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Cancer Stem Cell and Epithelial Mesenchymal Transition (EMT) in Canine Transmissible Venereal Tumour- A Preliminary Study

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ABSTRACT

Background: Cancer stem cell (CSC) and Epithelial to Mesenchymal Transition (EMT) transforms conversion of normal cells in the tumor microenvironment into CSC which is the main cause of embryogenesis, carcinogenesis and metastasis.

Methods: Nineteen canine patients with recurrent transmissible venereal tumor (TVT) and metastasis of inguinal lymph node were the subjects of present study. Preoperative work up and clinical staging involving radiography, ultrasonography, hematobiochemical analysis and histopathology or biopsy were done to confirm the type of cancerous tissue. Tumor tissues were collected in ice cold RPMI-1640 and phosphate buffer solution. Flow cytometry analysis was performed following established protocol from surgically excised tissue sample. Trypan blue dye exclusion test was done to quantify the live/dead cell count. CD24PE, CD44FITC, CD133APC and EpCAM antibody with conjugates were chosen markers for flow cytometry analysis.

Result: Nineteen canine patients were diagnosed with Transmissible Venereal tumor (TVT) (common sexually transmitted disease in male dog) through biopsy of excised surgical tissue following Haematoxylin and Eosin staining protocol. Recurrence was seen even after adjuvant chemotherapy with Vincristine injection @ 0.25 mg/m^2 at weekly interval for 5 weeks. Trypan blue dye exclusion test confirms 98% live cell count in transmissible venereal granuloma after digestion and incubation for flow cytometry. Flow cytometry analysis of the cells from the TVT showed surface biomarkers for EpCAM+ cells, EpCAM CD24+ cells, EpCAM CD44+ cells, EpCAM CD133+ indicating cells with CSC and EMT properties. Presence of CSC and EMT cells in this patient was responsible for chemoresistance, distant metastasis and recurrence. Identification of specific CSC in a malignant cancer will yields deeper insight into biological aspects of metastasis and formulation of targeted therapy in fighting with cancer.

Key words: Antibody marker, Cancer stem cell, Carboplatin, Epithelial-mesenchymal transition, Flow cytometry, Trypan blue.

INTRODUCTION

Solid tumor is heterogenous group of infiltrating and resident cells in the tumor microenvironment (TME), consisting of neutrophil, macrophages, B-cell, T-cell, fibroblasts, cancer associated stromal cells [cancer associated fibroblast (CAF)], dendritic cells, myeloid derived suppressor cells (MDSCs), innate lymphoid cells (ILCs), natural killer cell, (NKs) cancer stem cells (CSCs), Epithelial- Mesenchymal transition (EMT) cells and extracellular matrix (ECM) (Li *et al*., 2007; Giraldo *et al*., 2019; Hinshaw and Shevde, 2019; Mao *et al*., 2021). Cancer stem cells represent a small population of cells (0.01-2%) in the TME that possess the strong self-renewal ability, limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of apoptosis, sustained angiogenesis, tissue invasion (Gasch *et al*., 2017; Yang *et al*., 2020). Existence of CSCs in canine were initially reported in osteosarcoma (Wilson *et al*., 2008), glioblastoma (Stoica *et al*., 2009) and mammary gland carcinoma (Cocola *et al*., 2009; Pang *et al*., 2011). 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 (Dou *et al*., 2007; Ghani *et al*., 2011), Hoechst dye exclusion (SP cells) (Kitamura *et al*., 2009; Mannelli and Gallo, 2012), detection of enzymatic activity of aldehyde dehydrogenase1 (ALDH1) (Kim *et al*., 2011), detection of surface markers (Clarke *et al*., 2006; Dou and Gu, 2010; Mannelli and Gallo, 2012), signalling pathway identification (Elizabeth *et al*., 2012), serial colony-forming unit assays (replating assays) (Kitamura *et al*., 2009), label retention assays (Clarke *et al*., 2006) and migration assays (Biddle *et al*., 2011). Canine transmissible venereal tumor (CTVT) is a naturally occurring tumor transmitted by seeding of viable tumor cells through physical contact (Kumar *et al*., 2021; Cingi-cagri *et al*., 2020). Presence of CSC in this cancer have been evaluated in clinical sample and biological sample through culture and flow cytometry of the clinical samples, *in situ* polymerase chain reaction

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(PCR) and enumeration of stable sequence (Grandi *et al*., 2022; Liao *et al*., 2017). Among these detection of surface marker such as CD44, CD133, CD90 and CD34 has been more sensitivity and specificity owing to their role in cancer stemness and progression. (Calloni *et al*., 2013). The receptor for hyaluronan, CD44 is a member of the type I transmembrane glycoprotein family. It can also bind to collagen, fibronectin, or chondroitin sulphate. CD44 also function as a signalling platform that combines signals from growth factor and cytokine to regulate cell adhesion, migration, proliferation, differentiation and survival (Aubert *et al*., 2015; Orian-Rousseau *et al*., 2014). Cell surface antigen CD133 (also called AC133 and prominin-1) is the most commonly used marker to identify and separate CSCs from different types of solid tumors. The primary biological function of CD133 is to control the maintenance and proliferation of stem cells as well as their fate (KimandRyu, 2017; Calloni *et al*., 2013). CD24 is a sialoglycoprotein expressed on the surface of most B lymphocytes. CD24 can increase tumor cell proliferation, motility and invasiveness as well as tumor growth and metastasis *in vivo*. Aggressiveness of cancer, including breast, ovarian, lung and prostate cancers are attributed to CD24 overexpression (Bauman *et al*., 2005).

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 (Thiery *et al*., 2009). 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 (Kalluri and Weinberg, 2009). Cells undergoing EMT are identified through loss of epithelial marker such as E-cadherin, EpCAM and cytokeratin and gain of mesenchymal markers like fibronectin, vimentin and N cathedrin.

This study aims to establish the level of expression of epithelial and mesenchymal markers in commonly occurring cancers such as CTVT with mesenchymal-like phenotype (spindle shape, separation from groups of cells and front to back polarity). During EMT, epithelial cells acquire a mesenchymal-like phenotype, which enables them to move easier through surrounding tissues and vessels to migrate and invade different organs. The present study was carried out with objectives *viz*., (1) Preoperative work-up and clinical staging (TNM staging) including histopathology and cytology to diagnose the CTVT. (2) To analyze expression of surface marker of cancer stem cells (CSC) and Epithelial mesenchymal transition (EMT) cells such as CD24, CD44, CD133 and EpCAM in CTVT tumors through flow-cytometric analysis.(3) To correlate the presence of CSC and EMT Cells with patient clinical outcomes and to identify potential prognostic values of selected markers.

MATERIALS AND METHODS

The present research work encompasses nineteen intact male or female mongrel and mixed breed dogs with visible signs of cauliflower like growth with fresh blood discharge on the prepucial sheath/ anterior end of penis in male dogs and cauliflower growth on vagina or vulva of female dogs, presented to Veterinary Clinical Complex and Department of Veterinary Surgery and Radiology for chemotherapy or surgery between November 2021 to December 2023 for a period of 2 years. The surgically excised sample were processed for flow cytometry at the Institute of life science, Nalco square, Bhubaneswar. The present study excludes patients a) in which surgery was performed to remove hyperplastic growth or benign tumor. b) pregnant, lactating, or dogs unable to undergo general anaesthesia. c) severely debilitated and cachectic patients, not a suitable candidate for surgery. f) Patients from a long distance so that are difficult to do a follow up. The inclusion criteria were 1) patients where radical surgery was carried out for removal of primary tumor mass as a part of curative intent therapy and 2) those patients who undergo surgery and owner's consent was obtained for sample collection and chemotherapy treatment. Standard operating protocol (SOP) was followed for surgical procedure for removal or excision of the primary tumor mass. Patients were chosen according to signalment (breed, age, sex, body weight), cytology/histopathology of the tumor sample, location of the tumor and recurrence of tumor mass was there even after standard chemotherapy treatment or previous history of surgical excision of primary tumor mass (Table 1). Preoperative and clinical work up involving radiography (orthogonal view of lesion, 3-view thoracic radiography), abdominal ultrasonography for locating the changes in inguinal lymph node, hematological examination was carried out as part of initial patient preparation for chemotherapy and surgery. TNM staging was done basing on following criteria; (T1= tumor ≤2cm, T2= 2 cm <T <5 cm; T3> 5 cm; N0= Absence of metastasis; N1= Metastasis to regional lymph node; M= metastasis to other organ except regional lymph node (Hataka,2004). Dogs were treated with vincristine (0.025 mg/kg for 3-6 weeks) with palliative care at client owner's residence. Surgically excised tumor samples were processed for flow cytometry analysis as per the prescribed protocol (Zayas *et al*., 2019; Parys *et al*., 2023). Following amount of antibody and conjugates were used as surface marker for CSC and EMT identification in the flow cytometry machine (BD LSR Fortessa™ X-20 machine):

CD24 PE- IgG1 mouse=10 µg/ml= 8 µL.

CD44 FITC-IgG1 mouse = 50 µg/ml= 2 µL.
CD133 APC- IgG1 mouse= 12.5 µg/ml= 16 µL.
EpCAM BV 786 - 0.5 µL.

RESULTS AND DISCUSSION

Preoperative and clinical work up were performed in these nineteen dogs (3 mixed breed and 16 mongrel dogs) involving haematological examination, radiography and ultrasonography. Haematological examination (CBC, DC, TLC) was insignificant in most cases except in 2 cases with mild anaemia and bleeding from genitalia which were treated with Iron dextran injection (10 mg iron/kg body weight at weekly interval) and haematinics. Three view thoracic radiography revealed no metastasis. Lymphography technique with iohexol and blue dye was also practiced to verify possibility of metastasis to anatomical and/or first tier node *i.e* inguinal lymph node but was found negative. Abdominal B-mode ultrasonography was done to assess the functional status of the visceral organs and nodes and all were found within the normal sono-anatomy and insignificant. Histopathology of the biopsy sample showed sheets of individual round cells with round vesicular nuclei, cytoplasmic vacuoles and indistinct borders. Extracellular stroma was scanty with infiltration of lymphocyte, plasma cells (Fig 1). The most prominent cytological feature of TVT was that nucleus was eccentrically located with vacuole in the cytoplasm called strings of pearls. Cells had a high N/C ratio with the mitotic figure in different stages of development (Fig 2). Adjuvant chemotherapy with Vincristine (0.025 mg/kg body weight for 3-6 weeks at weekly interval) in 5 patients and rest of 4 dogs were treated with neoadjuvant chemotherapy and other 10 dogs were treated with single agent vincristine (0.025 mg/kg body weight) for atleast 5 cycles at weekly intervals. According to TNM staging, only 3 dogs had metastasis, but those were not detected either through radiography, ultrasonography or lymphography. Sample were collected from these unresponsive patients with atleast 5 dosage of post chemotherapy treatment. These tissue samples were processed into single cell suspension and live/dead cells

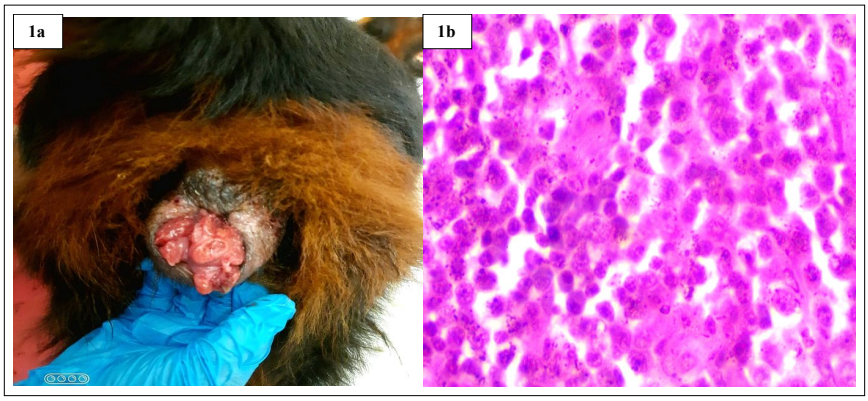
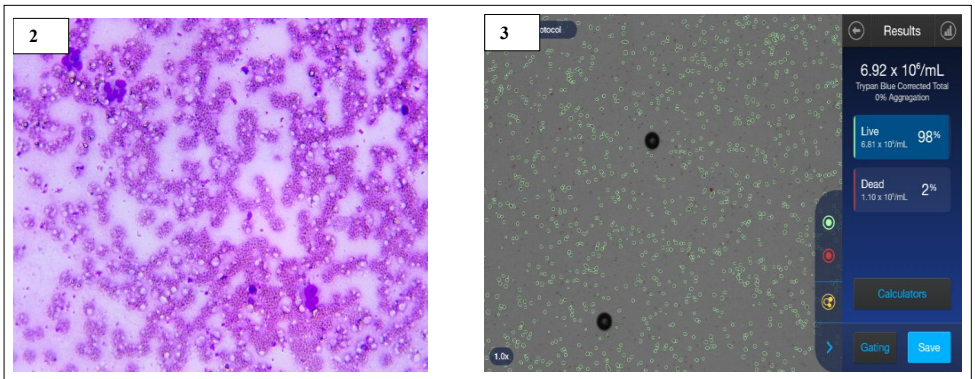


Fig 1: (a) Gross morphology of TVT in a labrador (case no.7, female, 7 yr, 23 kg) with cauliflower like growth covering the vagina. Mass was soft, vascularized, friable. Tumor biopsy was collected after surgical excision. (b) Histopathology of the surgically excised TVT sample reveals large, round, homogenous neoplastic cells scattered in clusters, with conspicuous nuclei and nucleoli, thick, eosinophilic cytoplasm, occasionally vacuolated and mitotic figure in the center. stained using eosin and haematoxylin. visualized using a 100× clear field microscope.



Cytological sample showed round neoplastic cells with characteristic clear cytoplasmic vacuoles with classical appearance of string of pearls. Stained with Giemsa stain (40X).

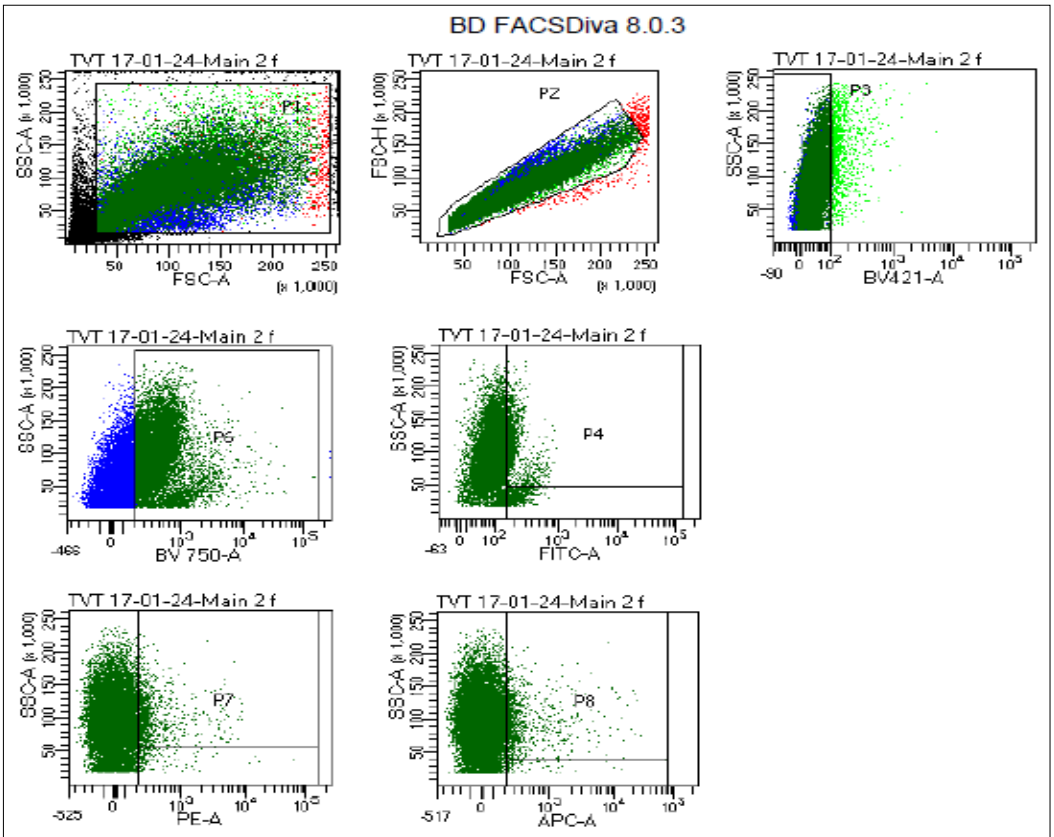
Fig 2: Cytology or impression smear of the cauliflower like growth on the penile part of a mongrel dog (male, 5 yr, 14 kg).

were stained with Trypan blue and checked under Dark field microscope. Live cells are unstained cells whereas dead cells are positively stained (blue colour) when counted in cell counter. Live cell count was 98% with 6.81 × 10^8 cells indicating very good sample size and quality that can be checked for flow cytometry (Fig 3). Cells suspensions were then processed and reacted with CD 24 PE, CD 44 FITC, CD 133 APC and EpCAM BV 786 mouse IgG antibody, then fixed with perm buffer and finally loaded in flow cytometry machines. Cells derived from malignant TVT were positive for CD 24 PE, CD 44 FITC, CD 133 APC and EpCAM BV 786 mouse IgG antibody indicating stemness criteria of the tumors and presence of EMT cells or Epithelial Mesenchymal Transition cells. The results were processed through BD FACSDiva™ software in BD LSR Fortessa™ X-20 machine. Initially cells were plotted on a dot plot and then they were screened and gated though forward scatter (FSC) and side scatter (SSC)for sorting based on size and complexity. Cell debris were excluded through gating. Then, live EpCAM$^+$ cells were gated at laser BV 750 and were around 54% in sample 1 and 57% of stem cell population in sample 2 (Fig 4). Live CD24 PE$^+$ cells were 3.1% in sample 1 and 6.3% in sample 2, CD 44 FITC$^+$ cells were 12.1% in sample 1 and 10.4% in sample 2 and live CD133+ APC cells were 1.2% in sample 1 and 5.7% in sample 2 when analysed in flow cytometer software.

Statistical analysis

Flow cytometer results were expressed through SPSS statistics in Fig 5. Bar graph were plotted in two sample for EpCAM, CD24, CD44 and CD133 antibody. From the graphs it can be observed that EpCAM+ CD24+, EpCAM+CD44+, EpCAM+CD133+ cells have stemness properties and the percentage of population of these types of cells are high, which leads to metastasis and malignancies. The overall survivability of the 19 canine patients after surgery, chemotherapy are as follows in Table 1.

The present research involved Cancer stem cell (CSC) and Epithelial- Mesenchymal transition (EMT) cell identification and systematic clinical staging with clinical outcome in the 19 canine TVT patients starting from history, physical and clinical examination to radiography, ultrasonography and diagnosis of the type of tumor through cytology and histopathology. Excisional or incisional biopsy



Live cell appears unstained in a background of blue stained dead cells. Above picture is from the monitor of cell counting machine (Countess, Invitrogen) with live cell count (6.92 x 10^6/ml). Dead cell appears as red dot in this machine.

Fig 3: Processed cell monolayer collected from canine TVT and stained with trypan blue.

and cytology or impression smear of lymph node were also performed to identify the node as positive or negative for nodal metastasis. As previously reported by Stockman *et al*. (2011), the CTVT cells in this study displayed histomorphological features that included a round shape, string-like arrangement in strings, a high nucleus: cytoplasm proportion, round-shaped nuclei with chromatin ranging from delicate to coarse and prominent nucleoli. The basis of the staging procedure adopted was TNM staging and the most relevant node to identify and the stage was the sentinel lymph node from the regional lymphocentrum. Sample was collected from chemoresistant patient which were positive for TNM staging indicating presence of certain cell types having stemness properties

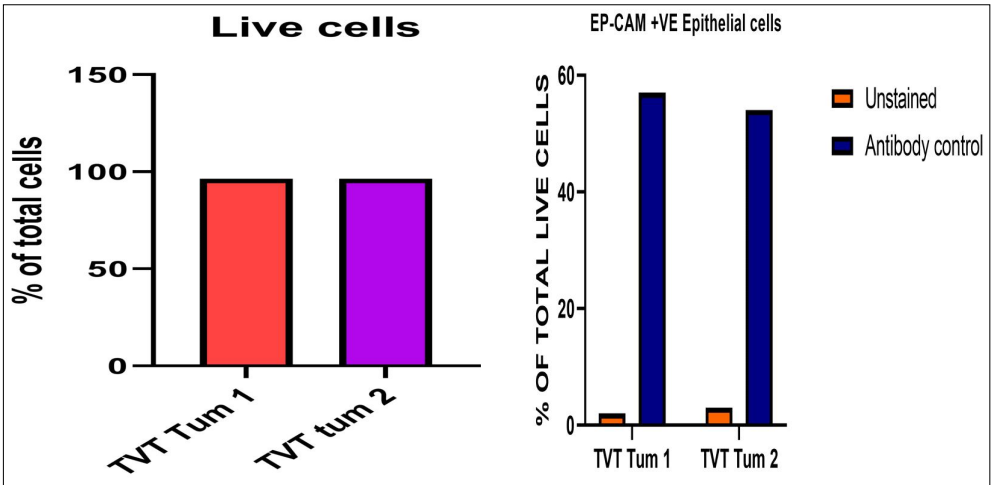


Fig 4: Dot plot for analysing the surface markers on the tumor cells through forward scatter and side scatter plotting for assessing the size and complexity of the cell types.

Table 1: Signalment and clinical outcome of canine transmissible venereal tumor (TVT).

Case no	Signalment (Sex, Age, Body wt)	Location of tumor	Clinical outcome (Overall survival time)
1	Male , 9 yr, 14 kg	Genitalia	370 days
2	Male, 2 yr, 13 kg	Genitalia	Complete clinical remission (CCR)
3	Male,10 yr, 11 kg	Preputial sheath	560 days (1 yr 6 m)
4	Female, 11 yr, 16 kg	Genitalia	CCR
5	Male, 7 yr, 15 kg	Preputial sheath	Lost to follow up
6	Male, 5 yr, 14 kg	Genitalia	CCR
7	Female, 7 yr, 23 kg	Genitalia	420 days (Fig 1a)
8	Female, 9 yr, 18 kg	Genitalia	720 days (2 yrs)
9	Male, 11 yr, 19 kg	Preputial sheath	Lost to follow up
10	Male, 12 yr, 13 kg	Genitalia	CCR
11	Female, 5 yr, 12 kg	Genitalia	Lost to follow up
12	Female, 6 yr, 22 kg	Genitalia	Lost to follow up
13	Male, 13 yr, 19 kg	Skin	Lost to follow up
14	Male, 11 yr, 14 kg	Preputial skin	CCR
15	Male, 8 yr, 13 kg	Genitalia	Lost to follow up
16	Male, 13 yr, 17 kg	Genitalia	CCR
17	Male, 12 yr, 18 kg	Skin	Lost to follow up
18	Female, 10 yr, 16 kg	Genitalia	CCR
19	Male, 4 yr, 12 kg	Preputial sheath	CCR

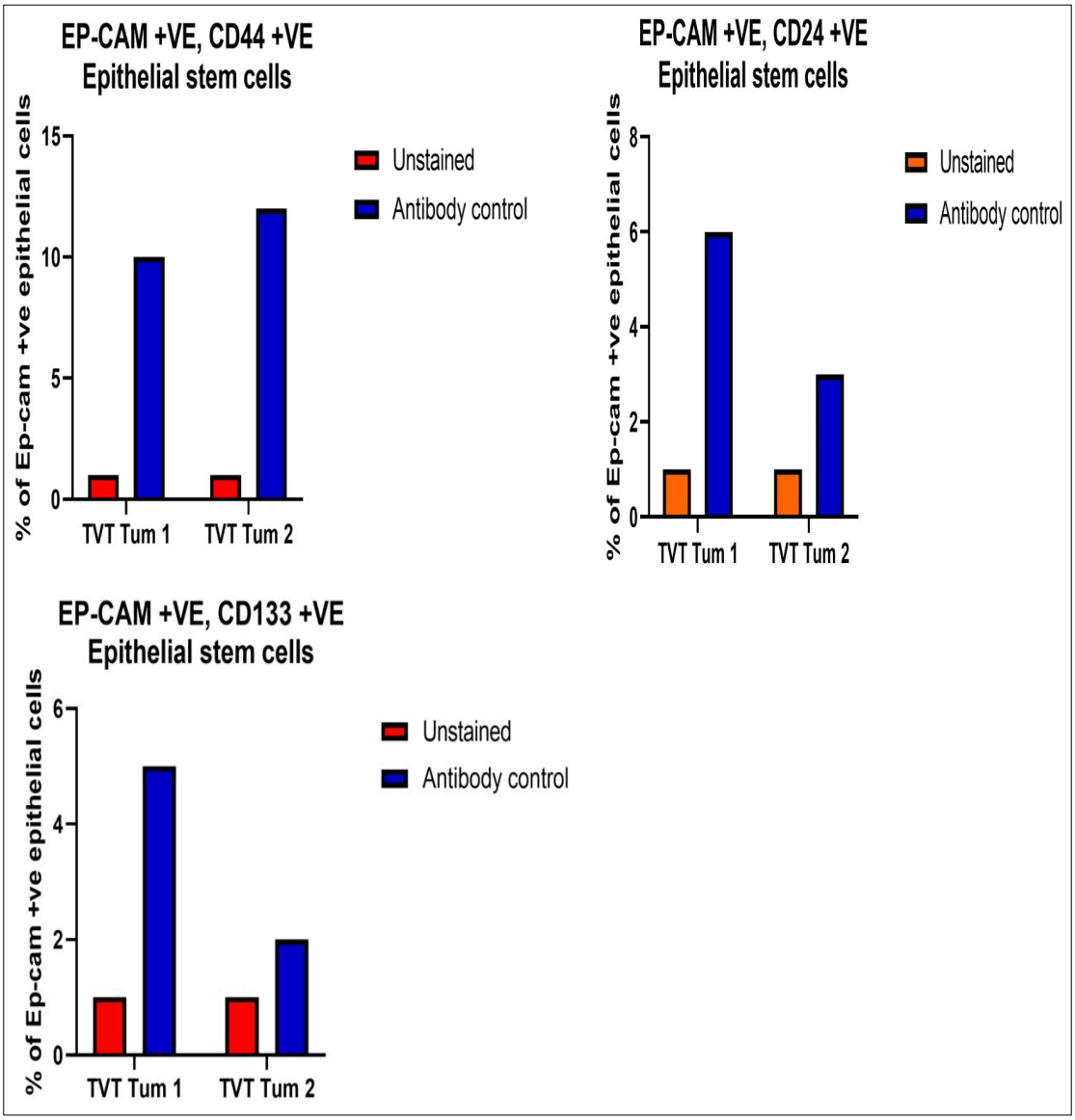


Fig 5: Bar diagram of EpCAM, CD24, CD 44 and CD133 representing percentage of live stem cells of different categorized present in TVT patient.

such as high prolificacy, unresponsive to external stimuli, self-renewal ability and resistant to chemotherapy. Among 98% of viable cells processed for flow cytometry, cells having stemness properties or CSC cells and EMT cells were approximately 50-60% which defines the above properties. The stemness marker such as CD24, CD44, CD133 and EpCAM cell surface marker used in the present study were used first time with the idea that tumor heterogenicity may be due to presence of certain cell types within the tumor microenvironment which were the continuous source of these cancerous cells. These cells are EMT cells and are EpCAM positive cells. Similar study was done by Zayas *et al*. (2019), but presence of mesenchymal cells (EMT cells) in our study may be a possible explanation for chemoresistance in these tumors. Expression of CD24+/- and CD44+/- cells constitute a valuable marker of poor prognosis is in accordance with the findings of Rogez *et al*. (2019) in their study on canine mammary carcinoma.

CONCLUSION

The ultimate goal of the present research was to identify the presence of cancer stem cells and Epithelial Mesenchymal transition cell types present in the tumor microenvironment in any types of malignant tumor through immunostaining and flow cytometry procedure which are less explored in canines as per the literature. Once, their presence is detected the newer and advance drugs (Chemotherapeutic drugs) and immunotherapeutic drugs can be tried to overcome cancer to some extent. As this is

a preliminary investigation of first of its kind undertaken to verify the presence of CSC, there were several drawbacks in tissue processing and specificity of the sample. Further research involving larger sample size and more no of flowcytometry and flow cytometry sorting may be carried out to identify different cancer stem cell and EMT cell and also matrix characterisation for clearly delineating different steps involved in tumorigenesis and metastasis in CTVT cases.

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Author's contribution

L. Killi and A.K. Sahoo have equal contribution in preparation of manuscript and processing of the sample, IN helps in supervision of the work, S.K. Panda and B.K. Patra helps in histopathology and cytology. L. Killi, A.K. Sahoo and S.S. Biswal helps in flow cytometry analysis.

Conflict of interest

The author declares no conflict of interest.

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