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Original Article
Özgün Araştırma

Tumor cells and microenvironmental interaction in natural course of canine transmissible venereal tumour

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Abstract: Canine transmissible venereal tumors (CTVT) are naturally occurring tumors that are mostly transmitted between dogs through coitus. This study aims to investigate the effect of CTVT on molecular expression and disease progression by studying the tumor microenvironment. For this purpose, biopsy samples taken from ten female dogs were evaluated histopathologically and CTVT was diagnosed. The expression of markers such as CD163, CD68, CD44, TGF-beta and bFGF was evaluated by immunoperoxidase tests. Histopathologically, CTVT cells exhibited pleomorphism, ranging from round to polygonal. Some cells exhibited prominent vacuoles and hypochromatic nuclei, while others exhibited hyperchromatic nuclei containing mitotic figures within the thin fibrovascular wall. Immunohistochemically, TGF-beta and CD44 expression was higher in CTVT cells compared to CD68 and bFGF, while bFGF expression was higher in fibrocytes and spindle cells compared to other markers. The results indicate that CD44 and TGF-beta may play a pivotal role in fibrovascular processes, CD163 and CD68 may facilitate interactions between stromal components and mesenchymal cells, and bFGF, TGF-beta and CD68 may contribute to the arrest of tumoral progression and the initiation of the regression phase. These findings underscore the necessity for further studies to elucidate the role of markers at different stages of CTVT progression.

Keywords: CTVT, dog, histopathology, immunoexpression, microenvironment

Köpek aktarılabilir venereal tümörlerinin doğal seyri sırasında tümör ile mikroçevre etkileşimi

Özet: Köpeklerde Aktarılabilen Aktarılabilir Venereal Tümörler (CTVT), doğal olarak oluşan ve çoğunlukla cinsel temas yoluyla köpekler arasında aktarılan tümörlerdir. Bu çalışma, CTVT'nin tümör mikroçevresini inceleyerek moleküler ekspresyon ve hastalık ilerlemesi üzerindeki etkisini araştırmayı amaçlamaktadır. Bunun için; on dişi köpekten alınan biyopsi örnekleri histopatolojik olarak değerlendirildi ve CTVT tanısı konuldu. CD163, CD68, CD44, TGF-beta ve bFGF gibi belirteçlerin immünoperoksidaz testlerle ekspresyonları değerlendirildi. Histopatolojik olarak; CTVT hücrelerinin yuvarlaktan poligonal şekle kadar değişen pleomorfizm gösterdiği, bazı hücrelerin belirgin vakuoller ve hipokromatik çekirdeklere sahip olduğu bazılarının ise ince fibrovasküler duvar içinde mitotik figürler içeren hiperkromatik çekirdeklerinin olduğu görüldü. İmmünohistokimyasal olarak, CTVT hücrelerinde TGF-beta ve CD44 ekspresyonu CD68 ve bFGF'ye kıyasla daha yüksek olduğu; bFGF ekspresyonunun ise fibrositlerde ve iğsi hücrelerde diğer belirteçlere göre daha yüksek olduğu gözlendi. Sonuçlar, CD44 ve TGF-beta'nın fibrovasküler süreçlerde önemli roller oynayabileceğini, CD163 ve CD68'in ise stromal bileşenler ve mezenkimal hücreler arasındaki etkileşimleri kolaylaştırabileceğini ve bFGF, TGF-beta ve CD68'in tümöral ilerlemenin durdurulmasına ve gerileme evresinin başlatılmasına katkı sağlayabileceğini göstermektedir. Bu bulgular, CTVT ilerlemesinin farklı aşamalarında belirteçlerin rolünü anlamak için daha fazla çalışma gerektiğini vurgulamaktadır.

Anahtar kelimeler: CTVT, histopatoloji, immünoekspresyon, köpek, mikroçevre

Introduction

Canine Transmissible Tenereal Tumor (CTVT), or Sticker's sarcoma, is a contagious and transplantable tumor by coitus between male and female dogs and other canidae. The mass grow rapidly in cauliflower-like shape and later regressed spontaneously. Its consistency may show friable to firmness. Ulceration and bleeding may occur. It is more encountered in mongrel dogs and kennels, where stay dogs are kept together. The tumor cells are almost round to polygonal and sometimes contain mesenchymal and histiocytic cells. CTVT cells contain sometimes distinct, clear, and cytoplasmic vacuoles (Cangul, 2001; Thangathurai et al., 2008; Ganguly et al., 2016; Alkan et al., 2017). The histoarchitecture of CTVT is distinct. - In the progressive

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(P) phase of CTVT, proliferating round to polygonal shape cells are closely packed in rows or cords by thin mesenchymal (fibrovascular) septum. During the stable (S) phase, the developing tumor parenchyma and stroma stop growing, and the inflammatory response is initiated. At the regressive (R) phase, fibroblasts and spindle cell transformation are rapidly developed at intercepts of mesenchymal bundles and the periphery of CTVT cells. (Hiblu et al., 2019; Setthawongsin et al., 2019).

The tumor is known to have some immunogenic properties, and the immunity provides the inhibition of tumoral progression for several decades (Cohen, 1973). But, there is limited knowledge regarding the interaction between CTVT cells and the tumor microenvironment (TME). Even though CTVT has been well studied (Hiblu et al., 2019; Ke et al., 2022). At that course, tumor cell behaviour (tissue invasion capability) by stromal interaction and molecular profiling during transmission phases of CTVT is unfortunately not known. In fact, there is a close interaction between host immune system and tumor cells. So, the tumor cells together with their microenvironment and molecular expression in CTVT are thought to be highly complex. Stem-like cancer cells are developed in a niche together by infiltrating inflammatory cells (TILs), immune cells, fibroblasts, and angioblast. In the cellular communication process, extracellular matrix proteins (such as MMP), cytokines (IFNγ, TNF-alpha, and interleukin) and chemokines, growth factors, singaling systems (Wnt/ß-catenin pathway), as well as genetic elements (miRNA, mRNA, MHC) and genetic mutations (LINEs) play roles as well. (Hsiao et al., 2004; Siddle and Kaufman, 2015; Kanca et al., 2018; Frampton et al., 2018; Zayas et al., 2019; Skytthe et al., 2020). Thus, the more understandable/comprehensible, highly complex changes may give more facilitate the insight on potential therapeutic strategies for treating contagious CTVTs.

In this context, macrophages (such as M2) involving drug-targeting of the surface marker CD163 and CD68 expresses. Macrophages are phagocytic cells showing versatile functions. They also have several roles in cellular architecture, tissue remodeling, and fibrosis, i.e., TME and tumorigenesis, as well as regulation of inflammation (induction of anti-inflammatory activity) as tumor-infiltrating cell against tumor cell proliferation (Skytthe et al., 2020; Zhang et al., 2022). CD44 is known as a complex transmembrane glycoprotein. Its expression has formerly showed formerly shown to have a close relationship with the morphogenesis of cancer stem cells (or cytoskeleton) and epithelial-mesenchymal plasticity as well as roles in tumor cell proliferation, metastasis, and resistance against chemotherapeutics. Thus, CD44 has a versatile molecule providing interaction between extracellular matrix (ECM) and matrix metalloproteinases (MMPs), several growth factors. It has also played an important function in the stimulation of receptor tyrosine kinases (RTKs) while tumor cells proliferating (Ponta et al., 2003; Xu et al., 2015; Primeaux et al., 2022). Basic Fibroblast Growth Factor (bFGF) over fibroblast proliferation has a role in fibroblast proliferation due to being mitogenic agent. There are also several essential roles in regulating cell growth, angiogenesis, and organizing the tumor microenvironment. bFGF binds to and activates its tyrosine kinase receptors during cancer progression. Additionally, there have been several similar roles as aforementioned in CDs (Turner and Grose, 2010; Ardizzone et al., 2022). Transforming Growth Factor-beta (TGF-β) stimulates tumor cell progression and recruits fibroblasts, other mesenchymal stem cells, and angioblasts while undergoing cells to apoptosis and inhibiting T cell activation, proliferation, differentiation, and migration. As such, it regulates tumor microenvironment and immunocyte infiltration (Hao et al., 2019; Chan et al., 2023).

This study aims to evaluate the microenvironment of CTVT tumor cells to understand how it affects molecular expression and disease progression.

Material and Method

Pathological Evaluation of Collected Samples

In total, ten collected samples were the biopsies fixed in buffered formalin solution, and 10% were sent to the Department of Pathology, Faculty of Veterinary Medicine at Kirikkale University, TURKIYE The samples were selected from eight vaginal and two clitoral sites, in accordance with the site of lesions in female dogs. Tumor suspected-materials were evaluated suitably to general macroscopic criteria including their size, color, shape, and appearance of the upper and sectional faces of the mass. Tissue samples were trimmed, passed through ethanol and xylol series and liquid paraffin series on a tissue tracking device (Leica TP1020, Germany), and blocked in paraffin (Thermo Shandon, EG1150, Germany). Sections were taken from blocks with a thickness of 5 microns (Shandon, AS325, Germany). Tissue sections were stained using the Hematoxylin-Eosin (H&E) staining method (Luna, 1968).

Immunohistochemical Analysis

The Avidin-Biotin Complex Peroxidase (ABC-P) method was utilized to observe changes in the progression and regression of CTVT and mesenchymal tumor components. The secondary antibody employed was the Avidin Biotin Complex Peroxidase (ABC-P, HRP/DAB, ab64264, Abcam, France). CD163, CD68, CD44, TGF-beta, and bFGF expression was evaluated (Table 1). The ABC-P staining method was used for the rest of the procedure, following the instructions provided in the kit. For this purpose, serial sections were taken on 5-micron-thick adhesive slides. Glass slides were deparaffinized and dehydrated in Phosphate Buffered Saline (PBS-pastile, Sigma) by passing through xylol and alcohol series. Then, they were gently placed in a citrate buffer (pH=6.0, 10× concentration, Bioptica-Italy) solution in the microwave oven to reveal the antigen in the tissue. They were kept at 800 watts for 25 minutes in a microwave oven. To remove the endogenous peroxidase activity, they were kept in a 3% hydrogen peroxide-methanol mixture at room temperature for 5 minutes; and then taken into a humid chamber. The sections were kept in a 37°C oven for 25 minutes by dripping a drop of normal blocking serum on. Primary sera containing antibodies (aforementioned immunomarkers) were dripped onto the sections and incubated for 60 minutes in an oven at 37°C. Then, appropriate secondary antibodies labeled with biotinized and Horse Radish Peroxidase (HRP) were used and incubated in the incubator (Nüve, EN055, Turkiye) at the specified temperature and time. At the end of each step up to herein, tissue sections were washed twice by PBS at 5 minutes except for post-primary antibody dripping. For the reaction to become visible, 3-3'Diaminobenzidine (DAB, Abcam) chromogen and buffer were gently mixed in good proportion. The chromogen mixture was dropped on the sections and waited for 5 minutes. The sections were washed with distilled water. Finally, Gill's hematoxylin (Bioptica, Italy) was used for background staining. The slides were passed through ethanol and xylene series. The slides were covered with a coverslip and nonaqueous mounting medium (Entellan®, Merck, Germany). Findings were evaluated under an optic Brightfield microscope (Olympus BX51, Japan) and photographed (Olympus DP25 camera, Japan) as in other histopathological examinations, and the results were scored.

Table 1. Antibody panel and detailed information using in immunohistochemical analysis

Markers	Trademark	Catalog number	Dilution	Properties	Antigen retrieval (pH 6.0, Citrate buffer)
CD163	Abcam	ab182422	1:200	**Polyclonal** rabbit IgG	Yes
CD68	Abcam	ab125212	1:200	**Monoclonal** rabbit IgG	Yes
CD44	Abcam	ab189524	1:200	**Monoclonal** rabbit IgG	Yes
TGF-beta	Santa Cruz	sc-130348	1:200	**Monoclonal** mouse IgG1	Yes
bFGF	Antibodiesonline	AA 143 250	1:200	**Polyclonal** rabbit IgG	Yes

Semiquantitative Scoring and Statistical Analysis of Pathological Data

Serial tissue histosections were examined at ×200 magnification to see neoplastic changes after diagnosis of CTVT. These criteria included CTVT neoplastic cell and clear cell, pleomorphism, mitotic index, polychromasia, inflammatory cell infiltrations (neutrophil, macrophage, lymphocyte, and plasma cell), and spindle cells to be like in fibrocyte and fibroblasts in neoplastic tissue in three different areas of high power fields (HPFs). Histologically, the cells in histostained sections were counted semiquantitatively. The results were divided into 0 (no finding), 1,2,3,4,5,6 (for cases with lesions, according to the degree of finding) index slices according to Cingi et al. (2020). After converting them into equally divided percentiles and scoring, these data were analyzed by unpaired-t-test and Wilcoxon test. For immunostaining sections, positive cells were counted in three different areas (or HPFs). Mean and standard error were calculated for all samples and analyzed with the one-way ANOVA method and posthoc Tukey test. Between the criteria of mean and standard error-values, the statistical difference was evaluated significantly for $p<0.001$ (GraphPad Prism 8.0, USA).

Results

Histopathological findings

CTVT cells had polymorphism varying from round to polygonal shape. These cells contained hypochromatic nuclei and distinct nucleoli or hyperchromatic nuclei. Mitotic figures were widespread. Some of the cells had clear vacuoles and hypochromatic

nuclei. But, these clear cells were not so evaded in proliferating tumor cells. Thin fibrous or fibrovascular stroma was interrupted by CTVT cells in chords or packages (**Figure-1a and b**). In some areas, the periphery of mass, in particular, fibroblasts and fibrocytes or spindle-shaped cells covered the microscopic fields. In such areas, of many capillaries and arterioles were dispersed in connective tissue. Inflammation gets accorded in CTVT proliferation and involved neutrophil leucocyte, lymphocyte, macrophage, and plasma cells, consecutively (**Figure1-c**).

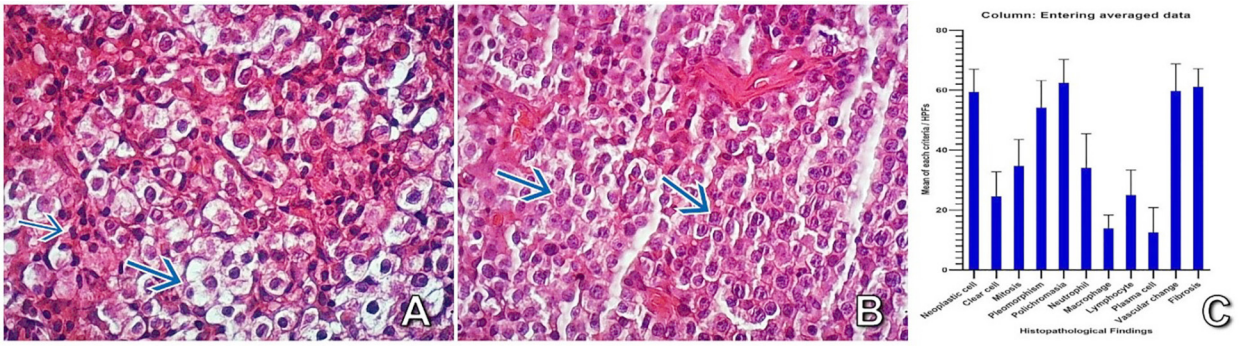

Figure 1. (A) In CTVT tumor, clear cells (thick arrow) and inflammatory cells (thin arrow). ×200 magnification H&E staining, **(B)** CTVT cells showing polygonal to round shape (arrows). ×200 magnification, H&E staining. **(C)** The bar graph represents histopathological findings scores of Canine Transmissible Venereal Tumor

Immunoexpression Results

CD163 and CD68 expressions were intensive in the cytoplasm of CTVT cells and clear cells (undergoing apoptosis or cellular alteration with clear vacuoles). Spindle-shaped cells were also reacted in cytoplasms with CD163 and CD68 immunomarker. Some of the reactions belonged to macrophage and macrophage-like cells. However, the reaction was not encountered in each microscope field by CD163 and in some microscope fields by CD68. However, there is no meaningful statistical difference. Thus, the reaction was not as much as in neoplastic cells. Polymorphonuclear cells, lymphocytes, and plasma cells did not react with both immunomarkers. The reactions in tumor cells and spindle cells were more dense in CD163 when compared to those in CD68 (**Figure-2a and b**). CD44 expressions were dense in both cytoplasms of CTVT cells and clear cells. The total reactions were more than those obtained from other CDs, TGF-beta, and bFGF markers. The reactions in these tumor cells were encountered in almost all microscope fields. However, the reactions in spindle-shaped cells were less when compared to reactions obtained from other markers. The last reaction was obtained by CD44 in spindle cells among all markers (**Figure-2c**). The immunohistochemical results by the TGF-beta marker were almost similar to the CD163 marker in CTVT cells, clear cells, and spindle-shaped cells. In other words, there is no meaningful statistical difference when the total cellular components were considered. However, the reaction in spindle cells is found in fibrocyte and fibroblast-like appearance due to being together with wide collagen bundles. The reaction by the bFGF marker was similar to the figure of the TGF-beta marker. However, fibrocyte-fibroblasts and spindle-shaped cells were more reacted with the bFGF marker (**Figure-2d and e**). Tumor cells were also reacted by bFGF marker as much reaction by CDs and TGF-beta markers as in tumor cells. Thus, there is no statistical difference at total positive cell counts. However, bFGF immunoexpression in clear cells undergoing apoptosis and / or cellular degeneration was not reacted as potential as in other immunomarkers in CTVT cells and clear cells. (see in **Figure-2f**).

Table 2. The mean, standard deviation, and p-values of the immunoperoxidase tests

	CD163			CD68			CD44			TGF-beta			bFGF			p
	n	m	SD	n	m	SD	n	m	SD	n	m	SD	n	m	SD	
Neoplastic Cell	10	7.44	4.55	10	4.44	1.49	10	11.88	5.16	10	7.77	4.21	10	9	4.23	
Clear Cell	10	10	5.04	10	10.55	4.55	10	12.55	4.51	10	12.55	5.49	10	3.11	1.85	0.0593
Inflammation	10	0	0	10	0	0	10	0	0	10	0	0	10	0.44	0.44	
Spindle Cell	10	5.22	3.15	10	3.55	2.33	10	1.44	0.62	10	4.66	2.39	10	6.44	1.57	

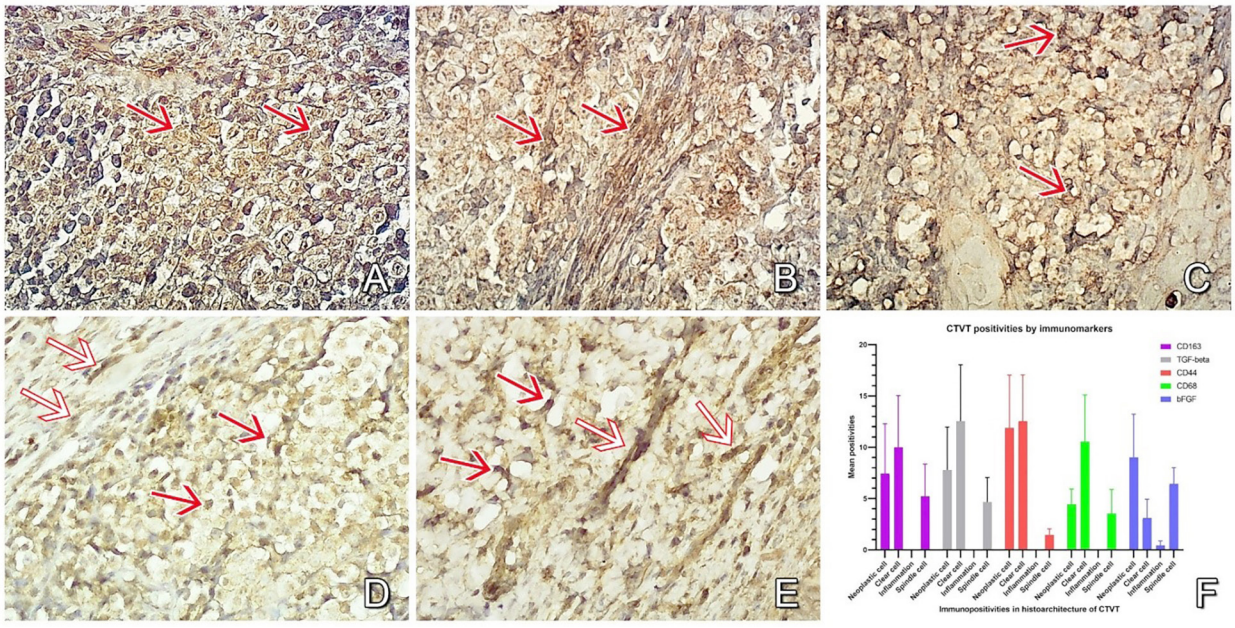

Figure 2. (A) CD163 positivity in polygonal to round shape tumor cells, immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(B)** CD68 positivity in round to spindle-like cells, immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(C)** CD44 positivity in polygonal to round shape cells, immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(D)** bFGF positivity in polygonal to round shape cells carrying clear vacuoles (red arrows) and fibrocytes and fibroblastic cells (white arrows), immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(E)** TGF-beta positivity in round shape cells having clear vacuoles (red arrows) and spindle-like cells (white arrows), immunoperoxidase staining, ×200 magnification, DAB chromogen and counterstaining with Mayer's hematoxylin. **(F)** The graph depicts the immunoexpression of CD163, CD68, CD44, TGF-beta, and bFGF according to cell type.

Discussion

Canine Transmissible Venereal Tumor is a transmissible neoplastic change during coitus between adult animals. It has been known for over one hundred years (Alkan et al., 2017; Birhan and Chanie, 2015). Although many studies are focusing on revealing several cell characteristics (such as cytogenetic, immunophenotypic, apoptotic, histopathologic, ultrastructural, and biochemical etc.) of the tumor, it has not been fully elucidated (Mukaratirwa et al., 2003; Sidha et al., 2015; Birhan and Chanie, 2015; Flórez et al., 2017; Frampton et al., 2018); So, there have been still some mysterious and dilemma to wait for its exploration. CTVT cells have ubiquitous morphology: spindle cells (fibroblast and histiocyte like-cells) and round to polygonal cells (Cangul, 2001; Thangathurai et al., 2008; Ganguly et al., 2016; Ujvari et al., 2016; Alkan et al., 2017). It is stated that its morphology has been related to plasmacytoid, lymphocytoid, and mixed-shaped cells (Flórez et al., 2016; Duzanski et al., 2017). We also observed this different morphology and cellular types in tumor parenchyma and microenvironment. The histoarchitecture of CTVT can be interchangeable from the progression to the regression phase. At initial, microscope fields are covered by polygonal-shaped cells. These cells are intercepted with fibrovascular septum. Later, the figure of the microscope field can transform into fibroblast and spindle cells. In our study, we also observed several round to polygonal-shaped cells and spindle cells or spindle-like cells in microscope fields of different cases. In particular, spindle-shaped cells were covered in the microscope field in a few cases. As expected, the other histomorphological components were accorded with CTVT cell riched-cases in that cases. Thus, we thought for the general view of cases constituted the subject of study that they were in either P or S phases rather than R-phases. We also thought that some CTVT cell poor-cases might be transformed into R-phases. Meanwhile, inflammatory cells (in particular lymphocytes and

macrophages) can infiltrate tumor parenchyma during the regressional process (Hiblu et al., 2019; Setthawongsin et al., 2019). On the other side, CTVT immunophenotype can be different from cellular morphology. Some cells represent a dilemma in immunoexpressions. It is shown that CTVT tissue can give immunoexpression in T-lymphocyte by CD8, tumor-infiltrating T-lymphocytes (TILs) by CD3, T-regulatory cells (Treg) by CD4, B-lymphocyte by CD79 as well as dendritic cells and bone-marrow-derived stem cells dendritic cells from bone marrow by CD1a, CD11c, CD40, CD80, CD83 and CD86 (Shankaran et al., 2001; Pai et al., 2011; Silveira et al., 2009; do Prado Duzanski et al., 2022).

Indeed, CTVT cells and tumor microenvironment (TME) are always found in complete interaction. Recent documents have been signed to powerfull evidence regarding cellular components and stromal companents including fibrocyte and vessels in tumor niche (Hiblu et al., 2019; Ke et al., 2022). In this study, we tried to show the role of induced inflammatory cells as well as CTVT cells and undergoing cellular degeneration in tumor niche in accordance with the knowledge (Skytthe et al., 2020; Zhang et al., 2022). Differently from previous documents, we picked out specific markers to show potent effects in both neoplastic and inflammatory cells. In the results, fibrovascular stroma, including spindle-like cells, also gave an expression. In this context, those macrophages expressed CD163 and CD68 in CTVT cells and stromal infiltrating cells. We obtained an expression with CD44 in those cells, showing formerly their roles in cancer stem cells (or cytoskeleton) and epithelial-mesenchymal plasticity. Basic Fibroblast Growth Factor (bFGF), mitogenic agent for fibroangioblast, and Transforming Growth Factor-beta (TGF-β) recruiting agent for both CTVT cells and fibroangioblasts gave an expression as mentioned in CDs (Shankaran et al., 2001; Silveira et al., 2009; Pai et al., 2011; Turner and Grose, 2010; Ardizzone et al., 2022; do Prado Duzanski et al., 2022). In all phases (P, S, and R) of current cases, we encountered high expressional levels in both CTVT cells and clear cells. In the P and S phases, clear cells undergoing apoptosis or degeneration gave relatively more expression by CD163, CD44, and TGF-beta according to CD68 and bFGF. Inflammatory cells including lymphocytes (TILs, Treg) and neutrophils, did not give an exact result, but histiocyte-like cells and spindle cells gave less expression by bFGF. Spindle cells comprising fibrocytes and fibroblasts and spindle-like cells gave relatively less expression by CD163, CD68, CD44, and TGF-beta despite much more in inflammatory cells when compared to in bFGF. This situation showed us that TGF-beta and CD44 might co-play a role in the fibrovascular process by triggering inflammatory reactions by means of CD68 expression. Induced- macrophages can facilitate the interaction between fibrous stromal components and possibly mesenchymal stem cells by CD44, CD68b, and FGF expressions. As such, the development of fibrous tissue of the tumor niche might realize a transition into R-phase from P and S phases.

In conclusion, we arrive come to the conclusions: First of all, the CTVT tumor niche is highly complicated. Round to polygonalshaped cells and spindle cells, spindle-like cells can interact. This situation selects destiny; either the tumor regresses or keeps stable according to undergoing degeneration and mesenchymal tissue development. Secondly, excepting out bFGF role, TGF-beta and CD68 can provide together for stopping tumoral progression and passing into regression phases. However, we thought that CD163 did not have a great role in inducible macrophages as much as CD68. Lastly, for CTVT cells, CD44 is thought to be more effective in recruiting of round to polygonal shaped-tumor cells. CD44 molecule can also be a determination factor for cells undergoing degeneration in clear cells. We strongly believe that this study's results show that there might be useful showing an important interaction between tumor cells and mesenchymal cells (such as fibroblasts, stem cells, and bone marrow-resourced inflammatory cells) in tumor stroma. We recommended researchers focus on such transmissible tumors so that further studies can be cross-checked together with these studied markers and several mesenchymal cell markers and stem cell markers in each of the progression, stable, and regression phases. The marker results should definitely be compared between those phases.

Ethics approval: The study samples consisted of tumor tissues sent to Kırıkkale University, Faculty of Veterinary Medicine, Department of Pathology. Ethical approval was not required.

Conflict of interest: No person or organization provided funding for this study, and the authors have no conflicts of interest.

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