Assessment of tumor-induced pain and C-reactive protein levels in dogs with canine transmissible venereal tumors

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Abstract: The aim of this study was to determine the impact of tumor volume on generalized behavior, pain perception, and serum C-reactive protein (CRP) in dogs with canine transmissible venereal tumors (CTVTs). Pain scoring was performed using the Glasgow Composite Measure Pain Scale Short-Form. Cytomorphology of the tumors was studied through fine-needle aspiration and histopathology. Molecular identification of CTVTs was carried out using PCR assays based on LINE-1/c-myc. Hematological profiles including complete blood count and CRP were analyzed using an automatic hematology analyzer and immunoturbidimetric assays, respectively. Chi-square and regression analysis statistics were used for data analysis. Results of the study revealed a statistically significant (R2 = 0.798) impact of tumor volume on pain scores in corresponding individuals. Hematological parameters and serum CRP values were observed in normal ranges and did not reveal any significant association with tumor volume. All of the samples were found positive for LINE-1/c-myc molecular rearrangement, revealing a 552-bp PCR product. On the basis of cytology tumors were differentiated into plasmacytoid, lymphocytoid, and mixed types. No significant association could be observed among cytological types and sex of the individuals. It may be concluded that increasing tumor size may result in localized pain and mild behavioral alterations in affected dogs. However, hematology profiles and serum CRP levels are independent of the localized tumor proliferation.

Key words: Pain scores, C-reactive protein, hematology, cytology, histopathology

1. Introduction
Cancer is generally not a contagious disease; however, in a few conditions, tumor cells may attain the substantial capacity to disseminate among the individuals of a population. Canine transmissible venereal tumors (CTVTs) (1) and Tasmanian devil facial tumor type 1 and type 2 (2) occur in dogs and Tasmanian devils, respectively, and are typical examples of contagious tumors. CTVT is frequently witnessed in dogs living in close contact with each other, in wild and stray dogs that show significant sexual activity (3). Most commonly it is transmitted through sexual intercourse but it could also transmitted through licking, sniffing, and biting of tumor-affected areas (4). Venereal tumors are mostly localized and grossly seen as single or multiple, large or small, soft or friable, pedunculated, nodular, or papillary and tubular masses (5).

C-reactive protein (CRP) is an essential prognostic marker in several malignant conditions (6,7) such as esophageal cancer (8), colorectal cancer (9), pancreatic cancer (10), lung cancer (11), and breast cancer (12). Therefore, CRP in correlation with leukocytic patterns is a determinate biomarker that has the potential to increase the predictive efficacy of prognostic models (13). Moreover, treatment of pain is a necessary aspect of clinical practices and requires the ability to recognize and assess pain in animals (14).

Therefore, the aim of the study was to determine the impact of tumor volume on generalized behavior, pain perception, and serum CRP levels in CTVT-affected dogs clinically diagnosed through cytological examination and PCR.

2. Materials and methods
2.1. Source of samples
The samples were collected from the Pet Clinic of the University of Veterinary and Animal Sciences of Lahore. A total of 30 dogs of different breeds, ages, and weights were included in the present study that fulfilled predetermined
criteria. Inclusion criteria were individuals not spayed or neutered, exhibiting clinical signs of CTVT, not exhibiting signs of any other fatal disease, and not having any reoccurring tumors (15).

2.2. Pain assessment
Assessments of pain, spontaneous and evoked behaviors, interactions with the animal, clinical observations, and behavioral changes in the individual animals were made according to a structured questionnaire following the standard protocols described for the Glasgow Composite Measure Pain Scale Short-Form (14).

2.3. Hematological analyses
Whole blood was collected from the respective individuals from the cephalic vein at the time of surgical procedure and transferred to EDTA-coated and clot activator vacutainers separately as described by Thrall et al. (16). Hematological parameters including total erythrocyte count (TEC), hemoglobin (Hb), packed cell volume (PCV), total leukocytic count (TLC), and differential leukocytic count (DLC) were analyzed using an automatic hematology analyzer (Beckman Coulter Ac-T diff) by standard procedures. The CRP concentration was determined by immunoturbidimetric method using a Randox Canine CRP Kit (Catalogue No. CP2798) according to the manufacturer’s instructions. Analyses of the samples were performed in duplicate. Prior to the processing of all samples, the equipment was calibrated with three levels of calibrators that allowed the calibration of the automated equipment. A reference material was used in order to minimize the occurrence of errors. The formation of antigen–antibody complexes was evaluated with a spectrophotometer at 340 nm for 25 s.

2.4. Tumor investigations
Tumor volume was calculated before the time of surgical excision using the following formula: volume = $L \times W \times H \times \pi/4$ (L: length; W: width; H: height in centimeters), as previously described (17). A 21-gauge hypodermic needle connected to a 10-mL disposable syringe was used for aspiration of tumor cells. The tissue aspirate was smeared to make a monolayer of CTVT on a microscopic slide (18). Cytomorphological analysis was completed by counting at least 100 cells from each sample. The observed samples from each slide were categorized as lymphocytoid or plasmacytoid according to cell morphology. Tumor samples were obtained by surgical removal under aseptic conditions. Pain management was done by injecting 5 mL of 2% lidocaine HCL locally at the site of excision (19). Each sample was divided into two parts; one part was stored at $-20^\circ$C for molecular studies while the other part was fixed in 10% neutral buffered formalin, dehydrated by a series of graded alcohols, cleared through xylene, embedded in paraffin, and sectioned for histopathological analyses (20).

2.5. Molecular identification
Fresh frozen samples were used for the molecular identification of extracted DNA. The Gene-Jet Genomic DNA Purification Kit (Catalogue No. K0722-250, Thermo Fischer Scientific) was used for the DNA extraction with modifications for tissue DNA isolation. Canine somatic DNA from skin biopsy was used as a negative control. A NanoDrop ND-1000 UV spectrophotometer at 260 nm was used to check purity and concentration of the DNA. Myc S-2 and LINE-AS1 primers were used to identify a 552-bp segment starting from the 5’ end of the LINE-1 insertion outside the first exon of c-myc (21):

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\text{MycS-2: ATTCCTACGAATGATTGGGCAGAAT}
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\[
\text{LINE AS-1: GACACATAGATCAGTGGAACAGAAT}
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DNA was amplified using a commercially available PCR master mix (Catalogue No. K0171, Thermo Scientific). The samples were processed in the thermocycler (programmable thermal cycler PTC-6) as follows: initial denaturation at $94^\circ$C for 5 min; followed by 35 cycles of denaturation at $94^\circ$C for 1 min, annealing at $64^\circ$C for 50 s, and extension at $72^\circ$C for 1 min; and then final extension at $72^\circ$C for 5 min. Thereafter, 10 µL of each PCR product was gel electrophoresed at 100 V for 20 min. The size of the PCR products was compared against a DNA ladder marker. The agarose gel was then visualized and photographed under a UV transilluminator (Vilber Lourmat).

2.6. Statistical analysis
All statistical analyses were performed using GraphPad Prism 7 (22). The influence of tumor volume on Glasgow Composite Measure Pain Scale Short-Form scores and serum levels of CRP in affected individuals was analyzed by regression analysis. Hematological parameters were described by descriptive statistics. Association between cytological type of CTVT and sex of the individual was tested by the chi-square test. The level of significance was 0.05.

3. Results
Median tumor volume observed in the study was 20.67 cm$^3$. Results of the regression analysis revealed a statistically significant ($R^2 = 0.798$, $P < 0.0001$) impact of tumor volume on pain scores in corresponding individuals. On the other hand, serum CRP values did not reveal any significant ($P = 0.0641$) association with tumor volume and were observed to be in normal ranges (3.587 ± 1.37 mg/L). Figure 1 shows the line fit plot of the observed data. Values of the hematological parameters including TEC, Hb, PCV, TLC, and DLC were in normal ranges. The Table shows the values of various hematological parameters.

Cytological examination of tumor masses through fine-needle aspiration revealed the presence of frequently vacuolated round cells having large nuclei, frequent mitotic figures, and noticeable nucleoli, supporting the diagnosis of
CTVT. Cytological types were classified into lymphocytic, plasmacytoid, and mixed populations depending upon the physical characteristics of the principal cell type. The lymphocytic group included cells count of 60% or more with round morphology, some degree of finely granular cytoplasm, and round nucleus having rough chromatin along with vacuoles present in the outside edges of the cell. The plasmacytoid group included 60% or more ovoid cells, having abundant cytoplasm (low nucleus to cytoplasm ratio) and centrally located nucleus. Those samples in which either cell morphology did not surpass 59% of the total cells were categorized into the mixed type group.

Out of a total of 30 samples, there were more females represented (73.33%) than male dogs (26.67%). The highest frequency was observed for plasmacytoid tumors (30%), followed by mixed tumors (23.33%) and the lymphocytoid type (20%), in female dogs. In males, the frequency of plasmacytoid tumors was comparatively higher (13.33%), followed by lymphocytoid and mixed types, both of which contributed 6.67%, respectively. Collectively, the plasmacytoid cytology was the most common, with 43.3% of samples, followed by mixed (30%) and lymphocytic (26.6%). However, statistically no significant (P < 0.05) association could be observed among the cytological types and sexes of the individuals.

Histopathological studies revealed large round or polyhedral cells with elliptical to circular nuclei having coarsely accumulated chromatin; most nuclei of cells had single, prominent, and bluish nucleoli. High nuclear : cytoplasmic ratio was observed in most of the cases. Cytoplasm was scanty, light blue to colorless (poorly stained), and finely granular and contained distinct clear vacuoles. However, on the basis of histological patterns the samples were differentiated into progression and regression phase tumors. The progression phase tumors revealed closely packed tumor cells along with a mild subcapsular inflammatory infiltrate, while plasma cells and tumor-infiltrating lymphocytes were notable in regression phase tumors. Nuclei of the neoplastic cells had eccentric, distinctive, and violet nucleoli. The neoplastic cells were structured diffusely or in the form of threads encapsulated by connective tissue. Figure 2 illustrates the needle aspiration cytology and histopathological patterns of tumorous masses.

Genomic amplification of the tumor samples through MycS-2 and LINE AS-1 primers produced a region of 552 bp characterizing the LINE-1 transposon insertion in the c-myc gene. All of the tumor samples were found positive for LINE-1/c-myc molecular rearrangement. Figure 3 shows the agarose gel electrophoresis pattern of an amplified product with approximately 552 bp from a CTVT.

4. Discussion
The importance of efficient pain alleviation for animals is being increasingly accepted in the veterinary profession. However, CTVT has remained neglected regarding pain management in combination with antiproliferative therapy. To our knowledge, the current study is the first to investigate the association of tumor volumes with pain scores in affected dogs. Reid et al. (14) demonstrated the cut-off points of pain scores as 7, 7, and 8 for analgesia. Moreover, the dogs that were not considered to require analgesia revealed median pain scores of 4, 2.5, and 3 with the Dublin, Glasgow, and North Carolina scores, respectively. The individuals with observed tumor volumes in the present study revealed varying degrees of pain but most animals did not require critical analgesia according to the guidelines of Reid et al. (14). In the
present study, frequency of CTVT was higher in females (73.33%) compared to male dogs (26.67%). The findings are in accordance with the studies of Araujo et al. (23), who reported a higher frequency observed in females compared to males. The histopathological findings of the current study are also in accordance with those of the studies of Ulcar et al. (24) and Murchison (25), who described pleomorphic round to ovoid shaped cells, with eccentrically amphiphilic round to ovoid nuclei with moderate amounts of cytoplasm. Furthermore, all tumor samples were confirmed as CTVT through LINE-1/c-myc-based PCR. The findings of the present study are in accordance with the previous studies performed by Liao et al. (21) and Setthawongsin et al. (1), who used similar sets of primers to amplify the 552-bp LINE-1/c-myc product for the confirmation of CTVT.

Hematological values observed in the present study were in normal ranges when compared with the previously published reference values (16). The normal values are indicative of the absence of any discrepancy in hematopoietic as well as in immune functioning (18). The findings are in accordance with previous studies conducted to find the association between elevated levels of biomarkers and several organ-specific malignancies (26–28). Proteins (matrix metalloproteinase-7 and matrix metalloproteinase-12) are generally related to tumor growth and contribute to sustained proliferation and invasion. CRP has been extensively studied as a biomarker of malignancies (26,29) and is supposed to support the tumor microenvironment by inducing tumor-promoting inflammation (30). Similarly, in canines, elevated levels of CRP have consistently been attributed to increased tumor progression and poor prognosis (31). However, the values of serum CRP levels in the CTVT-affected dogs were observed to be in the normal ranges and did not reveal any statistical associations with tumor volumes. The values are

Figure 2. a- Mixed type cells in Giemsa-stained aspiration smear, arrow showing plasmacytoid cells while arrowheads show lymphocytoid cell type (100×). b- Histopathological slide of progression phase tumor having frequent round cells with lesser amount of connective tissue (10×, H&E stain). c- Regression phase tumor revealing fewer round to polyhedral shaped cells along with greater amounts of connective tissue. Arrows show numerous tumor-infiltrating lymphocytes in the tumor microenvironment (40×, H&E stain). d- Typical round to polyhedral cells having vacuolated cytoplasm, larger nuclei, and single nucleoli (100×, H&E stain).
in accordance with the studies of Hillström et al. (32), who previously studied serum levels of CRP in normal canids using the immunoturbidimetric method. Furthermore, normal ranges of the total leukocyte count and differential leukocyte count observed in the present study support the normal levels of serum CRP levels (33). Lack of association between serum CRP levels and CTVT volume along with the normal hematological ranges in the present study are indicative of localized tumor proliferation with reduced risks of progression and fair prognosis. However, the role of CRP in metastasized conditions of such tumors is so far unstudied.

It could be concluded that CTVTs with increased volumes remain a source of localized pain and mild behavioral alterations in affected dogs. However, serum CRP levels are independent of the localized proliferation of tumor masses having different cytomorphological and histological patterns.

References


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