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New insights in veterinary cancer immunology

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Published in
Frontiers in Veterinary Science

ISSN 1664-8714
ISBN 978-2-8325-5923-9
DOI 10.3389/978-2-8325-5923-9

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New insights in veterinary cancer immunology

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Citation
Fonseca-Alves, C. E., Massoco, C., Queiroga, F. L., eds. (2025). *New insights in veterinary cancer immunology*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-5923-9

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frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 14 June 2024
DOI 10.3389/fvets.2024.1440527

OPEN ACCESS

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RECEIVED 29 May 2024
ACCEPTED 04 June 2024
PUBLISHED 14 June 2024

CITATION
Fonseca-Alves CE, Queiroga FL and Massoco CdO (2024) Editorial: New insights in veterinary cancer immunology.
Front. Vet. Sci. 11:1440527.
doi: 10.3389/fvets.2024.1440527

Editorial: New insights in veterinary cancer immunology

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KEYWORDS

dogs, immune system, immunotherapy, immuno-oncology, tumor-associated inflammation

Editorial on the Research Topic
New insights in veterinary cancer immunology

1 Introduction

Veterinary cancer immunology is an emerging area that has been growing in the past years and is focused on understanding how the immune system interacts with animal cancer cells (1). In humans, immunology is one of the most studied areas in cancer research with new technologies being created for patient treatment. One of the most notable discoveries in recent years was the creation of a CAR-T cell therapy for hematopoietic cancer treatment (2). Cancer immunology explores the mechanisms by which the immune system can detect and destroy cancer cells, as well as how tumors evade immune surveillance (3). Research in veterinary cancer immunology has revealed that, similar to humans, animals' immune systems can recognize cancer-specific antigens and mount a response (1, 4). However, tumors often develop strategies to suppress the immune response, such as producing immunosuppressive cytokines or recruiting regulatory T cells that inhibit anti-tumor activity. Understanding these interactions is crucial for developing effective immunotherapies for animal cancers (5).

Recent advancements in veterinary cancer immunology include the development of novel immunotherapeutic approaches. These include cancer vaccines that stimulate the immune system to recognize and attack tumor cells, monoclonal antibodies that target specific cancer antigens, and checkpoint inhibitors that block proteins used by cancer cells to evade immune detection (4, 6, 7). Clinical trials in veterinary medicine have shown promising results, particularly in treating canine cancers like lymphoma (4) and osteosarcoma (8). These therapies not only improve the prognosis and quality of life for animals but also provide valuable insights that can translate to human oncology.

Another important aspect of veterinary cancer immunology is the study of comparative oncology, which examines cancer across different species to identify common mechanisms and potential treatments (9). Animals, particularly dogs, develop cancers that are biologically similar to human cancers, making them excellent models for research. By studying cancer in animals, researchers can gain a better understanding of tumor biology, immune response, and the efficacy of new treatments. This comparative approach helps bridge the gap between veterinary and human oncology, leading to advancements that benefit both animal and human patients (10).

In recent years, significant progress has been made in understanding the relationship between the immune system, inflammation, and various cancer subtypes. For this reason, the Research Topic "*New insights in veterinary cancer immunology*" focused on the recent advances in veterinary cancer immunology and compilated original and review articles that provide the most recent advances in veterinary cancer immunology.

Lymphomas in dogs can be categorized based on their cellular origin (B-cells or T-cells) using specific surface markers like CD21/CD79a for B-cells and CD3/CD4/CD8 for T-cells. Bae et al. conducted an interesting investigation to examine the clinical implications of aberrant lymphoma phenotypes in canine patients. For this, 27 dogs with aberrant lymphoma diagnosed through flow cytometry were analyzed for paraneoplastic syndromes, prognostic factors, and clinical features. Common aberrancies included the immunophenotype MHCII- and $CD3^+/CD21^+$. B-cell lymphomas frequently exhibited $MHCII^-$, $CD3^+/CD21^+$, $CD34^+$, and $CD79a^-$, while T-cell lymphomas showed $CD3^+/CD21^+$, $CD4^-/CD8^-$, $CD5^-$, and $CD45^-$. The platelet-neutrophil ratio and serum albumin levels varied significantly among different immunophenotypes. CD34 expression correlated with cranial mediastinal mass, clinical substage, and fever, while MHCII expression was linked to chemotherapy reactions and fever. Despite these correlations, no significant differences in survival periods were observed among the phenotypic aberrancies. The study concludes that aberrant lymphomas are common in dogs, with specific clinical prognostic factors linked to these immunophenotypes.

Altamura and Borzacchiello published a valuable opinion article regarding feline oral papillomavirus-inducing oral squamous cell carcinoma in cats in comparison with human Head and neck squamous cell carcinoma (HNSCC). In humans, HNSCC is a prevalent cancer affecting the aerodigestive tract in humans, with tobacco, alcohol, and poor oral hygiene as primary risk factors. However, around 25% of these cases, particularly oral squamous cell carcinoma (OSCC), are linked to high-risk human papillomavirus (HPV), mainly HPV-16 and HPV-18. These HPV-associated cancers typically occur in the oropharynx, with viral oncogenes E6 and E7 disrupting tumor suppressors like p53 and pRb. Similarly, in veterinary medicine, papillomaviruses (PVs) are implicated in squamous cell carcinomas (SCC) in cattle and horses, and there is growing evidence of their role in feline OSCC (FOSCC), with various studies showing significant associations between feline PVs (FcaPVs) and FOSCC. In this manuscript, the authors described those initial studies identified FcaPV-2 in FOSCC, revealing its transforming properties similar to those of HPV in human cancers. Subsequent research confirmed the presence of FcaPV-2 in FOSCC-derived cell lines and actual tumor samples, with high viral loads and active viral gene expression linked to oncogenic processes. Studies from different regions, including Italy, Japan, and Germany, have consistently detected FcaPV-2 in FOSCC, reinforcing the virus's role in tumor development. Despite the variability in reported association rates, ranging from 5 to 58%, the evidence points to a significant co-causative role of FcaPVs in FOSCC, warranting further research and consideration in veterinary oncology. In conclusion, the association between FcaPVs and FOSCC highlights the need for additional studies to understand this relationship better and potentially reclassify FcaPV-positive FOSCC as a distinct clinical entity. This could lead to tailored diagnostic and therapeutic approaches, similar to HPV-related HNSCC in humans. Future research should focus on multicentric studies to monitor these tumors' biological behavior and response to treatments, ultimately improving feline patients' diagnosis, treatment, and prognosis.

Rodney et al. published a pioneer study that investigated the genomic landscape and alteration of gene expression in cats with feline oral squamous cell carcinoma (FOSCC). FOSCC is a prevalent and aggressive cancer in cats, accounting for up to 80% of feline oral cancers and typically having a poor prognosis. Using whole exome sequencing (WES) and RNA sequencing, these researchers have identified somatic mutations and gene expression changes linked to FOSCC. This study is the first to apply WES to feline cancer and revealed tumor-associated mutations in six cats, with notable similarities to mutations found in human head and neck squamous cell carcinoma (HNSCC). Mutations in the TP53 gene, common in many cancers, were found in four samples, each with unique mutations. Other mutations were discovered in genes related to cellular growth control, such as KAT2B and ARID1A. Gene expression analysis indicated molecular similarities to human oral squamous cell carcinoma (OSCC), including changes in pathways related to epithelial to mesenchymal transition and the IL6/JAK/STAT pathway. These findings enhance the understanding of FOSCC and support its use as a comparative model for studying human HNSCC.

Chimeric antigen receptors (CARs) have been used in human oncology and have shown exceptional potential for patient treatment. Over the last 20 years, the investigation of CARs has advanced precipitously in recent decades as a state-of-the-art treatment option for several cancer subtypes. However, the use of this technique in Veterinary Oncology remains under development and clinical trials have only recently been initiated in dogs. Cockey and Leifer contributed to this Research Topic with a relevant mini-review manuscript, highlighting the potential of CARs in veterinary immuno-oncology. CARs are artificially designed proteins that combine a specific tumor associated antigen-binding single-chain variable fragment with the signaling domain of a T cell receptor and its co-receptors (TCR signaling complex). T cells from patients are engineered to express a CAR, enabling them to identify and destroy target cells, typically hematological cancers. While the U.S. Food and Drug Administration (FDA) has approved several CAR T-cell therapies for human use, there are numerous obstacles to adapting these treatments for veterinary use. This review addresses key considerations for using CARs in veterinary settings, including CAR design and choice of cell carriers, and explores the potential future of CAR therapy in veterinary oncology. This manuscript brings a critical approach to CARs in veterinary oncology and provides new insights for future studies.

The tumor microenvironment (TME) plays a critical role in cancer development, progression, and response to immunotherapy. It significantly influences cancer cell behavior and can contribute to tumor aggressiveness. Recognizing the importance of TME proteins, Brambilla et al. conducted a relevant study that investigated the role of Periostin (POSTN) in canine patients with bladder urothelial carcinoma (BUC). POSTN is an extracellular

matrix protein involved in tissue regeneration and metastasis. In humans, high POSTN levels are linked to aggressive tumors and poor prognosis in various cancers, except for BUC, where POSTN is downregulated. Since the role of POSTN is unclear in veterinary medicine, these authors examined POSTN expression in canine BUCs, using 45 tumor samples and 6 non-neoplastic bladder samples. Results showed that POSTN expression was generally lower in tumors compared to normal tissue, with a significant inverse correlation between POSTN expression and mitotic count. These findings suggest that POSTN could be a prognostic marker and a potential target for anticancer therapies in canine BUCs.

The transformation from adenoma to carcinoma in human colorectal cancer (CRC) is well-known, but its transcriptomic features in canines remain unclear. Lin et al. assessed for the first time in veterinary medicine, the transcriptome data from normal, adenoma, and cancerous canine colon tissues to investigate a malignant progression. These authors identified several genomic alterations with differential analysis highlighting the role of PFKFB3 in this transformation. Enrichment analysis showed metabolic dysregulation, immunosuppression, and typical cancer pathways in canine colorectal tumors. Dynamic expression patterns of differentially expressed genes (DEGs) and network analysis revealed inflammatory and immune pathways' roles, with the S100A protein family involved in malignancy. They identified five novel CRC markers, with GTBP4 showing strong diagnostic and prognostic potential. This study provides new biomarkers and comparative evidence for CRC research in humans and dogs.

Klingemann, wrote a review article about the use of *Viscum album* in Veterinary Oncology. *Viscum album*, commonly known as mistletoe, is a plant with notable medicinal properties. This paper explores the potential of *Viscum album* L. (mistletoe) extract, which has been used in human cancer treatment, for canine cancer therapy. It contains compounds like lectins and viscotoxins, which have anti-cancer and immune-boosting effects. Additionally, the extract's lectin ML-1 can enhance patient wellbeing by triggering endorphin release. Given its cross-reactivity with canine cells and low side effect profile, mistletoe extract may be a viable treatment option for dogs with cancer. In oncology, mistletoe extract is used to inhibit cancer cell proliferation and improve patient wellbeing by stimulating endorphin release. Its therapeutic potential extends to both human and veterinary medicine due to its relatively low side-effect profile.

Understanding a tumor's immune context is crucial in cancer research. Canine oral melanoma serves as a good model for human immunotherapy, but more studies are needed to understand its immune landscape and its similarity to human melanoma. Vanhaezebrouck et al. performed a retrospective study analyzing RNA sequences from formalin-fixed paraffin-embedded (FFPE) tissue samples of canine oral melanomas using Nanostring Technology. The relevant study compared melanoma tumors restricted to the oral cavity (OL) and primary oral tumors with a history of metastasis (OM). Normal buccal mucosa samples were used as a reference. The OM group showed significant gene expression changes compared to the OL group, including decreased expression of genes like S100, BRAF, and BCL2, and increased expression of hypoxia-related genes (VEGFA), cell mobility genes (MCAM), and PTGS2 (COX2). Immune landscape analysis indicated a shift in the OM group from a potentially active "hot" tumor suppressed by immune checkpoints to heightened expression of checkpoints and immune blockades like PD1 and IDO2. The OM group also had reduced expression of Toll-like receptors (TLR4) and IL-18, aiding in immune escape, and signs of immune cell exhaustion were evident in both groups through increased TIGIT expression. Both groups showed higher CD4 expression compared to normal tissue, but CD4 expression decreased significantly in OM tumors compared to OL. This preliminary study highlights significant gene expression differences between localized and metastatic canine oral melanomas.

Razmara et al. wrote a pertinent review article focused on the role of natural killer (NK) cells in canine cancer immunotherapy. The field of cancer immunology has gained significant attention due to immunotherapies that effectively target immune cells, achieving notable anti-tumor effects. Despite this progress, successful cellular immunotherapy for solid tumors remains difficult. Dogs with naturally occurring cancers provide a valuable model to address these challenges, especially for NK cell-based therapies. This review highlights recent advancements in understanding canine NK cells, crucial for future translational NK immunotherapy research given their innate cytotoxicity against a broad array of targets and their potent cytokine production. Characterizing canine NK cells is important due to the challenges in defining them and the limited availability of specific reagents. The review also summarizes current clinical and translational studies using NK cell immunotherapy in canine cancer patients. These studies offer insights into efficacy and immune responses, paving the way for novel combinational therapies. This knowledge enhances the canine cancer model and supports translational efforts to improve cancer treatments for both dogs and humans.

Summing up, all the manuscripts published in this Research Topic offer a fresh insight into the use of the immune system for various therapeutic strategies in veterinary oncology. These advancements have the potential to broaden the adoption of immunotherapies in clinical practice and pave the way for more translational comparative studies. Ongoing research into immunotherapies approaches in veterinary oncology remains critical to refine cancer management and treatment options for canine patients.

Author contributions

CF-A: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. FQ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. CM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

We thank all the authors and reviewers of this Frontiers Research Topic for their excellent contribution. We also thank Frontiers editorial team for their valuable support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Mini Review
PUBLISHED 17 February 2023
DOI 10.3389/fvets.2023.1130182

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

RECEIVED 22 December 2022
ACCEPTED 31 January 2023
PUBLISHED 17 February 2023

CITATION
Cockey JR and Leifer CA (2023) Racing CARs to veterinary immuno-oncology. *Front. Vet. Sci.* 10:1130182. doi: 10.3389/fvets.2023.1130182

Racing CARs to veterinary immuno-oncology

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Chimeric antigen receptors (CARs) have demonstrated remarkable promise in human oncology over the past two decades, yet similar strategies in veterinary medicine are still in development. CARs are synthetically engineered proteins comprised of a specific antigen-binding single chain variable fragment (ScFv) fused to the signaling domain of a T cell receptor and co-receptors. Patient T cells engineered to express a CAR are directed to recognize and kill target cells, most commonly hematological malignancies. The U.S Food and Drug Administration (FDA) has approved multiple human CAR T therapies, but translation of these therapies into veterinary medicine faces many challenges. In this review, we discuss considerations for veterinary use including CAR design and cell carrier choice, and discuss the future promise of translating CAR therapy into veterinary oncology.

KEYWORDS

applied immunology, cancer, cell therapy, immunotherapy, translational medicine

1. Introduction

Cell-based immunotherapy has progressed exponentially over the past few decades as a cutting-edge treatment option for multiple cancers. Adoptive cell therapy (ACT) involves harvesting immune cells from the patient, expanding them under good manufacturing practice (GMP) conditions, and reinfusing a clinically relevant dose. One of the first human ACTs used isolated tumor infiltrating lymphocytes (TILs) and selected for cells with a T cell receptor (TCR) specific toward a tumor neoantigen presented on MHC I of the tumor (1–3). Although promising (4–6), a significant advance in ACT that takes advantage of the specificity and affinity of antibodies against a tumor surface antigen, rather than relying on endogenous T cell receptors (TCRs), is chimeric antigen receptors (CARs). The FDA has approved multiple CAR T therapies against human B cell maturation antigen expressed on antibody-secreting plasma cells (7, 8), and CD19, which is expressed on the surface of almost all B cells (9–12). Similar to humans, lymphomas are common in companion animals. Retrospective analysis of 171 canine and feline non-Hodgkin's lymphoma samples revealed 79.9% of canine cases were B cell lymphomas that were predominantly multicentric, while 64.6% of feline cases were T cell lymphomas that were predominantly alimentary (13). While chemotherapy remains the standard of care in veterinary medicine (14), CARs are an attractive alternative or add on therapy for refractory veterinary lymphomas. Clinical trials have only recently been initiated in dogs. In this review, we outline the design of CARs and the future outlook of the therapy for veterinary use.

2. CAR construct design

Development of a CAR therapy requires multiple steps, each of which presents unique challenges for translation to veterinary medicine (Figure 1). In this section, we summarize basic CAR design and methods of expressing the CAR in primary cells.

CARs are created by stitching together an ScFv, a hinge, a transmembrane domain, and one or more cytoplasmic signaling domain(s) derived from the TCR signaling complex (15, 16). ScFvs are developed from the variable light and heavy chains of a specific monoclonal antibody targeting a tumor-associated antigen. Some CAR approaches use endogenous ligands or receptors, rather than ScFvs, to target tumors and may be a good alternative when cross-reactive or veterinary-specific antibodies are not available (17). Newer high-throughput fluorescence-activated cell sorting (FACS) screens can also be used to identify potential antibodies or ScFvs (18), but it is unclear if this strategy would be practical for clinical manufacturing in veterinary medicine.

The cytoplasmic signaling domains are critical to drive T cell activation and can lead to different effector functions in the patient. Use of one signaling domain, CD3ζ, resulted in low-level signaling, and poor persistence or anergy in patients (19, 20). CAR T therapies approved for human use have additional costimulatory receptor signaling domains like 4-1BB (Kymriah®, Breyanzi®, Abecma®, and Carvykti™) or CD28 (Yescarta®, Tecartus®). Human primary T cells transduced with a CAR containing the CD28 signling domain preferentially generated effector memory T cells *in vitro* ($CCR7^{-}CD45RO^{+}$) while the 4-1BB signlaing domain drove a central memory phenotype ($CCR7^{+}CD45RO^{+}$) (21). Using NSG mice with a xenografted osteosarcoma, infused human CAR T cells with 4-1BB had lower expression of exhaustion markers than those with CD28 (22). Some CARs use two costimulatory domains and have increased efficacy in preclinical animal models (23, 24). Comparison of efficacy of different CAR components in veterinary oncology remains limited and will likely require additional empirical testing (25).

CARs are frequenty delivered to patient primary T cells using a replication-incompetent lentivirus or γ-retrovirus (26, 27). Pre-activation is required because the viruses can only (γ-retrovirus), or preferentially (lentivirus), integrate into dividing cells (26, 27).

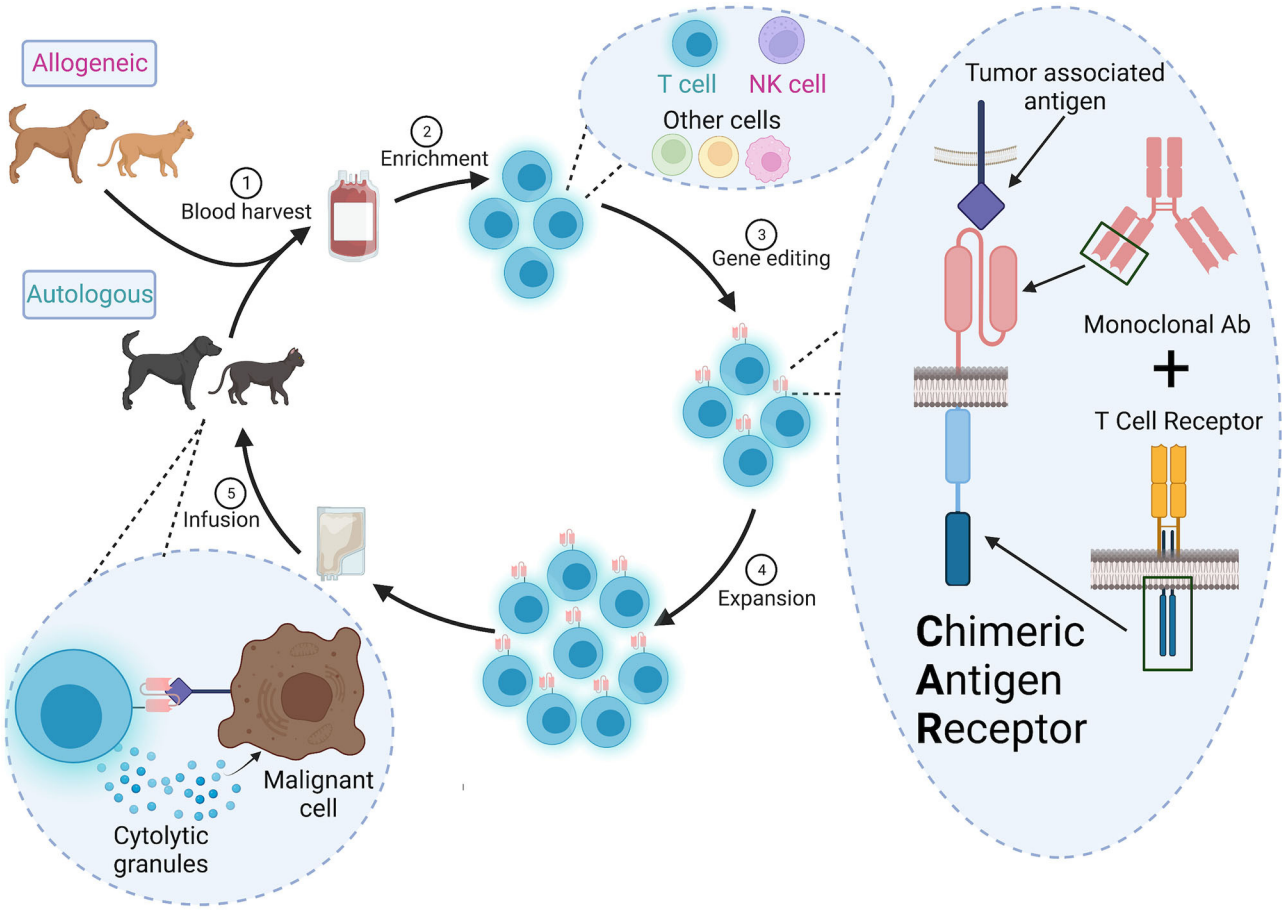

FIGURE 1
Overall scheme for CAR therapy in veterinary medicine. (1) Autologous (from the patient) or allogeneic (from a donor) cells are harvested from peripheral blood or apharesis, (2) enriched and (3) engineered to a express a CAR *ex vivo*. The CAR contains the variable heavy and light chains of a monoclonal antibody specific for a tumor-associated antigen and signaling domains from the TCR signaling complex. (4) The CAR^{+} cells are expanded to a clinically relevant dose and (5) infused into the patient. These CAR cells will detect and destroy cells expressing the target antigen.

However, other approaches have used transposons to integrate the CAR-encoding DNA (28, 29). To avoid delivery of viruses to patients, anti-canine CD20 CAR mRNA was directly electroporated into canine T cells (30, 31). However, CAR expression by mRNA delivery was transient and waned after 14 days (30). Lipid nanoparticles may enhance delivery of CAR mRNA and can be used *in vivo* (32). Transient CAR expression could be an advantage for veterinary therapy since it will limit immune reaction against the xenogeneic antibody components of the ScFv. Regardless of which CAR is developed, the sequences should be species-matched as much as possible to reduce host anti-CAR immune responses.

Gene editing tools like transcription activator-like effector nucleases (TALEN®) and clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 allow for simultaneous delivery of the CAR and reduced graft-vs-host and host-vs-graft responses (33–36). For example, CAR insertion into the TCR locus allows for expression of the CAR under the endogenous transcriptional regulation of the TCR promoter, which limits exhaustion, and elimination of TCR expression, which reduces graft vs. host disease (37). Conversely, deletion of β2-microglobulin, part of MHC I, reduces CAR T cell rejection by the host. However, loss of MHC I increases detection and destruction by natural killer (NK) cells, which can be mitigated in part by knock-in of human leukocyte antigen E into the *B2M* locus (38). Inhibitory receptors such as PD-1, which limit CAR T cytotoxicity, can also be deleted using these gene editing tools (39). However, CRISPR can induce unwanted mutations (40, 41), or multiple donor DNA insertions (42). Unpredicted translocations have also occured when TALEN® was used to delete the TCR alpha chain and CD52 to make "universal" CAR T cells (35). Fortunately, these off-target events are relatively rare (43).

3. Cell manufacturing

While GMP guidelines must be followed for clinical-stage ACT in both human and veterinary medicine, there are some specific considerations for veterinary application. In this section we will outline methods that are under investigation for veterinary CAR T cell expansion, as well as systems employed in human CAR T cell production that could be adapted for veterinary use.

Production of cells for clinical use requires validation of standard operating procedures and GMP-grade reagents and materials in all manufacturing steps with individual certificates of analysis. Growth of cells for human medicine requires serum-free, xeno-free, GMP-grade, commercial media formulations. There may not be commercially available GMP-grade species-specific sera for veterinary applications. Anti-canine CD20 CAR T cells failed to grow in OpTmizer™ serum-free media, and while there was some growth in LymphoONE™ serum-free media, CAR expression levels were low, suggesting empirical identification of optimal growth conditions for each veterinary CAR application may be necessary (44). Moreover, veterinary species cytokine supplements are limited (45) and thus validated cross-reactive reagents may be required (46). Feeder cells or special additives can enhance *ex vivo* expansion. For example, K562 cells can be engineered to express human CD32 and canine CD86, thereby acting as artificial antigen presenting cells (aAPCs). Co-culture of canine T cells with these aAPCs resulted in nearly six-fold expansion, and was even able to stimulate proliferation in T cells that were unresponsive to agonistic anti-canine CD3/CD28 beads (30). High $CD8^+$ subset expansion and reduced PD-1/PD-L1 expression on canine CAR T cells occurred when the cells were grown with thyroid adenocarcinoma aAPCs expressing CD80, CD83, CD86, and 4-1BBL in the presence of phytohemagglutinin (47). Phytohemagglutinin also increased retroviral transduction efficiency (44). Additional advancements in cell culture using closed-system bioreactors can further enhance *ex vivo* expansion yield, reduce contamination risks, and minimize technician handling (26, 48–52). These devices will likely be employed more frequently in future veterinary clinical trials.

4. Choosing the right "CAR driver"

Currently all FDA approved human CAR therapies are T cell-based, and T cells are also the "driver" for canine CAR therapy. However, many different immune cells could potentially be used to carry a CAR (Table 1). In this section, we describe the major advantages and limitations of each cell type, as well as the development and therapeutic potential in veterinary medicine. The focus is on canine CARs since they have advanced the furtherest in veterinary medicine, but we will discuss potential use in felines and highlight findings in human medicine that have potential for veterinary applicability.

4.1. T cells

The most advanced CAR therapeutics in veterinary medicine are T cells. The first clinical trial of CAR T cells in canine patients delivered a CD20 CAR mRNA by electroporation. The CAR contained a murine anti-canine CD20 ScFv with human CD8α leader, hinge, transmembrane, and a CD3ζ signaling domain (30). One canine patient with relapsed spontaneous B cell lymphoma was infused in three separate doses and had reduced $CD20^+$ cell numbers with no adverse events. A follow up study treated diffuse large B cell lymphoma with anti-CD20 CAR containing the same ScFv, but canine signaling domains (78). No adverse events were documented following infusion in three dogs, but this therapy had lower efficacy and *in vivo* persistence of the cells was poor. Eventually, an escape-variant of CD20 was detected on peripheral blood B cells post-infusion. Additionally, two of the dogs developed anti-mouse ScFv CAR serum antibodies, which peaked at day 50 post-infusion. These types of anti-CAR immune responses can be reduced by generating a "caninized" ScFv where all but the complementarity determining regions of the ScFv are canine.

Preclinical and clinical investigation of canine CAR T cells has also begun to target solid tumors, which have notoriously been resistant in human CAR therapy. A HER2 CAR T cell therapy (79) with canine CD28 and CD3ζ signaling domains secreted IFNγ and was cytotoxic against $HER2^+$ osteosarcoma and breast cancer target cell lines *in vitro* (80). IL13Rα canine CAR T cells secreted IFNγ when incubated with $IL13R\alpha^+$ targets (81). A canine glioma cell line implanted into mouse brains was effectively eliminated using canine CAR T cells against IL13Rα with either a human or a canine 4-1BB signaling domain. B7-H3 CAR T

TABLE 1 Summary of species-specific surface markers that define immune cells and can be used to enrich desired populations through FACS or magnetic bead enrichment.

Immune cell	Human phenotypic markers	Murine phenotypic markers	Canine phenotypic markers	Feline phenotypic markers	References
T cell	$CD3^+CD56^-$ $\alpha\beta TCR^+$	$CD3^+$ $\alpha\beta TCR^+NK1.1^-$	$CD3^+CD5^{bright}NKp46^-$ $\alpha\beta TCR^+$	$CD3^+CD56^-$ $\alpha\beta TCR^+$	(53–58)
NK cell	$CD3^-CD56^+CD7^+$	$CD3^-$ $NK1.1^+$ $\alpha\beta TCR^-$	$CD3^{-/+}$ $CD5^{dim}CD8^+TCR\alpha\beta^-TCR\gamma\delta^-$ $CD21^-CD4^-$ $CD94^+$ $NKp46^+$	$CD3^-CD56^+$	(53, 54, 59–64)
NKT cell	$CD3^+CD56^{+/-}iTCR^+$	$CD3^+NK1.1^+iTCR^+$	$CD3^+CD5^{intermediate}$ $NKp46^+CD94^+$ $iTCR^+$	$CD3^+CD56^+$	(53, 54, 59–61, 65–68)
γδ T cell	$CD3^+\gamma\delta TCR^+$	$CD3^+\gamma\delta TCR^+$	$CD3^+\gamma\delta TCR^+$	$CD3^+\gamma\delta TCR^+$	(69–71)
Macrophage	$CD68^+$: $CD80^+CD206^{dim}$ (M1) or $CD80^-CD206^{bright}$ (M2)	$F4/80^+$: $CD38^+$ (M1) or $CD38^-$ (M2)	$Iba1^+$: $CD204^-$ (M1) or $CD204^+$ (M2)	$Iba1^+$: $CD204^-$(M1) or $CD204^+$(M2)	(72–77)

cells (82) were more cytotoxic than HER2 CAR T cells toward canine osteosarcoma spheroids, but cytotoxicity was similar for the constructs incorporating CD28 or 4-1BB signaling domains (83). Two healthy canine subjects were then infused with either frozen or fresh autologous B7-H3 CAR T cells. The fresh infusion did induce a grade 2 toxicity but no other adverse events were observed, while the recipient of frozen cells had an allergic reaction 67 days later that was likely unrelated to the infusion. Together, these results show that canine CAR T cells are safe and well-tolerated, even for some solid tumors.

The most notable drawback of human CAR T therapy is cytokine release syndrome, which presents with pyrexia, delirium, hypotension, and increased serum IL6, and often requires administration of the IL6 receptor antagonist tocilizumab and steroids (84). To enhance safety and rapidly deplete infused cells in the event of an adverse reaction, drug-sensitive "kill switches" can be incorporated into the CAR (85–89). Since some adverse reactions have been observed in canine CAR T trials, including a case report of increased serum cytokines consistent with cytokine release syndrome (90), incorporating kill switches in the CAR construct may be needed in veterinary medicine as well.

4.2. Natural killer cells

NK cells have reduced risk of inducing a graft vs. host reponse and have shown promise in human preclinical studies. Moreover, human NK cells can be sourced allogeneically (91, 92), and infused at higher doses (93, 94). Allogeneic sourcing may allow mass production of an "off the shelf" product, reducing manufacturing costs, which is a significant concern in veterinary medicine.

Major challenges to using NK cells for veterinary CAR therapy include the lack of consensus on surface markers, limited antibody reagents, and lack of robust purification and expansion protocols. Feline NK cells are $CD56^+CD3^-$ (53, 59) and feline CD3 and CD56 antibodies exist (clones NZM1 and SZK1, respectively) (95, 96). However, there is not a consensus on canine NK markers. NKp46 is a common NK marker across species and $CD3^-NKp46^+$ cells enriched by FACS from canine peripheral blood mononuclear cells (PBMCs) exhibited cytotoxicity toward canine osteosarcoma and canine thyroid adenocarcinoma targets (60). Coculture of canine PBMCs with K562 cells expressing membrane bound IL15 and 4-1BBL, and added human IL2 and IL15, expanded large granular lymphocytes with cytotoxic activity (61). These presumptive NK cells were $CD5^{dim}CD3^+CD8^+TCR\alpha\beta^-TCR\gamma\delta^-CD21^-CD4^-$ and although they did not have mRNA for CD56, they did have mRNAs for other NK receptors like NKG2D, NKp30, and NKp46. CD5 depleted canine PBMCs cultured with IL2 alone or IL2 and IL15 for 14 days also had NK-like cytotoxicity yet were $CD56^-$ (97). $CD94^+$ cells enriched from canine PBMCs were $CD5^{dim}NKp46^+CD3^-$ (54). A first-in-canine clinical trial infused expanded cells with a similar phenotype into ten sarcoma patients in combination with intratumoral rhIL2 following focal radiotherapy (98). Five of the patients remained metastasis free at the 6-month primary endpoint (98). Despite NK cells being safe (99), their clinical efficacy does not yet match CAR T. Moreover, NK cells have a shorter *in vivo* lifespan than T cells. Addition of the *IL15* gene may provide sufficient signaling to overcome these limitations (100, 101).

4.3. Other cells

Immune cells such as natural killer T (NKT) cells, γδ T cells, and macrophages have been explored preclinically and clinically as human CAR drivers. Human NKT cells are rare $CD3^+$ lymphocytes expressing an invariant αβ TCR, and may coexpress CD56 (65, 102, 103). Feline NKT cells are $CD56^+CD3^+$ (53, 59); however, canine NKT markers are more controversial. Originally defined as $CD3^+$ lymphocytes that bound to complexes of α-galactosylceramide and murine CD1d (68), one group identified a $CD5^{intermediate}NKp46^+CD94^+CD3^+$ subset of large granular lymphocytes that may be NKT cells (54). Clinical isolation protocols for NKT cells may require dual CD56/CD3 enrichment for felines or NKp46/CD3 for canines, and there are currently no expansion protocols to obtain clinically useful numbers of these feline or canine cells. Regardless, human CD19 CAR NKT cells against lymphoma (104), and GD2 CAR NKT cells against neuroblastoma (105), have demonstrated preclinical efficacy, with

CD19 CAR NKT cells exerting anti-lymphoma activity through both the CAR and the invariant TCR interaction with CD1d. However, not all tumors express CD1d and much of the activity will be *via* the CAR (106, 107). Human GD2 CAR NKT cells, co-expressing IL15, infused in pediatric neuroblastoma patients, were well-tolerated and reduced metastasis in one patient. This study provided safety data for human CAR NKT cells co-expressing self-supporting growth factors (108). NKT cells may soon be explored for CAR therapy in veterinary medicine.

In veterinary medicine, γδ T cells play an important role in mucosal immunity (109), and can comprise nearly half of the PBMC compartment in young ruminants (110). γδ T cells express TCRs with broad specificity and are MHC independent, yet they have *in vitro* cytotoxic activity similar to NK and T cells. Human GD2 CAR γδ T cells demonstrated *in vitro* cytotoxicity to the LAN1 neuroblastoma cell line (111). Both canine and feline *TCRG* loci have been identified and subsets can be classified through PCR, but robust isolation and expansion protocols are lacking (69, 70, 112). Moreover, many γδ T cells are located in peripheral tissues and may be difficult to enrich from peripheral blood in sufficient numbers to expand for clinical use (113). Enrichment of human Vδ1 cells from peripheral blood and expansion in cell culture bags using IFNγ, anti-CD3, and IL4, for 2 weeks followed by IL15 for 1 week, did generate a clinically relevant product yield and upregulation of effector markers (NKG2D, DNAM-1, NKp30, NKp44, and 2B4) (114). However, further research is needed to determine if γδ T cells will be useful in veterinary CAR therapy.

Macrophages are abundant in tumors of many different species, can exhibit anti-tumor activity, and have therapeutic potential as CAR drivers (115, 116). Macrophages can polarize to many different functional states from the extremes of proinflammatory M1 to anti-inflammatory/immunosuppressive M2 cells. Tumor-associated macrophages also adapt to the tumor microenvironment in ways that promote rather than eliminate tumors (117). In dogs, high numbers of macrophages in tumors is correlated with increased aggressiveness and worse prognosis for mammary cancer (72). Human THP-1 monocytic cells engineered to express CD19, HER2, or mesothelin CARs, phagocytosed target cells *in vitro* (118). Primary human HER2 CAR macrophages extended survival in a mouse ovarian xenograft model, suggesting that they still demonstrated antitumor activity despite the immunosuppressive tumor microenvironment (118). Macrophage immunotherapy in veterinary oncology has largely focused on *in vivo* activation of macrophages rather than *ex vivo* manipulation and reinfusion, but there is potential to develop them as CAR drivers (119–122), A limitation is that macrophages, and their precursor monocytes, are notoriously difficult to genetically modify regardless of species. Some approaches to overcome this limitation include using a replication-incompetent adenovirus (118, 123). Despite their limitations, macrophages and other CAR drivers warrant a basic science investigation to understand their true potential for use in veterinary medicine.

5. Discussion

Cell-based immunotherapy has gained traction as a promising therapeutic modality for multiple cancers in both human and veterinary patients. Although clinical veterinary studies are still in the beginning phases, the potential for breakthrough therapies, like has happened for human hematologic oncology, is high. Veterinary clinical trials involving infusions of T cells and NK cells have demonstrated the feasibility and safety of harvesting and manufacturing cells for clinical use (30, 78, 83, 98). However, to fully break into the cellular immunotherapy sector the way human medicine has, veterinary schools or other hospitals will need appropriate infrastructure for cellular manufacturing and genetic modification, or identify industry partners. Current manufacturing systems are designed for clinical production of human cellular therapeutics, but as interest in veterinary cell therapy grows, so will the market for xeno-free GMP-grade media, reagents, and supplements to be used for species-specific cell isolation and clinical expansion. The potential cost of the therapy also presents a major hurdle, and possibly the biggest challenge toward translation to clinical veterinary use. Insurance coverages that can defray the six-figure prices of human CAR T cell therapies would not be an option in veterinary medicine. Thus, a significant focus of future veterinary CAR research must be to develop more generally tolerable therapies with low levels of side effects to create a product that could be administered at a general veterinary practice. These will likely include a product where endogenous TCRs are deleted and other modifications are made to reduce cytokine release syndrome. Overall, companion animal patients may greatly benefit from immunotherapies that have seen success thus far in human patients due to their shared spontaneous disease development. As the field progresses in veterinary medicine, future treatment modalities designed for companion animals may one day translate back to human medicine.

Author contributions

JC and CL conceived of the review, wrote, and edited the manuscript. JC performed searches in PubMed and Google Scholar databases including, but not limited to: CAR T therapy, chimeric antigen receptor, canine, feline, CAR NKT, macrophage markers, T cell phenotype, gamma delta T cell veterinary, and murine T cells. Both authors contributed to the article and approved the submitted version.

Funding

JC was funded by the Liz Hanson Graduate Scholarship. This work was supported by a Cornell Feline Health Center grant to CL.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Opinion
PUBLISHED 17 May 2023
DOI 10.3389/fvets.2023.1148673

OPEN ACCESS

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RECEIVED 20 January 2023
ACCEPTED 02 May 2023
PUBLISHED 17 May 2023

CITATION
Altamura G and Borzacchiello G (2023) Feline oral squamous cell carcinoma and *Felis catus* papillomavirus: is it time to walk the path of human oncology? *Front. Vet. Sci.* 10:1148673. doi: 10.3389/fvets.2023.1148673

Feline oral squamous cell carcinoma and *Felis catus* papillomavirus: is it time to walk the path of human oncology?

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KEYWORDS

papillomavirus, *Felis catus*, oral squamous cell carcinoma, head and neck cancer, comparative oncology

1. Introduction

In humans, head and neck squamous cell carcinoma (HNSCC) is among the most common cancers worldwide. This severe disease affects the aerodigestive tract, including oral cavity and oropharynx (1). Tobacco smoking, alcohol abuse and poor oral hygiene are believed to be the main risk factors. However, it is estimated that a subgroup of oral squamous cell carcinoma (OSCC), accounting for 25% of all HNSCCs, is associated with alpha high-risk human papillomavirus (HR-HPV) infection, with the HPV-16/-18 being the major responsible for cancer development. This particular type of tumor arises mainly at oropharyngeal sites, where persistent infection plays a key role toward neoplastic transformation. Viral oncogenes E6 and E7 drive carcinogenesis in infected cells by impairing the molecular pathways of two key tumor suppressors such as p53 and pRb (1). In veterinary oncology, association of PVs with SCC of the upper digestive tract has been ascertained in bovine and equine species (2–5). In cattle, bovine PV type-4 (BPV-4) is considered a main player in early steps of tumorigenesis leading to development of SCC of the esophagus, mouth, and oropharynx, along with environmental co-factors (2, 6). Furthermore, there is increasing evidence that *Equus caballus* PV-2, likely to be a co-causative agent of genital SCC in horses, is also involved in development of a subset of equine HNSCC, thus being considered an equine equivalent of HPV-16 (3–5). Studies in dogs have suggested a possible role of canine PVs in oral carcinogenesis, particularly in the transformation of oral papillomas into SCC (7–9). Similarly, a rising number of published work indicates that *Felis catus* papillomaviruses (*Fca*PVs) exhibit mucosal tropism, being consistently detectable in a subset of OSCC of cat and playing a co-causative role in the development of these tumors (10–16).

2. Association of FOSCC with *Fca*PVs infection

The first hints of a biologically significant association of feline OSCC (FOSCC) with *Fca*PVs came from the pioneering studies aimed at characterizing transcriptional activity of *Fca*PV type 2 (*Fca*PV-2) *in vivo* and its biological properties in feline living cells (10, 17). Here, viral DNA and gene expression were reported in one FOSCC case and *Fca*PV-2 E6 and E7 oncogenes appeared to exert transforming properties comparable to those of HR-HPVs associated with human HNSCC (10, 17). Additional clues were pointed out when *Fca*PV-2 was shown to be detectable and transcriptionally active in cell lines derived from cat gingival and tongue SCC (18). Moreover, these cell lines showed a molecular scenario compatible

with a *Fca*PV-2 E6-dependent p53 degradation with similarities to that reported for HPV-16 E6 (18). Stronger evidence came from two later independent studies that reported detection of *Fca*PV-2 in a relevant subset of FOSCC from different geographical areas, with a prevalence of 31% (10/32) in Italy and ∼58% (11/19) in Japan (12, 19). Importantly, the study conducted in Italy pointed out expression of *Fca*PV-2 oncogenes E6E7 in tumors and higher viral load compared to non-neoplastic oral mucosa harboring viral DNA, suggesting active infection and oncogenic functions (19). A later work from Germany confirmed expression of PV antigen in ∼21% (5/21) of FOSCC and the presence of PV-induced cellular changes (koilocytes and inclusion bodies) in a subset of samples (15). Furthermore, a recent multicentric study demonstrated the presence of at least one *Fca*PV type (among these *Fca*PV-1/-2/-3/-4/-5) in ∼21% (22/103) of a pool of FOSCC samples from Italy and Austria (11). Consistent data were presented in two independent published works conducted in USA and New Zealand, denoting the presence and high viral load of *Fca*PV-3 in 5% (1/20) of FOSCC but not in normal mucosa, and typical PV-induced cellular changes in a *Fca*PV-3 positive tumor sample (13, 14). Taking these studies together, the association rate of *Fca*PVs with FOSCC seems to fluctuate between 5 and 58%, however a conference paper from USA dated in 2015 reports that it can even reach 100% (12/12) (20).

In summary, the evidence of a co-causative role of *Fca*PVs in the development of FOSCC is as follows:

1) Different *Fca*PV types (-1/-2/-3/-4/-5) exhibit mucosal tropism (16, 21).
2) A subset of FOSCC samples is associated with *Fca*PVs DNA (11–14, 19, 20).
3) Detection of *Fca*PVs DNA in a subset of FOSCC is a common finding in different geographical areas, as per independent studies by different research groups (11–14, 19, 20).
4) There is histological, molecular and immunohistochemical evidence of PV active infection in a subset of FOSCC by different research groups (13, 15, 19).
5) *Fca*PV-2 displays high viral load and expression of E6E7 oncogenes in FOSCC samples (10, 19).
6) *Fca*PV-2 DNA is detectable and viral oncogenes are expressed in FOSCC-derived cell lines (18).
7) *Fca*PV-2 E6 and E7 oncoproteins exert transforming properties by impairing p53 and pRb pathways in feline living cells (10, 18, 22).
8) *Fca*PV-3 induces cellular changes compatible with PV-induced cancer and displays high viral load in FOSCC (13, 14).

Finally, as brilliantly summarized in a recent, excellent critical review of the literature, *Fca*PVs infection clearly emerges as a risk factor for a subset of FOSCC (∼16%). Interestingly, the authors even warn that the number of PV positive cases might be underestimated, due to: (I) DNA fragmentation occurring in formalin fixed-paraffin embedded samples causing false negative PCR results. (II) The use of consensus primers, which exert lower sensitivity than type-specific primers. (III) Infection by genotypes not detectable by the primers employed in elder studies. (IV) The possible occurrence of the "hit and run" mechanism by which the virus may initially induce cellular transformation, to then disappear and go no longer detectable (23).

3. Discussion

Among the numerous diseases classified as HNSCC (SCC of oral cavity, oropharynx, nasopharynx, larynx and upper esophagus), HPV-positive SCC shows different biological features, genetic background and molecular markers compared

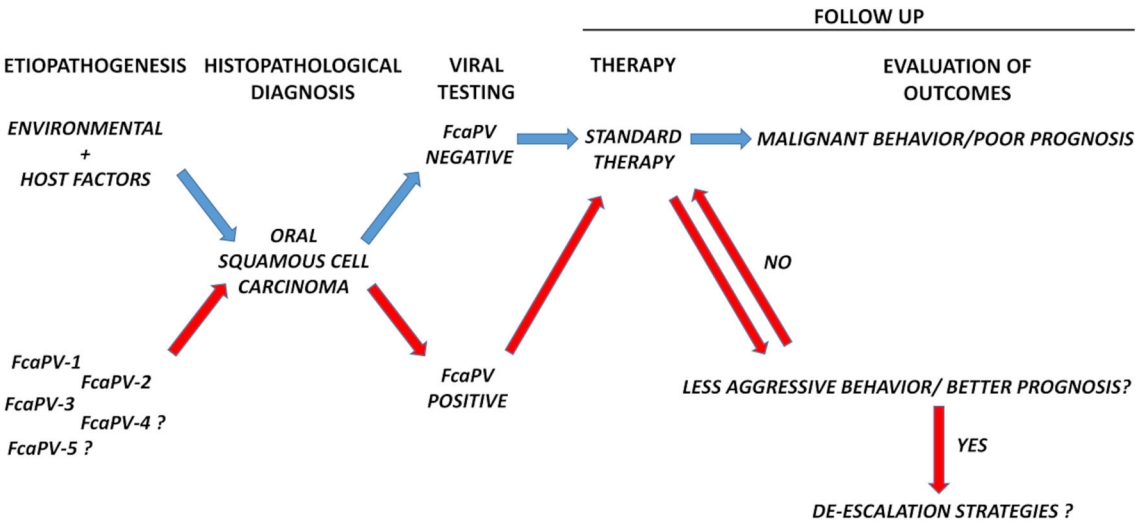

FIGURE 1
Schematic diagram showing the diagnostic workflow proposed in this article. Follow up of biological behavior and response to therapies of *Fca*PV-positive vs. *Fca*PV-negative tumors would help to understand whether, as in humans, the former constitutes a different oncological entity compared to the latter and possibly pave the way to de-escalation strategies. Viral testing would be a necessary, preliminary step before clinical handling.

to HPV-negative counterpart and is now considered as a distinct clinical entity (1, 24). Indeed, due to a less aggressive behavior combined with an improved response to therapies, the 3-year overall survival of patients bearing HPV-positive cancer is 82 vs. 57% of those affected by HPV-negative tumors (1, 24). Recent studies carried out in the context of global scale meta-analyses conducted by the International Agency for Research on Cancer (IARC) confirm improved survival in HPV positive patients, with oropharyngeal tumors driving this trend (25). These data further strengthen the rationale of numerous past, ongoing, and future studies aiming at de-intensificating therapeutic protocols against HPV-related OSCC (25, 26). The focus of these studies is to maintain high cure efficiency, reduce treatment-related toxicity, and preserve the quality of life at the same time (25, 26).

Therefore, HPV testing is a crucial step working as a link between the pathologist and the clinical oncologist toward a complete diagnostic, therapeutic and prognostic evaluation in the practice of cases of human OSCC. Immunohistochemistry (IHC) for p16 is considered a reliable test that serves as a surrogate for HPV detection, since it is a downstream effector of impaired pRb (24, 27). In doubtful cases, IHC may be further integrated by molecular tools such as *in situ* hybridization (ISH) for viral DNA and E6/E7 gene expression analysis (27).

Oral tumors are frequent in cats, among these SCC is the most common malignancy (28). Surgery, chemotherapy and radiation therapy are available as treatment options, however the prognosis is poor in most of the cases, leading to death or euthanasia (28). We still do not know whether *Fca*PV-related FOSCC may actually constitute a distinct oncological entity in terms of biological behavior as in human counterpart. There is a need to collect additional data; therefore, we ask ourselves and the broadest community of veterinary oncologists whether it is time to set-up large-scale strategies toward this goal by coordinating scientific efforts worldwide. This means to design multicentric follow up studies with the aim of monitoring the biological behavior and the evolution of these tumors over time, in order to understand whether they represent or not a different clinical subject compared to PV-negative disease (Figure 1). Evaluating the incidence of PV-related cancer in different oral sites would be of great interest as well, to see whether it preferentially develops at oropharynx also in felines. If so, this FOSCC subtype will further confirm its reliability as animal model of HPV-driven HNSCC and *vice versa* (29). Standard treatment for human HNSCC is surgery followed by high-dose cisplatin with adjuvant radiotherapy and it is widely ascertained that this protocol works particularly well for HPV positive tumors. Importantly, in IARC studies, surgery has been proven to be the most impactful treatment and further indications have emerged that chemo-radiotherapy might be de-escalated (25). Will a similar scenario be confirmed in feline species, it will be even more important to devise possible de-escalation strategies for *Fca*PV-positive patients (Figure 1). This would be achieved by setting up studies where modulating different combinations of surgery, chemo-, radio- and biological therapy to then compare primary (e.g., overall survival, disease-free survival) and secondary outcomes (e.g., loco-regional control) in differently treated experimental groups. Among the de-intensification strategies eligible for the treatment of human HNSCC, biological therapy based on the use of monoclonal antibody Cetuximab as adjuvant has been object of great attention (30). Hence, it is worth mentioning that our recent work in pre-clinical models of FOSCC has provided promising results, thus encouraging future studies in the feline counterpart (31). We imagine a future perspective where de-escalated therapies may provide advantages for cats, with a more favorable balance between therapeutic efficacy and welfare, and owners, in handling and economic terms. In such a context, viral testing would emerge as a necessary, preliminary step prior to the clinical management, as determined by the diagnostic algorithm in human practice. In this regard, a proportion of *Fca*PV-2 positive FOSCCs display p16 immunostaining, and this appears as a positive prognostic parameter in cats too. However, the role of p16 as marker of PV infection in this species is controversial (19, 23, 32), thus molecular tests (PCR and RT-PCR) would be recommendable (10, 19). Moreover, ISH would find possible application, considering that it is successfully employed for detection of *Fca*PVs DNA and mRNA in feline cutaneous SCC (33–35).

In conclusion, pending definitive data in humans, we believe it is time to begin studies in domestic feline, with the aim of studying the biology, clinical behavior and response to therapies of *Fca*PV-related FOSCC. In a future perspective, this would help to ameliorate the approach of the veterinary oncologists in terms of diagnosis, therapeutic and prognostic evaluation toward feline patients.

We are used to think of comparative oncology as the discipline in which studies on animal models pave new ways in human medicine. However, this would be the case were the "human model" traces the path for feline oncology, although we do not yet know what lies at the end of the road.

Author contributions

GA and GB drafted the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was financially supported by a grant from the Board of Directors, University of Naples Federico II.

Conflict of interest

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frontiers | Frontiers in Veterinary Science

TYPE Brief Research Report
PUBLISHED 17 May 2023
DOI 10.3389/fvets.2023.1079019

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Comparative and Clinical Medicine, a section of the journal Frontiers in Veterinary Science

RECEIVED 24 October 2022
ACCEPTED 29 March 2023
PUBLISHED 17 May 2023

CITATION
Rodney AR, Skidmore ZL, Grenier JK, Griffith OL, Miller AD, Chu S, Ahmed F, Bryan JN, Peralta S and Warren WC (2023) Genomic landscape and gene expression profiles of feline oral squamous cell carcinoma. *Front. Vet. Sci.* 10:1079019. doi: 10.3389/fvets.2023.1079019

Genomic landscape and gene expression profiles of feline oral squamous cell carcinoma

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Feline oral squamous cell carcinoma (FOSCC) is a cancer of the squamous cell lining in the oral cavity and represents up to 80% of all oral cancers in cats, with a poor prognosis. We have used whole exome sequencing (WES) and RNA sequencing of the tumor to discover somatic mutations and gene expression changes that may be associated with FOSCC occurrence. FOSCC offers a potential comparative model to study human head and neck squamous cell carcinoma (HNSCC) due to its similar spontaneous formation, and morphological and histological features. In this first study using WES to identify somatic mutations in feline cancer, we have identified tumor-associated gene mutations in six cats with FOSCC and found some overlap with identified recurrently mutated genes observed in HNSCC. Four samples each had mutations in *TP53*, a common mutation in all cancers, but each was unique. Mutations in other cellular growth control genes were also found such as *KAT2B* and *ARID1A*. Enrichment analysis of FOSCC gene expression profiles suggests a molecular similarity to human OSCC as well, including alterations in epithelial to mesenchymal transition and IL6/JAK/STAT pathways. In this preliminary study, we present exome and transcriptome results that further our understanding of FOSCC.

KEYWORDS

whole exome sequencing, feline oral squamous cell carcinoma, human head and neck cancer, variant calling comparisons, cancer

Introduction

Feline oral squamous cell carcinoma (FOSCC) is the fourth most common cancer, and the most commonly found malignant oral tumor in cats (1), with a 1-year survival rate of <10% (2). FOSCC arises from the normal squamous epithelium of the oral (1) (gingiva, tongue, and sublingual regions) cavity. FOSCC rarely metastasizes to distant locations; however, the lymph nodes can be affected in 13–31% of cases (3). Early studies indirectly implicated using flea collars, feeding predominantly canned foods in the diet to increase the risk of development of FOSCC up to 5.3-fold (3) as well as environmental tobacco smoke (4). There are currently no broadly effective treatment options for most cats with FOSCC. Surgical excision has shown to be an effective method to treat FOSCC and other oral tumors with cats having 1-year survival rates of over 80% (5, 6). Chemotherapy or radiation are additional alternatives, but most owners don't opt for these treatments due

to side effects and for most cats, the median survival time is only 2–6 months (7, 8). FOSCC presents with similar features as HNSCC, such as inflammation, spontaneous formation, heterogeneous cell environment, natural tumor, and host immune system interactions, and thus may present an opportunity to model comparative therapeutics (9, 10).

HNSCC is the sixth most common cancer worldwide, with 550,000 new cases per year, and has a 5-year survival rate of 50% (11, 12). Like FOSCC, if HNSCC is diagnosed in the early stages survival rates are much higher (2, 13, 14). Predicted risk factors for HNSCC include exposure to tobacco smoke, alcohol, and infection with HPV (13–15). Molecular similarities between the two species have been reported but these presumptions are only based on candidate gene studies in FOSCC. Both humans and cats show the perturbed function of p53, altering cell metabolism, preventing cell cycle arrest, and apoptosis (16). Gain of function in p53 has also been observed in HNSCC and is associated with enhanced tumor progression, invasive cell growth, and metastatic potential (17). Overexpression of *EGFR* is found in 69–100% of FOSCC and 90% of HNSCC cases, driving cycle progression, and facilitating the invasion of oral tissues (10, 18–20).

Naturally occurring companion animal models of cancer are becoming integral to the understanding of tumor evolution and progression (1, 6). The mouse is the standard model, yet those models lack critical factors such as spontaneous tumor formation as a result of acquiring somatic mutations in shared habitats, progressive tumor heterogeneity, and often a cancer-conditioned immune system. Understanding the genomic environment of both cat and human OSCC will help inform future translational studies. With many unknowns in the genetics of FOSCC, we aimed to characterize the mutational and transcriptional profile of FOSCC and contrast this with HNSCC. To accomplish this, FOSCC tumor tissue and matched blood samples were used for whole exome sequencing (WES), and RNA-seq was generated on FOSCC tumor tissue and oral cavity samples from healthy cats. Somatic mutations, their corresponding contribution to the tumor mutation burden (TMB), and differentially expressed genes (DEG) were assessed to compile a preliminary report of FOSCC genetics.

Materials and methods

Clinical samples

Genomic DNA isolated from six individual FOSCC tumors and matched normal peripheral blood were used for WES; RNA isolated from four of the tumors used for WES and two additional tumors, as well as three normal oral mucosa (NOM) samples from healthy cats were used for RNA-seq (Table 1). All NOM and seven of the eight FOSCC samples came from different cats archived by the Cornell Veterinary Biobank; the FOSCC and corresponding blood samples had been collected by Cornell University Veterinary Dentistry and Oral Surgery Service during standard-of-care surgical procedures (i.e., biopsy) in accordance with IACUC approved protocol #2005-0151. One FOSCC sample (605591) was collected at the Ontario Veterinary College Companion Animal Tumor Sample Bank in accordance with IACUC approved protocol #4409. FOSCC was diagnosed by histopathology in all tumor samples by a board-certified veterinary pathologist (Figure 1).

WES, variant calling, and annotation

WES was completed according to Rodney et al. (21). The Illumina NovaSeq6000 was used to generate paired-end 2 × 150 bp reads, producing an average of 80× depth of sequencing coverage. Raw sequence reads were mapped to Felis_Catus_9.0 reference using Burrows-Wheeler Aligner (BWA) v0.7.17 (22) and PCR or optical duplicates were marked using Picard tools v2.19.9 (http://broadinstitute.github.io/picard/). These files were then processed through the Genome Analysis Toolkit (GATK) v4.0.1 for base quality rescore calibration. Data is available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA844099. Mutect2 was used to identify somatic single nucleotide variants (SNVs) and insertions and deletions (indels) using the main filtering parameters of *t_lod_fstar* (filters out variants with insufficient evidence of the presence in the tumor sample) and *panel_of_normals* (filters out variants present in at least two samples in the panel of normal) (Supplementary Figure S1). The generated VCF files were further cleaned for missing data and variant allele frequencies <0.1% using VCFtools. SNVs were then annotated using Variant Effect Predictor (VEP) v101.0 (22) which classifies variants by their predicted impact on gene function. In our final call SNV and indel call set we focused on missense, frameshift (predicted changes to the amino acid), and non-sense (predicted to alter gene structure) variants. An estimation of false positives was completed using the manual viewing of IGV for a randomly chosen 20 variants per sample and confirming the presence of that variant in aligned sequences at a given base position.

Tumor mutational burden (TMB)

TMB was calculated for all six tumor samples. For each VCF file, Felis_Catus_9.0 is set as the reference and Ensembl (release 102) was used for annotation. Starting with only non-synonymous somatic mutations we followed a similar process as reported in HNSCC studies (23, 24) (Supplementary Table S1). TMB was estimated using the number of non-synonymous SNVs divided by the total feline exon probe size of 35 MB (21), which includes non-essential splice site regions (25).

Cross-reference of HNSCC mutated genes

Two resources of recurrently mutated genes in HNSCC were explored in this study: Driver Database version 3 (26) (DriverDBv3; accessed online on 3/15/21) and OcoKB (27) (version and access date of 3/15/21). The DriverDBv3 identified 263 genes total. For OncoKB substantially fewer genes ($n = 10$) we retrieved but this was due to filtering by druggable status. A table of all genes were collated and used for further investigation of the presence of mutations in these same genes within the FOSCC samples.

10.3389/fvets.2023.1079019

TABLE 1 Case description table that includes detailed patients information and assays performed.

Sample	Age (years)	Breed	Sex	Tumor/tissue location	Diagnosis	WES	Assays performed	
							RNA-seq	Library size (bp)
7741	16	DSH	MC	Mandible	FOSCC	Yes	Yes	453
9895	12	DSH	FS	Mandible	FOSCC	Yes	Yes	469
19791	20	DSH	MC	Maxilla	FOSCC	No	Yes	490
24147	12	DSH	MC	Mandible	FOSCC	Yes	Yes	532
23263	13	DSH	MC	Sublingual	FOSCC	Yes	Yes	499
28139	14	DSH	FS	Maxilla	FOSCC	No	Yes	498
26903	16	DSH	MC	Mandible	FOSCC	Yes	No	N/A
605591	10	UNK	M	Mandible	FOSCC	Yes	No	N/A
23962	1	DSH	M	Maxilla	NOM	No	Yes	505
24235	1	DSH	M	Maxilla	NOM	No	Yes	559
23927	1	DSH	M	Maxilla	NOM	No	Yes	479

DSH, domestic shorthair; UNK, unknown; M, male; MC, male castrates; FOSCC, feline oral squamous cell carcinoma; NOM, normal oral mucosa; FS, male castrated; RQN, RNA quality number; bp, base pairs; N/A, not applicable.

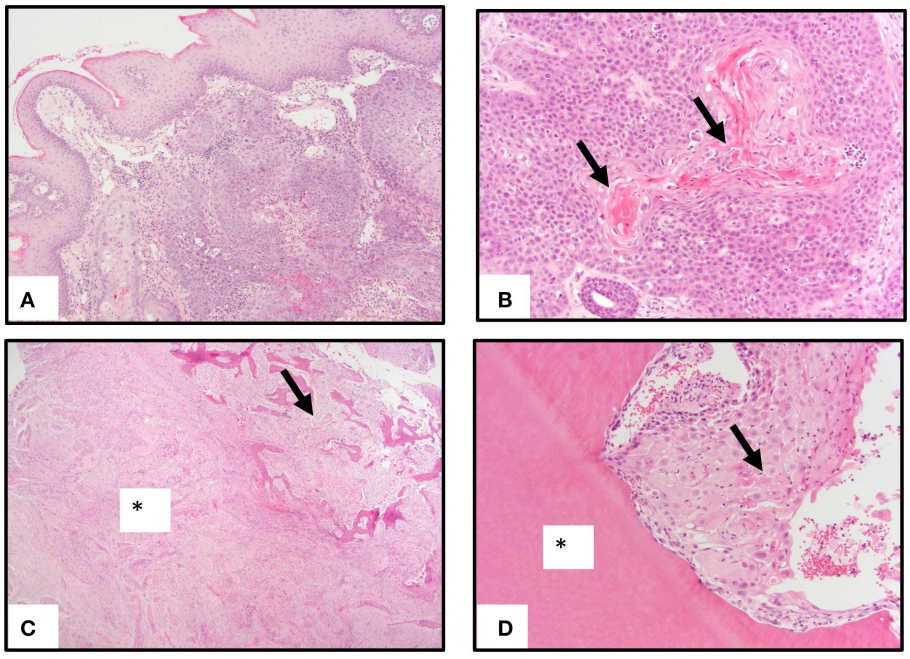

FIGURE 1
Pathology of oral squamous carcinoma. **(A)** Underlying a moderately hyperplastic gingival epithelium are ribbons, cords, and trabeculae of neoplastic squamous epithelial cells. **(B)** Neoplastic squamous epithelial cells surround and produce brightly eosinophilic keratin (arrows). **(C)** Neoplastic squamous epithelial cells that are enmeshed in abundant scirrhous responses (asterisk) are associated with marked bony invasion and remodeling (arrow). **(D)** Neoplastic squamous epithelial cells with dyskeratosis (arrow) invade the dentin layer of a tooth (asterisk).

RNA sequencing

Frozen tissue (~1 g) was homogenized in 2 mL of Trizol (Thermo Fisher) using 2.8 mm ceramic beads (Hard Tissue Homogenizing Mix, VWR). RNA was extracted from four FOSCC samples (9895, 24147, 23263, and 7741) following the Trizol protocol provided by the manufacturer (Thermo Fisher), and then treated with DNAse followed by cleanup with the RNA Clean and Concentrator-25 kit (Zymo Research). For all other samples, RNA was extracted with a modified Trizol method as follows: after the addition of chloroform and phase separation of the Trizol lysate, the aqueous phase was combined with an equal volume of 100% ethanol and loaded onto a Zymo-Spin column (Quick-RNA Prep Kit, Zymo Research). RNA samples were washed and eluted following the Quick-RNA Prep Kit protocol. For all samples, RNA concentration was measured with a Nanodrop (Thermo

Fisher), and integrity was determined with a Fragment Analyzer (Agilent). Because of variable RNA quality across samples (RQN range: 1.3–8.8), whole-transcriptome RNA-seq was conducted after depleting ribosomal RNA. rRNA was depleted with the NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat; New England Biolabs) using 500 ng input total RNA. RNA-seq libraries were prepared with the NEBNext Ultra II Directional library prep kit (New England Biolabs) and single-end 85 nt reads were generated on a NextSeq500 instrument (Illumina), resulting in an average of 25.1 M reads per sample (minimum 19.1 M).

RNA-seq analysis

Raw reads were trimmed for low-quality and adaptor sequences and filtered for minimum length with TrimGalore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), a wrapper for cutadapt (28) and fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) using parameters "–nextseq-trim = 20 -O 1 -a AGATCGGAAGAGC –length 50 –fastqc." Trimmed reads were mapped to the reference genome/transcriptome (Ensembl felCat9) with STAR (29) using these parameters: "–outSAMstrandField intronMotif, –outFilterIntronMotifs RemoveNoncanonical, –outSAMtype BAM SortedByCoordinate, and –quantMode GeneCounts," which also generated raw count outputs per annotated gene. Sample clustering and differential gene expression were analyzed with SARTools (30) and DESeq2 (31) using these parameters: "fitType parametric, cooksCutoff TRUE, independentFiltering TRUE, alpha 0.05, pAdjustMethod BH, typeTrans VST, and locfunc median." Feline gene symbols were converted to human gene symbols using Biomart (Ensembl) one-to-one orthology assignments to enable analysis of MSigDB (32) and custom human gene sets. The human ortholog gene symbols and log2-fold-change values for expressed genes (at least one group with average normalized counts > 50) were used for GSEA (33) "Preranked" analysis. Heatmaps of leading-edge genes were generated in R (d3heatmap) using row-normalized counts.

Results

SNV annotation

VCF files were annotated using VEP and filtered to remove any synonymous and intronic variants. In total, we found 809 non-synonymous SNVs in the FOSCC exome with a mean of 176 per sample (Supplementary Figures S2, S3). SNV annotation with VEP resulted in 56 non-sense, 19 frameshift mutations, 8 stop loss, and 731 missense calls. After a manual variant review, we estimated a false call rate of 5% (Supplementary Figure S4). Only one gene, *TP53*, the most commonly mutated gene in cancer, including HNSCC (16), was recurrently mutated with 5/6 of FOSCC samples containing a missense SNV at different positions in the DNA-binding domain (Figure 2).

Since TMB has emerged as a predictive biomarker of human patient stratification toward immunotherapy for some cancer types we evaluated TMB in FOSCC. Using our small cohort, the estimated TMB mean for each tumor was 3.7 with a range of 1.4–8.5. This was comparable to TMB calculations in HNSCC, with most tumors falling between three and seven (23). We were not able to determine if survival or response to immunotherapy was associated with TMB score due to a lack of data and immune therapeutics specific to the cat.

Differential gene expression, cluster analysis, and functional enrichment analysis

Of the 19,588 protein-coding genes annotated in Felis_catus_9.0 (Ensembl release 105), we found 2,372 differentially expressed genes (DEG; $p < 0.05$) in FOSCC ($n = 6$) when compared to normal oral mucosa ($n = 3$); of these, 1,388 genes were upregulated and 984 were downregulated (Supplementary Table S2). Principal component analysis demonstrated all samples clustered according to phenotype (Supplementary Figure S5), indicating that the primary global signal in the gene expression profiles distinguishes tumor from normal samples, regardless of anatomical location or variations in sample processing or quality. The most enriched gene sets using the GSEA MSigDB Hallmark collections are characteristic of pathways activated in cancer (Supplementary Table S3, Supplementary Figure S6). The epithelial-mesenchymal transition (EMT) gene set was the most enriched gene set, as well as signatures of pathway activation (TNFA, KRAS, IL6) and immune response was also observed in the TNFA, KRAS, hypoxia, and IL-6 signaling gene sets among others. Notably, a custom gene set consisting of the 200 most upregulated and downregulated genes in human OSCC (34) had higher normalized enrichment scores than any Hallmark gene set upregulated in human OSCC: NES 8.64, q-value reported as zero; downregulated in HOSCC: NES −4.07, q-value reported as zero; Figure 3.

FOSCC comparison with HNSCC

We compared the somatic mutations of FOSCC genes shown to have highly recurrent mutations in HNSCC to assess similarities. Genes harboring FOSCC non-synonymous SNVs, including frameshifts, were matched to the same genes in the HNSCC Driver Database 3 (35), and OncoKB (36). *TP53* was the most recurrently mutated somatic gene in HNSCC and FOSCC, with three FOSCC samples containing a missense mutation, and one sample containing a frameshift mutation (Figure 2). A missense mutation in *KAT2B, HOX3B, MED12L, ARID1A, and KMT2D* was present in one sample (Supplementary Table S4). Samples 9895 and 23263 had the most genes implicated in HNSCC with four genes in common each (Supplementary Table S4). This data indicates some evidence for overlap in the mutational background between HNSCC and FOSCC but is very preliminary at this stage.

Discussion

In this first study of the somatic mutations and gene expression variation present in FOSCC, we describe the similarities and differences when comparing to HNSCC, its most closely related

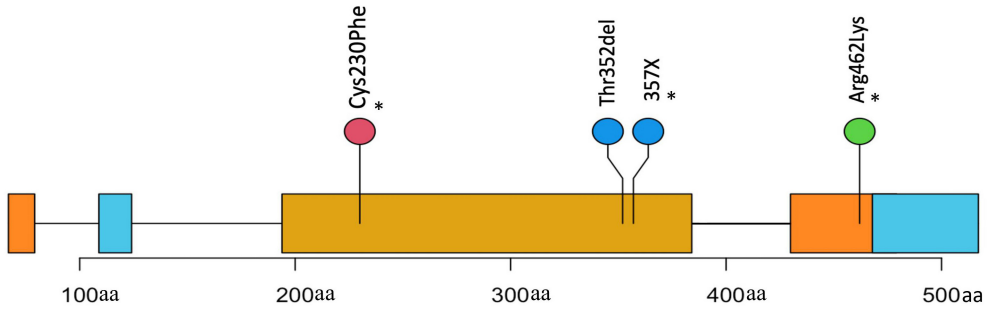

FIGURE 2
Positions and description of P53 somatic mutations in 4 samples. Three mutations(*) were missense causing an amino change in two samples and an unknown change in another (X). One mutation is a frameshift causing a deletion in the protein coding sequence.

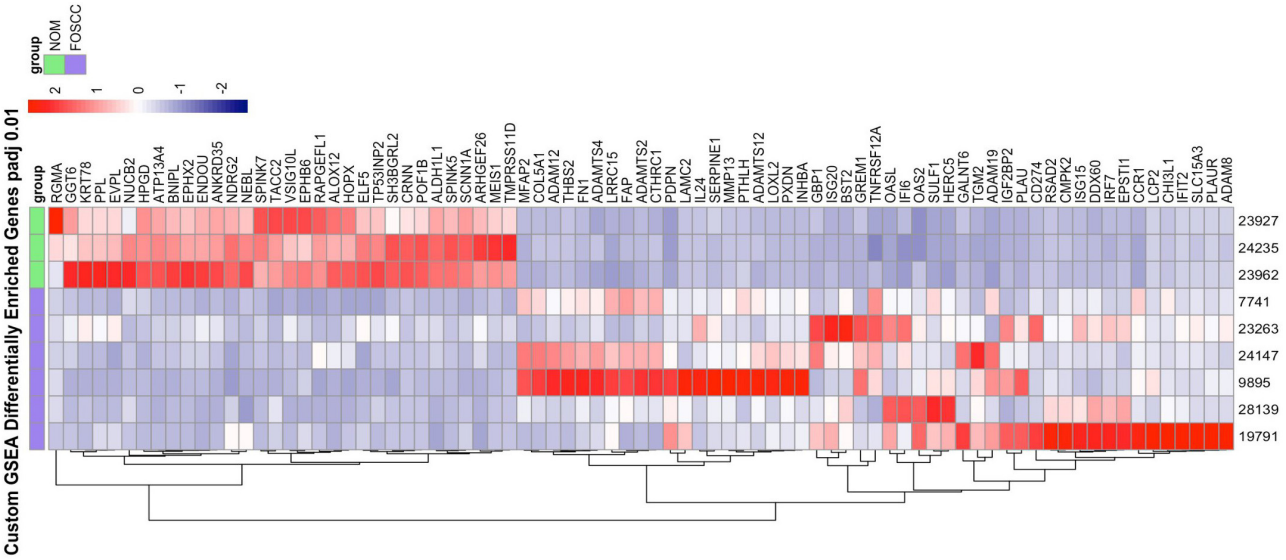

FIGURE 3
The heat map on the right illustrate the expression patterns of the top 50% leading edge genes known* to be upregulated and downregulated in human oral squamous cell carcinoma; the heat maps show clear differences between FOScc (first 6 columns) and control samples (last 3 columns), and to a much lesser extent, variation within group. NOM, normal oral mucosa; FOSCC, feline oral squamous cell carcinoma; GSEA, GENE set enrichment analysis. *Sun Y, Sang Z, Jiang Q, Ding X, Yu Y. Transcriptomic characterization of differential gene expression in oral squamous cell carcinoma: a meta-analysis of publicly available microarray data sets. Tumor Biol. 2016.

cancer type in humans. Although FOSCC is the most common oral tumor in domestic cats, our knowledge of its genetic properties is very limited. The few characterized molecular features of FOSCC include overexpression of EGFR and perturbed *p53* expression (19, 37). When examining the commonalities of FOSCC and HNSCC, overexpression of *EGFR* in 90% of 750 HNSCC tumors sequenced, and *TP53* mutated in 41% of cases is also observed (38). We hypothesized that altered genes in FOSCC would be known to be implicated in HSCC, and would present alternate candidates for hypotheses testing of mechanisms of action (35). We find several mutated genes in common (Supplementary Table S4) the strongest candidate being *TP53,* which is the most mutated in HNSCC and all cancer types (2, 39–41). Given that *TP53* is a tumor suppressor gene and variation in *TP53* and is a predictive marker for immunotherapy in HNSCC (16) it is reasonable to hypothesize that if feline immune checkpoint drugs were available their use in the rapidly growing FOSCC could be efficacious. In agreement with our findings, *p53* mutations have been seen in other FOSCC studies (4, 37).

Immunotherapy is not an option in feline cancer treatment today but may likely be available in the future. Since TMB has been shown to be useful in some human cancer types, e.g., HHNSCC non-small cell lung cancer and melanoma (42–44), we sought to compare FOSCC to HNSCC for this metric despite our small cohort size. Recent studies on HNSCC have found that mutations in *TP53* are associated with high TMB and low overall survival rates, and coincidently HNSCC patients with high TMB have higher response rates to immune checkpoint therapy (23, 24). Moreover, HNSCC with TMB values of >5.0 is associated with poor prognosis (45, 46). Two of our FOSCC samples fall into what we suggest is a high TMB value at 8.5 and 6 and if immune checkpoint therapy was available could be evaluated for tumor control. But we recognize this is

very speculative at this stage of evaluating FOSCC genetics. More FOSCC sequencing experiments to obtain better estimates of TMB are needed as well as the future availability of immune checkpoint inhibitors to improve outcomes for this very lethal cancer.

Other FOSCC recurrent or single gene mutations of interest we evaluated included *KAT2B, ARID1A, MED12L,* and *HOXB3* mostly due to their involvement in cell growth. *KAT2B* is a member of the lysine transferases that are responsible for the acetylation of a broad range of proteins that can function as tumor suppressors or oncogenes (47). HNSCC cell lines have shown universal loss of *KAT2B* (11) and HNSCC tumors show significantly lower *KAT2B* expression compared to normal tissue (48). One FOSCC sample had a missense variant in *KAT2B* (Supplementary Table S4) and it was significantly downregulated in our RNA-seq data (Supplementary Table S4) suggesting a candidate driver gene role in FOSCC. A *KMT2D* mutation was identified in only one sample but has similar epigenetic properties to *KAT2B* (49). Studies using The Cancer Genome Atlas database (TCGA) have associated mutations occurring in *KMT2D*, to the open chromatin state, thereby promoting gene expression (50, 51). Mutations in *KAT2B* and *KMT2D* may induce epigenetic changes in both HNSCC and FOSCC that could advocate for the treatment with epigenetic drug control of both (47, 51). A missense mutation in *ARID1A* was found in one sample (Supplementary Table S4), however, this gene did not show altered expression in FOSCC. *ARID1A* functions as a tumor stemness repressor by disrupting the function of the p53 or PTEN pathways (52, 53). This gene is often deleted in many human cancers (52) yet with no known role in feline cancers.

Several other candidate genes (*MED12L, HOXB3,* and *PXYLP1* with one gene mutation per sample) were found to overlap in HNSCC and FOSCC (Supplementary Table S4). Mediator Complex Subunit 12L (*MED12L*) works by activating the kinase activity of *CDK8* which regulates the growth and division of cells (54). *MED12L* is differentially overexpressed in many cancers however, the *MED12L* complex is altered in only 3.05% of HNSCC patients (55, 56). The *HOX* genes regulate a wide range of cell activity including proliferation and migration thus making their contribution to FOSCC beyond interpretation at this stage. HNSCC studies have shown an overall increased expression of all *HOX* genes, including *HOXB3* (57–59). No overlap in actionable genes mutated in FOSCC was found when searching OncoKB relative to HNSCC. We believe this is due to the low sample size of our cohort which only additional sequencing can address.

Altered gene expression is often used to discover and advance gene candidates for further study of their oncogenic roles. Transcriptional profiling showed that FOSCC is enriched with genes known to be upregulated in Human OSCC (34) suggesting conserved gene regulatory mechanisms. While these findings were predicted given the remarkable clinical, pathological, and genetic parallels between both tumors (9, 37, 60–62) they in no way confirm comparative origins or outcomes. The functional gene and canonical pathway enrichment analyses provided us further insight into the possible activation of several pathways that were seen in FOSCC that included EMT, hypoxia- and inflammation-related pathways (61–65), indicating the complementary value of gene expression analysis in this study. EMT typically involves the expression of transcription factors that can activate this cellular program (i.e., *SNAI1*, *TWIST1*, *ZEB1*, and *ZEB2*), and is characterized by the upregulation of mesenchymal-related genes (e.g., *FN1*, *VIM*, *CDH2*, and metalloproteinases) and downregulation of epithelial-related genes (i.e., *CDH1* and cytokeratins) (65–67). These studies are insightful into the aggressive biological behavior of FOSCC in part due to the activation of the EMT program. Not surprisingly activation of the hypoxia and angiogenesis pathways, as well as several inflammation pathways are predicted to be implicated in oral cancer with rapid tumor growth creating areas of ischemia and necrosis, and the production of reactive oxygen species (62). These events also induce the expression of transcription factors that can stimulate angiogenesis while simultaneously eliciting a local inflammatory response inducing expression of *PTGS2* (i.e., COX-2), with signaling *via* NF-kB, TNF, IL-6, and TGFB, all of which were found to be significantly enriched in FOSCC.

In the context of the recurrent *TP53* somatic mutations observed, it was interesting to note that the p53 pathway was not significantly activated across the set of FOSCC tumors compared to healthy controls (GSEA FDR > 0.2). This observation suggests that *TP53* mutations in FOSCC represent loss-of-function events and that the mechanisms that would normally result in p53 pathway activation are likely impaired. Consistent with cancer studies in people (68), *TP53* and relevant target genes (e.g., *CDKN1A*) known to be expressed in wild-type cells were found to be downregulated in FOSCC, while genes that would be expected to be upregulated in *TP53* mutated cells (e.g., *E2F1*, *MYBL2*, and *FOXM1*) were enriched in FOSCC. Overall, these findings strongly implicate *TP53* somatic mutations as driver events in FOSCC tumorigenesis.

Another aspect of FOSCC revealed by RNA-seq was the variation among tumor expression profiles. Indeed, although cluster analysis revealed a distinct molecular phenotype when compared to control samples, we observed differences among tumor samples among individual genes as well as for relevant biological pathway activity (Figure 3, Supplementary Figure S6). This tumor heterogeneity may reflect inter-individual differences (i.e., variations in tumor stage, anatomical location, breed, age, other clinical and environmental factors, etc.), and inherent variations among primary clinical samples of naturally occurring disease, which are comprised of heterogeneous cell types and frequencies and are typically collected in a clinical setting rather than under strict research protocols.

There are several limitations to this small study, with the most important factor being cohort size. Only six samples were available at the time of sequencing, therefore conclusions between potential causal variants in the cat in common with the human were found less frequently. Another limitation of the study is that healthy mucosal tissue from case animals was not available to use as matched RNA-seq controls, due to limitations of the clinical sampling protocol. Tumors also originated from different anatomical locations and were collected and processed at different times, which can contribute to transcriptional variation. Lastly, aside from *TP53*, the common mutations identified with functional effects have not been studied at length and the implication of variants in these genes are unknown. Further study is in progress in a larger cohort of cats where more significant conclusions may be drawn.

Conclusion

The first exome resource was used to evaluate the somatic mutational landscape of FOSCC and gene expression changes were identified using RNA-seq. We observed several mutations in *TP53*, consistent with what is seen in HNSCC, and several other genes also overlapped between FOSCC and HNSCC. Our study suggests that similar genes initiate tumorigenesis in both species and perhaps FOSCC may serve as a comparative model of treatment in HNSCC. Similarities in the mechanism of FOSCC and HNSCC shown in the RNA-seq studies, such as genes implicated in inflammation further demonstrate the possible use of the domestic feline as a model for HNSCC.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

WW and JB provided samples for analysis. AR completed variant calling and analysis. ZS, OG, and SC assisted in variant calling and analysis. JKG and SP completed sample collection as well as RNA-sequencing analysis. All authors contributed to the article and approved the submitted version.

Funding

This work was funded in part by the EveryCat Foundation under Award Number W21 002 (to SC). OG was supported by the National Cancer Institute (NCI) of the NIH under Award Number K22CA188163 and by the Siteman Cancer Center and The Foundation for Barnes-Jewish Hospital. The funders had no role in the study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

Some of the cryopreserved samples and associated phenotypic data were provided by the Cornell Veterinary Biobank, a resource built with the support of NIH grant R24 GM082910 and the Cornell University College of Veterinary Medicine.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1079019/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 October 2023
DOI 10.3389/fvets.2023.1254458

OPEN ACCESS

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RECEIVED 07 July 2023
ACCEPTED 03 October 2023
PUBLISHED 16 October 2023

CITATION
Bae H, Kim S-K and Yu D (2023) Comparative analysis of the aberrant immunophenotype and clinical characteristics in dogs with lymphoma: a study of 27 cases.
Front. Vet. Sci. 10:1254458.
doi: 10.3389/fvets.2023.1254458

Comparative analysis of the aberrant immunophenotype and clinical characteristics in dogs with lymphoma: a study of 27 cases

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Introduction: Aberrant lymphoma phenotypes are frequently found in dogs, but the clinical implications are sparse.

Methods: Twenty-seven dogs with aberrant lymphoma diagnosed using flow cytometry between 2017 and 2023 were analyzed. Major paraneoplastic syndromes, prognostic factors, and clinical features of lymphoma were compared to their immunophenotypes.

Results: Twenty-seven dogs had aberrant immunophenotypes, with MHCII- (48%) and CD3+/CD21+ (44%) being the most commonly identified aberrancies. In B-cell lymphoma, the most frequent aberrancies were MHC II- (53%), CD3+/CD21+ (41%), CD34+ (24%), and CD79a- (24%). Meanwhile, in T-cell lymphoma, CD3+/CD21+ (63%), CD4-/CD8-(50%), CD5- (50%), and CD45- (50%) were the most common. The platelet–neutrophil ratio was significantly higher in the CD3+/CD21+ group than in the other groups, where either one or both markers were not expressed (55.23±39.64; 18.72±14.95, respectively; $p=0.001$). Serum albumin concentration was significantly lower in the MHCII-group (2.59g/dL, 95% CI 2.31–2.87) than in the MHCII+ group (3.06g/dL, 95% CI 2.88–3.23; $p=0.009$). CD34 expression showed significant correlations with cranial mediastinal mass, WHO clinical substage, and fever ($p=0.028$, $p=0.041$, and $p=0.047$, respectively). MHCII expression was correlated with adverse reactions to chemotherapy, cranial mediastinal masses, and fever ($p=0.009$, $p=0.023$, and $p<0.001$, respectively). No statistically significant differences in the survival period were observed for any of the phenotypic aberrancies.

Conclusion: Aberrant lymphomas are common in dogs. Some clinical prognostic factors that significantly correlate with aberrant immunophenotypes have been identified and can be applied clinically.

KEYWORDS

lymphoma, immunophenotyping, flow cytometry, aberrancies, dog

1. Introduction

When compared to experimental animals, naturally occurring diseases in dogs could reflect human diseases such as cancer. Studying these similarities can provide valuable insights into disease mechanisms, treatments, and potential therapeutic targets for dogs as well as humans.

Immunophenotyping plays a crucial role in the accurate diagnosis and classification of canine lymphoma, similar to human lymphoma. Despite of limited availability of commercially specific dog antibodies, a significant prognostic factor based on the immunophenotyping in canine lymphoma has been established (1–3). The immunophenotypes of lymphoma in dogs is categorized according to the origin of B-and T-cells, with representative markers of CD21/CD79a and CD3/CD4/CD8 commonly used in dogs. Furthermore, various phenotypes have been identified in detail, such as CD45 in all leukocytes, CD34 in precursor hematopoietic cells, and major histocompatibility complex class II (MHCII) in antigen-presenting cells. Among these, aberrant phenotypes characterized by either increased or decreased expression of specific antigens are well established in both human and veterinary medicine (4–7). Several studies are being conducted to explore the possibility of utilizing immunophenotypes, including various aberrancies, for clinical purposes and prognosis prediction in humans (8–13).

Clinical prognostic factors such as World Health Organization (WHO) substage, mediastinal lymphadenopathy (14), and paraneoplastic syndrome (PNS) (15) have been studied in canine lymphoma, but the clinical implications of aberrant phenotypes have yet to be studied in dogs.

Thus, the aims of this study were [1] the identification of aberrant phenotypes in dogs with various types of lymphoma and [2] the investigation of associated types with the severity of clinical signs, PNS, and prognosis of the aberrant phenotypes.

2. Materials and methods

A retrospective *in vitro* analysis of the clinicopathological parameters and immunophenotypes of dogs with lymphoma was conducted using lymph node aspirates and peripheral whole blood samples collected at the time of diagnosis. This study was approved by the Institutional Animal Care and Committee (IACUC) GNU-230425-D0087.

Among the dogs diagnosed with lymphoma that visited Gyeongsang National University Veterinary Teaching Hospital between 2017 and 2023, 35 dogs that were immunophenotyped by flow cytometry were included in this study. The inclusion criteria were as follows: [1] dogs diagnosed with lymphoma through the following diagnostic procedures: cytology, histopathology, immunophenotyping, and clonality test (through fine needle aspiration (FNA) or a biopsy sample of enlarged lymph nodes or target lesions); [2] dogs without underlying diseases other than lymphoma that may affect hematological changes; and [3] dogs with naïve lymphoma who had not received chemotherapy prior to admission or dogs with relapsed lymphoma six months after the last chemotherapy. Both nodal and extranodal forms were included, and all dogs were staged according to the WHO staging system (16). Cytologic grading was evaluated according to the updated Kiel classification (17, 18). Histopathology and polymerase chain reaction for antigen receptor rearrangement were requested from an external laboratory (IDEXX, Westbrook, ME, United States), while cytology and immunophenotyping using flow cytometry.

The classification criteria for each lymphocyte lineage in immunophenotyping were as follows: [1] B-cell lymphoma, if the tumor cells were CD21+ (>70% of the major cells) and the T-cell marker was negative; [2] T-cell lymphoma, if the tumor cells were CD3+ (>70% of the major cells) and the B-cell marker was negative; and [3] non-B and non-T lymphomas, if the tumor cells were negative for both B-cell and T-cell markers.

Phenotypic aberrancies were defined as follows: [1] reduced or absent expression of pan-leukocyte or lymphocyte markers (CD45 and MHCII), [2] simultaneous expression of lymphocyte markers of different lineages (CD3 and CD21) or different stages of differentiation (markers of mature stage and CD34), [3] in T-cell, CD4 and CD8 markers were expressed simultaneously, or neither was expressed, [4] in B-cell, loss of CD79a, which is expressed in all maturation stages of B-cells (7, 19, 20).

At the time of diagnosis, the presence of PNS was assessed in all dogs to determine the clinical course and prognosis. The correlation between WHO substage 'b' (clinically ill), anemia, hypercalcemia, thrombocytopenia, and immunophenotype was analyzed. Survival time was defined as the period from the date of diagnosis at our hospital to the day of death due to lymphoma. Dogs that died from causes unrelated to the tumor or whose follow-up was discontinued were considered 'censored'. Treatment response was evaluated in dogs that had received chemotherapy or equivalent medication and was divided into complete remission (CR), partial remission (PR), progressive disease (PD), and stable disease (SD) according to previous literature (21).

Upon admission, laboratory examinations were performed to assess the overall clinical condition of the dogs and to detect any underlying diseases, including PNS. A complete blood count (Procyte Dx hematology analyzer, IDEXX, Westbrook, ME, United States) and blood film analysis, including platelet-to-lymphocyte ratio (PLR), platelet-to-neutrophil ratio (PNR), lymphocyte-to-monocyte ratio (LMR), and neutrophil-to-lymphocyte ratio (NLR) were performed. Acid–base balance and electrolyte concentrations (Nova pHOX analyzer, Nova Biochemical, Waltham, MA, United States), serum biochemical analysis (Catalyst Dx® Chemistry Analyzer, IDEXX, Westbrook, ME, United States), coagulation tests (Coag Dx™ analyzer with citrate PT and citrate aPTT cartridges, IDEXX), and complete urinalysis (VetLab UA Analyzer, IDEXX) were also performed. The fibrinogen levels were evaluated using the Millar's method (22).

FNA aspirates were collected from the prescapular or inguinal lymph nodes of dogs with generalized lymphadenopathy, and peripheral blood was collected to evaluate WHO staging. For cases of extranodal lymphoma, FNA aspirates were collected from regional lymph nodes suspected of involvement near the target lesions (stomach, intestinal segments, liver, spleen, and cutaneous lesions) and pleural or abdominal free fluids were also collected. All aspirates were suspended in 0.3–0.5 mL of 5% dextrose saline, and peripheral blood was collected in ethylene-diamine-tetraacetic acid tubes. All samples were analyzed within 24 h of collection by the same operator, and those with ambiguous diagnoses, low cellularity, or low viability were excluded.

The antibodies used in this study were based on previous studies (23). Multi-color flow cytometric analysis was conducted to evaluate the contemporary expression of different antigens within the same cellular population. The sample preparation and analysis method was similar to previous studies (6), with the exception that CD14-negative cells were sorted (CD14 is expressed on monocytes and macrophages), and only lymphocytes were selected using the difference in granularity and lymphocyte-specific markers. The major cells were first identified through cytology, and flow cytometric analysis was primarily performed on lymphocytes, which constituted the largest population (>60%). Lymphocytes exhibiting a tumorigenic phenotype, even at a small percentage, were also analyzed. The samples were acquired using the MACSQuant Analyzer 10 (Miltenyi Biotech, Bergisch Gladbach, Germany) and analyzed using FlowJo software version 10.8.0 (Ashland, OR, United States).

All statistical analyses (Student's t-test, Mann–Whitney U test, Fisher's exact test, and Kaplan–Meier curve) were performed using SPSS Statistics version 27.0, for Windows (SPSS Inc., Chicago, IL, United States). Clinical data on admission, including signalment, temperature, body weight, presence of abnormalities in hematological parameters, and clinical substages, were evaluated for their impact on survival time using Kaplan–Meier estimators and log-rank tests. For Fisher's exact test, clinical, clinicopathological, and immunophenotypic data were dichotomized and evaluated. The p values were two-sided and were considered significant at $p < 0.05$.

3. Results

3.1. Study population and prevalence of immunophenotypic aberrancies

The characteristics of the dogs included in this study are summarized in Supplementary Table S1. Among the dogs initially recruited for the study, 27 were included, all of whom showed immunophenotypic aberrancies with immunophenotyping by flow cytometry at the time of diagnosis. The mean (± standard deviation) age of 27 dogs was 9 ± 3.5 years (range, 3–15 years). The breeds included Maltese ($n = 7$), Mixed ($n = 5$), Shih-Tzu ($n = 4$), Golden Retriever ($n = 3$), Miniature Poodle ($n = 2$), Yorkshire Terrier ($n = 1$), Dogo Canario ($n = 1$), Dachshund ($n = 1$), Chihuahua ($n = 1$), Cocker Spaniel ($n = 1$), and Shiba ($n = 1$). The multicentric lymphoma was the most common ($n = 24$), followed by as an extranodal lymphoma, the alimentary ($n = 1$), tongue ($n = 1$), and liver ($n = 1$) forms were identified. When classified by WHO clinical stage, one was WHO stage I, two were stage III, 13 were stage IV, and 11 were stage V. Of the 27 dogs, 11 were WHO substage 'a' (asymptomatic) and 16 were substage 'b' (clinically ill).

A total of 21 dogs received chemotherapy after being diagnosed with lymphoma. Among these, 14 dogs underwent the L-CHOP protocol, three dogs were administered chlorambucil, and protocols CHOP, COP, and doxorubicin alone were applied to one dog each. Two of the dogs that received the L-CHOP protocol, died of tumor progression and were lost to follow-up immediately after chemotherapy in the first week (L-asparaginase and vincristine). One dog that was treated with chlorambucil showed a poor response and was treated with a combination of prednisolone, imatinib, and cyclophosphamide. Upon evaluation of the response after chemotherapy, five dogs with CR, six dogs with PR, four dogs with SD, and 8 with PD were identified.

On cytological examination, 12 dogs had large cells, 7 dogs had intermediate cells, and five dogs had small cells. In three dogs, intermediate and large cells were identified as heterogeneous. Among the 22 dogs that could be analyzed by the updated Kiel classification, five dogs (23%) were found to have low-grade lymphoma and 17 dogs (77%) were found to have high-grade lymphoma (Supplementary Tables S2, S3). In the low-grade lymphoma group, there were three clear cells, one prolymphocytic-like cell, and one centrocytic-like cell type. In the high-grade lymphoma group, there were seven centroblastic polymorphic (predominantly large cells), three Burkitt-type, three plasmacytoids, two pleomorphic, one centroblastic polymorphic (predominantly small cells) cell, and one anaplastic lymphoma. Of the eight dogs that could be diagnosed through histopathological and immunohistochemical methods, diffuse large B-cell lymphoma (DLBCL) was identified in five, TZL in two, and diffuse small B-cell lymphoma in one.

A total of 27 dogs were identified as having an aberrant immunophenotype (Table 1). Among the total aberrancies,

TABLE 1 Aberrant phenotype epidemiology.

Aberrancy	Percentage (aberrant/tested) (%)			
	Total (n = 27/35)	B-cell (n = 17/23)	T-cell (n = 8/10)	Non-B & non-T-cell (n = 2/2)
CD3+/CD21+	12/27 (44%)	7/17 (41%)	5/8 (63%)	0/2
CD3-/CD21-	2/27 (7%)	1/17 (6%)	0/8	1/2 (50%)
CD4+/CD8+	1/27 (4%)	0/17	1/8 (13%)	0/2
CD4-/CD8-	5/27 (19%)	–	4/8 (50%)	1/2 (50%)
CD5+	–	0/17	–	1/2 (50%)
CD5-	–	–	4/8 (50%)	1/2 (50%)
CD79a-	-	4/17 (24%)	–	0/2
CD45-	4/27 (15%)	0/17	4/8 (50%)	0/2
CD34+	5/27 (19%)	4/17 (24%)	1/8 (13%)	0/2
MHCII-	13/27 (48%)	9/17 (53%)	3/8 (38%)	1/2 (50%)

MHCII, major histocompatibility complex class II.

MHCII- (13/27, 48%) and CD3+/CD21+ (12/27, 44%) were the most frequently identified, whereas 19% of these dogs (5/27) did not express CD3 or CD21. All 22 dogs that could be analyzed by the updated Kiel classification showed immunophenotypic aberrancies. Among the dogs that showed aberrancy, five were found have low-grade lymphoma, and 17 were found to have high-grade lymphoma, with the high-grade lymphoma group showing more immunophenotypic aberrancies. CD3+/CD21+ (4/5, 80%) and MHCII- (7/17, 41%) were the most frequently identified in low-and high-grade lymphomas, respectively. The most represented aberrancies in B-cell lymphoma were MHCII- (9/17, 53%), CD3+/CD21+ (7/17, 41%), CD34+ (4/17, 24%), CD79a- (4/17, 24%), while CD3+/CD21+ (5/8, 63%), CD4-/CD8- (4/8, 50%), CD45- (4/8, 50%), CD5- (4/8, 50%) were expressed in T-cell lymphoma. In the two cases showing the non-B/non-T phenotype, CD3-/CD21-, CD4-/CD8-, expression of CD5, and MHCII-were confirmed at a rate of 50%. In both cases, the canine natural killer cell (NK cell) marker, NKp46, was highly expressed, suggesting NK cell-derived lymphoma.

3.2. Correlation with aberrant immunophenotype and clinicopathologic, paraneoplastic syndrome measurements

As a result of analyzing the clinical measurement in the CD3+/CD21+ group, which was identified as most frequent among the aberrant phenotypes, the PNR was significantly higher in the group identified as CD3+/CD21+ than in the other groups (either one or both were not expressed) (55.23 ± 38.52; 18.13 ± 17.12, respectively; $p = 0.003$), CD45-group than in CD45+ group (90.01 ± 43.30; 26.85 ± 22.66, respectively; $p = 0.007$). NLR was significantly lower in the CD45-group (7.45 ± 10.58; 0.47 ± 0.22; $p = 0.041$). The ionized calcium concentration was also significantly higher in the group identified as CD3+/CD21+ than in the other groups (1.32 mmol/L, 95% CI 1.29–1.35; 1.24 mmol/L, 95% CI 1.19–1.29, respectively; $p = 0.01$). Serum albumin concentration was significantly lower in the group of MHCII- (2.59 g/dL, 95% CI 2.31–2.87) than in the group of MHCII+ (3.10 g/dL, 95% CI 2.94–3.26; $p = 0.005$) (Figure 1).

When analyzing the association between phenotypic aberrancies and clinical measurements, the expression of CD34 showed significant correlations with cranial mediastinal lymphadenopathy, WHO clinical substage, and fever ($p = 0.017$, $p = 0.037$, and $p = 0.033$, respectively). Cranial mediastinal lymphadenopathy was present in four out of the five dogs (80%) that expressed CD34, whereas only three of the 18 dogs (16.7%) that did not express CD34 had cranial mediastinal lymphadenopathy. Five (41.7%) of 12 dogs with substage "b" expressed CD34, whereas none of the 11 dogs with substage "a" expressed CD34; four out of five dogs (80%) with CD34+ had fever, whereas only four of 18 dogs (22.2%) with CD34-had fever. The odds of cranial mediastinal lymphadenopathy were 20 times higher in CD34+ than those in CD34-dogs. CD34+ dogs were 2.6 times more likely to be evaluated as WHO clinical substage "b"

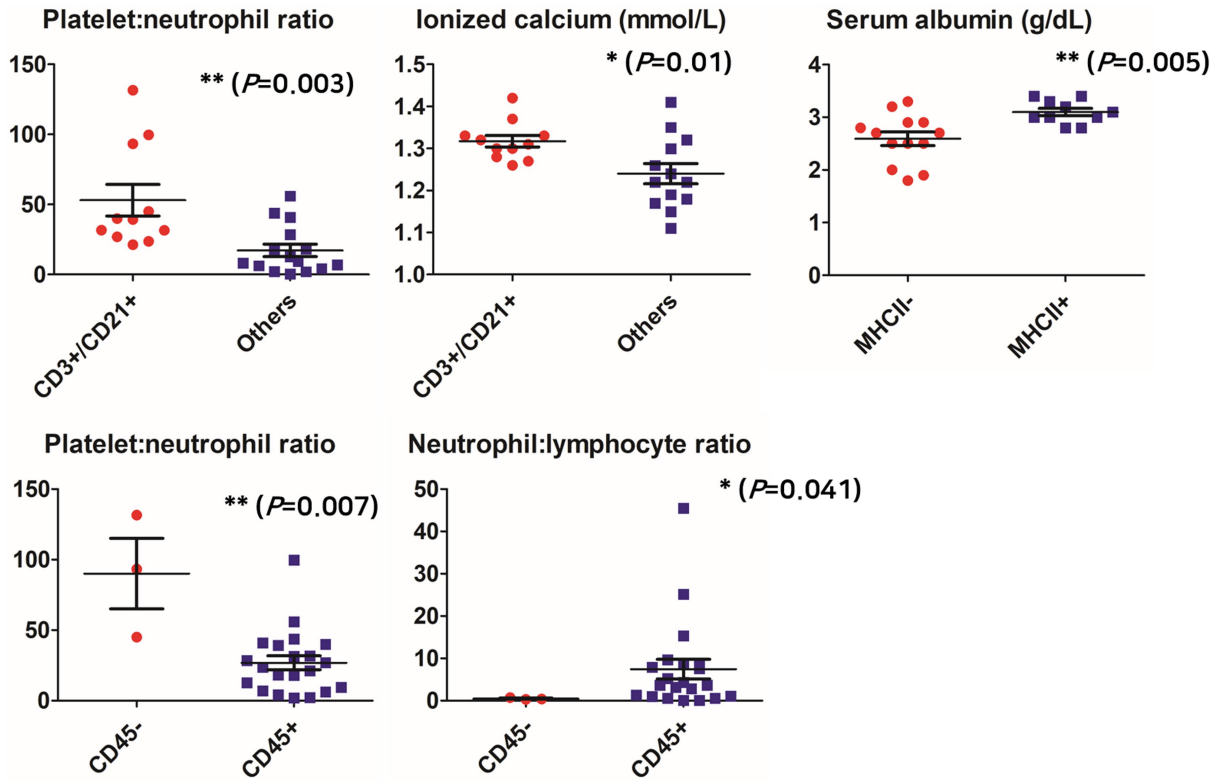

FIGURE 1
Comparison of clinicopathological parameters according to aberrant phenotypes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

TABLE 2 Association analysis between prognostic factors of lymphoma and aberrant immunophenotypes using Fisher's exact test.

	Frequency (%)		Total
Cranial mediastinal lymphadenopathy	CD34+	CD34-	
Yes	4 (57.1%)	3 (42.9%)	7
No	1 (6.3%)	15 (93.8%)	16
Fisher's exact test (*P*) [95% CI]	0.017 [1.613–247.981]		
WHO clinical substage	CD34+	CD34-	
a	0 (0%)	11 (100%)	11
b	5 (41.7%)	7 (58.3%)	12
Fisher's exact test (*P*) [95% CI]	0.037 [1.441–4.589]		
Fever	CD34+	CD34-	
Yes	4 (50%)	4 (50%)	8
No	1 (6.7%)	14 (93.3%)	15
Fisher's exact test (*P*) [95% CI]	0.033 [1.200–163.367]		
Chemotherapy adverse reactions	MHCII+	MHCII-	
Yes	7 (77.8%)	2 (22.2%)	9
No	2 (22.2%)	9 (81.8%)	11
Fisher's exact test (*P*) [95% CI]	0.022 [1.754–141.404]		
Fever	MHCII+	MHCII-	
Yes	7 (100%)	0 (0%)	7
No	3 (18.8%)	13 (81.3%)	16
Fisher's exact test (*P*) [95% CI]	<0.001 [1.923–14.790]		

than CD34-dogs, and their odds of having fever were 14 times higher.

A significant correlation was observed between the presence of MHCII expression and fever ($p < 0.001$), and the presence of chemotherapy adverse reactions ($p = 0.022$). Fever was present in seven of 10 MHCII+ dogs (70%), whereas none showed fever in the 13 MHCII-group. Chemotherapy adverse effects were present in seven of nine MHCII+ dogs (77.8%), whereas only two of 11 dogs with MHCII-showed chemotherapy adverse reactions. The odds of adverse reactions to chemotherapy were 16 times higher in the MHCII+ group than in the MHCII-group, and the odds of expressing MHCII were 5.3 times greater when fever was present than when fever was absent (Table 2).

3.3. Survival analysis

The overall median survival time was 365 days (range 1–1,138 days). Ten dogs died due to lymphoma, while two dogs died due to other reasons unrelated to the tumor. Twelve dogs survived the entire study duration, and follow-up was discontinued for three dogs.

No statistically significant differences in the survival period were observed for any of the phenotypic aberrancies. The survival time was significantly shorter in the substage "b" group ($p = 0.006$) and in the group with anemia and monocytosis among those with PNS ($p = 0.028$ and $p = 0.024$, respectively) (Supplementary Table S4).

4. Discussion

Companion animals serve as excellent models for human diseases, particularly spontaneously occurring cancers, which reflect similar pathobiologies and comorbidities. Dogs and humans share common cytogenetic and clinical features, pathology, tumor biology, tumor behavior, and genetic aberrations in the case of lymphoma (24, 25). In this study, flow cytometry was used to analyze immunophenotypes, an important prognostic factor in dogs diagnosed with lymphoma, and aberrancies were identified in 77% of the dogs (27/35 dogs). Dogs with high-grade lymphoma classified by the updated Kiel classification showed much more immunophenotypic aberrancies than ones with low-grade lymphoma. Furthermore, correlations between clinical, hematological, and serological findings were identified in dogs with aberrant phenotypes. As hypothesized, aberrancies associated with prognostic markers of lymphoma and PNS were identified, but no significant difference in survival time was observed according to the aberrant phenotype.

Previous studies have reported a slight difference in the incidence of aberrant phenotypes depending on the definition used. Specifically, Celant et al. found that 12% (310/2,612) of dogs had specific antigen aberrancies (20), while Wilkerson et al. reported an incidence of 22% (5). The incidence of loss of MHCII expression or low expression, which was the most frequently identified aberrancy in our study, has been reported to be approximately 14–72% in previous studies (2, 11). Additionally, the co-expression of CD3 and CD21 has been reported to range from as low as 0.7% (20), to as high as 31–50% (5, 19), while one study reported no cases of CD3/CD21 co-expression (26).

The MHCII proteins are specifically expressed on professional antigen-presenting cells such as B lymphocytes, monocytes, and dendritic cells (27). The MHCII gene expression signature suggests that antigen presentation to the immune system plays a significant role in therapeutic responses (27). The reduced MHCII expression may hinder sufficient tumor immunosurveillance, which could have contributed to the unfavorable outcome (27). In this study, the loss of MHCII expression was identified in the seven dogs with high-grade morphotypes (7/17, 41%). Although this study did not find a significant difference in survival time depending on MHCII expression, several previous human and dog studies have stablished its potential use as a prognostic factor.

CD3 is a complex molecule associated with the T-cell receptor and is expressed during maturation in early thymocytes (5). It is a representative marker expressed in T-cell lymphocytes and is present in all stages of T-cells from early precursor T-cells to mature T-cells that enter the circulation and lymph nodes. On the other hand, CD21 is a marker of mature B-cells, and when immature B-cells naïve to antigen exposure are released from the bone marrow, CD21 antigen is expressed on the surface (28).

In this study, the most frequently identified aberrancies in all lineages were double positive for CD3 and CD21. The co-expression of different lineage markers is a characteristic feature of tumors that cannot be identified in reactive lymph nodes (19). Four of the five dogs with low-grade lymphoma showed CD3+/CD21+, and three of them showed clear cell types and were diagnosed with TZL in histopathology. Eight dogs co-expressed CD21 among T cells, of which four dogs were presumed to have TZL and one dog had clinically aggressive multicentric T-cell lymphoma (no biopsy available). These were expected results since TZL is low grade indolent

lymphoma and expresses CD21, as previously reported (29). However, in the case of the other dog, it was not clinically indolent. Six out of seventeen dogs with high-grade lymphoma showed CD3+/CD21+ expression, which is assumed to be aberrant expression.

CD21-positivity has already been described in canine T-cell neoplasms in the past (5, 19). Since it is a tumor cell, it is possible that the phenotype of the antigen expressed during the maturation stage of lymphocytes is altered. It is also possible that these two types of tumors occurred concurrently. One dog with CD3-expressing B-cell lymphoma was histopathologically diagnosed with DLBCL. However, in the clonality test, both T and B cells were confirmed to be clonal, suggesting that both tumor lineages occurred concurrently.

The loss of CD45, CD5, and CD4/CD8 double negativity was the second most common abnormality in T cells. CD45 is a transmembrane protein tyrosine phosphatase that serves as a common leukocyte marker due to its expression in all leukocytes irrespective of lineage (30). CD45 loss has also been regarded as a tumor hallmark in previous studies (19, 31). Among the histologic subtypes of lymphoma, CD45 is a characteristic finding of TZL, and as mentioned above, is clinically indolent. In our study, two dogs with CD45-were histopathologically diagnosed with TZL. One of the two dogs without histopathology had a tongue mass as the primary complaint and exhibited an immunophenotype of CD3+/CD4-/CD8+/CD21+/CD45-in the regional lymph nodes. Although the phenotype of the tongue mass was not analyzed, cytology confirmed that the main population was small lymphocytes (data not shown). The possibility of TZL originating from the tongue is also high in this dog, considering a previous case of TZL in the tongue (32). However, in another study, 5.3% of T-cell not otherwise specified dogs were identified as CD45- (20). Although this study did not find any aggressive lymphoma with CD45-, it is expected that the frequency of this aberrant type will increase as the population size increases.

T lymphocytes differentiate from double-negative (CD4-/CD8-) thymocytes to double-positive (CD4+/CD8+) cells before leaving the thymus and evolving into CD4+ and CD8+ cells (7). Since CD4 and CD8 are not expressed in mature T-cells, which are marked by CD3+, the phenotype observed in this study was considered aberrant. The CD4-/CD8-phenotype has been in various anatomical forms of T-cell lymphoma. For example, the phenotype of neoplastic lymphocytes in cutaneous epitheliotropic lymphoma was CD4-/CD8+ or CD4-/CD8-(33), and CD4-/CD8+ or CD4-/CD8-patterns were also identified in hepatosplenic and hepatocytotropic lymphoma (34). Regarding the survival period, a recent study found that dogs with a CD4-/CD8-/MHCII+ phenotype had a relatively long progression-free interval (2), while another study reported a more aggressive progression (35). Among lymphoma in dogs, lymphoblastic lymphoma, which has the most aggressive progression, has been shown to express CD4-/CD8-or CD4+/CD8+ (36). In this study, there was no significant difference in survival time, and no significant association with prognostic factors was confirmed. The four dogs with the CD4/CD8 double-negative phenotype did not show any common anatomical tumor regions.

In B-cells, MHCII-was identified in approximately 53% of cases, followed by CD3+/CD21+ in 41%, CD34+ in 24%, and CD79-in 24%. As previously mentioned, low MHCII expression is well known as one of the most reliable indicators of poor outcomes in human B-cell lymphoma (27, 37). Studies in dogs with B-cell lymphoma have also demonstrated an association between low MHCII expression and high mortality and relapse rates (11). There is evidence to suggest that MHCII expression is correlated with more robust immunosurveillance in B-cell lymphomas, as well as longer survival in T-cell lymphomas (12). However, in studies investigating MHCII expression in T-cell lymphoma, it remains unclear whether it can be used as a prognostic factor, as dogs with strong MHCII expression have been shown to have shorter survival times (2). Additionally, no difference in survival time was found depending on MHCII expression in a cohort of DLBCLs in a later study (38). Nevertheless, in this study, there was a strong correlation between MHCII expression and clinical signs and PNS caused by lymphoma, such as fever and cranial mediastinal lymphadenopathy, which suggests that MHCII expression may impact the severity or prognosis of the disease.

CD34 is a well-known hematopoietic precursor cell marker that has been clinically used to distinguish between acute lymphoblastic leukemia, chronic lymphocytic leukemia, and the leukemic stage (Stage V) of lymphoma (28). Although aberrant expression of CD34 has been observed in lymphoma without bone marrow involvement, its biological significance remains unclear (5, 6, 39). In case of precursor lymphoma, antigens expressed in lymphocytes at a relatively early stage of differentiation, including CD34, may also be identified. In this study, 19% (5/27) of dogs showed CD34 expression, and all showed high-grade morphotypes in the updated Kiel classification. Four of them were positive for CD21, a mature B-cell marker, suggesting aberrant expression in mature B-cell lymphoma. Among the T cells, one dog was identified as CD34+ and CD4-/CD8-, suggesting the possibility of precursor lymphoma. In previous studies, the expression rate of CD34 was 10–29%. When CD34 is co-expressed with CD21, a mature B-cell marker, as in this study, it is considered an aberrant phenotype (5, 11). Unfortunately, due to the lack of histopathology and a bone marrow analysis, the WHO classification and bone marrow infiltration was unknown. However, in the case of CD34+ B-cell lymphoma, considering various clinical and immunophenotypic characteristics, and tumor course, the possibility of acute leukemia or precursor lymphoma was estimated to be low. The response to chemotherapy of dogs with CD34+ B-cell lymphoma was poor, as it recurred during the induction (L-CHOP) protocol. Despite conducting a rescue protocol, the disease was considered progressive with a survival time of 99 days.

Next, the relationship between the phenotypic aberrations and clinical measurements was analyzed. The clinical significance of blood cell ratios as a biomarker has already been accepted for several diseases in humans, and investigations are being conducted on tumors of various origins in dogs. Inflammation plays a fundamental role in lymphomagenesis and tumor progression, and vice versa, and can lead to changes in peripheral blood leukocyte composition (neutrophil, monocyte, and lymphocyte, especially), depending on the severity and extent of inflammation (40, 41). PNR was confirmed as an independent prognostic factor in dogs diagnosed with DLBCL, with a cutoff value of 0.032; a higher value increases the risk of tumor progression before 180 days (42). Platelet–neutrophil interactions in malignant conditions are directly related to PNR, especially in humans and dogs with lymphomas presenting malignant hypercoagulability as a frequent PNS (42–45). In our study, PNR was significantly higher in the CD3+/CD21+ and CD45-group than in the group without this phenotype. Although there was no statistically significant difference in survival time among the PNR, CD45, and CD3+/CD21+ phenotypes, dogs with a low PNR generally tended to be 'clinically ill' upon admission. However, this was not related to tumor progression,

and the relationship with phenotypic aberrancies could not be confirmed. Additionally, NLR was higher in the CD45+ group than in the CD45 + group. In human non-Hodgkin's lymphoma, an NLR of 3.5 or higher was identified as a negative prognostic factor (46, 47). In this study, two out of four dogs with CD45-were diagnosed with TZL, and both had lymphocytosis at diagnosis, with neutrophils within the reference range. The difference in NLR is speculated to be largely due to the influence of lymphocytosis. However, the low NLR compared to lymphomas showing aggressive clinical features (e.g., DLBCL, peripheral T-cell lymphoma not otherwise specified) is probably due to the mild degree of tumor-induced inflammatory response (neutrophil change) and host immunity change (lymphocyte change) compared to aggressive lymphoma. This is presumed to be in line with the clinically indolent type and clinical signs similar to those of aggressive lymphomas, such as generalized lymphadenopathy and lymphocytosis, but different progression is thought to be related to changes in the blood cell ratio. The TZL population was too small to detect differences between TZL and other lymphomas or between TZL and healthy dogs.

Compared to the serum chemistry data, the concentration of ionized calcium was significantly higher in the CD3+/CD21+ group. However, their clinical relevance remains unknown. Among the dogs studied, two had hypercalcemia, which was evaluated as PNS due to parathyroid hormone related hormone, while one was found to have hypocalcemia. Several significant results were found in the Fisher's exact test, most of which were related to PNS. Neoplastic fever is thought to be due to the innate immune response to a tumor antigen or the development of necrotic cells within the tumor, and is particularly common in hematopoietic cancers such as lymphoma (14). In this study, a significant difference in the presence or absence of fever was identified according to MHCII expression ($p < 0.001$). This is thought to be related to the function of MHCII and the mechanism of its upregulation in dogs with tumors. Cytokines such as TNF-α, IFN-γ, and IL-1 upregulate MHCII expression (48), and these cytokines also activate the arachidonic acid cascade to produce prostaglandin E_2, which acts on the thermoregulatory center of the hypothalamus to regulate the development of fever (49). Although the direct causal relationship between MHCII expression and fever is unknown, further research is needed to determine the clinical relevance of MHCII as a negative prognostic factor and fever as a PNS. There was a correlation between MHCII expression and adverse reactions to chemotherapy as well. When MHCII was expressed, the risk of chemotherapy adverse reactions was higher than when it was not expressed. Most adverse reactions were myelosuppression, mainly neutropenia. Grade III or IV chemotherapy-induced neutropenia is known as a favorable prognostic factor of lymphoma in dogs (50, 51). Likewise, although the direct relationship between MHCII expression and chemotherapy adverse reactions could not be identified, it is presumed that there is an unknown mechanism between them.

In the case of CD34+ lymphoma, it can be presumed to be a precursor-derived or remaining aberrancy in the differentiation stage of tumor cells or leukemic involvement in high-grade lymphoma (52). WHO clinical substage "b" refers to a state with clinical signs such as lethargy, inappetence, weight loss, polyuria/polydipsia, or fever due to lymphoma, and it is a negative prognostic factor for lymphoma (1, 53). In this study, all 11 dogs in WHO substage "a" did not express CD34, while five out of 12 dogs (41.7%) in substage "b" expressed CD34. One of the five dogs expressing CD34 was of T-cell origin, and the other was of B-cell origin. There was no statistically significant difference in survival time, likely due to the small sample size, but four of the CD34+ dogs showed rapid tumor progression after or during the induction protocol (L-CHOP). Chemotherapy response in all four dogs was assessed as PD, and euthanasia was performed, or the dogs died due to tumor progression. When the correlation with cranial mediastinal masses, another negative prognostic factor, was analyzed, CD34 was expressed in four out of seven dogs (57.1%) with masses and only one (6.3%) out of 16 dogs without masses. Again, a causal relationship between these factors could not be confirmed, but it is presumed that there is a possible clinical association between CD34 expression, response to chemotherapy, and the progression-free interval.

When the Kaplan–Meier curve was used to analyze survival time based on the aberrant phenotype, no statistically significant difference in median survival time was observed for any phenotype. Previous studies have shown that the prognosis of T cells is poorer than that of B cells, but this study did not identify any differences in survival time according to immunophenotype. Although the dogs with high-grade lymphoma in the updated Kiel classification showed more immunopheonotypic aberrancies, it was not confirmed if it is related to the prognosis. There are prior studies that show that the prognosis is related to specific morphotypes with the updated Kiel classification (54). However, this study did not confirm the association with detailed morphotypes, immunophenotypic aberrations, and prognosis due to the small population. The survival period was significantly shorter in the PNS group, particularly in dogs with anemia and monocytosis. These results align with previously known negative prognostic factors for lymphoma. The small sample size and cases where tumor progression was not confirmed due to loss during follow-up may have contributed to these findings.

This study had some limitations associated with its retrospective nature. First, due to the small number of individuals, there were limitations in analyzing various aspects of phenotypic aberrations, which are known to have a particularly low frequency. In this study, two out of 19 dogs were identified as having non-B, non-T cell origin, and both were confirmed to be positive for the NK cell marker NKp46. Therefore, NK cell lymphoma could be diagnosed. However, it was difficult to compare the clinical characteristics because there were only two dogs. Second, there are still a few antibodies produced that target dogs for flow cytometry, and the majority of those used in this study are antibodies that cross-react with human, rat, or mouse cells. In this case, a false expression could be observed because of the risk of nonspecific binding. However, in this study, this was unlikely because the background cells, including neutrophils, monocytes, and reactive lymphocytes, did not show nonspecific binding to each antibody. Additionally, there are few antibodies available to further subdivide the phenotypes within the same lineage. By identifying phenotypic changes according to lymphocyte maturation stage and comparing them with clinical measurements, subtypes that are not yet widely known in veterinary medicine, such as precursor lymphoma, can be identified. Third, the WHO classification based on histopathology and bone marrow analysis remains a key tool for the diagnosis and classification of lymphoma and distinguishing it from bone marrow-originated diseases such as leukemia. In particular, a bone marrow analysis is necessary to determine whether the CD34 expression identified in this study is an aberrant immunophenotype, precursor lymphoma, or acute lymphoblastic leukemia, and peripheral blood

tests are insufficient. Although it is not possible to evaluate the treatment response or prognosis using these methods alone, a clear evaluation is important as histological classification and grading is a strong prognostic factor. In clinical practice, surgical resection is not often attempted as diagnosis can typically be achieved to some extent with less invasive cytology and immunophenotyping. Future studies in populations with secured pathology results may enable accurate analysis between prognostic factors and clinical measurements. Such studies are expected to help our understanding of various subtypes of lymphoma in dogs by analyzing how the functions that are changed by aberrant antigen expression are clinically expressed at the gene expression level.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by Gyengsang National University, IACUC no. GNU-230425-D0087. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

HB: Conceptualization, Resources, Software, Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. S-KK: Data curation, Formal analysis, Resources, Writing – review & editing, Funding acquisition, Validation. DY: Funding acquisition, Resources, Validation, Writing – review & editing, Conceptualization, Project administration, Software, Supervision.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture and Forestry (IPET) through Companion Animal Life Cycle Industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (322092-04-1-HD030).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1254458/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 November 2023
DOI 10.3389/fvets.2023.1258247

OPEN ACCESS

EDITED BY
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RECEIVED 13 July 2023
ACCEPTED 12 October 2023
PUBLISHED 16 November 2023

CITATION
Brambilla E, Ciaputa R, Crepaldi P, Dzimira S, Nowak M, Dziegiel P, Piotrowska A, Mollica Govoni V, Fonseca-Alves CE, Laufer-Amorim R, Stefanello D, Romussi S and Grieco V (2023) Canine urothelial carcinoma: expression of Periostin in spontaneous canine urothelial carcinoma and its correlation with histological features. *Front. Vet. Sci.* 10:1258247. doi: 10.3389/fvets.2023.1258247

Canine urothelial carcinoma: expression of Periostin in spontaneous canine urothelial carcinoma and its correlation with histological features

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The tumor microenvironment is considered one of the main players in cancer development and progression and may influence the behavior of cancer cells. Periostin (POSTN) is an extracellular matrix protein, and its main functions are induction of fibrillogenesis, fibroblastic cell proliferation and migration, enhancing regeneration in normal tissue, and promoting metastasis in case of neoplasia. POSTN has already been studied in humans in several normal tissues, inflammatory processes, and neoplasms, revealing an important role in tumor progression in various types of cancer, such as colon, lung, head and neck, breast, ovarian, and prostate. In these latter, high levels of POSTN are usually associated with a more aggressive tumor behavior, tumor advanced stages, and poor prognosis, while in human bladder urothelial carcinoma (BUC), unlike in most tumors, POSTN expression seems to be downregulated. The expression of this marker has been poorly investigated in veterinary medicine; thus, this study aimed to immunohistochemically investigate the presence and the intensity of POSTN expression in canine BUCs and to determine a possible relationship between POSTN expression and histopathological features such as mitotic count and muscular and vascular invasions. For the present retrospective study, archived samples from 45 canine BUCs and 6 non-neoplastic canine bladders were considered for histological evaluation and immunohistochemical examination for the expression of POSTN. POSTN expression was semi-quantitatively assessed considering both the percentage of the neoplastic stroma positive for POSTN and the intensity of the immunohistochemical labeling. Histologically, 38 out of 45 tumors were papillary and 7 out of 45 were non-papillary. All tumors were infiltrating, being that 21 were muscle-invasive, and a significant correlation between this feature and vascular invasion emerged ($P = 0.0001$). In normal bladder tissue, as reported in humans, a thick and strongly positive belt of POSTN was visible, and in canine BUCs, stating that the expression is comparable with

human benign as well as malignant bladder tissue, a general decrease in POSTN expression was observed except for a strongly labeled ring of POSTN observed around some neoplastic nodules infiltrating the muscle layer. Moreover, POSTN expression and mitotic count were significatively inversely correlated ($P = 0.0015$). The fact that POSTN protein is less expressed in urothelial carcinomas than in the normal bladder supports what was reported in human BUCs and, together with the negative correlation between mitotic count and protein expression that emerged in the present retrospective study, encourages further prospective follow-up studies to verify the possible role of POSTN in canine BUCs as a prognostic marker, and also as a possible target for the development of future anticancer therapies.

KEYWORDS

POSTN, dog urothelial carcinoma, urinary bladder, canine urothelial carcinoma, cancer associate fibroblasts (CAFs)

1 Introduction

The tumor microenvironment can be defined as everything that surrounds tumor cells that are not the tumor cells themselves and is considered nowadays as one of the main players in cancer development and progression (1). The tumor microenvironment is highly complex and consists of non-tumor cells (i.e., cancer-associated fibroblast, endothelial cells, or infiltrating leukocytes) and a large list of proteins and soluble factors such as extracellular matrix (ECM) proteins, and soluble components such as hormones, growth factor, and cytokines (1, 2). The way that microenvironment components interact among them and with the tumor cells is very complex and only partially understood (1, 3).

Tumor cells can alter the microenvironment by changing the properties of the host tissue and vice versa, and the composition of the tumor microenvironment may influence the behavior of cancer cells (2).

Periostin (POSTN) is a non-structural protein of the (ECM), previously named osteoblast-specific factor-2, which was first identified in 1993 as a putative adhesion protein for preosteoblasts in a mouse osteoblastic MC3T3-E1 cell line (4–6). This protein was then named POSTN since it was identified in the periosteum and in periodontal ligament, teeth, and cardiac valves that physiologically undergo mechanical stress during tissue regeneration and development (3). Investigation into heart diseases such as myocardial infarction and cardiac hypertrophy revealed two key POSTN functions. The first one is represented by the induction of fibrillogenesis stimulating type one collagen production and cross-linking and then inducing fibrosis (7). The second main action consists of the activation of cell migration through the integrin binding, which stimulates fibroblastic cell proliferation and migration (8), enhancing regeneration in normal tissue and promoting metastasis in case of neoplasia.

POSTN plays a central role in the homeostasis of normal tissues by regulating cell differentiation and proliferation (1). POSTN is physiologically mainly produced by stromal cells such as myofibroblasts, osteoblasts, and bone marrow-derived mesenchymal stromal cells. POSTN is present in a wide variety of normal adult and fetal tissues, such as embryonic periosteum, periodontal ligament, placenta, cardiac valves, adrenal glands, lung, thyroid, stomach, colon, vagina, ovary, testis, prostate, breast, and urinary bladder (6, 9, 10). In particular, a belt of periostin in the bladder stroma beneath the surface epithelium has been identified (11). POSTN is also produced by cancer-associated fibroblasts (CAFs), and its expression seems to be upregulated in several pathologies, such as inflammation, tissue repair, and malignant transformation (12). Tumor cells, especially cancer stem cells, can also produce POSTN, which has been shown to regulate multiple biological behaviors of tumor cells, including proliferation, survival, invasion, angiogenesis, metastasis, and chemoresistance (13).

In human medicine, studies on several cancer types, such as non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), malignant pleural mesothelioma (MPM), breast cancers (1, 14, 15), and others, have demonstrated that high POSTN levels are usually associated with a more aggressive tumor behavior, advance stage, and poor prognosis, suggesting that POSTN levels could be a useful prognostic biomarker (1, 9, 16–21).

Unlike in most of these latter tumors, POSTN expression appears to be downregulated in human BUCs compared with normal tissue (11).

The expression of this marker has been poorly investigated in canine-bearing tumors. Only two studies focusing on canine mammary tumors (22, 23), one on squamous cell carcinoma and one on osteosarcoma, are present in the literature (24, 25). One of the two studies regarding canine mammary tumors (22) demonstrated a positive correlation between the expression of POSTN in CAFs in mammary carcinomas, the tumor grade, and the expression of the Ki-67 proliferative antigen, suggesting a role of POSTN in the pathogenesis of canine mammary tumors as in humans. Similarly, in canine squamous cell carcinoma and osteosarcoma, an increased expression of POSTN was detected in the neoplastic stroma.

Bladder cancer comprises 1.5–2% of all naturally occurring cancers in dogs, a rate similar to that reported in humans (26). Dogs with invasive BUCs recently were proposed as a "large animal" model of invasive BUCs in humans because they show similar invasive behavior, morphology, and metastasis location (26).

State of that, this study aimed to immunohistochemically investigate the presence and the intensity of POSTN expression

in canine BUCs to determine an eventual relationship between levels of POSTN expression and histopathological features such as tumor type, tumor infiltration, vascular invasion, mitotic count, and tumor grade.

2 Materials and methods

2.1 Samples and histology

For the present retrospective study, histological slides and related paraffin blocks from 45 cases of canine BUCs and 6 non-neoplastic canine bladders, the latter from necropsied dogs that died from trauma, were retrieved from the archives of the Pathology Unit of the Veterinary Medicine and Animal Sciences Department of the University of Milan, from the archives of the Pathology Department of the Veterinary Faculty of the Wroclaw University of Environmental and Life Sciences, and from the Veterinary Pathology Service of the São Paulo State University in Brazil. BUC samples belonged to 45 dogs. Breed and sex were known in 44 out of 45 cases examined (98%). Breeds were so represented: mixed breed dogs (18) (41%), Yorkshire terriers (3) (7%), poodles (3) (7%), West Highland White terriers (3) (7%), Miniature Schnauzers (2) (5%), Labrador retrievers (2) (5%), Dachshunds (2) (5%), Bernese mountain dogs (2) (5%), Drahthaar (1) (2%), hound (1) (2%), American cocker spaniel (1) (2%), Beagle (1) (2%), Siberian husky (1) (2%), Large Swiss shepherd dog (1) (2%), Bull terrier (1) (2%), and English bulldog (1) (2%). A total of 18 (41%) out of 44 were male dogs (one of which was neutered), while 26 (59%) were female dogs (spayed in 7 cases). The age of the dogs, which was known in 43 out of 45 (96%) cases, ranged from 7 to 17 years with an average of 11 years (1st quartile = 10 years; 3rd quartile = 12 years).

Samples were derived from excisional biopsies (31 cases), cystoscopic biopsies (13 cases), and necropsy (1 case). Biopsies smaller than 5 mm were excluded from the caseload. All samples had been fixed in 10% buffered formalin, and during trimming, a complete longitudinal section was routinely processed for histology. In brief, samples were dehydrated in graded alcohols, clarified in xylene, and embedded in paraffin. From the paraffin block of each sample, serial 5 microns thick sections were obtained. One section was stained with hematoxylin–eosin to be histologically evaluated and classified using the WHO 2004 classification of domestic animal tumors (27). During the histological examination, tumors were graded based on the two more cited grading systems for canine BUCs: Valli et al. (28) and Meuten and Travis (29). Moreover, tumor extension (through the thickness of the bladder), whether it invaded only the chorion or extended to the muscular layer, mitotic count (number of mitoses in 10 high-magnification fields, corresponding to an area of 2.37 mm^2), and presence of neoplastic cell within vessel lumina (vascular invasion) were recorded.

2.2 Immunohistochemistry

Staining was performed on a LEICA BOND-MAX (Leica Biosystems, UK) according to the following protocol. First, tissues were deparaffinized (Bond Dewax Solution, Leica Biosystems, UK) and pre-treated with the Bond Epitope Retrieval Solution 1 (Leica Biosystems, UK) for 20 min. The activity of the endogenous peroxidase was blocked by peroxide block using the BOND Polymer Refine Detection System (Leica Biosystems, UK). A polyclonal antibody directed against human POSTN (NBP1-82472, Novus Biologicals) was used as the primary antibody. The protein BLAST analysis using the human POSTN sequence against the canine database demonstrated 97.76% identity (274 of 377 amino acids), with zero gaps (0%), between the human and canine POSTN sequences, being the genes for *POSTN* (POSTN) conserved in different species including dogs and humans. The anti-POSTN antibody, diluted 1:100 in the Bond Primary antibody Diluent (Leica Biosystems, UK), was applied for 15 min at room temperature. Next, the samples were incubated with Post Primary and Polymer using the BOND Polymer Refine Detection System (Leica Biosystems, UK). The 3,3-diaminobenzidine (DAB Chromogen) was applied as the substrate for the reaction, and then all the sections were counterstained with Hematoxylin (BOND Polymer Refine Detection System, Leica Biosystems, UK). A section of a canine mammary tumor was used as a positive control, while negative controls were obtained by substituting the primary antibody with rabbit normal serum at the same protein concentration as the primary antibody.

TABLE 1 The semiquantitative score applied for the assessment of the immunohistochemical extension and intensity of the expression of POSTN in the tumor stroma.

Percentage of neoplastic stroma positive for POSTN	Points
0	0
1–5%	1
6–20%	2
21–50%	3
51–60%	4
>60%	5

Immunohistochemical results were assessed within each complete longitudinal tumor section using a semiquantitative score (Table 1), considering the percentage of tumor stroma positive for POSTN in relation to the total tumor stroma included in the complete longitudinal tumor section. Furthermore, the intensity of the immunohistochemical labeling was defined as absent (0 points), weak (1 point), moderate (2 points), and intense (3 points). Where the signal intensity was not homogeneous, the percentage of tumor stroma expressing POSTN with the highest intensity was considered.

2.3 Statistical analysis

Statistical analysis was performed by JMP. We calculated the descriptive statistics (absolute and relative frequencies) of the categorical variables collected as breed, sex, tumor type, muscular infiltration, vascular invasion, tumor grading, and POSTN expression score. We also described the distribution of

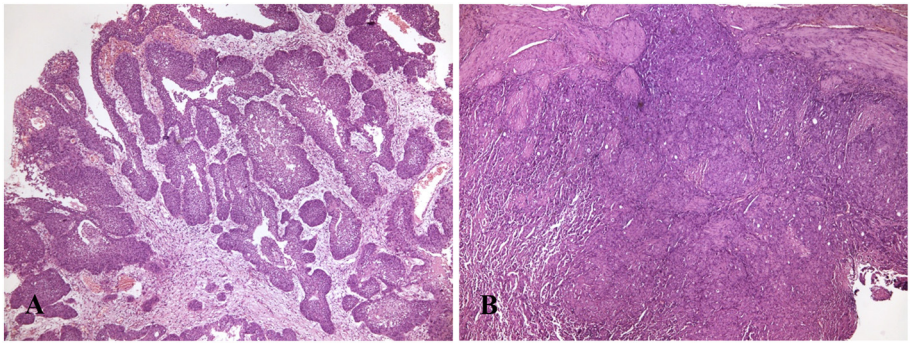

FIGURE 1
(A) Dog with papillary and infiltrating urothelial carcinoma. Papillary projections supported by a central fibrous stalk, varying in thickness, are covered by multiple layers of neoplastic urothelium (H&E, 40X). **(B)** Dog, urinary bladder with non-papillary and infiltrating carcinoma appeared as a thick plaque (H&E, 40X).

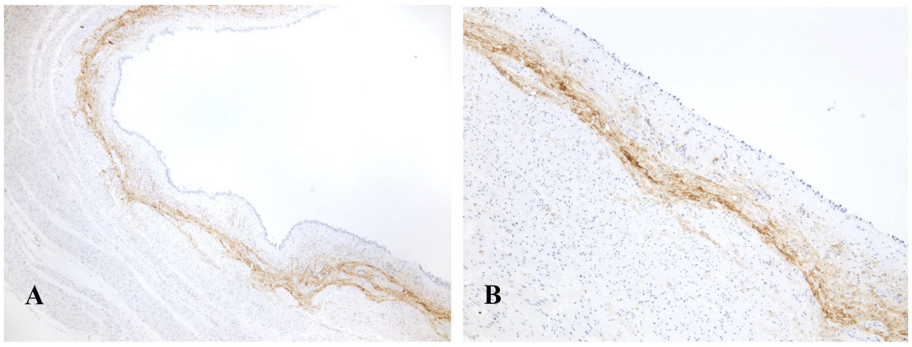

FIGURE 2
Normal urinary bladder of a dog. The immunohistochemical labeling for POSTN is constantly and extensively visible as a compact intensely positive belt located in the supporting connective tissue beneath the urothelium [Immunohistochemistry: **(A)** 40X, **(B)** 100X].

TABLE 2 POSTN expression extension—immunohistochemical results.

Number of cases	Percentage of neoplastic stroma positive for POSTN	Points
5	0	0
8	1–5%	1
16	6–20%	2
13	21–50%	3
3	51–60%	4
0	>60%	5

TABLE 3 POSTN expression intensity—immunohistochemical results.

Points	Intensity	Number of cases
0	Absent	5
1	Weak	22
2	Moderate	17
3	Intense	1

continuous variables such as age and mitotic count with the average + SD and/or first and third quartile for age and mitotic count. The goodness of fit to a normal distribution was assessed for the mitotic count with the Shapiro–Wilk W-test. The presence of a significant relationship between categorical variables (i.e., type of tumor—infiltrating or not infiltrating lamina propria, muscular invasion—present or absent, and vascular invasion—present or absent) was then analyzed with the Pearson and exact chi-square tests to evaluate the presence of a significant relationship. Similarly, the presence of any statistical relationship between the extent of the immunohistochemical signal with the intensity in terms of score and any correlation of the two parameters with the categorical variables above was assessed.

To evaluate the effect of the immunohistochemical POSTN expression score, as a categorical variable with four levels, on the continuous variable mitotic count, a one-way ANOVA model was used after checking the assumption of normality for the mitotic count variable using the Shapiro–Wilk test, and the assumption of equal variances using the Levene test.

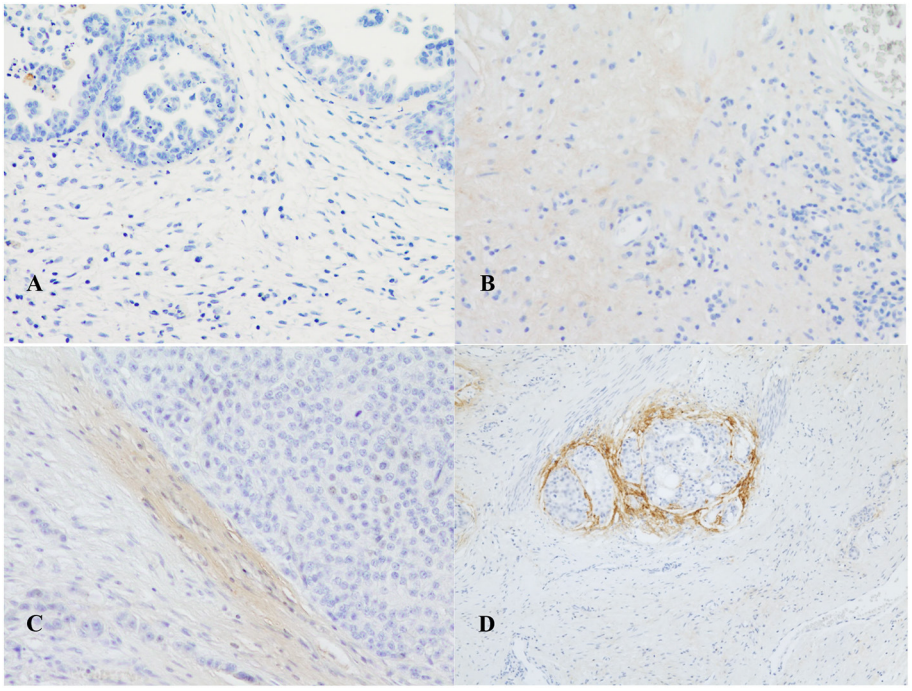

FIGURE 3
Immunohistochemical expression of periostin in canine urothelial carcinomas: **(A)** absence of expression; **(B)** weak; **(C)** moderate (40X); and **(D)** intense (20X). In **(D)**, an intense ring of POSTN was visible around some groups of neoplastic cells infiltrating the urinary bladder muscle layer.

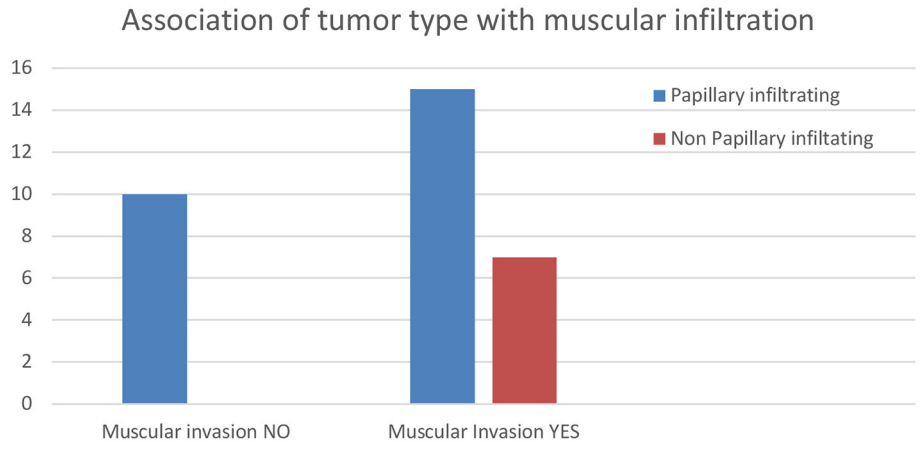

FIGURE 4
Histogram of the results of the association of tumor type with muscular infiltration.

3 Results

3.1 Histology

According to the last WHO (2004) classification of domestic animal tumors in use, 38 tumors were diagnosed as papillary (84%) and 7 (16%) cases as non-papillary (30).

All tumors had an infiltrative growth pattern, but the infiltration of the surrounding tissue occurred at different levels: in 12 out of 45 (27%), it was limited to the bladder lamina propria, while in 21 out of 45 (47%), the tumor extended to the muscle layer. However, in other 12 out of 45 (27%) cases, the depth of the infiltration was not completely evaluated since samples (being collected as cystoscopic biopsies) lacked the muscle layer.

Aggregates of neoplastic cells in the vessel lumina (vascular invasion) were detectable in 26 out of 45 (58%) neoplastic cases. In 7 of these 26 cases, the tumor infiltrated only the lamina propria; in 18 cases, the neoplasm also involved the muscle layer; while in one case, the depth of tumor extension was not evaluable.

In the 38 papillary BUCs, papillary or cauliflower-like structures projecting into the bladder lumen were visible. These projections showed a central fibrous stalk, varying in thickness, covered by multiple layers of neoplastic urothelium characterized by mild-to-severe cellular atypia. Secondary or

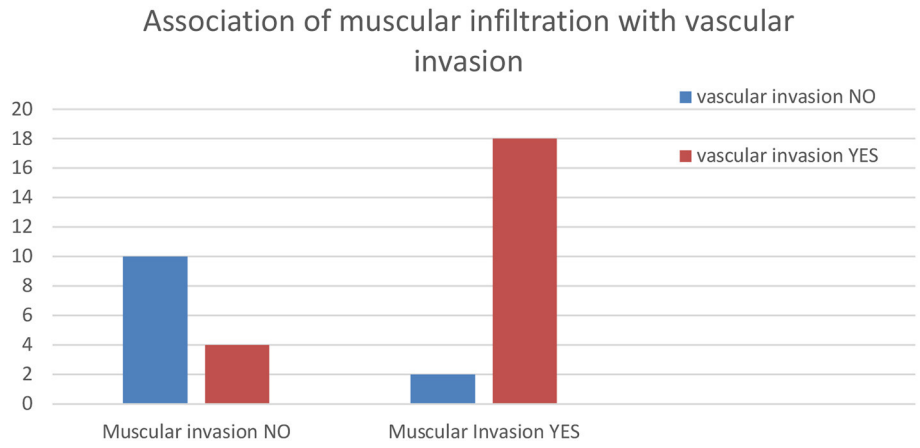

FIGURE 5
A histogram of the results of the association of muscular infiltration with vascular invasion.

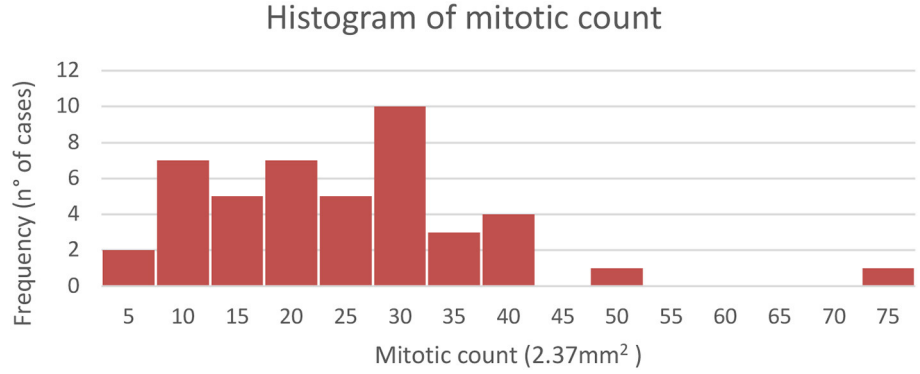

FIGURE 6
Distribution of mitotic count in the analyzed canine urothelial carcinomas $n = 45$.

branching villous projections from the main tumor emerged in advanced tumors.

Non-papillary and infiltrating BUCs appeared as plaques, raised masses, or flat nodules. These tumors were often variably ulcerated and, in all seven cases, infiltrated into the deeper muscle layers.

In both papillary and non-papillary tumors (Figure 1), neoplastic cells were polygonal, with sharp cell borders, a variable amount of eosinophilic cytoplasm, characterized by the presence of colorless to eosinophilic cytoplasmic inclusions called Melamed–Wolinska bodies (27, 29).

The round to oval nuclei were generally large and vesicular, and nucleoli were often prominent. Varying degrees of differentiation and anaplasia were present, and atypical nuclei were frequently observed. Mitoses ranged from 4 to 73 in 2.37 mm^2, averaging 22.5. Bizarre mitotic figures were variably seen in tumors with a high mitotic count. Within the tumor, limited areas of squamous and/or glandular metaplasia and desmoplastic reaction were variably observed (27, 29).

Tumors were often characterized by superficial ulceration and areas of coagulative necrosis and multifocal hemorrhages variably in width; moreover, BUC's lamina propria and submucosa were frequently infiltrated by numerous small mature lymphocytes and plasma cells scattered or variably arranged in aggregates or follicular structures.

Based on the Valli et al. (28) grading system, which considers the nuclear shape and position, the appearance of the chromatin, and the presence of prominent nucleoli, 11 out of 45 (24%) tumors were graded as grade 1, 26 out of 45 (58%) as grade 2, and 8 out of 45 (18%) tumors as grade 3.

Based on the Meuten and Travis (29) grading system, which also focuses on the invasiveness of the tumor, considering invasive tumors as high grade and non-invasive tumors as low grade, all the tumors (100%) were graded as high grade.

3.2 Immunohistochemistry

In the three non-neoplastic bladders examined, POSTN was intensely and diffusely (>60%) expressed (Table 1) within the connective tissue of the lamina propria and submucosa just beneath the bladder epithelium (Figure 2).

Conversely, the extension of the POSTN expression in BUCs generally ranged from 0% to 60% (0 to 4 points, Table 2) and, when present, it was haphazardly localized in the neoplastic stroma and

varied in intensity from weak to intense (0 to 3 points, Table 3). In 5 out of 45 (11%) tumors, POSTN was absent (0 points). In 8 out of 45 (18%) cases, the extension of the immunohistochemical label was from 0 to 5% (1 point) and, among these latter, in 6 cases, the intensity was weak (1 point), and in two was moderate (2 points). Extension of the POSTN expression ranged from 6 to 20% in 16 (36%) cases (2 points), 9 with weak intensity (1 point) and 7 with moderate intensity (2 points). Moreover, an intense ring of POSTN was visible around some groups of neoplastic cells infiltrating the urinary bladder muscle layer (Figure 3).

3.3 Statistical relationships between histological variables and POSTN expression

For the 33 BUCs, including muscle in the histological samples, a significant ($P = 0.04$) relationship between tumor histological type and muscular invasion was observed. Non-papillary infiltrating tumors were all muscle-invasive, while in the case of papillary infiltrating BUCs, 60% of tumors invaded muscle and 40% did not (Figure 4).

The Chi-squared test showed a significant relationship ($P = 0.0001$) between muscular infiltration and the presence of neoplastic aggregates in the vessel lumina (interpreted as vascular invasion). BUCs with muscular infiltration and vascular invasion (in blue in the right column of the mosaic plot reported above—Figure 5) were significantly more than expected. On the contrary, tumors with no muscular infiltration and no vascular invasion were less than expected (Figure 5).

The histogram of mitotic counts is reported in Figure 6. Mitoses ranged from 4 to 73 in 2.37 mm^2, with an average of 22.5. The histogram shows, among the highest frequencies, that the number of mitoses was 10 or 20 in 2.37 mm^2 in seven cases, while in 10 cases, 30 mitoses were counted. The mitotic count was normally distributed with an average ± SD of 21.4 ± 10.7 mitotic count in the Shapiro–Wilk test, excluding an outlier with 73 mitoses in 2.37 mm^2 in one dog with a non-infiltrating tumor.

The ANOVA model ($n = 45$, $r^2 = 0.35$) of mitotic count with the extension of the immunohistochemical expression of POSTN in BUC stroma (score—Table 1) shows a statistically significant ($P = 0.0015$) mitotic count score, so mitotic count is negatively correlated with increased extension of POSTN expression. Tukey-Kramer's HSD *post-hoc* test highlighted that score 3 had a significantly lower average of mitotic count equal to 12.3 with respect to the average of the scores 0, 1, and 2 that showed an average, respectively, equal to 37, 26, and 25 mitoses. The score 4 showed an average of 19.3, not statistically different from the other three score levels.

Conversely, the Chi-square analysis to verify a possible association between the results obtained with the extension of the immunohistochemical expression of POSTN in BUC stroma and its intensity or with other categorical variables, such as the presence of vascular invasion or muscle infiltration, did not show any significant results.

Moreover, no relationship was observed between the intensity and extension of the POSTN immunohistochemical labeling.

4 Discussion

POSTN regulates multiple biological behaviors of tumor cells, including proliferation, survival, invasion, angiogenesis, metastasis, and chemoresistance. Moreover, its expression seems associated with aggressive tumor behavior, advanced stage, and poor prognosis in most human tumors (1, 9, 15–20), except for BUCs in which POSTN seems to be downregulated (21). Since the growing importance of canine BUC as a model for human disease, and since POSTN has been poorly investigated in veterinary medicine, in the present study, POSTN immunohistochemical expression was evaluated in 45 spontaneous canine BUCs.

Considering patients, four of them were from predisposed breeds, such as West Highland White terriers (3 cases) and Beagle (1 case) (31, 32), while the remaining patients belonged to other breeds or, in 18 cases, were mixed-breed dogs. These findings are in line with recent research (13), which suggested that, independently from the predisposed breed, other factors, such as the possible mutation of the *BRAF* gene and epigenetic factors, can be involved in urinary bladder carcinogenesis.

On the other hand, the 2-fold higher BUC risk in spayed/neutered dogs, if compared with intact dogs (33–35), was not confirmed in the present study. These findings deserve to be further investigated on a larger number of samples, especially spayed dogs.

Regarding cell morphology, varying degrees of differentiation and anaplasia were present, and atypical nuclei were frequently observed reflecting what is reported in the literature (29). Mitoses ranged from 4 to 15 in 14 out of 45 (31%) cases, from 16 to 30 in 22 out of 45 (49%) cases, and were more than 30 in 9 out of 45 (20%) cases, reaching 73 mitoses in one non-infiltrating tumor. The mitotic count in 31 (69%) cases was >15 mitoses, which demonstrates that BUCs have a particularly high replicative potential which could indicate a consequent high malignancy of these tumors.

Moreover, all tumors were infiltrating, and their growth pattern was papillary or not papillary in 38 (84%) and seven cases, respectively (16%). These findings are in line with the literature stating that the most common BUC variant in canine species is the papillary and infiltrating one (27, 31). Regarding tumor histological classification and extension, all the non-papillary tumors were muscular invasive, revealing that non-papillary tumors are more prone to invade the muscle layer. Moreover, the Chi-square analysis revealed a significant correlation between muscular invasion and the presence of neoplastic vascular emboli. A recent meta-analysis reviewing several studies on both human and canine BUCs revealed that in most studies on canine BUC, histologically important features such as muscular or vascular invasion were not assessed. In addition, the assessment of tumor local invasion in dogs with BUC has not already been standardized (35). In humans, the muscle-invasive BUC represents only 30% of the cases (36), while in canine patients, most BUCs are invasive, reaching the muscle layer and often invading the lymphatic vessels or even affecting adjacent organs such as the urethra and prostate (29). This is probably due to the advanced stage of the disease at the time of diagnosis (28, 31, 37, 38). Even if, in the present study, all BUCs examined had an infiltrative growth pattern, in the 33 samples in which the muscle layer was also evaluable, the level of the

infiltration was limited to the bladder lamina propria in 12 out of 45 (27%) while the tumor extended to the muscle layer of the urinary bladder wall in 21 out of 45 (47%) cases. This is an important finding to be considered in future prospective studies because non-invasive diagnostic tools, nowadays used also in veterinary, such as ultrasound, could contribute to discovering tumors before a deep invasion, and prognosis could be different depending on tumor local extension (chorion or muscle).

However, in other 12 out of 45 (27%) cases, the depth of the infiltration was not completely evaluable, reflecting the fact that the biopsies collected by cystoscopy frequently do not include all muscle layers, making impossible, in most cases, the assessment of local tumor invasion (35, 39).

Another interesting histological feature was the presence of aggregates of neoplastic cells in the vessel lumina (vascular invasion) that was statistically associated with the extension of the tumor in the bladder muscle layer and observed in 58% of the tumors examined. The distinction between pseudo-vascular invasion and true vascular invasion has been assessed based on the identification of criteria proposed by Meuten et al. (40). Namely, at least one of the following criteria is required: the most important criteria used to define vascular invasion in human tumors are thrombus adherent to the intravascular tumor, tumor cells invading through the vessel wall and endothelium, and neoplastic cells within a space lined by lymphatic or blood vascular endothelium. Our results confirm that vascular invasion is a frequent finding in canine BUCs, which are frequently diagnosed at a late stage of development. Noteworthy, a recent study highlighted vascular invasion as a significant indicator of poor patient outcomes in canine BUCs (35); thus, this important finding deserves to be included in the pathology diagnostic report, which could also include the tumor grade.

However, although various grading systems have been formulated for canine BUCs over the decades, none of them is routinely used in veterinary pathology, and the two main grading systems, proposed by Valli et al. (28) and by Meuten and Travis (29), are still not validated with prognostic studies in veterinary medicine (30). However, both these latter grading systems were applied to the tumor included in the present study, and, based on Valli's grading system, 11 out of 45 (24%) tumors were graded as grade 1, 26 out of 45 (58%) as grade 2, and 8 out of 45 (18%) tumors as grade 3. These findings are in line with what has already been reported in the literature (30), indicating that the use of three-tier grading systems, such as that proposed by Valli et al. (28), causes the inclusion of most tumors in grade 2, the intermediate grade.

On the other hand, Meuten's two-tier system application showed that all tumors were high-grade, as they were all infiltrating. Nevertheless, this latter grading system does not consider the level of the tumor extension, and infiltrating neoplasm are included in high grade independently from their extension level, which can be limited to the bladder chorion or involve the deep muscle. In any case, the inclusion of all examined tumors in high grade following Meuten's grading system and the inclusion in grade 2 of most of them when Valli's grading system was applied demonstrates that further studies, considering a larger number of patients and related follow-up, are required to individuate the best grading system for canine BUCs.

However, in human medicine, tumor histological appearance and extension are not the only features considered, and, frequently, immunohistochemical markers are also employed to better characterize the neoplasm behavior and aggressiveness. The present study is the first to evaluate the expression of POTN in the normal canine urinary bladder and BUC, which represent an important tumor in this species and has been suggested as a possible animal model for studying the human counterpart. Regarding the expression of POSTN in the normal canine bladder, in our study, it was constantly and extensively present and was visible as a compact intensely positive belt located in the supporting connective tissue beneath the luminal urothelium. These findings consistently reflect what has been observed in humans and represent an interesting comparative feature demonstrating structural similarities between the canine and human urinary bladder (21). In all neoplastic specimens, the normal POSTN front was interrupted by the tumor growth, but it was still visible in the adjacent non-neoplastic mucosal portions of the bladder.

As far as tumors are concerned, in the neoplastic stroma, POSTN was always less extensively and less intensely expressed than in the normal bladder, and it was absent in 6 cases. Also, these findings align with what Kim et al. (41) reported for human urothelial carcinoma cell lines in which a general decrease in POSTN expression was reported compared to normal bladder tissue. Kim et al. (41) also demonstrated that inhibition of AKT (protein kinase B) by POSTN induced the upregulation of E-cadherin and suppressed the invasiveness of bladder cancer cells (21, 42), hypothesizing that, conversely, the POSTN downregulation could be involved in cancer progression. On the other hand, Silvers et al. (43), performing quantitative real-time PCR in 10 human bladder cell lines, observed high POSTN expression in muscle-invasive bladder cancer cell lines J82, TCC-SUP, and UMUC3.

This is an interesting finding and could reflect what was noted in our samples that had a generally weak or absent expression of POSTN but in which an intense ring of POSTN was visible around some group of neoplastic cells infiltrating the urinary bladder muscle layer. This feature, suggesting a possible protective role of POSTN secreted by CAFs with the aim of containing the neoplastic infiltration, was also noted in a recent study on human urothelial carcinomas, even if the research was focused on the tumor affecting the upper urinary tract (42).

The statistical analysis did not reveal any correlation between the extent and intensity of stromal positivity for POSTN and between these two variables and categorical variables such as type of tumor, tumor infiltration, and muscular and vascular invasion.

However, the statistical analysis demonstrates that POSTN expression and mitotic count were significatively inversely correlated. Although score 3 was defined by the highest range of stromal POSTN expression (21–50%), an influence of this range on the mitotic analysis did not emerge. The interesting relation between POSTN expression and mitotic count, emerging for the first time, was not checked in human BUCs and deserves to be investigated in further studies since it seems to suggest a relationship between POSTN expression and a greater rate of neoplastic cellular proliferation (negative prognostic factor). Mitotic count, although a negative prognostic factor, is, however,

not considered a distinguishing criterion for histological grading, so there are no cutoffs for mitotic count in veterinary medicine any more than there are for human medicine (30). In a recent study on human urothelial carcinoma of the urinary tract, the statistical relationships with periostin expression and epidemiological and histological features have been investigated, revealing that there was no significant difference in the mean age, sex, tumor laterality, or histological grade between patients with low and high stromal periostin expression, while the frequencies of tumors with non-papillary gross findings were significantly higher in patients with high stromal periostin expression than in those with low expression (42). The latter correlation between increased periostin expression and papillary tumor morphology is not confirmed by the present study.

On the other hand, the lack of statistical correlation between POSTN expression and tumor grading could instead be due to an inadequacy of the BUC canine grading systems, as already highlighted (31).

From statistical evaluations, the presence of neoplastic emboli does not seem influenced by the expression of POSTN. This finding is not surprising because, as described in the literature, vascular invasion seems to be correlated to other factors, such as complex interactions among tumor cells, stromal cells, and endothelial cells. Many molecules are involved in this cellular crosstalk, including growth factors, cell surface receptors, and cytokines, as described particularly in human breast cancer (43–46). Another aspect observed histologically was the inflammatory infiltrates, especially lymphoplasmacytic, often present within the stroma of the tumors included in the study as described in the literature (28, 29). However, these inflammatory infiltrates were never associated with a variation in POSTN expression compared to the remaining tumor stroma. Moreover, POSTN was arranged, in some cases, to surround muscular infiltrates. These features suggest that POSTN, involved in the biochemical mechanisms of collagen synthesis and fibrillogenesis, could be produced by cancer-associated fibroblasts (CAFs) with a protective purpose against tumor infiltration in deeper layers and vascular invasion.

5 Conclusion

In conclusion, in canine BUCs, a general decrease in stromal POSTN expression was observed compared to the normal bladder, and POSTN levels were significantly inversely proportional to mitotic count, suggesting a relationship with a greater rate of neoplastic cellular proliferation (negative prognostic factor). Moreover, as for human BUCs, in some cases examined, a ring of POSTN was observed surrounding the muscle-invasive neoplastic infiltrates. As the extent of POSTN expression inversely correlates with mitotic count, this protein could have a role as a prognostic marker. However, this should be verified in future follow-up studies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by the Comitato Etico Tutela Degli Animali Dell'universita' Degli Studi di Milano (Dipartimento di Medicina Veterinaria e Scienze Animali). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

EB: Conceptualization, Data curation, Methodology, Writing—original draft. RC: Conceptualization, Data curation, Methodology, Writing—review & editing. PC: Formal analysis, Writing—original draft. SD: Conceptualization, Writing—review & editing. MN: Conceptualization, Writing—review & editing. PD: Conceptualization, Writing—review & editing. AP: Methodology, Writing—review & editing. VM: Writing—review & editing, Conceptualization. CF-A: Conceptualization, Writing—review & editing. RL-A: Conceptualization, Writing—review & editing. DS: Data curation, Writing—review & editing. SR: Data curation, Writing—review & editing. VG: Conceptualization, Methodology, Supervision, Writing—review & editing.

Acknowledgments

The authors acknowledge the support of the APC central fund of the University of Milan.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1258247/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 29 November 2023
DOI 10.3389/fvets.2023.1192525

OPEN ACCESS

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RECEIVED 23 March 2023
ACCEPTED 11 October 2023
PUBLISHED 29 November 2023

CITATION
Lin Z, Zhang J, Chen Q, Zhang X, Zhang D, Lin J and Lin D (2023) Transcriptome analysis of the adenoma–carcinoma sequences identifies novel biomarkers associated with development of canine colorectal cancer. *Front. Vet. Sci.* 10:1192525. doi: 10.3389/fvets.2023.1192525

Transcriptome analysis of the adenoma–carcinoma sequences identifies novel biomarkers associated with development of canine colorectal cancer

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The concept of adenoma-to-cancer transformation in human colorectal cancer (CRC) is widely accepted. However, the relationship between transcriptome features and adenoma to carcinoma transformation in canines is not clear. We collected transcriptome data from 8 normal colon tissues, 4 adenoma tissues, and 15 cancer tissues. Differential analysis was unable to determine the dynamic changes of genes but revealed that PFKFB3 may play a key role in this process. Enrichment analysis explained metabolic dysregulation, immunosuppression, and typical cancer pathways in canine colorectal tumors. MFuzz generated specific dynamic expression patterns of five differentially expressed genes (DEGs). Weighted correlation network analysis showed that DEGs in cluster 3 were associated with malignant tissues, revealing the key role of inflammatory and immune pathways in canine CRC, and the S100A protein family was also found to be involved in the malignant transformation of canine colorectal tumors. By comparing strategies between humans and dogs, we found five novel markers that may be drivers of CRC. Among them, GTBP4 showed excellent diagnostic and prognostic ability. This study was the first systematic exploration of transformation in canine CRC, complemented the molecular characteristics of the development and progression of canine CRC, and provided new potential biomarkers and comparative oncologic evidence for biomarker studies in human colorectal cancer.

KEYWORDS

adenoma–carcinoma sequences, canine colorectal cancer, dynamic expression model, PFKFB3, GTPBP4

1. Introduction

The third leading cause of cancer-related deaths worldwide is colorectal cancer (CRC) (1). In human patients, most cases of colorectal cancer were sporadic, originating from polyps inside abnormal recesses (2, 3), and canine CRC showed a similar pattern (4–6). Tumorigenesis began with the mutation of intestinal epithelial stem cells in the colon or rectal mucosa, then adenoma, and finally colorectal cancer. Over a period of more than 10 to 15 years, tubular adenoma transformed into adenocarcinoma, and alterations in the Wnt,

RAS, TP53, and PI3K-AKT pathways all occurred sequentially (7). The effects of Wnt, P53, and TGF-β signaling pathways have been identified in canine CRC studies (8–11). The development of multi-hit gene models of carcinogenesis, together with a deeper understanding of chromosomal instability, microsatellite instability, and the CpG island methylator phenotype of CRC (12, 13), will help clarify the various genetic and epigenetic alterations underlying the adenoma–carcinoma sequence (14). In canine CRC, Jack Russell Terriers were found to have the same germline APC genetic mutation as humans, and this breed was characterized by a high incidence of hereditary colorectal cancer (15, 16). All studies at this stage showed a high degree of similarity between CRC in dogs and humans. And canine CRC may reveal new molecular mechanisms in human (11, 17). Canine colorectal cancer has similar copy number abnormalities to human colorectal cancer, revealing a strong degree of genetic homology between sporadic canine and human CRCs (18). Similar to human colorectal cancer, overactivation of the WNT signaling pathway is also present in canine colorectal cancer, accompanied by repeated mutations in genes associated with CTNNB1 and TGF-β signaling pathways (17). APC was the most significantly mutated gene in both canine adenomas and adenocarcinomas (Frequent Alteration of the Tumor Suppressor Gene APC in Sporadic Canine Colorectal Tumors). The emergence and development of high-throughput omics technologies have facilitated systematic studies comparing normal and tumor tissues at the gene, mRNA, and protein levels. Four consensus molecular sequences (CMS) of CRC-CMS1 (immunotrait, microsatellite instability), CMS2 (canonical trait), CMS3 (metabolic trait), and CMS4 (mesenchymal trait) – and transitional combinations of traits classification into distinct subtypes – established using gene expression data from 4,151 tumor samples (19). The transcriptome analysis of canine CRC also elucidated the molecular signatures specific to proliferative and aggressive canine tumors, revealing that CMS4 human colon cancer consisted of two subtypes, EMT and crypt-like invasion, with differences in TGF-β signaling pathways and microbial content (17).

Although a variety of differentially expressed genes (DEGs) have been found in CRC, the specific expression patterns of these genes in the pathogenesis of colorectal cancer remain unclear. Furthermore, the mechanisms by which CRC arose and evolved are not fully understood. So, it is essential to identify gene expression patterns associated with CRC development and progression.

In this study, transcriptome analysis of normal colon, adenoma, and colorectal cancer identified changes in signaling pathways during canine colorectal tumor formation, and dynamic analysis revealed five specific gene dynamic expression patterns. These findings correlated with three known markers of cancer: dysregulation of cell metabolism, avoidance of immune disruption, and activation of cancer-related pathways. WGCNA analysis identified the gene modules significantly related to the malignant phenotype of the tumor. It was found that the genes in the malignant module were mainly upregulated, and these genes mainly affected the tissue inflammation and immune process, thus revealing the important role of immunosuppression in the malignant transformation of canine CRC. By using a human-canine comparison strategy, we compared genes with a persistent upregulation pattern in human CRC. Five core genes were identified through analysis, and GTPBP4 was further screened as an adverse prognostic marker for colon cancer with optimal diagnostic ability. None of these genes have been studied much in human tumors and neither in canine tumors. Our analysis and comparative strategies identified these novel potential markers that should be continuously monitored and explored during tumor formation.

2. Materials and methods

2.1. Transcriptome data acquisition and differential analysis

We downloaded transcriptomic data (number PRJNA418842, PRJNA396033) from the SRA database for canine colorectal tumors and normal canine colon tissue. To obtain high-quality clean reads, fastp (v0.20.0) software was used to trim the adaptor and remove low-quality reads. We used STAR (v2.7.9a) software to compare high-quality clean reads to the Canis_lupus_familiaris reference genome (CanFam3.1). Raw read counts of mRNA genes were obtained as mRNA expression values using featurecount (v2.0.2). Normalization was performed using DESeq2 (v1.30.1), retaining only uniquely mapped reads for HTseq counting. The ComBat-seq method of R Package sva was used to remove the batch effect of RNAseq (20).

DEG was identified using the R package DESeq2 with a false discovery rate (FDR) <0.05 and an absolute log2|fold change| > 1. The GTF annotation database (Ensembl v104) was used for mRNA annotation. Gene Ontology and KEGG Pathway enrichment analysis of differentially expressed mRNAs was performed using the clusterProfiler R package (v3.18.1).

2.2. Gene set enrichment analysis

Gene Set Enrichment Analysis (GESA) was performed using GSEA software version 3.0[1] with the Molecular Signature Database gene set version 6.2. All gene expression data were phenotypically arranged with a number of 1,000. Use the FDR value threshold ($p < 0.05$) to identify pathways corresponding to genes enriched at the top or bottom of the gene set and sort according to the normalized enrichment scores.

2.3. Dynamic expression model analysis

The core algorithm of the "Mfuzz" R package is based on Fuzzy C-Means Clustering (FCM), which is used to analyze the time trend of gene expression in the transcriptome data with time series characteristics, and cluster the genes with similar expression patterns. To help understand the dynamic expression patterns of these biological molecules and their connection to function. According to the instruction of R package Mfuzz, soft cluster analysis is performed on transcriptome data (21). C-means clustering was performed using R-pack Mfuzz to map reads per million (FPKM) fragments of LRT-identified deg. to assess dynamic changes in expression pattern.

1 https://www.gsea-msigdb.org/gsea/index.jsp

2.4. Establishment of co-expression networks and identification of pathological phenotype-related hub modules using weighted gene co-expression network analysis

Co-expression analysis was performed using the Weighted Gene Correlation Network Analysis (WGCNA) R package, guided by published tutorials. Genes with FPKM < 1 of 22 samples were screened and samples (sample type and the number of samples) were clustered hierarchically based on Euclidean distances calculated from gene expression data and combined with (patient clinical information and trait information). Network topology analysis ensured a scale-free topological network and defined a soft-threshold power of (8). A total of (number of modules without counting gray modules) modules were identified based on a dynamic tree-cutting algorithm with a minModuleSize parameter of 30, and mergeCutHeight parameter of 0.25. For each module, the signature gene (the first component expression of the gene in the module) was identified, and then the correlation between the signature gene and clinical phenotype-related description, and clinical features such as tumor staging and grading were calculated. Genes with high connectivity in each module were considered pivotal genes. The co-expression relationships of each module were analyzed and visualized by Metascape (v3.5.20230101).

2.5. Analysis of immune cell infiltration in CRC

In this study, we applied the CIBERSORT algorithm to determine the immune cell subsets of canine tissues. Since the CIBERSORT algorithm is able to analyze nonspecific data and noise, it is superior to conventional deconvolution approaches for evaluating infiltrating immunity and for determining the abundances of specialized cells in the mixed matrix.

2.6. Cross-validation with human data sets

Due to the lack of veterinary databases containing information about colorectal tumors in dogs, we selected the proteins most relevant to human CRC and validated them using human samples from the Bioinformatics database. Transcriptome data for human colorectal cancer were obtained from the GEO database (GSE164541). Transcriptome data for ROC analysis and differential expression verification were derived from the TCGA database.[2]

2.7. Receiver operating characteristic

The pROC package was used for receiver operating characteristic (ROC) analysis of the data, and the results were visualized with ggplot2. The Area Under Curve (AUC) is often used to evaluate diagnostic tests, and the value range of AUC is generally between 0.5 and 1. The closer the AUC is to 1, the better the diagnostic effect of this variable in predicting the outcome.

2.8. Survival analysis

The Kaplan–Meier curve is a graphical method used to present the results of survival analyses and is commonly used to analyze the relationship between survival time and event incidence in patients. The optimal cutoffs for classifying patients into high and low gene expression groups were automatically calculated and survival curves were drawn by employing the survival package and survminer package in R3.5.3.

2.9. Statistical analysis

To assess the prognosticative efficacy of the biomarkers, the receiver operating characteristic (ROC) curves were generated depending on the levels of gene expression and sample types. The log-rank test p values were calculated, and a p value of 0.05 or less was regarded as statistically significant. Software packages such as GraphPad Prism version 8.0 or SPSS version 20.0 were used to conduct the statistical analysis. Statistical significance was defined as a two-tailed p 0.05.

3. Results

3.1. Identification of DEGs during canine CRC formation

In total, 2,578 DEGs were identified by comparing adenoma with normal colon (AN), 3,701 DEGs were identified by comparing carcinoma with a normal colon (CN), whereas 14 DEGs were identified by comparing carcinoma with adenoma (CA), suggesting that adenomas were more similar to carcinoma than to the normal. 3,750 DEGs were found after comparisons of all three tissue types (ACN) by LRT (DEseq2) (Supplementary Tables S1–S4). Figure 1A displayed the overlapping DEGs between the normal colon, adenoma, and carcinoma tissues. PFKFB3 was the only gene that crossed across. Although comparisons can be performed between any two tissue types to identify DEGs that exhibit relative up- or down-regulation, it is challenging to identify changes in expression patterns across the adenoma–carcinoma sequence.

3.2. Early and late stages of canine CRC onset were related to three hallmarks

GSEA was used to compare the transcriptome patterns of each of the three different tissues. After reviewing previously published gene sets related to canonical pathway activity, and metabolic, and immunological processes, enriched gene sets ($p < 0.05$) were chosen to produce heatmaps, as shown in Figure 1B.

The stages of tumor formation were generally divided into the early stage or advanced stage. The early stage referred to the transformation of normal tissue into an adenoma, and the late stage referred to the

2 https://portal.gdc.cancer.gov/

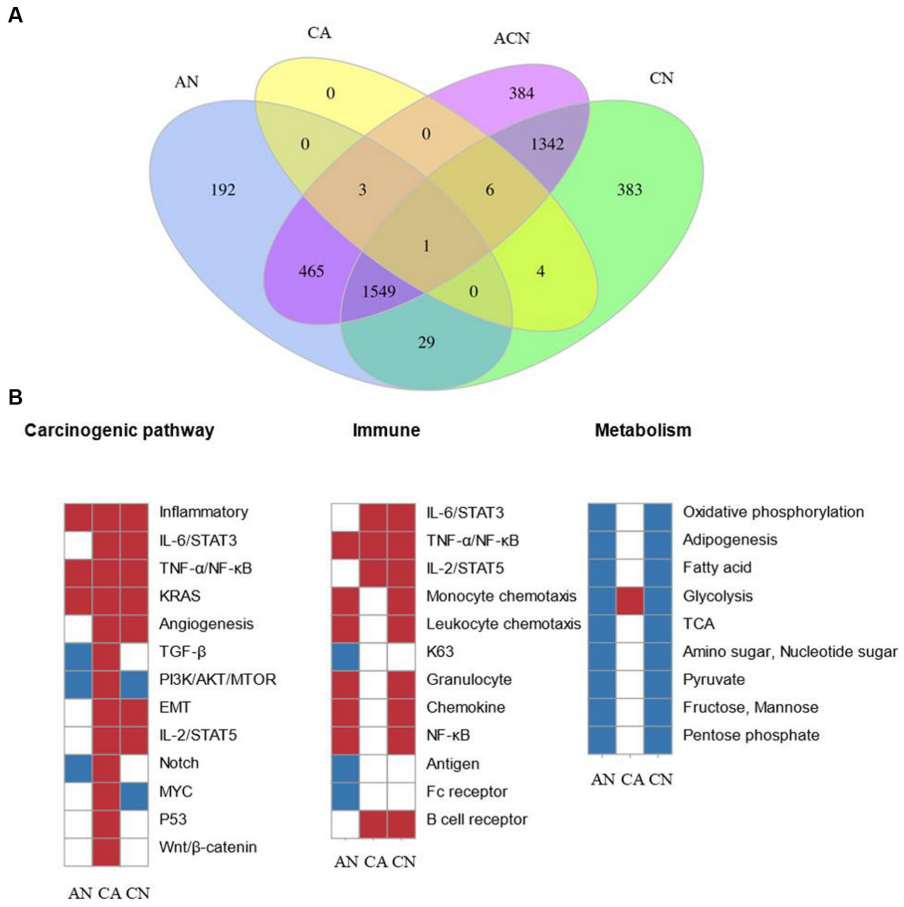

FIGURE 1
Comparing the transcriptome data at three types of tumor-identified DEGs and molecular characteristics of CRC formation. N, normal tissue; A, adenoma tissue; C, carcinoma tissue; AN, adenoma vs. normal colon; CA, carcinoma vs. adenoma; CN, carcinoma vs. normal colon; ACN, three tissue types. **(A)** Venn diagram showing the overlap of DEGs identified by AN (blue), CA (yellow), CN (green), and ACN (purple) comparisons. **(B)** GSEA heatmap showing significant changes in canonical pathway activity, metabolic, and immunological activities during CRC formation.

transformation of adenoma tissue into carcinoma. Most typical pathways were activated during the transition from normal colon epithelial cells to adenomas to carcinoma, but only the TGF-β, PI3K/AKT/mTOR, and Notch pathways were downregulated during normal to adenoma transition and activated during the transition from adenoma to carcinoma. The inflammatory, TNF-α/NF-κB, as well as the KRAS pathways, were activated early during the transformation of normal to adenoma, and the IL-6/STAT3, angiogenesis, EMT, IL-2/STAT5, p53, and Wnt/β-catenin pathway were activated late during the transformation of adenoma to carcinoma.

Dysregulated metabolic activity was a feature of tumor formation. GSEA showed that the activity of most metabolism-related pathways decreased during tumor formation. Notably, glycolysis increased during the transformation of adenomas into cancerous ones.

During the adenoma to adenocarcinoma transition, genes affecting epithelial-mesenchymal transition (EMT) were significantly upregulated. Secondly, genes related to angiogenesis were significantly upregulated, which was consistent with the biological characteristics of the tumor. In addition, many classical pathways were up-regulated, including the response of genes regulated by NF-κB to TNF-α, the up-regulation of the STAT5 gene under the stimulation of IL2, and the up-regulation of genes after the activation of Notch, p53, and Wnt signaling pathway.

In the development and spread of malignancies, the effect of immune system was indispensable. Signaling pathways closely related to immunity, including IL-6/STAT3, TNF-α/NF-κB, and IL-2/STAT5, were activated during the transition.

Results from the GSEA showed that typical pathways, metabolism, and immune responses varied over time but were not always consistent. Different pathways underwent a variety of modifications at various transitions, some of which were engaged during the change from normal to adenoma and others involved in the transition from adenoma to carcinoma. We subsequently investigated the DEG expression patterns during tumor formation to shed further light on these findings.

3.3. Identification of dynamic expression patterns of adenoma to carcinoma processes

Based on the comparison of the DEGs discovered by the three tissue types, unsupervised hierarchical clustering was carried out, and heat maps of 3,750 DEGs in 27 samples were created to comprehend the dynamic changes of DEGS in the three phases of CRC formation. As seen in Figure 2A, various tissue types were enriched for gene

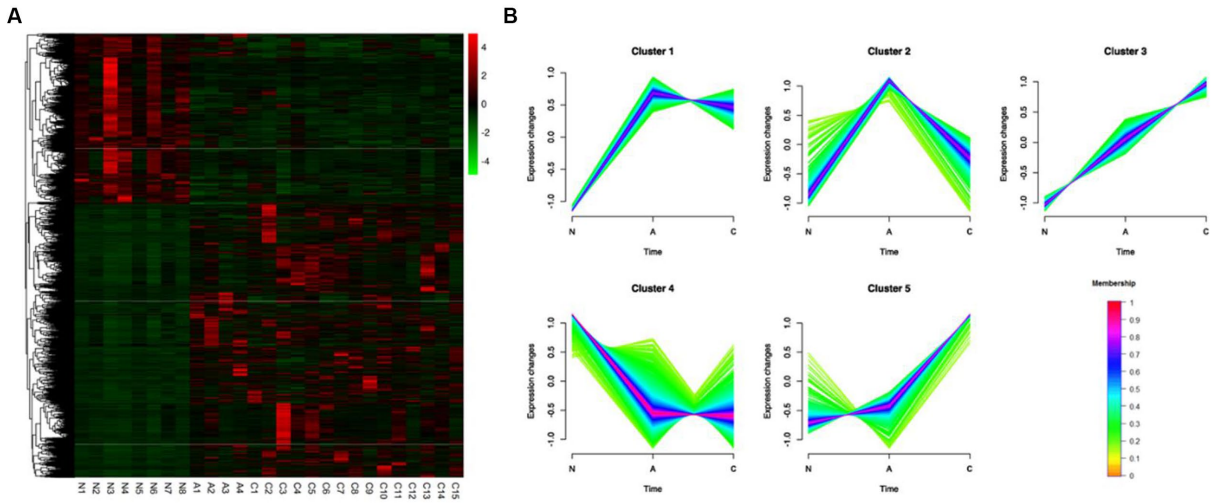

FIGURE 2
Identification of 3,750 DEGs dynamic expression profiles and chemical features from the ACN comparison. **(A)** 3,750 DEGs were heatmapped and clustered hierarchically. N, normal; A, adenoma; C, carcinoma. **(B)** The diagrams showed the patterns of dynamic changes in DEGs that were discovered using Mfuzz during the development of CRC.

clusters with various degrees of expression. Using Mfuzz, the 3,750 DEGs were divided into five clusters in order to evaluate the dynamic expression patterns of DEGs during the beginning of CRC (Figure 2B) (Supplementary Table S5). The gene expression pattern of cluster 5 changed significantly in the later stage of tumor formation, while the upregulation of DEGs only occurred during the transformation of adenoma to malignancy. While DEGs were only up- or down-regulated during the transition from adenomas, the expression pattern of DEGs in clusters 1 and 4 changed considerably during the early stages of tumor formation. During the change from normal to cancerous tissue, DEGs in cluster 3 continued to steadily increase (Figure 2B).

3.4. Enrichment analysis associated with dynamic expression patterns

We hypothesized that different expression patterns of genes served diverse purposes throughout the development of CRCs. The five clusters' GO and KEGG analysis revealed distinctive traits of the genes in each cluster.

GO analysis revealed that genes in cluster 1, which were upregulated during normal to adenoma transition, were associated with inflammation and immunity, including neutrophil chemotaxis, inflammatory response, chemokine-mediated signaling pathway, cellular response to interleukin-1, and the interleukin-17-mediated signaling pathway. Similar outcomes were found by KEGG analysis, which showed that genes were mainly involved in cytokine–cytokine receptor interaction, pathways in cancer, chemokine signaling pathway, inflammatory bowel disease, and MAPK signaling pathway (Supplementary Figure S1).

Genes in cluster 4, which were downregulated during normal to adenoma transition, were mainly associated with the metabolism (GO terms included hydrogen ion transmembrane transport, tricarboxylic acid cycle, lipid metabolic process, fatty acid beta-oxidation, aerobic respiration, mitochondrial ATP synthesis coupled proton transport, cholesterol biosynthetic process). KEGG analysis indicated that genes in cluster 4 were involved in pathways associated with the metabolic pathways, carbon metabolism, citrate cycle (TCA cycle), oxidative phosphorylation, chemical carcinogenesis – reactive oxygen species, among others (Supplementary Figure S4).

As shown in Figure 2D, genes in cluster 5, which were upregulated during the adenoma to carcinoma transition, were mainly involved in canonical pathways associated with cancer, including the Ras, PI3K-Akt, Proteoglycans in cancer, MAPK, and Hippo signaling pathways, among others (Supplementary Figure S5).

GO analysis revealed that genes in cluster 3, which were monotonously upregulated during the normal-adenoma–carcinoma sequence, were associated with immunity and cancer, including inflammatory response, positive regulation of cytokine production, signal transduction, chemotaxis cytokine-mediated signaling pathway, positively regulated inflammatory responses, IL-6 production, and cell–cell signaling. Similar conclusions were drawn from KEGG analysis, which revealed that genes were primarily engaged in the pathways for NF-κB signaling, IL-17 signaling, TNF signaling, pathways in cancer, and JAK-STAT signaling. Supplementary Figure S3 displayed the findings of GO and KEGG analysis of the other clusters (Supplementary Figure S3).

3.5. Construction of the co-expression network and identification of hub modules related to pathological phenotype using weighted gene co-expression network analysis

WGCNA was applied to 12,866 genes with an expression of at least 0.1 FPKM in order to investigate the association between DEGs

and the pathophysiology of tissues. This process produced 33 modules of highly co-expressed gene modules. Three of the modules' DEGs had a strong correlation with tissue phenotype. DEGs were closely linked to malignancy in the darkgreen module (cor = 0.57, $p = 0.002$) (Supplementary Table S6), benign in the lightyellow module (cor = 0.65, $p = 3e\text{-}04$), and normal tissues in the turquoise module (cor = 0.75, $p = 6e\text{-}06$) (Figure 3A). DEG expression patterns were dynamic in these three modules. Clusters 4 (1,261/2684) in the turquoise module were enriched in DEGs that were linked to adenoma tissues. The genes in the darkgreen module that were linked to malignant tissues were more prevalent in clusters 1 (15/150), 3 (44/150), and 5 (25/150).

The differentially expressed genes in cluster 4 may play a key role in the progression of normal tissue to adenoma. The proportion of differentially expressed genes in cluster 4 is the largest in the normal module, but the proportion in both benign and malignant modules is very small. Meanwhile, the proportion of genes in cluster 2 is very small in both normal and malignant modules, but the proportion in the benign module is very high. This suggests that genes in clusters 2 and 4 could be engaged in the essential early events of tumor formation but may not be the key clusters affecting tumor progression.

The genes in cluster 3 and cluster 5 had the highest proportion in the malignant module and a low proportion in both the normal and benign modules, suggesting that these genes could be crucial for the eventual deterioration of the organization.

Through the analysis of this gene enrichment, this finding implies that in the development of colorectal cancer in dogs, the first is the dysregulation of metabolic activity (metabolic reprogramming), combined with the loss of cell proliferation and tight connections, and the development of normal tissues into adenomas. In further development, the Wnt signaling pathway, IL-6/STAT3 signaling pathway, changes in tumor immune microenvironment, and epithelial-mesenchymal transformation play an important role in the malignant transformation process of tissues.

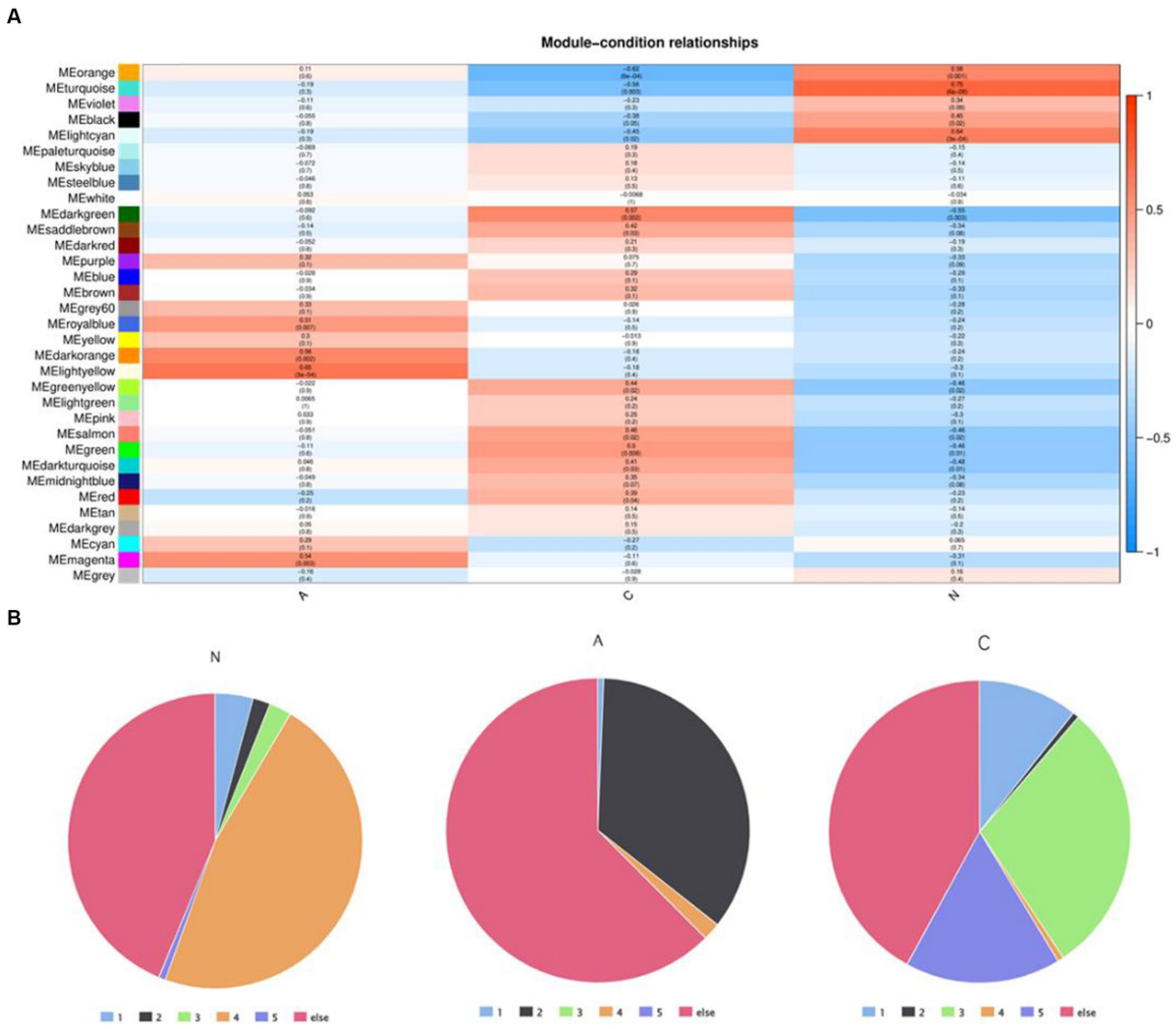

FIGURE 3
(A) Weighted gene co-expression network analysis identified the module genes closely related to tumor pathologic phenotypes and **(B)** distribution of DEGS in core module genes in five dynamic expression modes.

3.6. Dynamic expression patterns associated with pathological phenotype in the adenoma–carcinoma sequence

The monotonous changes following the formation of CRC indicated that these genes may be suitable as specific tracking biomarkers or driving factors. DEGs in cluster 3 were selected for further analysis as markers of CRC formation. The Venn diagram identified 44 genes that were consistently up-regulated in the modules significantly related to the malignancy of tumors, and the proteins represented by these genes had complex interactions (Figures 4A,B). Enrichment analysis found that these genes were mainly related to inflammation and immunity, suggesting that during the occurrence and development of colorectal cancer in dogs, changes in the tumor immune microenvironment may play the most important role. Through the core modules identified by MCODE, we found two main communities, one of which is the immune function community with IL6 as the core, and the other is the community with the S100 protein family as the core (Figure 4C). The biological functions of these core genes have been extensively studied in human tumor diseases, but need to be further explored in human and canine colorectal tumors. Cibersort was used to analyze the infiltration of immune cells in the three types of tissues. It was found that the infiltration of monocytes and neutrophils in the tumor tissues was increased, and the decrease of activated NK cells, the decrease of $CD8^+$T cells, and the increase of regulatory T cells all suggested the phenomenon of immunosuppression in canine colorectal tumors (Supplementary Figure S6).

3.7. Dog–human comparison strategy reveal core genes

Based on a new strategy for comparing humans and dogs across species, it could help clarify a central goal of cancer research, namely cancer driver–passenger distinctions. We compared to cluster 3 with clusters with the same expression pattern in the dynamic pattern of human colorectal adenoma-cancer and obtained 42 differential genes (Figure 5A). These differential genes are based on cross-species comparisons and are more likely to drive the development of cancer. The enrichment analysis of 42 differential genes revealed a variety of biological processes closely related to cancer. At the metabolic level, it involves the biosynthesis, decomposition and metabolism of fatty acids. It is involved in TH17 differentiation, IL17 signaling pathway and cytokine signaling pathway at the immune level. In addition, several classical cancer-related signaling pathways are involved, including the toll-like receptor, chemokine, Wnt, TGF-β, and MAPK signaling pathways (Figure 5B). A PPI network of 42 differential genes was constructed according to the STRING database, and 5 core genes that may play a key role were obtained by further analysis of MCODE (Figure 5C).

3.8. Diagnostic and survival analyses revealed the efficacy of five potential markers

Utilizing expression data and survival data from the TCGA database, we assessed the diagnostic and prognostic utility of the five

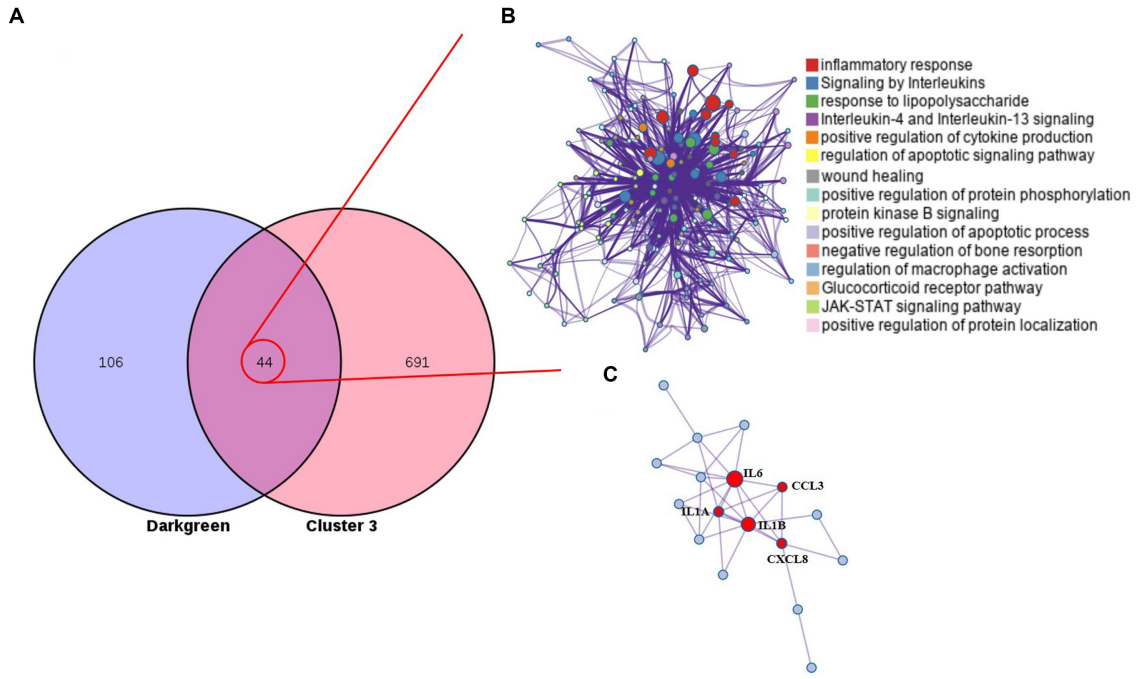

FIGURE 4
Cluster 3 genes in the core module. **(A)** The Venn diagram found 44 overlapping genes. **(B)** PPI network and enrichment analysis of 44 genes. **(C)** The core genes identified by MCODE.

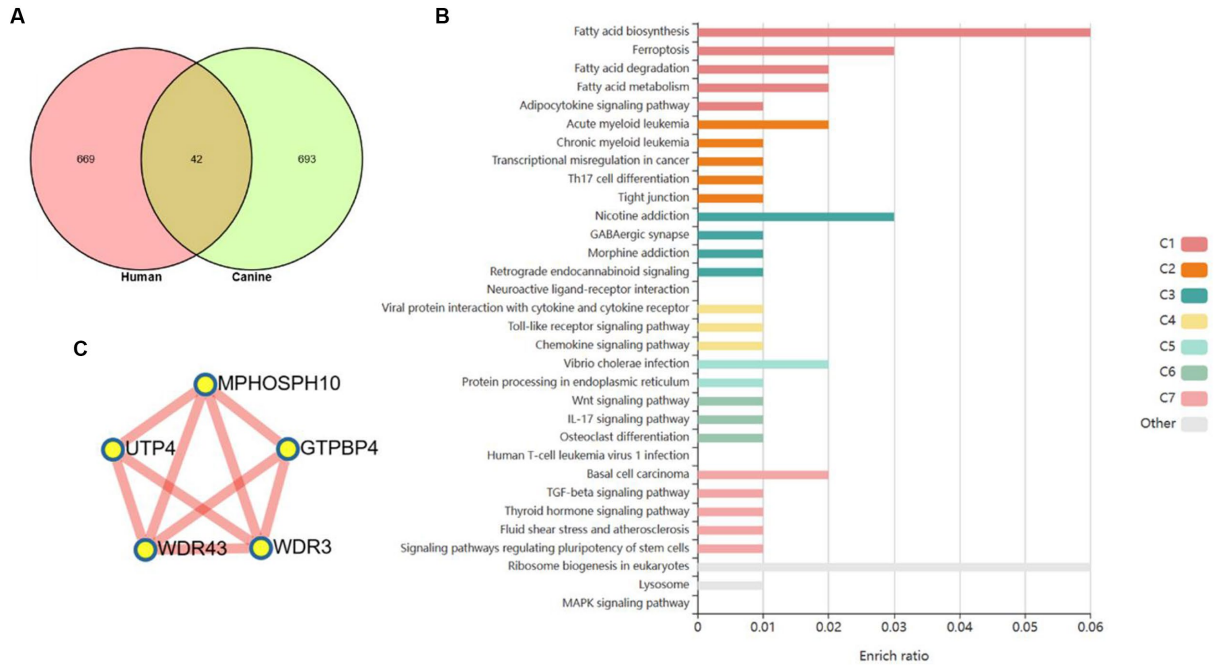

FIGURE 5
Core genes were screened based on human–dog comparative strategy. **(A)** Venn diagram to get the core genes. **(B)** Enrichment analysis reveals a variety of pathways and biological processes. **(C)** PPI network was used to screen out more core differential genes.

DEGs. The likelihood of OS was calculated using a Kaplan–Meier analysis, and groups with various levels of gene expression were compared using the log-rank test. High expressions of GTPBP4 were correlated with poor survival (Figure 6A). ROC curve analysis showed that all of them could be used as accurate biomarkers to assist in the identification of colorectal tumors. And GTPBP4 had the highest AUC score and the best diagnosis effect (Figure 6B).

4. Discussion

This evolutionary process of adenoma–carcinoma is widely accepted in human colorectal cancer studies (22). Based on the numerous molecular homologies and clinical characteristics of colorectal tumors in humans and dogs, canine colorectal tumors are likely to follow this evolutionary process (23). The dynamic expression pattern of human colorectal cancer has been extensively studied, but not reported in dogs.

We found that in adenoma and carcinoma, the expression of the number of gene activation is generally more than the number of gene expression suppression and four kinds of DEG eventually involve PFKFB3, which aroused our interest. Targeting glycolytic fluxes by metabolizing PFKFB3 for the treatment of glucose-dependent cancers. PFKFB3 increased IL-1β and TNF-α in intestinal epithelial cells to promote colitis-related colorectal cancer tumorigenesis. Interleukin-6 stimulated aerobic glycolysis by regulating PFKFB3 at the early stages of colorectal cancer (24). Mir-488 alleviated chemical resistance and glycolysis in colorectal cancer by targeting PFKFB3 (25). There are no studies exploring the biological role of PFKFB3 in canine colorectal tumors, nor even a single paper on canine topics, which is a good direction for future exploration.

The genes in cluster 5 of the five dynamic expression patterns were only altered when an adenoma turned into a carcinoma, and they were particularly enriched in the usual cancer pathway. Any gene that exhibited cluster 5's dynamic expression pattern during the development of a tumor may be crucial to the malignant transformation process. This methodology might make it easier to find new targets for cancerous transformation. GO and KEGG's analysis found that immune dysregulation played a key role in the development of tumors. Many immune pathways were primarily related to innate immunity. In the past decades, tumor immunity research has ignored the contribution of innate versus adaptive immunity. Additionally, mounting data indicates that innate immunity may be crucial to the carcinogenic effects of bowel cancer (26). Therefore, the application of adaptive and innate immune systems to fight with CRC cells could overcome the specificity problem, which was a significant concern in chemotherapy and radiotherapy (27).

Among the five dynamic expression patterns, the genes in cluster 5 were only changed during the transition from adenoma to cancer and were enriched in the typical cancer pathway. Any gene that follows the dynamic expression pattern of cluster 5 during tumor formation may play a key role in malignant transformation. This model may help facilitate the discovery of new targets for malignant transformation. GO and KEGG's analysis found that immune dysregulation played a key role in the development of tumors. Many immune pathways were primarily related to innate immunity. In the past decades, tumor immunity research has ignored the contribution of innate versus adaptive immunity. Increasing evidence also suggests that innate immunity may play an important role in the carcinogenic effects of bowel cancer (26). Therefore, the application of adaptive and innate immune systems to fight with CRC cells could overcome the specificity problem, which was a significant concern in chemotherapy and radiotherapy (27).

Combined with WGCNA analysis, we found that immune-related genes may play a key role in the malignant progression of colorectal

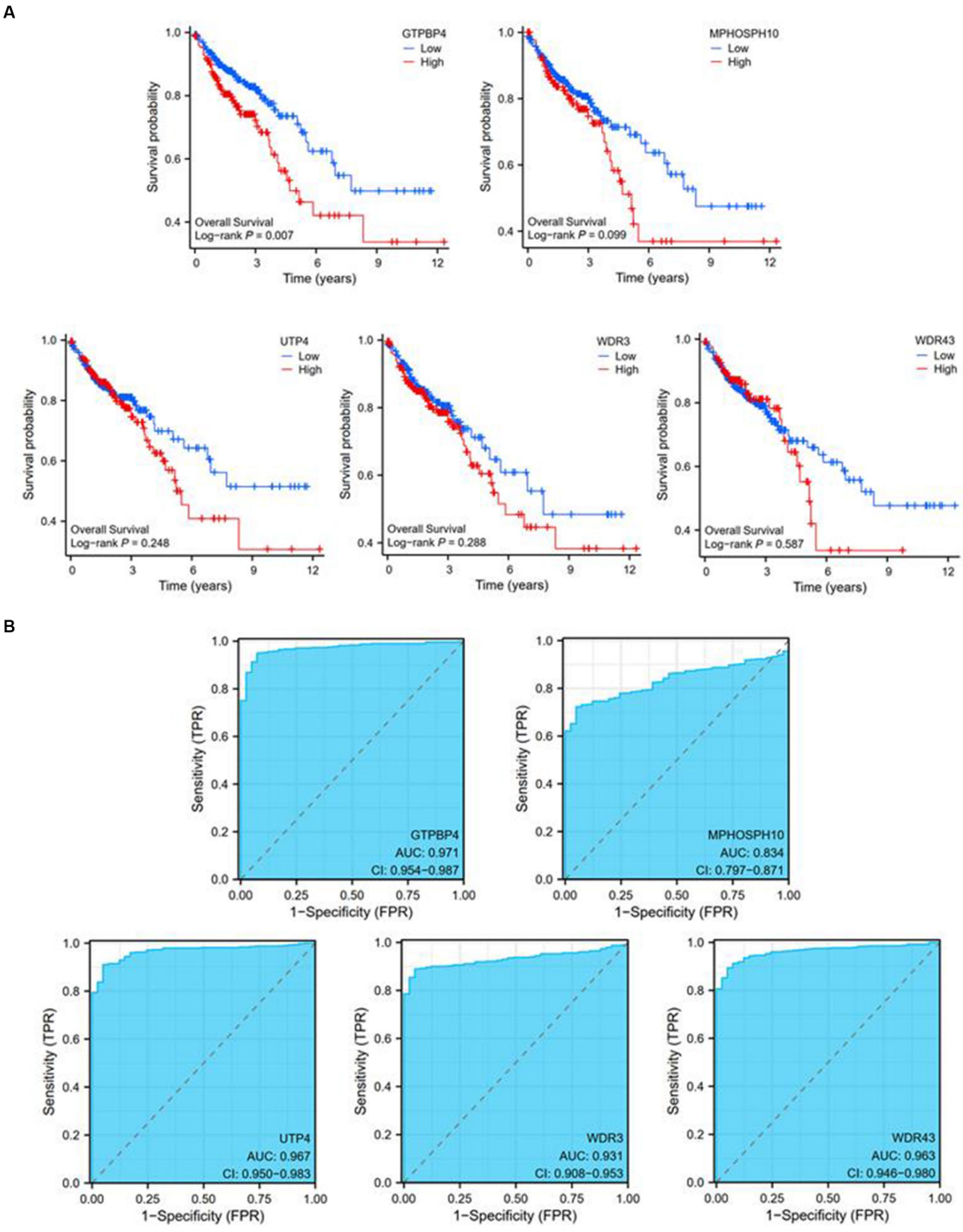

FIGURE 6
Diagnostic and prognostic ability tests of five genes. **(A)** Survival curve of GTPBP4, MPHOSPH10, UTP4, WDR3, and WDR43. **(B)** ROC curve of GTPBP4, MPHOSPH10, UTP4, WDR3, and WDR43.

tumors. As a weighted target, IL-6 has been extensively studied in human colorectal cancer. We were also surprised to find that the S100A protein family played an important role in the malignant development of canine colorectal tumors.

Strategies based on cross-species comparisons are more likely to identify cancer drivers, and we followed the new strategy of human–dog comparisons. In fact, a similar gene expression pattern exists in human colorectal cancer, and the consistently up-regulated gene

community is more likely to play an important role in cancer. Through comparative screening, we found 42 common differential genes, and enrichment analysis found that these genes have a wide range of biological roles related to classical cancer pathways, metabolism and immunity are covered.

Five core genes were identified by further analysis of the 42 genes, which were GTPBP4, MPHOSPH10, UTP4, WDR3, and WDR43.

A critical regulator of cell cycle progression and MAPK activation is guanosine triphosphate binding protein 4 (GTPBP4), a member of the GTPBP family that is highly conserved throughout eukaryotes from yeast to humans (28). It was demonstrated that GTPBP4 promoted hepatocellular carcinoma (HCC) growth and metastasis both *in vivo* and *in vitro* and promoted aerobic glycolysis by inducing dimeric pyruvate kinase M2 (PKM2) formation (29). A higher level of GTPBP4 was detected in CRC metastatic tissues, and GTPBP4 has been proven to promote CRC metastasis by disrupting RhoA activity (30). GTPBP4 has shown superior diagnostic and survival effects through human colorectal cancer survival analysis and diagnostic tests.

M-phase phosphoprotein 10 (MPHOSPH10) belonged to categories of cellular physiologic response and signal transduction (31), however, its role in tumor progress has still been little studied. It was found that hypoxia markedly down-regulated cell survival-related genes such as, MPHOSPH10, IMP-3, ITGA2, SDCBP, and IGBP3 in SK-N-MC cells (32).

One of the subcomplexes in the small subunit (SSU) processome, the U three proteins (Utps), was essential for the formation of ribosomal chromatin (r-chromatin) and for the efficient production of rDNA (33). Seven proteins made up the t-Utp subcomplex: Utp4, Utp5, Utp8, Utp9, Utp10, Utp15, and Utp17 (34). Utp4 was a ribonucleoprotein complex required for ribosomal RNA processing and small subunit assembly (35).

The tryptophan-aspartate repeat (WDR) domain was engaged in a large number of cellular processes, such as the ubiquitin-proteasome system (UPS), the G protein-coupled receptor signaling pathway, DNA damage perception and repair, epigenetic chromatin regulation, and the immune system (36). WD repeat domain 3 (WDR3), also known as DIP2 or UTP1, a member of the WD-repeat family, was engaged in a number of cellular processes, including signal transmission, apoptosis, gene regulation, and cell cycle progression (37, 38). By interacting with GATA4, WDR3 activated the Hippo signaling pathway, demonstrating that it was crucial in promoting the advancement of pancreatic cancer (39). Additionally, prostate cancer (PCa) tissues were found to have a substantially higher WDR3 level, and WDR3 overexpression increased markers of stem cell-like characteristics (40). These studies above showed that WDR3 may assist some malignant cancers to grow and proliferate, nevertheless, the biological function of WDR3 in CRC and its associated mechanism were still unknown.

Tryptophan-aspartate repeat domain 43 (WDR43), the ortholog of yeast Utp5, could interact with the Pol II machinery in embryonic stem cells (ESCs) (41, 42). By examining the data from the Gene Expression Omnibus (GEO) dataset and The Cancer Genome Atlas (TCGA) database, WDR43 was identified as a potentially significant oncogenic factor in the pathogenesis of CRC and a marker for predicting the efficacy of chemotherapy (43), which was consistent with our results. Additionally, cancer cells with higher WDR43 expression were more resistant to chemotherapy-mediated cell death and therefore the overexpression of WDR43 was related to the poor prognosis of CRC patients. *In vitro* studies have revealed that WDR43 knockdown increased apoptosis, inhibited CRC cell proliferation, migration, and invasion, and slowed carcinogenesis in animal models (44).

In pan-cancer analysis, these genes were significantly expressed differently in multiple cancers (Supplementary Figure S7), which reinforced our belief in the significance of this finding. The important role of GTPBP4, MPHOSPH10, UTP4, WDR3, and WDR43 in human tumors has been discovered, but more in-depth research on the mechanism is still lacking. However, in the study of canine CRC, the molecular functions and clinical diagnostic predictiveness of these markers have not been proven, which deserved further exploration.

5. Conclusion

Our study was the first to explore this dynamic expression pattern of normal-adenoma–carcinoma in canine CRC, explaining the molecular characteristics of colorectal tumor development in dogs. In conclusion, GTPBP4, MPHOSPH10, UTP4, WDR3, and WDR43 could serve as valuable biomarkers of cross-species values and provide new selections for the future diagnosis and treatment for both human and canine CRC.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because this study only analyzed publicly available sequencing data and did not involve animal testing.

Author contributions

ZL designed experiments and analyzed the data. JZ drafted the manuscript. QC and XZ conducted the experiments. JL reviewed the manuscript. JL and DL supervised the project. ZL and DZ conceived the project. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the The 14th Five-Year Plan National Key Research and Development Program of China (2022YFD1801104).

Acknowledgments

We thank Yeyizhou-Fu from Peking University for selfless help.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1192525/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Mini Review
PUBLISHED 03 January 2024
DOI 10.3389/fvets.2023.1285354

OPEN ACCESS

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RECEIVED 29 August 2023
ACCEPTED 09 November 2023
PUBLISHED 03 January 2024

CITATION
Klingemann H (2024) *Viscum album* (mistletoe) extract for dogs with cancer? *Front. Vet. Sci.* 10:1285354. doi: 10.3389/fvets.2023.1285354

Viscum album (mistletoe) extract for dogs with cancer?

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Compared with the options available to human patients with cancer, treatment choices for dogs are often more limited. Chemotherapy is frequently the first-line treatment for many cancers. However, its efficacy can be limited, and its side effects can affect the quality of the remaining life. This paper briefly summarizes the experience with *Viscum album L.* (mistletoe) extract in human patients as a stipulation to consider treatment with mistletoe extract for canines with cancer. The mistletoe extract contains -among others - lectins and viscotoxins that have documented anti-proliferative effect on cancer cells as well as immune-stimulatory function. Importantly, it also improves the well-being of patients with cancer due to its lectin ML-1 content, which can trigger the release of endorphins. Being cross-reactive with canine cells and having a relatively low side effect profile, it raises the question of whether mistletoe preparations might be considered as part of the treatment approach for dogs with cancer.

KEYWORDS

dogs, cancer therapy, mistletoe, *Viscum album*, immunotherapy

1 Introduction

1.1 What is mistletoe?

It is a semi-parasitic plant that grows on trees and uses their sap to thrive (Figure 1). In fact the trees it grows on, often die off over time. Mistletoe has been around as a cancer therapeutic for more than a century especially in the German speaking part of Europe (1). It was introduced by Rudolf Steiner and Dr. Ita Wegman, who treated the first patients with cancer with *Viscum album* mistletoe extract around 1920. In addition to some tumor responses, it was also noted throughout the years that the quality of life of patients with cancer even during chemo/radiotherapy, could be improved with mistletoe (2–4). In fact, mistletoe extracts are currently the most frequently prescribed non-conventional cancer treatment in central European countries; 70–80% of patients with cancer will receive it at some point.

Most bioactive ingredients of mistletoe have been identified and characterized such as lectins (I, II, and III), polypeptides (e.g., viscotoxins), and immunostimulatory glycoproteins (5–7). The extracts are also enriched in biologically active flavonoids, phenolic acids, sterols, lignans, terpenoids, and phenylpropanoids (8). There is strong evidence that the *complete* mistletoe extract is more potent than when isolated compounds are administered (9, 10). Mistletoe preparations are made from extracts of leaves, stems, buds, and ripe berries during the fall or winter harvest (11). Although some 1,500 species of plants are denoted as mistletoes, only white-berry mistletoe from Northern Europe (*Viscum album* L.) is used in cancer treatment. The tree *origin* of the mistletoe is also relevant (pine, apple, oak, or ash) and so is the *time of harvest* (fall vs. winter) as the concentration of the different active components

FIGURE 1
Mistletoe growing on host tree.

varies with the time of the year (12). For example, green berries in the fall have more viscotoxins, whereas white berries in winter carry more lectins (13). The extract can also be fermented, a process that can add bacterial metabolites to the extract, which can function as pathogen-associated molecular patterns [PAMP] (14). *Iscador*^{R} is fermented, whereas *Helixor*^{R} and *abnoba Viscum*^{R} are not.

Numerous *in vitro* studies have confirmed the direct inhibitory effect of mistletoe on malignant cell proliferation and apoptosis (15–17). Researchers from MD Anderson Cancer Center determined in a liver cancer model that this effect is related to certain components in mistletoe that downregulate the expression of the c-myc oncogene in cancer cells (18). In addition to anti-proliferative /apoptotic effects, mistletoe stimulates the secretion of immune-active cytokines (19) and augments the function of immune cells, such as T-lymphocytes (20, 21) and natural killer cells (22, 23). It also supports the maturation of dendritic cells and macrophages (24, 25). Mistletoe ingredients have an anti-angiogenic effects in cancer tissues and neutralize tumor-induced immunosuppression (17). Importantly, mistletoe can improve the quality of life of cancer patient (2–4). Even in the advanced stages, it mitigates cancer-related symptoms and reduces the side effects of chemotherapy and radiation. This beneficial effect is related to the lectin ML-1 content, which stimulates the release of endorphins (26).

Despite the widespread use of mistletoe preparations in central Europe, it has not found its place in the US and Canada as the FDA has not granted its stamp of approval largely due to the fact that the commercial mistletoe extracts have multiple ingredients which makes it difficult to standardize each batch for a given ingredient. However, the production of mistletoe follows a standardized manufacturing process and its batch to batch consistent biological activity is guaranteed.

Although there are numerous reports that mistletoe extracts have a therapeutic benefit in cancer patients in terms of response rate, overall survival, and quality of life, many of these studies have a major challenge: when mistletoe is administered concurrently with chemotherapy or radiation, it becomes difficult to define its contribution. Since the use of mistletoe is so prevalent in Germany, Troger et al. (27) went outside of the country to perform a randomized trial with mistletoe in patients with locally advanced pancreas cancer. Patients in the mistletoe arm had a significantly higher tumor response rate and longer survival time than those in the control group who received standard chemotherapy. In another study, mistletoe extract was given to 23 patients with advanced, chemotherapy naïve liver cancer with five patients (22%) achieving a complete or partial response (28). Acute myeloid leukemia is also responsive to *Viscum album* as reported from *in vitro* and *in vivo* (murine) studies (29). It is noteworthy that mistletoe has quite limited side effects when administered at the recommended dose, and the side effects that have been reported more frequently (inflammatory reaction at the injection site, fever, malaise) are the ones that physicians want to see with any immunotherapy as signs of an active immune response.

Owing to the initiative of a patient who had received mistletoe treatment for metastatic cancer, a Foundation (*"Believe Big"* https://www.believebig.org/what-is-mistletoe/) was initiated with the goal of supporting the clinical exploration of mistletoe in the US in a well-controlled clinical study setting. With funding from that initiative, a phase I study was conducted at Johns Hopkins in 21 patients with various advanced cancers who received mistletoe intravenously (600 mg) thrice a week until disease progression or toxicity occurred. The results of this safety/feasibility study were recently published (30). Side effects were minor, and 25% of the patients were reported to have stable disease with a median follow-up of 15.3 months. A reduction in tumor size occurred in three patients remaining stable for 2–5 months. Importantly, most patients reported an improvement in their quality of life. Further clinical trials are planned to determine the efficacy of mistletoe in different cancer sites.

For human patients with cancer, mistletoe (whole plant extracts) is usually administered *intravenously* at the beginning of the treatment cycle, followed by *subcutaneous* administration (i.e., 3 times a week). Intra-tumor application is particularly attractive in early stage cancer (even during or after surgery) when the tumor has not yet spread to other organs. Intra-tumor injection of ash tree- derived mistletoe into a human pancreatic cancer xenograft resulted in significant tumor response, with 75% of treated mice having either a partial or complete response (31). In a safety study, Steele et al. (32) treated 123 patients with cancer with intra-tumor mistletoe injections from various providers. The side effects were relatively mild, and consisted only of fever and local inflammatory reactions. In this context, a promising indication for local mistletoe administration is bladder cancer (33–35). It is important to note that mistletoe supplements in the form of

capsules, liquid extracts, teas, and powders have no scientific or clinical support for efficacy.

1.2 Rationale for using mistletoe for dogs with cancer

Despite the evidence of an anti-cancer effect in humans and its ability to improve the quality of life of patients with cancer, there is very limited well-documented experience with *Viscum album* in treating cancer in dogs. There may also be a perception that the berries are poisonous to dogs (they contain Viscumin). However, dogs have to eat a fair amount and even after accidental ingestion, signs and symptoms are limited (36). There is also a difference between uncontrolled and accidental ingestion of the plants/berries and administration of a medicinal preparation, which is well defined and prepared in pharmacological doses. Kienle et al. (36) reviewed the safety of various mistletoe preparations and doses in animals (mostly mice, one horse, one cat, no dogs) and noted minimal or only low grade side effects even at higher doses.

Although surgery, radiation, and chemotherapy are considered first-line treatments for most canine patients with cancer, there are many scenarios in which this approach fails, or the dog cannot tolerate it at some point. Not infrequently, owners cannot see the dog suffering from the side effects of chemotherapy, and the quality of life becomes a consideration. In fact, in a recent survey, it was found that about two-third of dog owners would not elect to treat their dog with chemotherapy due to the negative impact of the associated side effects (37).

Although Immunotherapy has become the fourth pillar of cancer therapy for humans, it is far less developed for dogs (38, 39). Considering its beneficial reports in human patients with cancer, it is surprising that *Viscum album* extracts have not received more attention as immune-active cancer treatment for our "best friend." The United States Department of Agriculture [USDA] regulates drug use in the veterinary space, and as long as mistletoe is not officially licensed, reimbursement will be limited. In fact, since all the companies that produce clinical-grade mistletoe are located in Europe, imports into the US are also regulated. However, there are some veterinarians in the US who can provide mistletoe treatment despite limited access and logistical challenges.

1.3 Current status of mistletoe use for cancer treatment in dogs

A literature search for studies on canine cancer cell lines exposed to mistletoe resulted in only one study that confirmed its cytotoxicity against canine astrocytoma cells (40). There have been less than a handful of clinical studies exploring the use of mistletoe in dogs. Biegel et al. (41) treated dogs with mammary tumors with mistletoe subcutaneously in the adjuvant setting after surgery. Compared to the non-treated control group, there was a trend ($p = 0.07$) toward a decrease in tumor-related death while maintaining a stable quality of life for a prolonged time. The same investigators treated dogs with oral malignant melanoma with mistletoe after radiation in a non-randomized study (42, 43). Eighteen dogs received mistletoe subcutaneously, while eight did not. The median survival time in the treatment group was 236 days versus 49 days in dogs that did not receive mistletoe.

Where to go from here? To convince veterinarians that mistletoe can have some benefits for dogs with cancer, the first step would be to conduct some comprehensive *in vitro* studies with canine cancer cell lines and tumor biopsy material to define which canine tumors are more sensitive to the cytotoxic and immunomodulatory effects of mistletoe. The next step would be to conduct phase I studies that would test the safety of escalating subcutaneous injections in dogs using the three times/week schedule adopted from humans. Ideally some pharmacokinetic studies can be included to assure that the dosage derived from human administration applies equally to dogs. Although mistletoe preparation have many components, it appears that the lectin plasma level can be reliably measured (44). With this knowledge, clinical trials could determine in which diseases mistletoe is most effective for dogs with cancer even as an alternative in situations where owners decide against more aggressive treatment. Despite the challenges of obtaining funding for veterinary trials, it would be relevant to look at the effect of mistletoe administered intra-tumor or locally, such as in melanoma and bladder cancer, if the tumor is accessible and has not metastasized. It may be more challenging to quantify the effect of the treatment on improving the quality of life of canine patients, as there are fewer well-established parameters in place (45). Considering the available facts though, *Viscum album*/mistletoe is a treatment option that should not be withheld for dogs considering the unequivocal benefits reported in human patients with cancer for more than a century.

Author contributions

HK: Conceptualization, Writing - original draft, Writing - review and editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 08 January 2024
DOI 10.3389/fvets.2023.1285909

OPEN ACCESS

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RECEIVED 30 August 2023
ACCEPTED 24 November 2023
PUBLISHED 08 January 2024

CITATION
Vanhaezebrouck IF, Bakhle KM, Mendez-Valenzuela CR, Lyle LT, Konradt K and Scarpelli ML (2024) Single institution study of the immune landscape for canine oral melanoma based on transcriptome analysis of the primary tumor.
Front. Vet. Sci. 10:1285909.
doi: 10.3389/fvets.2023.1285909

Single institution study of the immune landscape for canine oral melanoma based on transcriptome analysis of the primary tumor

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Introduction: Understanding a tumor's immune context is paramount in the fight against cancer. Oral melanoma in dogs serves as an excellent translational model for human immunotherapy. However, additional study is necessary to comprehend the immune landscape of dog oral melanomas, including their similarity to human melanomas in this context.

Methods: This retrospective study utilizes formalin-fixed paraffin-embedded (FFPE) tissue samples to analyze RNA sequences associated with oral melanoma in dogs. Nanostring Technologies was used for conducting RNA sequencing. The focus is on understanding the differences between melanoma tumors restricted to the oral cavity (OL) and the same primary oral tumors with a history of metastasis to the lymph nodes or other organs (OM). Normal buccal mucosa samples are also included as a normal tissue reference.

Results: In the OM patient group, gene signatures exhibit significant changes relative to the OL patient group, including significantly decreased expression of S100, BRAF, CEACAM1, BCL2, ANXA1, and tumor suppressor genes (TP63). Relative to the OL tumors, the OM tumors had significantly increased expression of hypoxia-related genes (VEGFA expression), cell mobility genes (MCAM), and PTGS2 (COX2). The analysis of the immune landscape in the OM group indicates a shift from a possible "hot" tumor suppressed by immune checkpoints (PDL1) to significantly heightened expression not only of those checkpoints but also the inclusion of other immune blockades such as PD1 and IDO2. In addition, the OM group had significantly reduced expression of Toll-like receptors (TLR4) and IL-18 relative to the OL group, contributing to the tumor's immune escape. Additionally, signs of immune cell exhaustion are evident in both the OM and OL groups through significantly increased expression of TIGIT relative to normal tissue. Both the OM and OL groups had significantly increased expression of the immune cell marker CD4 expression relative to normal tissue. Further, CD4 expression significantly decreased in OM relative to OL; however, this study cannot determine the specific cell types expressing CD4 in OM and OL tumors.

Discussion: This preliminary study reports significant changes in gene expression for oral melanoma between canine patients with localized disease relative to those with metastatic disease. In the future, a more in-depth investigation involving

immunohistochemistry analysis and single-cell RNA expression is necessary to confirm our findings.

KEYWORDS

dog, oral melanoma, immune landscape, transcriptome, cancer

Introduction

Oral melanoma is relatively common in canine companions, accounting for 7% of all malignancies. It is known for its aggressive nature (1). The cancer tends to metastasize first to the regional lymph nodes, then to the lungs, and less commonly to the liver, brain, and adrenal glands. The prognosis for affected dogs depends on the stage (TNM classification), pathology grade, mitotic index of cells, and Ki-67 expression (2). Pathologists have drawn comparisons between oral melanoma in canine and human melanoma due to similar gene dysregulations in the NRAS, AKT, and PTEN pathways, mutations in c-Kit (found in about 10% of cases), and overexpression of Cox-2. However, it's worth noting that Braf mutations are highly prevalent in human melanoma and occur less frequently in canine melanoma (3–7).

The oral cavity is the most common location for melanoma in dogs, and it can affect the lips, oral mucosa, the tongue, or the jawbone (maxilla or mandible). Treatment usually involves a multidisciplinary approach, which includes surgical excision to ensure healthy margins and radiotherapy. Chemotherapy has shown disappointing results similar to those seen in human subjects (8). Overall survival for most dogs with advanced local disease (tumor size >2 cm) or disseminated form is poor, with an average of 7–10 months (9, 10).

For the last 15 years, the veterinary community has been focused on researching and making progress in immunotherapy (11–14). Cancer cells can suppress the body's immune response and utilize surrounding cells, including immune cells, to promote their own growth and survival gain. Promoting an adequate immune response is paramount in the fight against cancer (15). Veterinary oncologists cannot achieve therapeutic improvement for these patients without understanding the complex interaction between cancer cells and surrounding immune cells.

In a preliminary veterinary pathology study using immunohistochemistry (IHC), the lymphocyte density in 32 oral tumors was analyzed, and it was found that a low B cell count was associated with a better prognosis. However, the authors could not comment on the T cell population based on its diversity, including pro-immune versus immunosuppressive Tregs (16). In addition, a recent study in canine melanoma reported on 25 biopsies using IHC and RNA extraction along with qualitative real-time polymerase chain reaction techniques to analyze the expression of immunosuppression markers FoxP3, IDO, and CTLA4. An analysis of all samples showed gene and protein expression correlated with poor prognoses (17).

Galon et al. (18) introduced the concept of Immunoscore® while studying human colorectal cancers in 2006. He quantified different populations of lymphocytes, CD8, CD3, and CD45R, their collocation at the center of the tumor versus the periphery, and their functional immune orientation (based on gene expression profiling). A scoring scheme was also introduced, establishing a patient's prognosis based on the immune landscape. The classification was more accurate than standard staging and pathological classification for predicting patient survival. The concept has gained international recognition and has become a benchmark for predicting prognosis.

Preliminary work on human skin melanoma reveals a complex immune landscape. For human melanoma patients treated with immune checkpoint inhibitor ipilimumab, a prominent CD8 infiltration and PDL1 negative status for lymph node metastasis is considered a favorable prognostic factor (19).

Human pathologists have increasingly employed digitalization and artificial intelligence software to facilitate the estimation of large-scale cell counts. A comprehensive understanding of cell type, density, count, and immune cells distribution within the tumor is crucial (20). Moreover, coupling this information with genomic analyses encompassing chemokines, checkpoints, and interferon activity can provide a more insightful evaluation of the tumor immune landscape and microenvironment. This integrated approach holds significant promise in accurately assessing a patient's prognosis and guiding treatment strategies. Ultimately, it could pave the way for a personalized treatment tailored to the patient's cancer's unique characteristics (21). Similar analyses in veterinary pathology could benefit our canine patients to determine the progression of the disease.

This single-institution clinical research study aimed to characterize the immune landscape of oral canine melanoma. We present an analysis of the immune contexture within the tumor microenvironment based on Nanostring technology (800 genes- IO canine- Ncounter®, Nanostring, Seattle, WA, 98109, United States) derived from 18 FFPE oral tumors. Dogs are characterized by spontaneous disease, a natural progression of cancer growth, and adaptation to the host environment. Understanding the immune context for canine melanoma could stimulate further research supporting translation to human melanoma (22).

Materials and methods

Tissue samples

Eighteen primary melanoma tumor samples and five normal tissue samples from buccal mucosa were procured from the pathology laboratory at Purdue University Veterinary College. All melanoma samples came from the primary oral tumor. All normal tissue samples were derived from the oral labial mucosa in healthy dogs. FFPE samples were obtained from biopsy, surgical excision, or autopsy between 2000 and 2020. Previous studies at our institution and others have reported the pathology approach for analyzing melanoma with IHC identification and classification (23, 24). To ensure accuracy, a board-certified pathologist (TL) performed a second review for each sample included in the study. Tumors were classified as local malignant (OL) if no evidence of metastasis was mentioned (staging obtained from the referring veterinarian or data from the pathology laboratory)

or oral malignant (OM) for primary tumors with known metastases. Given the specific nature and objectives of this study, clinicopathological characteristics were not included in the analysis.

Sample processing and RNA sequencing technique

For each sample, between 50 and 100 ng was shipped to Nanostring® for RNA extraction. The extracted RNA was solubilized and mixed in a hybridization solution. The 50 kb segment of interest was hybridized to a capture probe, and the distal part (50 kb) was linked to a fluorescent probe. Each type of fluorescence obtained was associated with a unique gene. The Nanostring® canine Immuno-Oncology (IO) chip targeting 800 genes related to cancer and immune markers was used for this analysis.

Following 24 h of hybridization, the samples were purified and placed in a cartridge. The fluorescent probes were immobilized using a current, and the samples were then ready for optical scan counting. The resulting data were presented approximately as the amount of fluorescence relative to housekeeping genes.

Normalization and statistical analysis

Data were normalized using the geNorm algorithm to identify suitable housekeeping genes. After removing potential candidates such as G6PD, OAZ1, TBC1D10B, TFRC, and UBB, the final set of housekeeping genes included ABCF1, DNAJC14, ERCC3, GUSB, MRPL19, NRDE2, POLR2A, PSMC4, PUM1, SDHA, SF3A1, STK11IP, TBP, TLK2, and TMUB2.

For Statistical analysis, a non-paired *t*-test with a false discovery rate *post-hoc* test was performed to determine significant differences among samples. Quality control metrics employed by the NanoString platform encompass Imaging QC, Binding Density QC, Positive Control Linearity, and Limit of Detection QC. These internal controls assess the technical success of the assay.

The quantification of targets was achieved through direct digital counting of a hybridized fluorescent barcode, which was bound to a streptavidin-coated imaging surface. These raw counts were normalized to housekeeping targets, and data were expressed as Log2 normalized counts.

Data analysis

A set of genes representing a potentially clinically relevant immune profile, cellular and microenvironment dysregulation, was identified through gene expression analysis. The significance of the expression was determined by assessing fold changes and adjusted *p*-values. The visualization of the data was accomplished by generating heat maps using R software [R Foundation for Statistical Computing, Vienna, Austria, Version 4.2.3.] with the utilization of the "heatmap" function as illustrated in Figures 1–3.

Furthermore, the significance of the expression levels was depicted through tables comparing overexpressed versus under-expressed genes. This comparison was made across various scenarios: local tumor (OL) versus normal buccal mucosa tissues (Table 1), local tumor with known metastasis (OM) versus normal buccal mucosa tissues (Table 2), and local tumor with known metastasis (OM) versus local tumor known to remain at the local level (OL) (Table 3). For each tissue sampled, the biopsy included the epithelial part and the stromal part. Statistically significant differences across groups was determined based on a ±1.5-fold threshold indicating differential changes with an adjusted *p*-value 0.05.

Results

Population characteristics

A total of 18 canine patients with a documented history of malignant melanoma between 2000 and 2020 were included in this study as described in Table 4. The average age of these patients was 10.5 years. Of the 18 patients, nine were castrated males, one was an intact male, six were spayed females, and two were intact females. Regarding breed distribution, most patients were of mixed breed (8/18), followed by three Golden Retrievers, two Labrador retrievers, two Schnauzers, one Cocker Spaniel, one Poodle, and one Scottish terrier (Table 4).

The oral primary tumor location is reported in Table 4. It was observed that the mitotic index was higher than 10 (per 10 Hpf) for oral tissues with a known history of metastasis. In contrast, for local tumors without available information on metastasis, the mitotic index was no more than three. Overall, 12 OL cavity samples were compared to six OM samples and five standard canine buccal tissue samples as shown in Table 4.

Heat maps providing a comparison of marker expressions that were significantly different between the three different groups are shown in Figures 1–3. In addition, Tables 1–3 summarize clinically relevant markers with significant changes in expression. Supplementary Tables S1–S3 show changes in expression for clinically relevant markers regardless of significance level.

Melanoma cell markers

Melan A, tyrosinase, and S100 are commonly used genes by pathologists to identify melanoma-associated proteins. The genes encoding proteins S100 A4, S100A8, S100A9, S100 A10, and S100A12 showed significantly decreased expression in OM relative to normal tissue (Table 1). Conversely, the expression of Sox 10 was significantly elevated in both OL and OM groups relative to normal tissue. Sox 10 is present during embryonal life and is a nuclear transcriptional factor in melanogenesis (25).

BRAF is significantly under-expressed in OL tumors compared to normal tissues (Table 1) and in OM tumors compared to normal samples (Table 2). When comparing OL tumors vs. OM tumors, BRAF was significantly under-expressed in OM compared with OL (Table 3). BRAF inhibitors are frequently used for human skin melanoma. However, regarding the under-expression of BRAF in canine oral melanoma, BRAF-targeted therapy may not be an option.

CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1) is significantly under-expressed in OL vs. normal tissues (Table 1). Furthermore, the decreased expression becomes even more significant when comparing OM vs. normal tissue (Table 2). These findings suggest a progressive decrease in this marker when transitioning from OL to OM phenotype. This tumor-associated

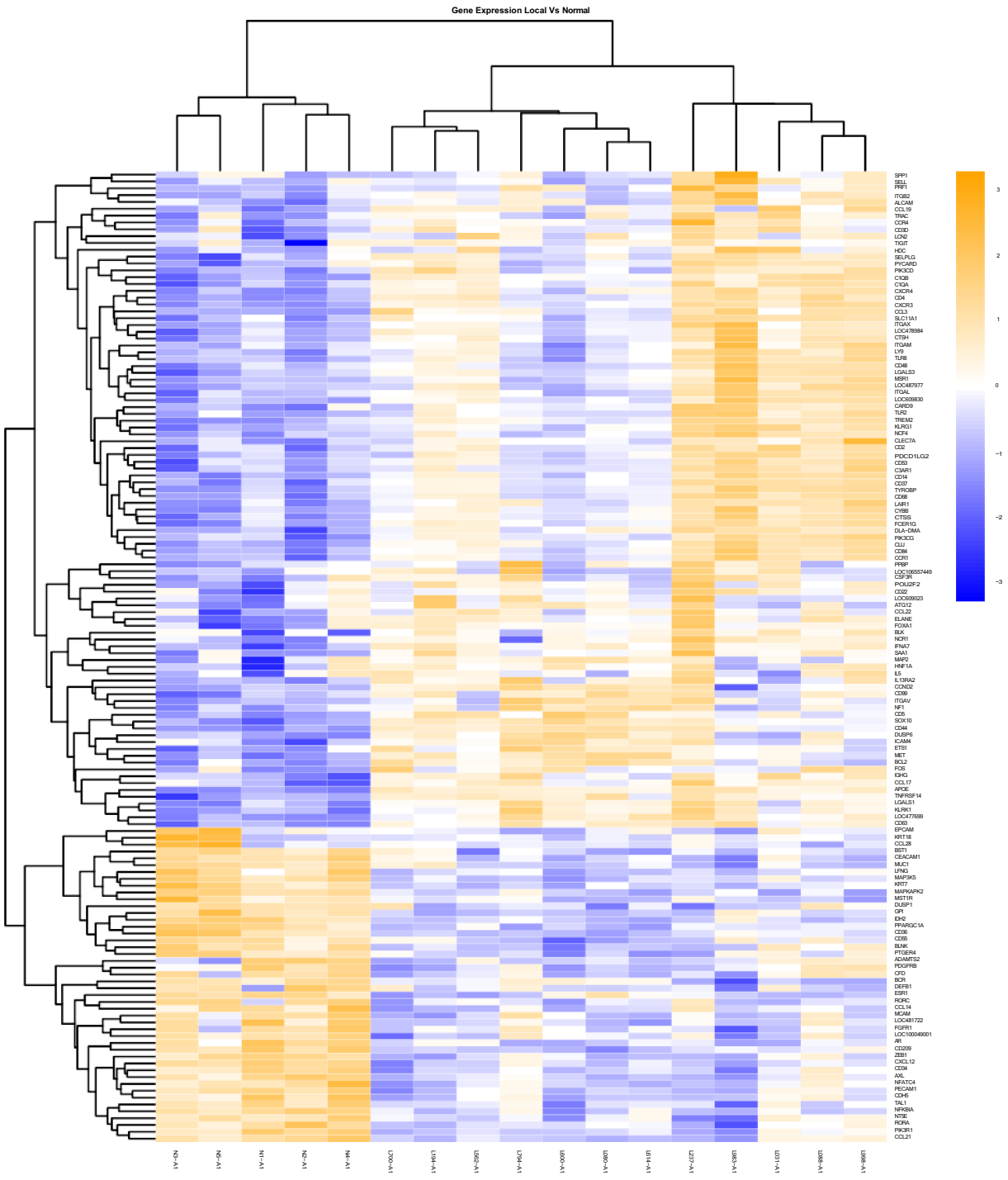

FIGURE 1
Gene Expression Comparison Patterns of Local (OL) Vs. Normal Oral Mucosa: 142 genes were found significantly expressed in the comparison of Local vs Normal mucosa. The list of genes is presented on the right "Y" axis of the heat map. Patients are indicated on the horizontal plane. Samples that start with an "N" come from normal dogs, and those that start with an "L" are from patients with local tumors. The color blue indicated low expression compared to high expression by orange.

antigen serves as a prognostic biomarker in several human cancers; for example, low CEACAM expression is linked with poor survival for patients with clear cell renal carcinoma (26).

DMBT1 was under-expressed in all melanomas in this study, albeit the decreases were not statistically significant (Supplementary Tables S1–S3). In human patients, DMBT1 is present in anorectal melanoma but not in the skin and functions as a suppressor gene. DMBT1 gene deletion or loss of function is associated with tumor progression in glioblastoma, medulloblastoma, and lung, gastric, and colorectal tumors (27).

NOS2 was elevated in OM samples compared to normal tissue (Table 2). Elevated NOS2 in human melanoma amplifies P13/AKT, HIF, and Ras pathways, TGF beta expression, and lower immune function. Moreover, NOS2 gene expression has been linked to a poor prognosis (28).

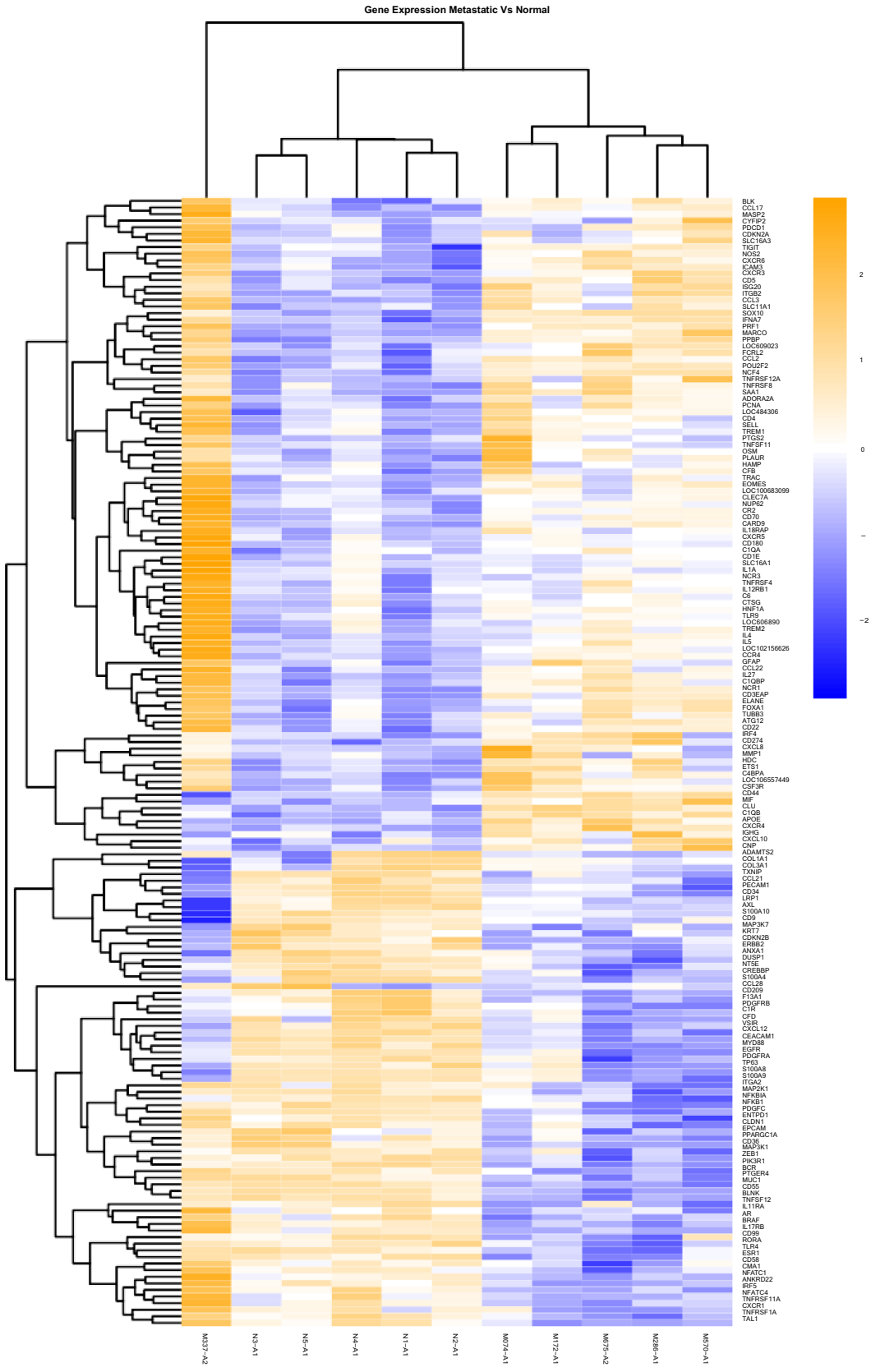

FIGURE 2
Gene Expression Comparison Patterns of Metastatic (OM) Vs. Normal Oral Mucosa: 171 genes were found significantly expressed in the comparison of Metastatic vs Normal mucosa. The list of genes is presented on the right "Y" axis of the heat map. Patients are indicated on the horizontal plane. Samples that start with an "N" come from normal dogs, and those that start with an "M" are from patients with metastatic tumors. The color blue indicated low expression compared to high expression by orange.

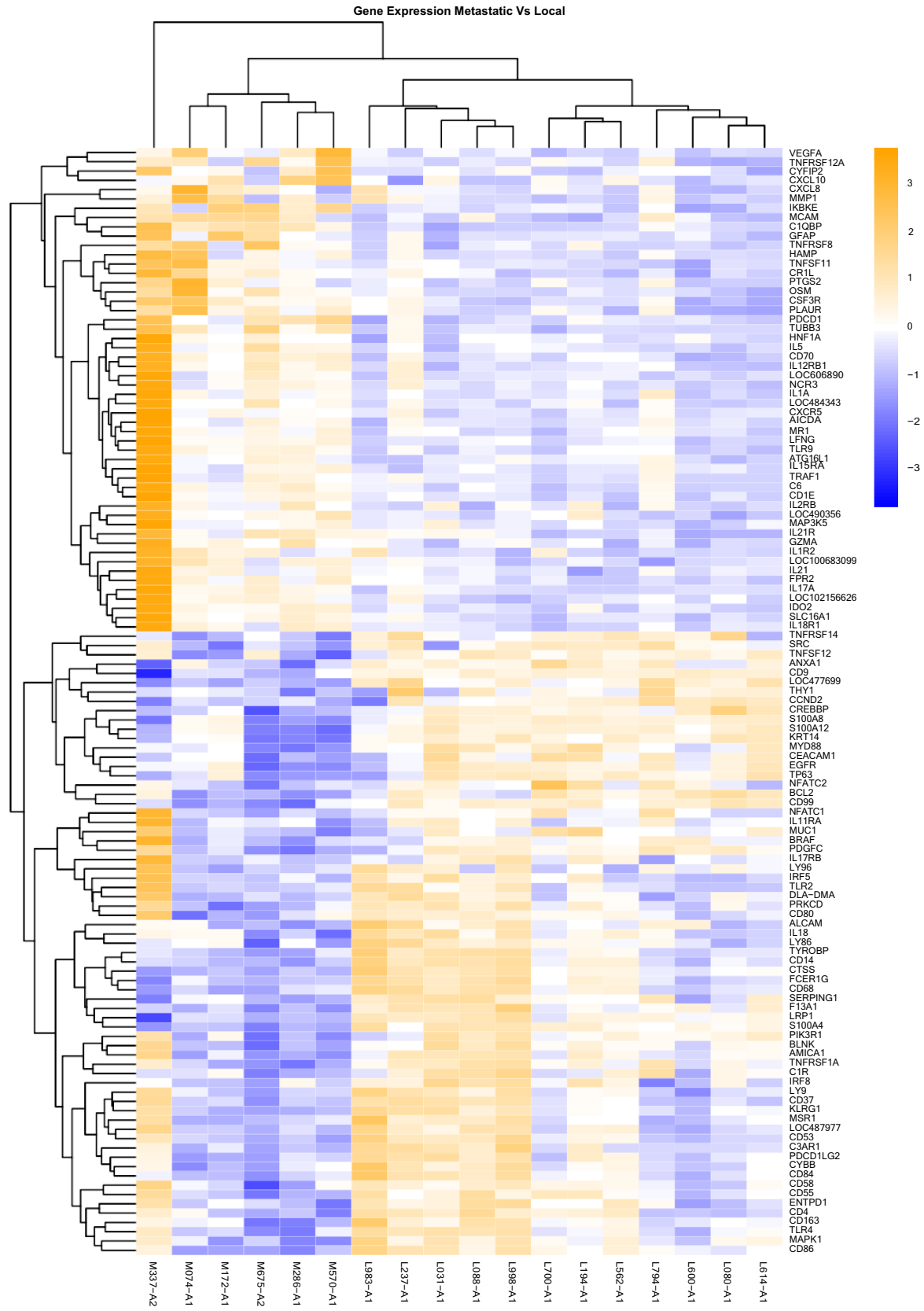

FIGURE 3
Gene Expression Comparison Patterns of Metastatic (OM) Vs. Local (OL) Tumors: 119 genes were found significantly expressed in the comparison of Metastatic vs Normal mucosa. The list of genes is presented on the right "Y" axis of the heat map. Patients are indicated on the horizontal plane. Samples that start with an "M" come from metastatic dogs, and those that start with an "L" are from patients with local tumors. The color blue indicated low expression compared to high expression by orange.

TABLE 1 Significantly expressed genes *of* local (OL) vs. normal.

Significantly expressed genes local (OL) vs. normal				
Cell type	Gene symbol	Protein description	Fold	*P*-value adj.
T cell	CD4	CD4 molecule	15.4079	0.0000646
NK cell	KLRG1	Killer cell lectin-like receptor subfamily G, member 1	11.231	0.000627
B cell	BLK	B lymphoid tyrosine kinase	3.35318	0.034682
	TYROBP	TYRO protein tyrosine kinase binding protein	5.18089	0.001619
Macrophages	CD14	CD14 molecule	5.8061	0.002133
	CD68	CD68 molecule	4.89344	0.009834
	CD84	CD84 molecule	13.3984	0.000931
	CD99	CD99 molecule, Protein MIC2	1.82053	0.032208
Cytokines	IFNA7	Interferon, alpha 7	4.59887	0.000105
Melanoma cells	BCL2	B-cell CLL/lymphoma 2	2.46065	0.002709
	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	−3.42594	0.006294
	MCAM	Melanoma cell adhesion molecule	−2.39599	0.023284
	SOX10	SRY (sex determining region Y)-box 10	26.6667	0.000106
Immune checkpoints	TIGIT	T cell immunoreceptor with Ig and ITIM domains	3.05555	0.04822
	PDCD1LG2	Programmed cell death ligand 2	5.49496	0.006176

The genes of local vs. normal that show an adjusted *p*-value below 0.05 with a fold change considered relevant (±1.5) are shown in the table. In addition, the possible associated cell types expressing the gene, gene symbol, and encoded protein description are presented.

For OM, Annexin (ANXA1) apoptosis marker expression was significantly lower relative to normal tissue (Table 2) and OL samples (Table 3). The BCL2 apoptosis regulator was significantly overexpressed in OL relative to normal tissue but significantly under expressed in OM relative to OL. Significantly less expression of the tumor suppressor TP63 was evident in OM relative to normal tissue (Table 2) and OL (Table 3). A comparison between OM and OL (Table 3) showed a significant elevation in expression for PTGS2 (Cox2) in OM. In addition, PTGS2 was also found significantly elevated in OM vs. normal tissue (Table 2) but not in OL vs. normal tissue (Supplementary Table S1). The angiogenesis marker, VEGFA, expression was downregulated in OL vs. normal tissue (albeit not significant change; Supplementary Table S1) and VEGFA was upregulated in OM vs. normal tissue (albeit not significant change; Supplementary Table S2). VEGFA was significantly upregulated when comparing OM vs. OL (Table 3). The cell mobility marker, MCAM, was significantly downregulated in OL vs. normal tissue (Table 1) and upregulated in OM vs. normal tissue (albeit not significant change; Supplementary Table S2). MCAM was significantly upregulated when comparing OM vs. OL (Table 3). To summarize, the OM expression pattern relative to the OL expression pattern, included reduced apoptosis, tumor suppressor gene repression, promotion of angiogenesis, and increased cell mobility, which are typically associated with disease progression and are considered hallmarks of cancer (15).

Immune landscape

Immune cell markers

T cell markers

The analysis revealed a significantly increased expression of CD4 in both OM and OL groups compared to normal tissue. However, CD4 and TH1 are significantly under-expressed in OM compared to OL (Table 3). The source of upregulated CD4 in OM and OL relative to normal tissues cannot be determined from this analysis but could be due to increased T effectors, T regulatory cells, and or neutrophils. More definitive analysis would require single-cell RNA analysis. The reasons for upregulated CD4 may also be different between OL and OM groups.

The expression of OSM (Oncostatin M) was significantly elevated in OM relative to OL (Table 3). Previous studies have reported a synergistic association between IL1Beta, IL-6, and OSM in human breast cancer, which is linked to a poor prognosis (29). Regarding the expression levels of pro-immune factors, analysis revealed non-significant expression of IL-2, IL-15, interferon beta1(IFNB1), and interferon gamma. Only IL-18 had a significant expression in OM compared to OL (Table 3).

NK cell makers

KLRG1 was significantly overexpressed in OL vs. normal samples (Table 1). However, KLRG1 was not significantly overexpressed in OM vs. normal samples and KLRG1 was significantly under-expressed in OM compared to OL (Table 3). KLRG1 acts as an inhibitory signal (checkpoint) for NK and T cells but is also expressed in Tregs (30). Granzyme A activity was significantly increased for OM relative to OL (Table 3). Additionally, expression of NCR3 was significantly higher in OM compared to normal and OL samples (Tables 2, 3). NCR3 is of prime importance for maintaining the cytotoxic function of NK cells (31). The increased Granzyme A and NCR3 in OM suggests a cytotoxic phenotype of NK cells in OM, whereas NK activity appears to be inhibited in OL, based on the increased expression of KLRG1.

B cell markers

The BLK gene is responsible for B cell proliferation and is significantly overexpressed in OM and OL relative to normal tissue

TABLE 2 Significantly expressed genes *of* metastatic (OM) vs. normal.

Significantly expressed genes metastatic (OM) vs. normal				
Cell type	Gene symbol	Protein description	Fold	*P*-value adj.
T cell	CD4	CD4 molecule	4.38595	0.015951
	OSM	Oncostatin M	40.1851	0.000374
NK cell	NCR3	Natural cytotoxicity triggering receptor 3	1.96637	0.049649
B cells	BLK	B lymphoid tyrosine kinase	4.32539	0.019839
Macrophages	CD99	CD99 molecule, MIC2	−2.3836	0.008101
	MIF	Macrophage migration inhibitory factor	2.59845	0.023702
	TLR4	Toll-like receptor 4	−2.79411	0.021418
	TLR9	Toll-like receptor 9	2.61574	0.012437
Cytokines	IFNA7	Interferon, alpha 7	7.10178	0.0000242
	IRF4	Interferon regulatory factor 4	6.83485	0.00872
	IRF5	Interferon regulatory factor 5	−4.06668	0.008101
	ISG20	Interferon stimulated exonuclease gene 20kDa	6.39423	0.012437
Melanoma cells	ANXA1	Annexin A1	−5.8559	0.002254
	BRAF	v-raf murine sarcoma viral oncogene homolog B1	−2.21539	0.0000242
	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	−9.41297	0.000127
	ICAM3	Intercellular adhesion molecule 3	3.7162	0.035191
	NOS2	Nitric oxide synthase 2, inducible	3.22223	0.038384
	PCNA	Proliferating cell nuclear antigen	1.91176	0.049501
	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase-2)	7.92719	0.003146
	S100A10	S100 calcium binding protein A10	−5.74469	0.032769
	S100A4	S100 calcium binding protein A4	−5.11119	0.000385
	S100A8	S100 calcium binding protein A8	−11.5099	0.012437
	S100A9	S100 calcium binding protein A9	−14.2991	0.007404
	SOX10	SRY (sex determining region Y)-box 10	48.6906	0.0000425
	TP63	Tumor protein p63	−4.62351	0.018215
Immune checkpoints	PDCD1	Programmed cell death 1	3.24433	0.010195
	CD274	Programmed cell death ligand 1 (PDL1)	3.57006	0.025641
	TIGIT	T cell immunoreceptor with Ig and ITIM domains	5.60184	0.006844

The genes of metastatic vs. normal that show an adjusted *p*-value below 0.05 with a fold change considered relevant (±1.5) are shown in the table. In addition, the possible associated cell types expressing the gene, gene symbol, and encoded protein description are presented.

(Tables 1, 2). Notably, the tertiary lymphoid structure has been associated with a positive response to immunotherapy checkpoint inhibitors in numerous cancer types (32).

Macrophage makers

Relative to normal tissues, OL tumors had significant upregulation of macrophage markers (CD14, CD68, CD84, and CD99). On the contrary, for OM tumors, these macrophage markers were not significantly upregulated relative to normal tissues (Table 2 and Supplementary Table S2). Further analysis reveals a notable significant decrease in the expression of markers associated with macrophages when comparing OM vs. OL tumors (Table 3). This includes decreased expression of both M1 (e.g., CD14) and M2 (e.g., CD163) markers in OM relative to OL. These results suggest OM tumors may contain fewer macrophages than OL tumors.

IRF4 expression has been associated with the presence and activity of M2 macrophages in the tumor, which are known to promote tumor development and immunosuppression (33). In OM, there was significantly increased IRF4 expression relative to normal tissue (Table 2) but not in OL vs. normal tissue. IRF4 is known to act as a negative regulator of TLR synthesis (34). In OM, we also observed significantly reduced expression of TLR 4 relative to normal tissue and OL (Tables 2, 3). In addition, reduced expression of IRF5, associated with interferon synthesis, was observed in OM relative to OL and normal tissue (Tables 2, 3). Macrophage inhibitory factor (MIF) levels significantly increased in OM vs. normal tissue (Table 2) but not in OL vs. normal tissue (Table 1). MIF is associated with progressive disease in human melanomas and is a potential target for advanced stages. Additionally, in OM, TLR9 expression is

TABLE 3 Significantly expressed genes *of* metastatic (OM) vs. local (OL).

Significantly expressed genes metastatic (OM) vs. Local (OL)				
Cell type	Gene symbol	Protein description	Fold	*P*-value adj.
T cell	CD4	CD4 molecule	−3.51299	0.015868
	IL18	Interleukin 18 (interferon-gamma-inducing factor)	−2.37245	0.048949
	OSM	Oncostatin M	9.48633	0.003424
	THY1	Thy-1 cell surface antigen	−2.20355	0.036482
NK cell	GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	2.53334	0.036301
	KLRG1	Killer cell lectin-like receptor subfamily G, member 1	−9.5645	0.001186
	NCR3	Natural cytotoxicity triggering receptor 3	1.93525	0.027144
B cells	TYROBP	TYRO protein tyrosine kinase binding protein	−4.22687	0.003424
Macrophages	CD14	CD14 molecule	−4.86962	0.003424
	CD163	CD163 molecule	−2.55398	0.017287
	CD68	CD68 molecule	−4.35219	0.011847
	CD84	CD84 molecule	−6.68181	0.005718
	CD86	CD86 molecule	−4.75581	0.001295
	TLR4	Toll-like receptor 4	−2.7451	0.011192
	TLR9	Toll-like receptor 9	2.29442	0.009216
Major histocompatibility complex (class 1)	MR1	Major histocompatibility complex, class I-related	1.7485	0.047368
Cytokines	IRF5	Interferon regulatory factor 5	−3.23611	0.011934
	IRF8	Interferon regulatory factor 8	−2.55556	0.035517
Melanoma cells	ANXA1	Annexin A1	−2.83874	0.022851
	BCL2	B-cell CLL/lymphoma 2	−3.46134	0.00013
	BRAF	v-raf murine sarcoma viral oncogene homolog B1	−1.68219	0.000982
	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	−2.74756	0.016576
	MCAM	Melanoma cell adhesion molecule	3.01003	0.004324
	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase-2)	5.63185	0.003637
	S100A12	S100 calcium binding protein A12	−5.39106	0.0396
	S100A8	S100 calcium binding protein A8	−5.05685	0.046783
	S100A9	S100 calcium binding protein A9	−4.83414	0.050571
	TP63	Tumor protein p63	−3.77941	0.018472
	VEGFA	Vascular endothelial growth factor A	5.24266	0.006493
Immune checkpoints	PDCD1 (PD1)	Programmed cell death 1	3.97031	0.001295
	IDO2	Indoleamine 2,3-dioxygenase 2	2.83516	0.004725
	PDCD1LG2	Programmed cell death ligand 2	−5.95454	0.003424

The genes of metastatic vs. local that show an adjusted *p*-value below 0.05 with a fold change considered relevant (±1.5) are shown in the table. In addition, the possible associated cell types expressing the gene, gene symbol, and encoded protein description are presented.

significantly increased relative to OL and normal tissue (Tables 2, 3). TLR9 has been linked to myeloid-derived suppressor cell (MDSC) activity in several cancers (35). Overall, the transcriptome analysis for macrophages suggests for advanced oral melanomas, there is less macrophage infiltration within the tumor and a transition from a pro-inflammatory, immune status toward an immunosuppressive status.

Immune checkpoints

Immune Checkpoints are expressed by different cell types such as T cells (PD1, TIGIT), macrophages, dendritic cells, and tumor cells (PDL1 and IDO2). The current methodology associated with this study cannot differentiate which cells are leading to changes in expression. Transcriptome analysis of immune checkpoints revealed a significant increase in the expression of PDL1 (gene CD274 in

10.3389/fvets.2023.1285909

TABLE 4 Clinical data from tissue samples.

Classification table of patient pathology							
ID	Species	Breed	Sex	Age	Tissue location	Mitotic index	Metastasis
L031-A1	Canine	Poodle	M	10	Left canine gingiva	0	No
L080-A1	Canine	Golden Retriever	MN	7	Hard palate	3	No
L088-A1	Canine	Schnauzer	FS	11	Oral Mucosa	1	No
L194-A1	Canine	Mixed Breed	FS	13	Right maxillary labial gingiva	0	No
L237-A1	Canine	Mixed Breed	MN	10	Hard palate	2	No
L562-A1	Canine	Mixed Breed	F	6	Oral Mucosa	0	No
L600-A1	Canine	Labrador Retriever	MN	9	Right Lip and hard palate	2	No
L614-A1	Canine	Mixed Breed	FS	6	Hard palate	1	No
L700-A1	Canine	Golden Retriever	MN	11	Oral Mucosa	0	No
L794-A1	Canine	Mixed Breed	MN	12	Lip	3	No
L983-A1	Canine	Mixed Breed	MN	11	Base of tongue	1	No
L998-A1	Canine	Mixed Breed	MN	9	Oral Mucosa	0	No
M074-A1	Canine	Labrador Retriever	MN	11	Left Mandibula	12	Lungs, Tracheobronchial lymph node, Pulmonary artery
M172-A1	Canine	Coker Spaniel	MN	12	Hard palate	15	Lymph node, Lung, Liver, Kidney, Heart, Esophagus
M286-A1	Canine	Golden Retriever	FS	12	Tonsils	15	Regional lymph nodes, lung
M337-A2	Canine	Mixed Breed	FS	13	Left Maxilla, bone involvement	40	Lungs
M570-A1	Canine	Schnauzer	F	14	Jaw region and pharynx	15	Lungs
M675-A2	Canine	Scottish Terrier	FS	12	Right Maxilla, Peri-orbital	12	Lung, Sub Mandibular and medial ileac lymph nodes, mesocolon
N1-A1	Canine	NA	NA	NA	Oral labial mucosa	NA	No
N2-A1	Canine	NA	NA	NA	Oral labial mucosa	NA	No
N3-A1	Canine	NA	NA	NA	Oral labial mucosa	NA	No
N4-A1	Canine	NA	NA	NA	Oral labial mucosa	NA	No
N5-A1	Canine	NA	NA	NA	Oral labial mucosa	NA	No

Clinical information from the 18 pathology samples, ID samples that start with an L correspond to local tumors, ID samples that start with an M correspond to metastatic samples, and ID samples that start with N correspond to normal tissue samples. Breed, sex, age, and metastasis status are presented with the primary tumor location for all samples. The mitotic index corresponds to the number of cells undergoing mitosis per ten histopathology high power fields as indicated per pathology report from each patient sample. NA, Not available.

Table 2) in OM tumors compared to normal tissues. PDL1 is a transmembrane protein expressed by tumors and immune cells (lymphocytes Treg, macrophages type 2, MDSCs). It acts as a negative regulator of tumor rejection by the adaptive T-cell response. PD1 expressed on T cells, is the receptor for PDL1 and is also significantly overexpressed in OM vs. normal (Table 2) and OM vs. OL (Table 3). PDCD1LG2 (PDL2) is significantly over-expressed in OL compared to normal tissues (Table 1) but not significantly over-expressed in OM compared with normal tissues. Furthermore, PDL2 is significantly under-expressed in OM vs. OL (Table 3), which could be associated with immune exhaustion in OM tumors. PDL2 expression has been associated with immunosuppression and it is generally expressed on antigen presenting cells as well as tumor cells (36). CTLA4 was overexpressed in OM relative to both OL and normal tissue, but the differences were not statistically significant (Supplementary Tables S1–S3).

IDO1 and IDO2 are members of tryptophan catabolic pathways expressed by tumor cells and tumor microenvironment cells (dendritic cells, macrophages, endothelial cells, tumor-associated Fibroblasts). These proteins exert a suppressive effect on CD8 cytotoxic lymphocytes, CD4 effectors, and NK cells while stimulating immune suppressor cells like Tregs and MDSCs (37). IDO1 and IDO2 were both downregulated in OL vs. normal tissues (albeit not significantly downregulated; Supplementary Table S1). IDO1 and IDO2 were both upregulated in OM vs. normal tissues (albeit not significantly upregulated; Supplementary Table S2). When comparing OM vs. OL, IDO2 was significantly overexpressed in OM (Table 3).

The immunosuppressive receptor, TIGIT is significantly overexpressed in both OM and OL relative to normal tissues (Tables 1, 2). TIGIT expression can be found on CD4 cells (including Tregs), NK cells, and or CD8 cells. Targeting TIGIT is an option for treating other advanced, cancers and based on these results may be an option for canine melanoma (38, 39). Overall, the data shows that the expression of immune checkpoints was significantly higher in both OL and OM tumors compared to normal tissues, with the OM type demonstrating

even higher expression levels of specific immune checkpoints (PD1, IDO2), and lower expression of PDL2 (Table 3).

In addition to the global analysis, one of the OM samples, M337-A2, is different from the rest of the OM group (Figures 2, 3). This oral tumor expresses immune blockade (IDO2) but unlike other OM tumors, conserves interferon response (IRF5 and IRF8), TLR activation (TLR2 and TLR4), and active immune cell markers (CD4). Of note, this was the only tumor in the group documented to have partial bone involvement. Analyzing the heat map for the OL group, four dogs (L794, L600, L080, and L614), had altered expression of the first 57 genes, starting with SPP1 and ending with CCR1, relative to the other OL dogs (Figure 1). This expression pattern suggests a lower inflammatory profile for these four tumors in comparison to the rest of OL tumors (including lower CD4, CD14, CD22, CD37, CD48, CD63, CD68,TLR2, TLR8, cCCL3, chemokines receptor CCR1, and Osteopontin Gene SSP1). All four dogs had tumors located at the hard palate and or lip. Consequently, tumor location might at least partially dictate expression of markers.

Discussion

Our study has limitations primarily due to its retrospective aspect, the reduced number of samples available, and the absence of strict patient follow-up. In the future, we recommend implementing rigorous enrollment of patients with advanced staging procedures and ensuring regular follow-up. A comprehensive pathology evaluation and genetic investigation should be performed to support these findings further.

The transcriptome analysis of oral melanoma suggests the presence of immune effectors such as NK and CD4 cells, which might be suppressed by negative regulators or immune checkpoints such as the PD1/PDL1/PDL2 axis, TIGIT, or IDO2. Our results suggest that as the disease progresses toward metastasis, some negative regulators increase further, including PD1, PDL1, and IDO2. As a result, exploring immune checkpoint inhibitors as potential treatment may represent a therapeutic opportunity in the veterinary world once the disease is diagnosed and the drugs available, similar to human melanoma. Immune checkpoint-targeted therapy has been a groundbreaking advancement in human cancer therapy over the past decade. The use of antibodies against these immune checkpoints has significantly improved the prognosis of numerous cancers in humans (36). Dog patients would benefit from receiving ICI inhibitors when available in the veterinary world as soon as the disease is diagnosed. Checkpoint inhibitors against immune exhaustion TIGIT could be of particular therapeutic interest for oral melanoma as it was significantly overexpressed in both the OM and OL groups relative to normal tissue in this study.

Our results suggest reduced macrophage infiltration in OM relative to OL tumors, including reduced expression of CD14 in OM relative to both OL and normal tissue. Moreover, there is a low expression of TLR4, and high expression of IRF4 with OM relative to normal tissue. This suggests any residual macrophage population in OM tumors might represent the M2 immunosuppressive type.

With disease progression, changes within the tumor microenvironment, such as hypoxia and an increase in VEGF production, stimulate the TGF beta pathway and trigger an upregulation in inflammatory cytokines and activated pathways (NOS), contributing to immune exhaustion. In this study, VEGFA expression was significantly upregulated in OM tumors relative to OL tumors. As a result, alternative therapeutic strategies for OM tumors could be explored targeting the microenvironment through hypoxia. In both OM and OL tumors there was downregulation of CEACAM1 relative to normal tissue. Furthermore, CEACAM1 was downregulated further in OM compared with OL tumors, suggesting progressive loss of this marker that is associated with a shift from OL to an OM phenotype. This suggests it could be a useful prognostic marker for canine oral melanoma, similar to previous reports describing its prognostic utility in human cancers. Finally, Oncostatin M (OSM) was found to be significantly overexpressed in OM compared with both OL and normal tissue.

The results of this study suggest reactivating immune T lymphocyte cells with interleukins (e.g., IL-2 and IL-15) and TLR agonists for macrophages could benefit the patient. Similarly, radiotherapy for its "vaccine *in situ* effect" or specific targeted RNA vaccines (SOX 10,) could contribute to enforcing the immune response. To establish the credibility of these hypotheses, further evaluation is necessary considering pathology characteristics, such as cell distribution, density, immunochemistry markers, possibly spatial cell communication networks, and single immune cell function assessed through flow cytometry and single-cell RNA seq strategies instead of Nanostring analysis.

In summary, this study utilized historical fixed samples to evaluate the RNA expression of various clinically relevant immuno-oncology genes in canine melanoma. Various genes were found to be significantly altered in patients with metastatic disease relative to patients with local disease, suggesting targeted therapeutic strategies may differ for these patient groups. Overall, the findings have potential value for guiding further studies in canines and developing immunotherapy strategies for melanoma. Future work will aim to develop a landscape score specific to each melanoma patient, enabling the identification of a tailored therapeutic option based on individual immune profiles.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found in the NCBI repository under accession number: GSE 228574.

Ethics statement

Written informed consent was obtained from the owners for the participation of their animals in this study ID PRJNA1014399.

Author contributions

IV: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. KB: Investigation, Methodology, Project administration, Writing – original draft. CM-V: Data curation, Formal analysis, Visualization, Writing – review & editing. LL: Conceptualization, Data curation, Investigation, Methodology,

Writing – original draft. KK: Supervision, Writing – review & editing. MS: Supervision, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank J. Robinson from Nanostring© Company and Debbie Knapp's lab (Purdue University) for their assistance with the project.

Conflict of interest

KK was employed by the Pathology, Cook Research, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1285909/full#supplementary-material

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frontiers | Frontiers in Veterinary Science

TYPE Mini Review
PUBLISHED 06 February 2024
DOI 10.3389/fvets.2024.1336158

OPEN ACCESS

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RECEIVED 10 November 2023
ACCEPTED 11 January 2024
PUBLISHED 06 February 2024

CITATION
Razmara AM, Gingrich AA, Toedebusch CM, Rebhun RB, Murphy WJ, Kent MS and Canter RJ (2024) Improved characterization and translation of NK cells for canine immunotherapy.
Front. Vet. Sci. 11:1336158.
doi: 10.3389/fvets.2024.1336158

Improved characterization and translation of NK cells for canine immunotherapy

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The field of cancer immunology has seen a meteoric rise in interest and application due to the discovery of immunotherapies that target immune cells, often leading to dramatic anti-tumor effects. However, successful cellular immunotherapy for solid tumors remains a challenge, and the application of immunotherapy to dogs with naturally occurring cancers has emerged as a high yield large animal model to bridge the bench-to-bedside challenges of immunotherapies, including those based on natural killer (NK) cells. Here, we review recent developments in the characterization and understanding of canine NK cells, a critical springboard for future translational NK immunotherapy research. The characterization of canine NK cells is exceptionally pertinent given the ongoing challenges in defining them and contextualizing their similarities and differences compared to human and murine NK cells compounded by the limited availability of validated canine specific reagents. Additionally, we summarize the current landscape of the clinical and translational literature employing strategies to capitalize on endogenous and exogenous NK cell immunotherapy in canine cancer patients. The insights regarding efficacy and immune correlates from these trials provide a solid foundation to design and test novel combinational therapies to enhance NK cell activity with the added benefit of motivating comparative work to translate these findings to human cancers with extensive similarities to their canine counterparts. The compilation of knowledge from basic canine NK phenotype and function to applications in first-in-dog clinical trials will support the canine cancer model and enhance translational work to improve cancer outcomes for both dogs and humans.

KEYWORDS

NK cell, canine (dog), immunotherapy, clinical trials, markers

Introduction

The advent of immunotherapy has propelled the field of oncology beyond the standard therapies of surgery, radiation, and chemotherapy. While the vast majority of successful immunotherapy methods to date have been T-cell based, such as PD-1/PD-L1 inhibition and CAR-T cells, such strategies are not universally successful in all patients. Thus, researchers have broadened their focus to harness and manipulate other immune cell types. Of these,

natural killer (NK) cells have emerged as an attractive candidate given their innate cytotoxicity against a diverse array of targets and their potent cytokine production. NK cells are considered sentinels of the innate immune system, capable of identifying and killing virus-infected and cancer-transformed cells via mechanisms that do not require antigen-specific recognition.

NK cells have been a focus of potential therapy for decades, since initial trials by Rosenberg et al. in the 1980s, among others (1–3). However, severe toxicity was seen in early attempts, largely due to amplified cytotoxic lymphocyte responses and the concomitant use of high-dose IL-2 to support adoptive transfer of these cells into patients. These findings emphasized the need to minimize such responses while still harnessing NK cells' anti-tumor effects. Companion canines have emerged as a useful model for studying novel cancer therapies given that dogs are a large, outbred species which develop spontaneous cancers in the setting of an intact immune system. To study NK cells in particular, the canine model is invaluable since the complex interplay between neoplasia development and a functional immune system can be evaluated. Here, we review recent canine immunotherapy trials which directly or indirectly act via NK cells while also summarizing the progress made and hurdles which still exist to advance canine NK immunotherapies.

Identification and characterization of canine NK cells

Populations of innate lymphoid cells (ILCs) have been extensively studied in humans and mice for decades. By comparison, canine ILCs are less defined, although recent studies have sought to advance our understanding (4). The identification and clarification of the canine NK cell populations based on surface markers has been a longstanding effort (5–8). Early work established that they are CD4-/CD20-, as these are the canine T and B cell markers, respectively (9). A detailed review regarding the evolution of the collective understanding of canine NK cell identification was published by Gingrich et al. (10).

To summarize, many early efforts focused on phenotypic identification of canine NK cells using surface markers, as such markers differ from those in humans and mice (10). Huang et al. were the first to describe canine NK cells using the surface density of the CD5 marker, a member of the scavenger receptor cysteine-rich superfamily and typically classified as a T cell marker (11). This study noted important differences in lymphoid phenotype based on CD5 density, as cells with CD5^{dim} expression were larger, contained more cytoplasmic granules, and demonstrated antigen-independent cytotoxicity, especially in the setting of IL-2 enrichment (11).

Further studies by Shin et al. continued to focus on the density of the CD5 receptor as an NK marker, particularly contrasting CD3+CD5^{dim}CD21− with CD3+CD5−CD21− cells (12). Following expansion and co-culture with K562 feeder cells and cytokines for 21 days, CD5^{dim} expressing cells did not express TCRαβ nor TCRγδ (12). Additionally, CD3+CD5^{dim}CD21− cells exhibited significantly higher IFN-γ cytokine production compared to CD3+CD5−CD21− (12). Based on these findings, the authors proposed that each population represents canine NK cells at different levels of maturation (12), although the stages of canine NK cell maturation and development remain a poorly understood topic in contrast to key discoveries in mouse and human studies (13–15).

The "pan-mammalian" NK cell receptor, NCR1/NKp46, has also been identified as a marker of canine NK cells (10). Studies by Grondahl-Rosado et al. noted that CD3-NCR1+ cells comprised 2.5% of canine PBMCs, a proportion much lower than NK cells seen in other mammals (6, 7). Foltz et al. developed a novel antibody to canine NKp46 for use in flow cytometry (5). Their work also identified a CD3-NKp46+ NK subset, representing approximately 2–3% of PBMCs (5). These cells were found to be highly cytotoxic against multiple canine cancer lines. Using a novel expansion technique, the authors also identified a population of CD3+TCR+NKp46+ cells (5). The CD3 positivity in canine NK cells was postulated to represent a different stage of maturation, although a conclusive trajectory has not been described to date (5, 10).

More recently, Grudzien et al. established a canine NK cell line (CNK-89) derived from a dog with NK cell neoplasia (16). This cell line is CD5+CD8+CD45+CD56+CD79a+NKp46+. Although CD79a is classically a B cell marker, the presence of the NKp46 protein and mRNA expression of NKG2D, NKp30, NKp44, NKp46 and perforin suggested NK cell properties for this cell line. Following treatment with IL-12, IL-15, IL-18 and IL-21, increased expression of granzyme B, perforin and CD16 was observed (16). Secretion of TNFα and IFNγ was also noted. These findings were not observed following treatment with IL-2, suggesting these neoplastic-derived cells are an IL-2 independent cell line and potentially useful for studying alternate pathways of canine NK cell activation (16).

Gingrich et al. detailed differential gene expression analyses of the two most widely accepted canine NK cell populations: CD3-CD5^{dim} and CD3-NKp46+ cells (17). Marked differences were seen in steady-state cells, including non-detectable mRNA expression of granzyme B, perforin, IFNγ and KLRD1/CD94 in CD3-CD5^{dim} cells, but detectable expression in CD3+NKp46+ cells (17). Remarkably, following co-culture with radiated human feeder cells [K562cl9, (18)], the two cell populations converged on a nearly identical mRNA expression phenotype (17). The findings suggested each population likely contains NK cells that are selected for rapid and dominant growth under stimulatory co-culture conditions.

The authors then conducted single-cell RNA sequencing of FACS-sorted CD3-CD5^{dim} and CD3-NKp46+ cells to explore overlap between the two populations. In these studies, at steady-state the CD3-CD5^{dim} population was found to be more heterogeneous than the CD3-NKp46+ one. Gene expression driving the variance for CD5^{dim} cells was predominantly non-NKC gene expression, reinforcing that CD5^{dim} appears to be a less specific marker. Further single-cell studies following the activation of the two cell populations in co-culture demonstrated a conserved trajectory to activation based on uniform, discreet changes in gene expression in canonical NK transcription factors as well as marked changes in expression of granzyme A, IL2RB, and KLRB1. These data described the transition in both CD3-CD5^{dim} and CD3-NKp46- canine NK cells from a resting state to an activated state which may lend insight to the stages of NK cell maturation in dogs, a physiologic process which has yet to be clearly elucidated.

Overall, the precise identification of canine NK cell populations remains elusive, likely in part due to a lack of understanding of the maturation process as well as variable gene expression and protein surface markers associated with different stages of development. However, based on the studies above, populations of innate, canine lymphocytes capable of cytokine-dependent, antigen-independent

cytotoxicity have been demonstrated to exist, paving the way for clinical applications of NK-based immunotherapies in dogs.

NK cells in canine immunotherapy

Ultimately, immunotherapy needs to be tested in immunocompetent hosts. This underscores a strength of the dog model, especially when novel immunotherapies are combined with serial immune correlates (19). The investigation of immune populations before, during, and following immunotherapy can not only provide insight to the presence or absence of clinical benefit in the relevant study but can also be hypothesis generating in the pursuit of improving efficacy. Additionally, immune correlates can bring to light potential biomarkers of response, leading to improved selection of dogs that are likely to respond to treatment and the identification of canine patient subsets that require innovative interventions. A concerted effort to combine the lessons learned from canine clinical trials performed or in progress is essential to the future of the field. To date, several canine immunotherapy trials have been completed with either direct or indirect effects on putative NK cell populations. Trials have used adoptive cell therapy, cytokine therapy, virus-based therapy, radio- and chemo-immunotherapy, and checkpoint blockade to treat dogs with spontaneous cancers with various methods of NK cell identification and analysis (Figure 1 and Table 1).

Our group has completed several first-in-dog trials using adoptive NK cell transfer to treat dogs with spontaneous cancers. In 2017, we treated dogs with unresectable limb osteosarcoma (OSA) using palliative radiotherapy (RT) and two intratumor injections of autologous NK cells (20). NK cells were expanded from CD5-depleted PBMCs over a 14 day co-culture with irradiated K562-C9-mIL21 feeder cells and 100 IU/mL recombinant human IL-2 (21). We observed a significant increase in CD45+GZMB+ cells in PBMCs post-treatment by flow cytometry suggesting systemic immune effects of the treatment, although there did not appear to be an association between survival and frequency of GZMB+ or IFNγ+ cells in peripheral blood (30). Given the intra-tumor route of administration, we also analyzed tumor biopsies by flow cytometry and observed that approximately 50% of intratumor CD45+ cells stained positive for an intracellular dye label consistent with persistence of the adoptively transferred NK cells for one-week post-transfer in the tumor microenvironment (TME) (20). We analyzed tumor tissue by qPCR and showed gene expression varied greatly by patient, with no difference in fold change gene expression between dogs that were alive or dead at 6 months (30). Though, it is interesting to note that the longest surviving dog, 18 months, showed the greatest fold-change in the expression of CD3, CD8, and IDO1 genes following RT and intratumor NK transfer (30).

Immunotherapies can also stimulate endogenous NK cells through cytokines that are responsible for the activation, migration, and expansion of NK cells *in vivo*. Our group also completed a first-in-dog dose escalation trial in dogs with pulmonary metastatic melanoma and osteosarcoma using inhaled recombinant human IL-15 to stimulate NK cells in the lung at metastasis sites (31). Seven of the initial enrollees were also analyzed in a preliminary assessment of peripheral NK cells using flow cytometry and RNA sequencing (17, 22). The proportion of total NK cells and NK cells expressing Ki67 increased during inhaled IL-15 treatment and had a significant increase in Granzyme B fold change (22). Conversely, there was evidence of upregulation of TIGIT gene expression, an inhibitory marker, at both day 7 and 14 post enrollment (22). The increase in both Granzyme B and TIGIT suggests

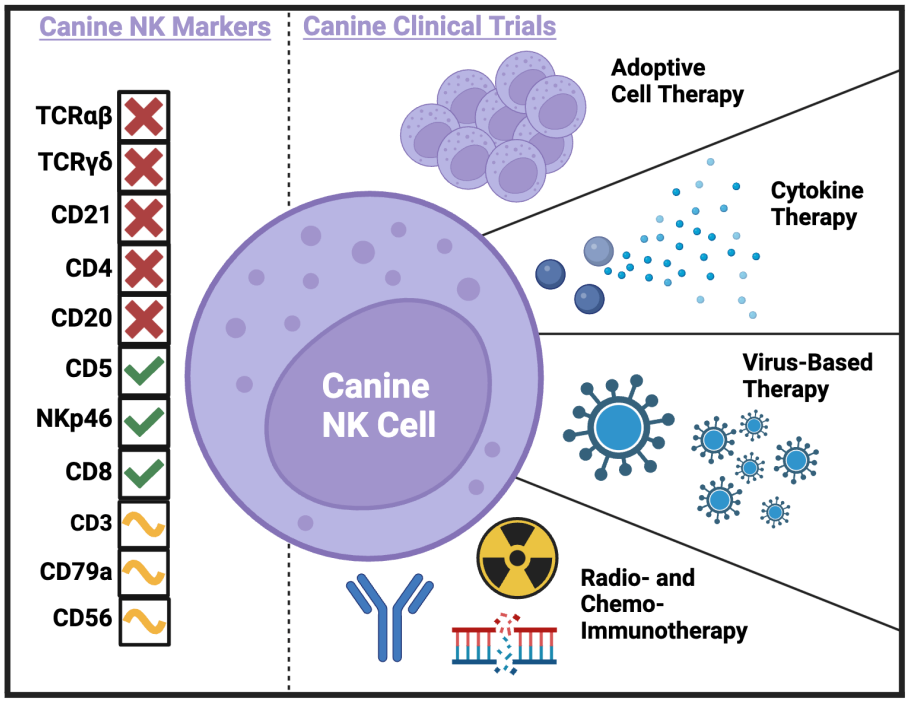

FIGURE 1
Current and potential canine NK markers and canine clinical trials completed to-date with NK cell correlates. Created with BioRender.com.

10.3389/fvets.2024.1336158

TABLE 1 Canine clinical trials with NK immune correlates.

General therapy	Specific therapy	Cancer diagnosis	NK correlates	Method of analysis	References
Adoptive cell therapy	Autologous NK cell transfer	Osteosarcoma	Number of NK cells	Flow cytometry	(20, 30)
			Serum cytokines	ELISA	
			Activation markers	Flow cytometry	
			Gene expression	qPCR	
Cytokine therapy	Inhaled IL-15	Osteosarcoma, melanoma	Number of NK cells	Flow cytometry	(31, 17, 22)
			Serum cytokines	ELISA	
			Activation markers	Flow cytometry	
			Gene expression	RNA sequencing	
			Cytotoxicity	Killing assay/flow cytometry	
Virus-based therapy	Oncolytic virus	Carcinoma, Adenocarcinoma	Number of NK cells	Flow cytometry	(24)
	Genetically modified HSV	Glioma	Gene expression	RNA seqencing/nanostring	(25)
	eCPMV	Inflammatory mammary cancer	Gene expression	RNA seqencing/nanostring	(27)
	eCPMV	Mammary cancer	Gene expression	RNA sequencing	(33)
			Number of NK cells	Flow cytometry	
	Cellular virotherapy	Various	Number of NK cells	Flow cytometry	(34, 35)
			Gene expression	RNA seqencing/CIBERSORT	
Radio- and chemo-immunotherapy	RT, TRT, IT-IC	Melanoma	Number of NK cells	Flow cytometry	(37)
			Gene expression	RNA sequencing	
	Chemotherapy, anti-CD20, SMI	B cell lymphoma	Gene expression	RNA sequencing	(38)

HSV, Herpes Simplex Virus; eCPMV, Empty Cowpea Mosaic Virus; RT, Radiotherapy; TRT, Targeted Radionuclide Therapy; IT-IC, intratumoral immunocytokine; SMI, Small Molecule Inhibitor.

concurrent stimulation of activating and inhibitory pathways, the balance of which potentially determines response to treatment. RNA sequencing of patient PBMCs offers preliminary evidence that the activating/inhibitory balance may be patient specific, since principal component analysis (PCA) variance was driven largely by two dogs that responded to treatment (17). At the completion of trial, among 21 dogs total, we observed a 39% clinical benefit rate (31). Cytotoxicity of patient PBMCs against osteosarcoma (OSA) and melanoma (M5) targets significantly increased from pre- to post-therapy and maximal cytotoxicity was significantly correlated with patient survival (31). The finding of increased peripheral blood cytotoxicity across the entire cohort post-treatment suggests that tumor cell death is occurring, but only leading to improved survival in certain patients.

There are many immunotherapies that are not traditionally NK-targeting or are non-specific in nature, which still result in NK activation, making them attractive candidates for multimodal treatments (23, 32). For instance, oncolytic viruses are a unique type of immunotherapy in that their primary function is to invade and replicate within cancer cells, leading to lysis, but it was soon recognized that this process also increases the immunogenicity of cancer cells, leading to the recruitment of and elimination by immune cells. This is a similar mechanism through which viruses, like cowpea mosaic virus, are used as therapy to bind non-specific receptors and stimulate the induction of an immune response in the TME.

Martín-Carrasco et al. tested an intratumor oncolytic virus based on canine wild-type adenovirus which was engineered to selectively replicate in mutated cells to treat canine patients with cancer (24). The strength of the study was the availability of serial sample collections from patients before and up to one year after treatment. At least four of the eight patients had an increase in peripheral NK cells within the first month after treatment as assessed by flow cytometry (24). However, CD56 was used as the identifying marker, which is not known to be expressed on canine NK cells, highlighting the importance of validating both the reagents used as well as the underlying biology given the extensive cross-species differences in NK cells.

In another trial, the authors treated canine oligodendroglioma and astrocytoma using intratumor injections of M032, a genetically modified herpes simplex virus. The authors observed enrichment of tumor mRNA gene signatures associated with NK cells in four of six patients with available specimens (25). In this study, NK cell gene signatures were labeled as belonging to "NK CD56dim cells" and assessed by the NanoString Technologies gene expression panel (25). The classification of NK cells as CD56dim by this method is described as based on expression of IL21R in an evaluation of nearly 10,000 samples from The Cancer Genome Atlas (TCGA) (26). So, while CD56 or $CD56^{dim}$ are not validated as canine NK markers, IL21R is thought to be expressed on canine NK cells and capable of being activated by its respective IL-21 ligand.

The same immune profiling method was used to investigate the abundance of NK cells in the TME of dogs with canine inflammatory mammary cancer treated with intratumor delivery of immunotherapy using empty cowpea mosaic virus-like particles (eCPMV) (27), which are recognized by toll-like receptors (TLRs). They found no significant change in cells labeled as "NK CD56dim cells," or cells enriched for IL21R, and the fold change of other genes associated with NK cells including KLRA1, KLRD1, and GZMB were increased in treated tumor tissue, but not significantly (27). Conversely, there was

significant upregulation in treated versus untreated tumor tissue of IL18R1 and significant downregulation of IL12RB2, which are both implicated in NK cell functions (27), supporting the pattern across canine immunotherapies in which both activation and inhibition are observed simultaneously. Another study treated patients with canine mammary cancer using eCPMV (33). This trial gave two injections into the largest tumor followed by tumor resection. In line with findings from dogs treated with eCPMV alone, NK-related genes were not differentially expressed using RNA-seq to analyze the tumors treated with neoadjuvant immunotherapy. Additionally, flow cytometry was used to define CD45+CD21−CD3−GZMB+ PBMCs as NK cells. This analysis showed insignificant changes in peripheral NK cells in response to eCPMV and surgery (33).

To bypass hurdles associated with non-surgical tumors and improve systemic effects, autologous canine mesenchymal stem cells can be infected with a canine oncolytic adenovirus and administered to patients. This was performed on 27 dogs with extracranial cancer and assessed by the same group in 10 subsequent dogs with high-grade gliomas (34, 35). In the initial trial, peripheral immune cells including NK cells increased after each dose, although the changes were not statistically significant (34). In the subsequent trial, dogs with gliomas received eight weekly treatments of cellular virotherapy (35). Using CIBERSORT analysis of bulk-RNAseq on tumor tissue, they found that NK cell fractions were not changed between responders and non-responders post-treatment (35).

Together, these virus-based therapies have shown preliminary efficacy in dogs but inconsistent association with NK cell numbers and related gene signatures as biomarkers of clinical effects. Changes in NK cells are more likely to be seen in the TME rather than in peripheral blood, especially with intra-tumor immunotherapies (28). These studies also expose the real and ongoing difficulties in identifying canine NK cells, with virtually every trial using different markers.

The future of canine NK immunotherapy is likely a combinatorial approach that enhances multiple anti-tumor methods. Several groups have spearheaded combination radioimmunotherapy and chemoimmunotherapy trials in dogs with spontaneous cancer with varying involvement of NK cells or related cytokines and genes (29, 36).

Four dogs with advanced stage melanoma were treated with trimodal immuno-radiotherapy which included sub-ablative external beam radiation therapy (EBRT), targeted radionuclide therapy (TRT), and intratumor immunocytokine (IT-IC) (37). Numbers of NK cells in circulating blood identified by flow cytometry using CD3−CD5^{dim} did not change significantly with treatment (37). However, RNA-seq analysis of tumor tissue from dogs before and following treatment indicated significant upregulation of KLRA1, KLRB1, NCR3 IL18R1, and TNFα at selected timepoints (37). The small sample size precludes conclusions regarding survival outcomes but may aid in contextualizing the contribution of NK cells in response to therapy.

The importance of placing immune changes in the framework of progression or survival is well-illustrated in an unrelated trial which enrolled 18 dogs with B cell lymphoma that were treated with doxorubicin chemotherapy, anti-CD20 monoclonal antibody, and a small molecule inhibitor (38). Lymph node aspirates analyzed by RNA-seq demonstrated genes associated with NK function as the most significantly upregulated gene set in dogs with poor survival, but samples were obtained from only one time point limiting conclusions regarding immune changes in response to therapy (38). Serial sampling of patient lymph nodes and tracking of changes in response to treatment would help clarify the conclusions of the study and identify predictive in addition to prognostic biomarkers.

Prognostic biomarkers of response were similarly investigated in a trial treating dogs with oral malignant melanoma using checkpoint blockade (39). The Ohashi Laboratory pioneered PD-L1 antibody therapy in dogs and have completed two clinical trials to date (40–42). The mechanism of anti-PD-L1 therapy is based on the understanding that PD-L1 on tumor cells binds to PD-1 on T cells, providing an inhibitory signal that interferes with anti-tumor T cell functions. In the context of NK cells, binding of anti-PD-L1 antibody to PD-L1 expressed on NK cells can increase activation and effector function (43). Thus, the treatment has the potential to both remove T cell inhibition and enhance NK cell function. While the initial canine anti-PD-L1 trial publications did not include immune correlates of response, a follow-up investigation of serum biomarkers by the same group found that overall survival following treatment was positively correlated with low PGE2, higher IL-2, and higher IL-12 in pre-treatment sera, helping to identify COX-2 as a potential target for future trials (39). NK cells were not the primary focus of the trial, but the authors noted that PGE2 is capable of suppressing the function of NK cells and IL-12 is well-established as necessary for the release of IFNγ by NK cells, the most prominent producers of the inflammatory cytokine (39). These studies illustrate the potential for clinical trials to inform future studies, identifying dogs exhibiting high baseline PGE2 serum levels as candidates for the addition of a COX-2 inhibitor. Given the successful development of anti-canine PD-1/L1 antibodies, determining whether these and other immune checkpoints are found on canine NK cells would have a profound impact on prospective targets and combinatorial approaches. Taken together, these data provide preliminary support for future investigations into combination NK immunotherapies to holistically impart anti-tumor effects.

Discussion

NK immunotherapy in dogs is progressing at an escalating rate with larger sample sizes and collaboration between university hospitals and specialty centers. At the time of this publication, the American Veterinary Medical Association (AVMA) Animal Health Studies Database lists five trials currently recruiting, and 35 trials with completed recruitment based on a search for "immunotherapy" in dogs with cancer. Studies from the University of Minnesota have demonstrated increased Antibody-Dependent Cellular Cytotoxicity (ADCC) efficacy in engineered human NK cells expressing recombinant CD64, opening the doors for the development of engineered canine NK cells that have similar effector capabilities (44). Investigators at The Ohio State University have simultaneously made progress in attempting to improve adoptive NK cell products for canine immunotherapy by imprinting NK cells with TGF-β during expansion to override potential inhibition in the TME (45). This group is using this method of TGFβ-imprinted NK cell therapy combined with carboplatin chemotherapy to treat dogs with OSA in an ongoing phase I clinical trial. Our own group has sought to improve the canine NK product using the expansion of unmanipulated PBMCs from healthy donors for allogeneic adoptive NK cell transfer. These works provide the infrastructure from which canine NK cells can be manipulated to enhance persistence and efficacy in future immunotherapy trials and this multi-institutional, rapid innovation in canine NK immunotherapy is indicative of the growing interest and recognized potential in the field.

By reviewing recent trials with available NK cell correlates, we begin to elucidate an intricate framework of NK responses to treatment. Overall, there is evidence of both NK activation and inhibition in canine immunotherapy with moderate and irregular impacts on NK cell proportions which vary based on intratumor versus peripheral sampling. Timing of sampling is also highly relevant, given that NK correlation to improved response can be negative or positive based on the treatment stage, a concept that can be expected in the context of limited NK cell half-life. Resolution of the role of NK cells in canine immunotherapy requires additional trials with intra-tumor and peripheral immune serial sampling and adequate enrollment for response assessments. The current literature clearly points to the potential promise of NK cell targeting, especially in combination therapies, to benefit both dogs and people for whom novel immunotherapies are needed.

Author contributions

AR: Writing – original draft, Writing – review & editing. AG: Writing – original draft, Writing – review & editing. CT: Writing – review & editing. RR: Writing – review & editing. WM: Writing – review & editing. MK: Writing – review & editing. RC: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the V Foundation Victory over Cancer T2021-016 (RC), NIH/NCI U01 CA224166-01 (RC), NIH/NCI T32 CA251007-01 (AR), and F30CA275317 (AR).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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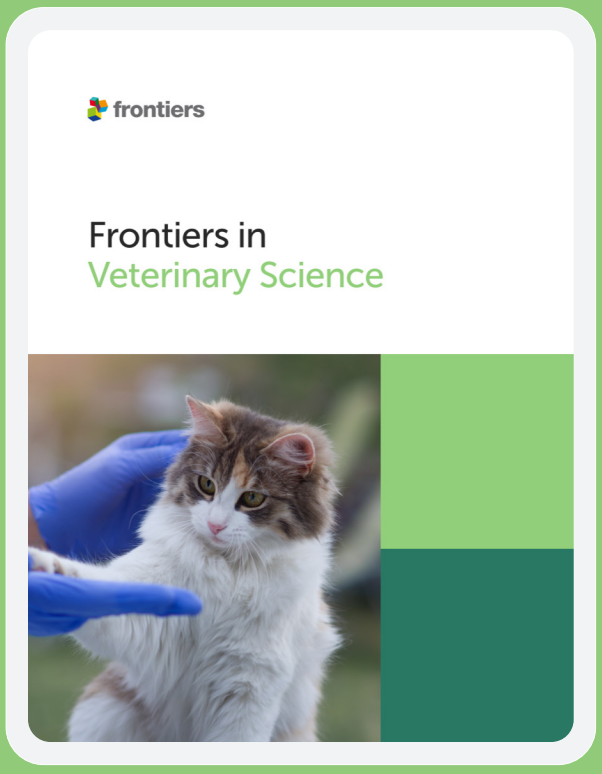

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