoor et al., 2019; Kim & Ryu, 2017; Livak & Schmittgen, 2001; Matou-Nasri et al., 2009; Olofsson et al., 2014; Rege & Hagood, 2006; Savani et al., 2001; Su et al., 2015; Teye et al., 2016; Tirino et al., 2013; Wang et al., 2001; Yan et al., 2015; Yan et al., 2013; Yin et al., 1997; Yu & Stamenkovic, 2000; Zöller, 2015). We used ex vivo tumour samples in order to minimise alterations in the

| Case | Staining intensity | Percentage of positive cells | IHC score |
|------|--------------------|------------------------------|-----------|
| 1    | 1                  | 2                            | 2         |
| 2    | 1                  | 3                            | 3         |
| 3    | 1                  | 2                            | 2         |
| 4    | 1                  | 3                            | 3         |
| 5    | 1                  | 2                            | 2         |
| 6    | 1                  | 1                            | 1         |
| 7    | 1                  | 3                            | 3         |
| 8    | 2                  | 2                            | 4         |
| 9    | 1                  | 2                            | 2         |
| 10   | 1                  | 1                            | 1         |
| 11   | 2                  | 4                            | 8         |
| 12   | 1                  | 1                            | 1         |
| 13   | 2                  | 4                            | 8         |
| 14   | 2                  | 4                            | 8         |
| 15   | 1                  | 3                            | 3         |
| 16   | 2                  | 3                            | 6         |
| 17   | 2                  | 4                            | 8         |
| 18   | 2                  | 3                            | 6         |
| 19   | 1                  | 1                            | 1         |
| 20   | 1                  | 3                            | 3         |
| 21   | 2                  | 3                            | 6         |
| 22   | 2                  | 2                            | 4         |
| 23   | 2                  | 4                            | 8         |
| 24   | 1                  | 2                            | 2         |
| 25   | 3                  | 4                            | 12        |
| 26   | 2                  | 4                            | 8         |
| 27   | 1                  | 2                            | 2         |
| 28   | 1                  | 1                            | 1         |
| 29   | 1                  | 1                            | 1         |
| 30   | 1                  | 1                            | 1         |
| 31   | 1                  | 2                            | 2         |
| 32   | 1                  | 3                            | 3         |
| 33   | 2                  | 2                            | 4         |
| 34   | 2                  | 1                            | 2         |
| 35   | 1                  | 1                            | 1         |
| 36   | 3                  | 1                            | 3         |
| 37   | 2                  | 2                            | 4         |
| 38   | 1                  | 4                            | 4         |

Note: IHC score was acquired by multiplying the percentage of positive cells and staining intensity.
Although a few efforts towards MSC identification and extracellular matrix characterisation have been carried out for CTVT (Clarke et al., 2006; Liu et al., 2012; O’Neill, 2011). This finding may be because of the very low levels of CD44+ CSC in human and canine tumours, usually 0.1–1%, which may be below the immunohistochemistry detection level (Ailles & Weissman, 2007; Blacking et al., 2012; Buishand et al., 2016; Bustin et al., 2009; Calloni et al., 2013; Flórez et al., 2012; Greve et al., 2012; Guth et al., 2014; Huang et al., 2015; Ito et al., 2011; Kapoor et al., 2019; Kim & Ryu, 2017; Livak & Schmittgen, 2001; Matou-Nasri et al., 2009; Olofsson et al., 2014; Rege & Hagoood, 2006; Savani et al., 2001; Su et al., 2015; Teye et al., 2016; Tirino et al., 2013; Wang et al., 2001; Yan et al., 2015; Yan et al., 2013; Yin et al., 1997; Yu & Stamenkovic, 2000; Zöller, 2015).

In our study, CD34 levels were considered absent to very low, similar to levels observed in other tumours (Clarke et al., 2006; Liu et al., 2012; O’Neill, 2011). This finding may be because of the very low levels of CD34+ CSC in human and canine tumours, usually 0.1–1%, which may be below the immunohistochemistry detection level (Ailles & Weissman, 2007; Blacking et al., 2012; Buishand et al., 2016; Bustin et al., 2009; Calloni et al., 2013; Flórez et al., 2012; Greve et al., 2012; Guth et al., 2014; Huang et al., 2015; Ito et al., 2011; Kapoor et al., 2019; Kim & Ryu, 2017; Livak & Schmittgen, 2001; Matou-Nasri et al., 2009; Olofsson et al., 2014; Rege & Hagoood, 2006; Savani et al., 2001; Su et al., 2015; Teye et al., 2016; Tirino et al., 2013; Wang et al., 2001; Yan et al., 2015; Yan et al., 2013; Yin et al., 1997; Yu & Stamenkovic, 2000; Zöller, 2015). Likewise, CD34 has been detected at the transcript level in several cancers, including but not limited to renal, thyroid, breast and stomach cancer. However, at the protein level, CD34 is detected in fewer cancer types such as colorectal, thyroid and melanoma (Kapoor et al., 2019), and this condition may be similar in animals, which could be another explanation for the findings. CD34+ has been correlated with vasculogenic and angiogenic processes, essential to the development of cancer (Kulmann-Leal et al., 2020), in the tumour niche upon angiogenesis might exert an adverse role, leading to tumour metastasis (Kulmann-Leal et al., 2020). In CTVT, it is possible that absent to very low CD34+ levels may be related to low levels of metastasis described in this tumour, or that the low flow cytometric levels observed might be related to normal endothelial cells present in CTVT.

Although a few efforts towards MSC identification and extracellular matrix characterisation have been carried out for CTVT (Balles-tero et al., 2020; Mukaratirwa et al., 2004), data on CD34 expression by MSC is currently non-existent. Curiously, no stromal cells displayed CD34 staining in our study.

However, a few studies have referred to the potential role of CD90 as a CSC marker in canine tissues. Low percentages of CD90+ cells in canine melanomas, osteosarcomas and hepatocellular carcinomas have been reported (Ailles & Weissman, 2007; Ballester et al., 2020; Blacking et al., 2012; Buishand et al., 2016; Bustin et al., 2009; Calloni et al., 2013; Flórez et al., 2012; Greve et al., 2012; Guth et al., 2014; Huang et al., 2015; Ito et al., 2011; Kapoor et al., 2019; Kim & Ryu, 2017; Kulmann-Leal et al., 2020; Livak & Schmittgen, 2001; Matou-Nasri et al., 2009; Michishita et al., 2014; Mukaratirwa et al., 2004; Olofsson et al., 2014; Rege & Hagoood, 2006; Savani et al., 2001; Su et al., 2015; Teye et al., 2016; Tirino et al., 2013; Wang et al., 2001; Yan et al., 2015; Yan et al., 2013; Yin et al., 1997; Yu & Stamenkovic, 2000; Zöller, 2015). Similarly, very low CD90 expression levels were found in the majority of CTVT tested in our study. CD90 expression has been correlated with increased tumourigenicity. Chen et al. demonstrated that the signal axis of CD90-integrin-mTOR/AMPK-CD133 is critical for promoting liver carcinogenesis. Jiang et al. demonstrated that expression of CD90 in gastric CSCs was correlated with ERBB2 expression. An in vitro study demonstrated that the presence of anti-CD90 AMb reduced the viability and/or metastatic potency of CM-RED cells. In CTVT, it is possible to hypothesise that the low level of CD90 decreases the potential metastasis of cancer cells and further study is necessary on this aspect.

Considering CD133, the expression in CTVT was 30%. However, some authors verified lower average scores in fresh melanoma and osteosarcoma samples, which could be attributed to tumour heterogeneity (Guth et al., 2014).

These discrepancies may be related to in vitro selective pressures towards more adapted CSC subpopulations (Guth et al., 2014) or even CSC heterogeneity commonly seen in human glioblastomas (Chen et al., 2010). In addition, the cell glycosylation state can interfere with CD133 expression levels and detection by specific antibodies (Mizra et al., 2008).

Several lines of evidence have suggested CD133 as a prognostic marker in many types of cancers, including breast cancer, lung cancer, gastric cancer and colorectal cancer, as well as in cancer initiation, development and metastasis (Liou, 2019). Accumulating evidence has shown that CD133 might be responsible for chemoresistance of CSCs (Behrooz et al., 2019). In glioma cells, the expression of CD133 has been shown to increase the drug resistance of camptothecin and doxorubicin via upregulation of p-glycoprotein 1 (multidrug resistance protein 1/MDR1) transcription and ABC transporter activity (Angelastro & Lane, 2010). Given that CD133 plays a role in regulating therapeutic resistance; in CTVT, it is necessary to know if this expression is related to a resistance case described in the literature, especially with MDR-1 overexpression.

Despite the evidence regarding the accuracy of using CD133 as a CSC antigen, it is still controversial, as the results can vary significantly depending on a number of factors, including cancer subtype, cell line variability, tumour microenvironment etc. (Glumac & LeBeau, 2018), and the overexpression of CD133 has been associated with non-metastatic disease and longer survival in renal carcinoma (Costa et al., 2012). However, in other types of tumour, including ovarian, the expression has been associated with increased metastasis (Angelastro & Lane, 2010; Behrooz et al., 2019; Costa et al., 2012; Liou, 2019; Roy et al., 2018). In CTVT, it is necessary to know what the function of CD133 is as its expression has also been associated with non-metastatic cells.

Additionally, in CTVT, it is possible that CD133 may provide a distinction between proliferative and quiescent cells and thus should be used for distinct cell populations in CTVT in relation to the progression stage.

In contrast, positive specific subpopulations for the CD44 marker were observed in CTVT. The tumour uniformly expressed high levels of positive cells, commonly greater than 90%. This result is in accordance with the literature, which describes CD44 as highly expressed in cancer cells (Yan et al., 2015). Its expression has been observed to be associated with a poor prognosis for many cancer types (Zhu et al., 2018) and different studies have revealed a positive relationship
between CD44 expression and microvessel density (MVD), which is a characteristic of angiogenesis (Chen et al., 2020; Han et al., 2015; Irani & Dehghan, 2018). In CTVT, it is possible that CD44 could promote tumour angiogenesis as in other types of tumour, however, without important metastatic function or prognosis. It is necessary to elucidate the specific action in this tumour, because this could give answers to development of tumour angiogenesis.

Cytological subgroups did not demonstrate different CSC profiles, despite the common association of plasmacytoid subtypes with a worse prognosis (Flórez et al., 2012). Nonetheless, we were unable to include lymphocytoid subtypes in our study since they are commonly seen in earlier stages of disease as small or inapparent lesions (personal observation) and thus go unnoticed by owners until they become obvious. We speculate that their inclusion could reveal substantial differences in CSC marker expression.

Canine transmissible tumours demonstrate different levels of classical CSC markers, which suggests the possible presence of CSC sub-populations. Additional studies involving functional CSC assays, such as side population analysis and ALDH activity should emphasise the characterisation of CSC in CTVT.

These results are very important in CTVT and new research is necessary to investigate the biological role of CSC in tumours. This analysis, together with clinical data, may help in elucidating the role in many ways, especially in angiogenesis and metastasis in this specific context, because the CSC hypothesis follows a hierarchical model in which only a small subset of the cells within the tumour are able to self-renew, differentiate and ultimately drive tumour growth (Ebben et al., 2010). Heterogeneous populations of CSCs are present among different types of cancer according to their protein expression profiles.

Thus, heterogeneity in the behaviour, and in some cases, in the treatment described in the tumour, could have a relationship with CSCs, as cells possess multilineage differentiation potential; they are thought to be the driving factor for intratumoural heterogeneity. Therefore, in CTVT or transmissible tumours, the detection and targeting of CSCs could be considered as a therapeutic strategy in the future.

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CONFLICT OF INTEREST

The authors do not have any financial or personal relationships with other people or organisations that could inappropriately influence (bias) their work.

AUTHOR CONTRIBUTIONS

Fabrizio Grandi: Conceptualization, Investigation, Methodology, Writing original draft, Writing review & editing. Helio Miot: Supervision, Validation, Visualization. Rafael Malagoli: Formal analysis, Methodology, Supervision. Gabriela Massoco: Conceptualization, Resources, Validation, Visualization. N. Queiroz-Hazarbassanov: Data curation, Formal analysis, Writing review & editing. Mauricio Montoya: Formal analysis, Writing original draft, Writing review & editing. Bruno Cogliati: Methodology, Writing review & editing. N. Rocha: Conceptualization, Project administration, Software.

ETHICAL APPROVAL

This study was approved by the Committee on Animal Research and Ethics under protocol 93/2014.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available at https://repositorio.unesp.br/bitstream/handle/11449/136206/grandi_f_dr_bot.pdf?sequence=3&isAllowed=y.

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REFERENCES

Ailles, L. E., & Weissman, I. L. (2007). Cancer stem cells in solid tumors. Current Opinion in Biotechnology, 18, 460–466.

Angelastelo, J. M., & Lame, M. V. (2010). Overexpression of CD133 promotes drug resistance in C6 glioma cells. Molecular Cancer Research, 8, 1105–1115.

Baccelli, I., & Trumpp, A. (2012). The evolving concept of cancer and metastasis stem cells. Journal of Cell Biology, 198, 281–293.

Baez-Ortega, A., Gori, K., Strakova, A., Allen, J. L., Allum, K. M., Bansse-Issa, L., Bhutia, T. N., Bisson, J. L., Briceño, C., Castillo Domacheva, A., Corrigan, A. M., Cran, H. R., Crawford, J. T., Davis, E., De Castro, K. F., De Nardi, B. A., De Vo, A. P., Delgado, L. Keenan, L., Donelan, E. M., ... Murchison, E. P. (2019). Somatic evolution and global expansion of an ancient transmissible cancer lineage. Science, 365(6452), eaau 9923.

Ballestero, F. H., Montoya, L. M., Yamatogi, R. S., Duzanski, A. P., Araújo Junior, J. P., Antonio de Oliveira, R., & Rocha, N. S. (2020). Does genetic alterations alter tumorigenesis in transmissible venereal tumor in dogs? Ciencia Rural, 50, 11.e20200082.

Behrooz, A. B., Syahir, A., & Ahmad, S. (2019). CD133: Beyond a cancer stem cell biomarker. Journal of Drug Targeting, 27(3), 257–269. https://doi.org/10.1080/1061186X.2018.1479756

Blacking, T. M., Waterfall, M., Samuel, K., & Argyle, D. J. (2012). Flow cytometric techniques for detection of candidate cancer stem cell subpopulations in canine tumour models. Veterinary and Comparative Oncology, 10, 252–273.

Buishand, F. O., Arkesteijn, G. J. A., Feenstra, L. R., Oorsprong, C. W. D., Mestemaker, M., Starke, A., Speel, E.-J. M., Kirpensteijn, J., & Mol, J. A. (2016). Identification of CD90 as putative cancer stem cell marker and therapeutic target in insulinomas. Stem Cells and Development, 25(11), 826–835.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for
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Tirino, V., Desiderio, V., Paino, F., De Rosa, A., Papaccio, F., Noce, M. L., Laino, L., Francesco, F. D., & Papaccio, G. (2013). Cancer stem cells in solid tumors: An overview and new approaches for their isolation and characterization. The FASEB Journal, 27, 13–24.

Wang, H., Zhan, Y., Xu, L., Feuerstein, G. Z., & Wang, X. (2001). Use of suppression subtractive hybridization for differential gene expression in stroke: Discovery of CD44 gene expression and localization in permanent focal stroke in rats. Stroke, 32, 1020–1027.

Yan, X., Luo, H., Zhou, X., Zhu, B., Wang, Y., & Bian, X. (2013). Identification of CD90 as a marker for lung cancer stem cells in A549 and H446 cell lines. Oncology Reports, 30, 2733–2740.

Yan, Y., Zuo, X., & Wei, D. (2015). Concise review: Emerging role of CD44 in cancer stem cells: A promising biomarker and therapeutic target. STEM CELLS Translational Medicine, 4, 1033–1043.

Yin, A. H., Miraglia, S., Zanjani, E. D., Almeida-Porada, G., Ogawa, M., Leary, A. G., Olweus, J., Kearney, J., & Buck, D. W. (1997). AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood, 90(12), 5002–5012.

Yu, Q., & Stamenkovic, I. (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes & Development, 14, 163–176.

Zöller, M. (2015). CD44, hyaluronan, the hematopoietic stem cell, and leukemia-initiating cells. Frontiers in Immunology, 6, 235.

Zhu, B., Wang, Y., Wang, X., Wu, S., Zhou, L., Gong, X., Song, W., & Wang, D. (2018). Evaluation of the correlation of MACC1, CD44, Twist1, and KISS-1 in the metastasis and prognosis for colon carcinoma. Diagnostic Pathology, 13(1), 45.

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