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Expression and Clinicopathological Relevance of Fibroblast Growth Factor Receptors in Canine Mammary Gland Tumors

Kabiru Sahabi[1], Gayathri Thevi Selvarajah*[1,2], Gurmeet Kaur Dhaliwal[3], Reuben Sunil Kumar Sharma[4] and Noordin Mohamed Mustapha[5]

[1]Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia
[2]CanRes MAKNA Laboratory, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia
[3]Companion Animal Medicine & Surgery, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia
[4]Veterinary Laboratory Diagnostics, Faculty of Veterinary Medicine, Universiti Putra Malaysia, Malaysia
[5]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
***Corresponding author:** gayathri@upm.edu.my

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ABSTRACT

This study was conducted to determine the expression of fibroblast growth factor receptors (FGFRs) in canine mammary gland tumors (CMT) and to investigate the expression relationship with clinical and histopathological parameters. Forty-six CMT tissues were immunohistochemically probed for the expression of FGFR2, FGFR3 and FGFR4 using rabbit polyclonal antibodies. The expression of each receptor was analyzed (Fisher's exact test) for its relationship with clinical parameters (breed size, age, neuter status, involvement of inguinal mammary gland, number of glands involved) and histopathology (mitotic index, tumor size, tumor grade, PCNA and Vimentin expression). Kaplan-Meier survival and Cox-Regression were performed for survival analysis. The proteins (FGFR2, -3 and -4) were localized to the membrane and cytoplasm. Forty-five tumors (97.8%) expressed both FGFR2 and FGFR3. The FGFR4 was expressed in 42 (91.3%) of the tumors. The expression of FGFR2 was significantly associated with histopathology grade 3 of the tumors (P=0.027). FGFR3 expression was not associated with any clinical or histopathology parameters. FGFR4 expression was associated with large breed dogs (P=0.044), and large tumor size (>3cm) (P=0.045), but none of the proteins expressed predicted post-surgical survival in the dogs. In this study, FGFR2 expression has indicated its usefulness in CMT as an indicator of increased tumor malignancy, while FGFR4 expression has demonstrated the ability to identify high stage tumors. Based on these findings, FGFR2 and 4 can be used as markers for advanced and aggressive CMT.

Key words: Canine mammary tumors, FGFR, Immunohistochemistry, Western blot.

INTRODUCTION

The incidence of canine mammary gland tumors (CMT) is increasing and the only protective measure is early (before first oestrus) spaying of dogs (Schneider et al. 1969). Several factors such as age, breed, exposure to ovarian hormones, type of diet and obesity have been associated with an increased risk of developing CMT (Sonnenschein et al. 1991; Perez Alenza et al. 2000; Canadas et al. 2019). Metastasis in these tumors is high as more than 50% are diagnosed as malignant (Klopfleisch et al. 2011; Zuccari et al. 2011; Canadas et al. 2019) and the most fatal location of metastasis is in the lungs (Klopfleisch et al. 2011). Due to the low success rate of treatment using conventional drugs (Lavalle et al. 2009), research is now geared towards identifying genes and proteins that are involved in tumorigenesis and tumor progression to serve as biomarkers that can accurately predict prognosis and possibly be targeted for therapy in canine and even human cancers (Selvarajah et al. 2009; Queiroga et al. 2011; Saijo, 2012; Lin et al. 2022; Atmane et al. 2023).

Among the recent biomarkers involved in growth signalling which have been investigated in human medicine are the fibroblast growth factor receptors (FGFRs) (Fearon et al. 2013; Sridharan et al. 2022; Furugaki et al. 2023). The four fibroblast growth factor receptors (FGFR 1-4) belong to the receptor tyrosine kinase group of receptors, which are transmembrane receptors with an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase

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domain (Haugsten et al. 2010; Murugesan et al. 2022). Recently, FGFR5 lacking the intracellular tyrosine kinase domain, but serves to regulate excessive activation of the pathway by the ligand has been identified (Yue et al. 2021). Activation of the receptors by ligand; Fibroblast growth factor (FGF) binding leads to the activation of a series of cascades, which are regulatory to cell growth, differentiation, migration and survival in cells derived from mesoderm and neuroectoderm (Haugsten et al. 2010; Qin et al. 2019; Suzuki et al. 2023). Aberrant expression of FGFRs has been reported to be involved in carcinogenesis and progression of selected human neoplasia including breast cancer (Blanckaert et al. 1998; Haugsten et al. 2010; Kuroso et al. 2010; Helsten et al. 2016; Qin et al. 2019; Wu et al. 2022;Bou Zerdan et al. 2023), with FGFR2 suggested as a breast cancer susceptibility gene (Meyer et al. 2008; Sun et al. 2012). Alterations in molecular structure such as axons 7 and 8 deletion in FGFR3 in breast cancer, and single nucleotide polymorphism (SNP) (Gly/Arg) in FGFR4 in head and neck squamous cell carcinoma causing aberrant expression has also been demonstrated as useful for prognosis prediction (Blanckaert et al. 1998; Marsh et al. 1999; Jang et al. 2001; Zammit et al. 2001; Ansell et al. 2009). Other FGFR alterations reported in cancer include gene fusion and copy number amplification (Krook et al. 2021). Although FGFRs are localized in the cell membrane and cytoplasm (Zammit et al. 2001), certain splice and/or mutated variants have been found to be localized in the nucleus, where they are implicated in cancer initiation and progression (Johnston et al. 1995; Zammit et al. 2001; Lu et al. 2008; Martin et al. 2011). Additionally, experimental FGFR1 nuclear localization promoted drug resistance in breast cancer cells (Servetto et al. 2021) and recently, FGFRs expression in cancers have been associated with drug resistance in the cancer cells, making the protein expression a hurdle that must be circumvented for effective cancer therapy (Sahabiet al. 2022; Mahapatra et al. 2023).

Despite the apparent significance of FGFRs proteins in cancer initiation, progression and survival, their expression and its association with clinical and pathological factors as well as its potential relevance in predicting prognosis is not adequately investigated in CMT, as only FGFR2 was reported as having highest expression in canine mammary complex carcinoma compared to other subtypes (Gentile et al. 2017). The study did not however correlate the FGFR2 expression with clinical or histopathological parameters of dogs. Additionally, FGFR3 is reported to have a five-fold increase in expression in a CMT sub-line with acquired doxorubicin resistance, compared to the original cells(Sahabi et al. 2022). If the CMT has similar FGFRs expression pattern as those reported in human breast cancer, then dogs with spontaneous mammary gland tumor could be used as a 'large animal model' for evaluation of novel therapies targeting the FGFRs. Insights on therapeutic strategies targeting the FGF receptor signaling in CMT models may hasten clinical trial approvals and speed up development of novel drugs for human breast cancer application. Additionally, dogs with FGFRs as drivers of tumorigenesis could benefit from the United State Food and Drug Administration (FDA) approved tyrosine kinase inhibitors for use in dogs with cancer capable of targeting FGFRs, including mutated proteins, in canine tumors (Marech et al. 2014; Fisher et al. 2021; Wu et al. 2022).

This study will investigate the expression and localization of FGFR2, FGFR3 and FGFR4 proteins in CMT, and present the first report of FGFRs expression in CMT tissues and their potential as prognostic factors in dogs with CMT. This study will also investigate the relationship between the specific FGFR expression and selected clinical and histopathological parameters.

MATERIALS AND METHODS

Sample Collection

Mastectomy tissues from the University Veterinary Hospital (UVH), Universiti Putra Malaysia (UPM) removed as part of routine therapy for dogs with CMT were collected in form of formalin-fixed and paraffin-embedded (FFPE) for this study. Fresh CMT samples from the UVH were snap-frozen in liquid nitrogen and subsequently stored at -80°C until further processing. Normal or non-diseased canine mammary gland and kidney tissues were collected from local (*canis lupus familiaris*) dogs that were euthanized. From the tissues, FFPE tissue blocks were prepared for routine histology and a small part of the tissue was snap-frozen in liquid nitrogen and subsequently stored at -80°C until further processing. The FFPE tissues were sectioned at 4µm thickness for routine histopathology and immunohistochemistry.

Retrospective Clinical Data Retrieval

The signalment of the dogs was retrieved from the sample submission forms available at the histopathology laboratory, while some other clinical data were retrieved retrospectively from the dog case records at the UVH. The information obtained includes age, breed, neuter status, tumor size, number and type of mammary gland(s) involved and histopathology subtype. The survival information of the dogs was obtained from medical records of UVH and via phone conversations with the dog owners. The owners gave their consent to use the information provided for this research. The survival time is the time interval between mastectomy and data analysis.

Tumor Grading

Tumors were graded histopathologically as Grade 1, 2 or 3 according to the tumor grading systems previously described (Pautier et al. 2000; Goldschmidt et al. 2011; Yanofsky et al. 2011).

Protein Isolation and Quantification

Protein was extracted from about thirty milligrams of the snap-frozen tissues using a protein extraction kit (Machery-Nagel, Germany) according to manufacturer's protocol. The extracted protein was quantified using bicinchoninic acid assay (BCA), (Pierce BCA Protein Assay Kit, Thermoscientific USA) according to the manufacturer's protocol. A standard curve was plotted (concentration against absorbance) using Microsoft office Excel 2007 and was then used to calculate the concentration of the protein samples. The protein samples were stored at -30°C until further processing.

Western Blotting

Western blot was used to validate the cross reactivity between the human antibodies with the canine FGFR proteins. The western blot was done according to a previously published protocol on canine tissues (Selvarajah et al. 2012). Briefly, 20µg of protein from each sample were separated on three 10% SDS gels using SDS-PAGE system (mini-PROTEAN Tetra Cell electrophoresis system, Bio-Rad Laboratories, CA USA), by applying 130V for 2 hours in 1X running buffer and transferred onto a methanol-pre-soaked polyvinylidine difluoride (PVDF) membrane by applying 100V for 1 hour in transfer buffer. Protein extracted from human nasopharyngeal tumor cell line was used as positive control.

The membranes were blocked for nonspecific binding with 5% BSA for 1 hour at room temperature and then incubated with polyclonal rabbit anti-FGFR2, FGFR3 and FGFR4 (Abcam^{R} Cambridge, U.K) at a dilution of 1:800, 1:300 and 1:200 respectively in blocking buffer (5% BSA) at 4^{o}C overnight in 50mL tubes on a roller. The membranes were incubated with horseradish Peroxidase-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, Germany) at 1:10,000 in blocking buffer for I hour at room temperature. The membranes were then washed, incubated and viewed with chemiluminescent substrate (Amersham^{TM} GE Healthcare, U.K) and the images were captured using a CCD camera in FluorChem^{TM} 5500 imaging system controlled by AlphaEase^{R}FC software (Alpha Innotech Corp. USA). A reference protein: β-actin was probed on the membranes with mouse monoclonal β-actin antibody (Santa Cruz biotechnology inc. Europe) and images captured as described earlier. All washing between steps were done with 1% Tween 20 in tris buffered saline slight or tris buffered saline alone, with agitation, at room temperature.

Immunohistochemistry

Three sets of the slides were used for primary antibodies incubation, while the remaining set was used as control for the experiment. The procedure was according to a previously published protocol (Peña et al. 1998). The tissue slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was by heating the slides in microwave at medium voltage for 15min in 10mM citrate buffer (pH 6). A peroxidase blocking solution (Dako, S2023 Denmark) was incubated on the slides for 30min to block endogenous peroxidase. Nonspecific tissue staining was blocked with 10% BSA (Sigma Aldrich, USA) for 30 minutes. The slides were then incubated with rabbit polyclonal anti-FGFR2, FGFR3, and FGFR4 antibodies (Abcam) in 0.1% BSA in 0.1% tween 20 in phosphate buffered saline (PBST), at dilutions of 1:250, 1:400 and 1:50, respectively. On the control slides, rabbit IgG in PBST at 1:400 dilutions replaced the primary antibodies. The slides were incubated overnight in a humidified chamber at 4°C. The slides were rinsed with PBST and incubated with anti-rabbit secondary antibody (DAKO, K4002, Carpentaria, CA USA), for 30 minutes at room temperature. The binding complex developed colour using 3,3$^{'}$-diaminobenzidine tetrahydrochloride (DAB) as substrate (Dako, K3468, Carpentaria, CA USA), incubated for 5 minutes. All washing was done with PBST (pH 7.4). However, for washings after rehydration and chromogen incubation (DAB), only PBS was used. The slides were counter stained with hematoxylin for one minute, dehydrated in increasing concentrations of alcohol and cleared in xylene before a cover slip is placed.

Tissue Scoring Method and Statistical Analysis

Tissues were scored by determining the proportion of positive cells from the total number of cells in three random high-power fields (X400). The expression of each receptor was analyzed using Fisher's exact test for its relationship with clinical parameters (breed size, age, neuter status, involvement of inguinal mammary gland, number of glands involved) and histopathology parameters (mitotic index, tumor size, tumor grade, PCNA and Vimentin expressions).

Survival Analysis

Survival analysis was performed to estimate survival rates of FGFR expression. Death was considered 'due to disease' if the dog died or was euthanized due to conditions related to the disease. A dog was considered "censored" for survival analysis if it died due to other causes or was lost to follow up. Kaplan- Meier curves were created for this analysis. Survival analysis was reported as Hazard Ratio where $P<0.05$ was considered statistically significant within a 95% confidence interval. All data analysis was done using SPSS version 20 (IBM Corporation, U.S. 1989, 2011).

RESULTS

A total of 7 protein samples were quantified using the standard curve form the BCA quantification assay. Four of the samples were canine mammary gland tumors, one normal canine mammary gland tissue, one normal canine kidney and one human nasopharyngeal carcinoma cell line (TW04). Western blot images reflected the approximate molecular weight of the antibodies suggested by the manufacturers (FGFR2: 110kDa; FGFR3: 110-120kDa and FGFR4: 87kDa) (Fig. 1). Forty-five tumors (97.8%) expressed both FGFR2 and FGFR3; only a single CMT was negative for both FGFR2 and FGFR3. FGFR4 was expressed in 42 (91.3%) of the tumors. The FGFR2 -3 and -4 expressions were localized to the cytoplasm and membrane, with few cells demonstrating nuclear localization (Fig. 2A, 2B and 2C, respectively).

The intensity of FGFR4 expression in the tissues was lower than those of FGFR2 and FGFR3. Twenty-one (45.7%) of the tumors demonstrated low (less than 50%) FGFR2 expression while 25 (54.3%) had high expression (more than 50%).

Twenty-one (45.7%) of the tumors had high (more than 50%) FGFR3 expression while 25 (54.3%) had low (less than 50%) expression. Twenty-four (52.2%) tumors had high (more than 50%) expression for FGFR4 while 22 (47.8%) had low expression (more than 50%). For both FGFR2 and FGFR3, the expression of the proteins was noted to be higher in the kidney epithelial cells; positive control tissue (Fig. 3A and 4A), than in the tumor tissues, while the normal mammary glands had the least expression (Fig. 3B and 4B). However, for FGFR4, the protein expression observed in the kidney was lower than the

Table 1: Association between FGFRs expression in tumor tissues and clinicopathological parameters

	FGFR2 Expression			FGFR3 Expression			FGFR4 Expression		
Parameters and categories	Low (<50%)	High (>50%)	P-value	Low (<50%)	High (>50%)	P-value	Low (<50%)	High (>50%)	P-value
Age									
<5yrs n=6	3	3	n.a	5	1	n.a	4	2	n.a
>5yrs n=40	18	22		20	20		18	22	
Breed									
Small n=34	13	21	n.a	18	16	n.a	13	21	0.044
Large n=12	8	4		7	5		9	3	
Neuter status									
Intact n=41	17	24	n.a	23	18	n.a	20	21	n.a
Spayed n=5	4	1		2	3		2	3	
Tumor size									
<3cm n=4	1	3	n.a	3	1	n.a	4	0	0.045
>3cm n=42	20	22		22	20		18	24	
Inguinal mammary gland									
Inguinal gland n=34	15	19		20	14		18	16	
Others[a] n=12	6	6	n.a	5	7	n.a	4	8	n.a
Number of glands									
Single n= 31									
Multiple n=15	15	16	n.a	19	12	n.a	18	13	n.a
	6	9		6	9		4	11	
Histologic grade									
Low n=32	11	21	0.027	15	17	n.a	14	18	n.a
High n=14	10	4		10	4		8	6	
Mitotic index									
Low n=35	13	8	n.a	19	16	n.a	15	20	n.a
High n=11	22	3		6	5		7	4	
PCNA expression									
Low n=16	8	8	n.a	8	8	n.a	9	7	
High n=30	13	17		17	13		13	17	n.a
Vimentin expression									
Low n=33	17	16	n.a	19	14	n.a	17	16	
High n=13	4	9		6	7		5	8	n.a

n.a = not associated, a = Others include Cranial thoracic, caudal thoracic, cranial abdominal and caudal abdominal mammary glands.

Fig. 1: Western blot image for fibroblast growth factor receptors and Beta actin: **Legend**: A = Loading buffer, B = Normal canine mammary gland, C,D,E,F = CMT, G = TW4 cell line and H = Normal canine kidney

expression observed in seven of the tumor tissues (Fig. 5A). The normal mammary gland also had very low expression of the FGFR4 protein (Fig. 5B).

The expression of FGFR2 was significantly associated with histopathology grade 3 of the tumors (P=0.027), but not with other clinical and pathological parameters (Table 1). Fibroblast growth factor receptor 3 expression was not associated with any clinical or histopathology parameters (Table 1). Fibroblast growth factor receptor 4 expression in the tissues was significantly associated with large breed dogs (P=0.044), and large tumor size (more than 3cm), (P=0.045) but was not associated with the other clinical and pathological parameters (Table 1). The expressions FGFR2, FGFR3 and FGFR4 in the tissues could not predict postsurgical survival of the dogs (Fig. 6).

DISCUSSION

The FGFRs are key players in normal embryonic development due to their involvement in processes that are essential to cell proliferation, differentiation and survival. However, their activities are not limited to normal development alone, as they are involved in cancer development and progression (Blanckaert et al. 1998; Haugsten et al. 2010; Francavilla and Obrien 2022), with FGFR2 been suggested as a breast cancer susceptibility gene by some of the studies (Meyer et al. 2008; Sun et al. 2012). Several studies that have been done on FGFRs expression in human breast cancers (Johnston et al. 1995; Zammit et al. 2001; Kuroso et al. 2010; Furugaki et al. 2023), however, only one study has investigated FGFR2 expression in CMT, but the study did not correlate the expression with clinical and/or histopathological parameters (Gentile et al. 2017). To the best of our knowledge, this is the first study to investigate and correlate the expression of these proteins in CMT to clinical and histopathological parameters.

The antibodies used to detect the FGFRs proteins on the CMT tissues were successfully validated on western blot analysis using canine proteins. The high percentage of

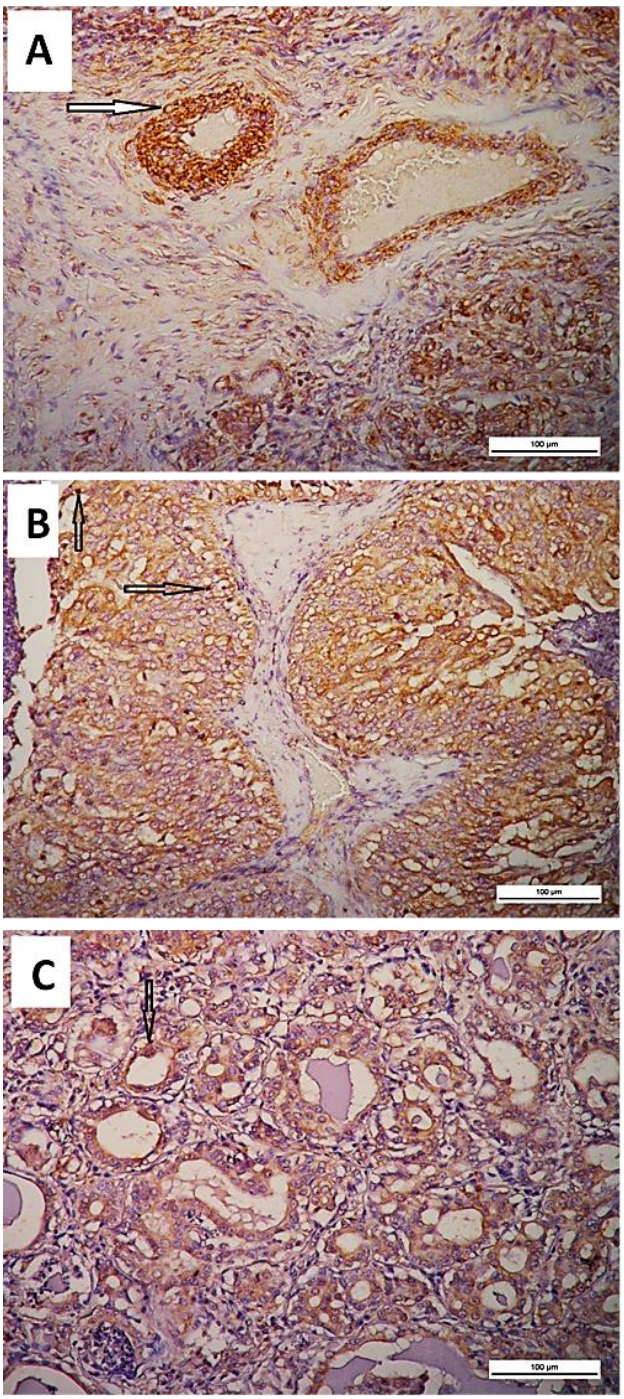

Fig. 2: Fibroblast growth factor receptor 2, -3 and -4 expression and localization in canine mammary gland tumors; **A)** showing cytoplasmic FGFR2 localization (arrow) **B)** showing nuclear FGFR3 localization (arrows) **C**) showing cytoplasmic FGFR4 localization (arrow).

tissues with positive FGFRs expression is comparable with human breast cancers (Zammit et al. 2001; Kuroso et al. 2010). Since the expression of FGFR2 and FGFR3 is present in majority of the mammary gland tumors and higher than in the normal mammary gland tissue, it confirms that just like in human breast cancer, these receptors play crucial roles in tumorigenesis and fibroblast growth factor signalling is an important pathway in CMT as previously suggested in other canine malignancies (Selvarajah et al. 2009; Fisher et al. 2021), and makes them worthy targets of continuous studies for

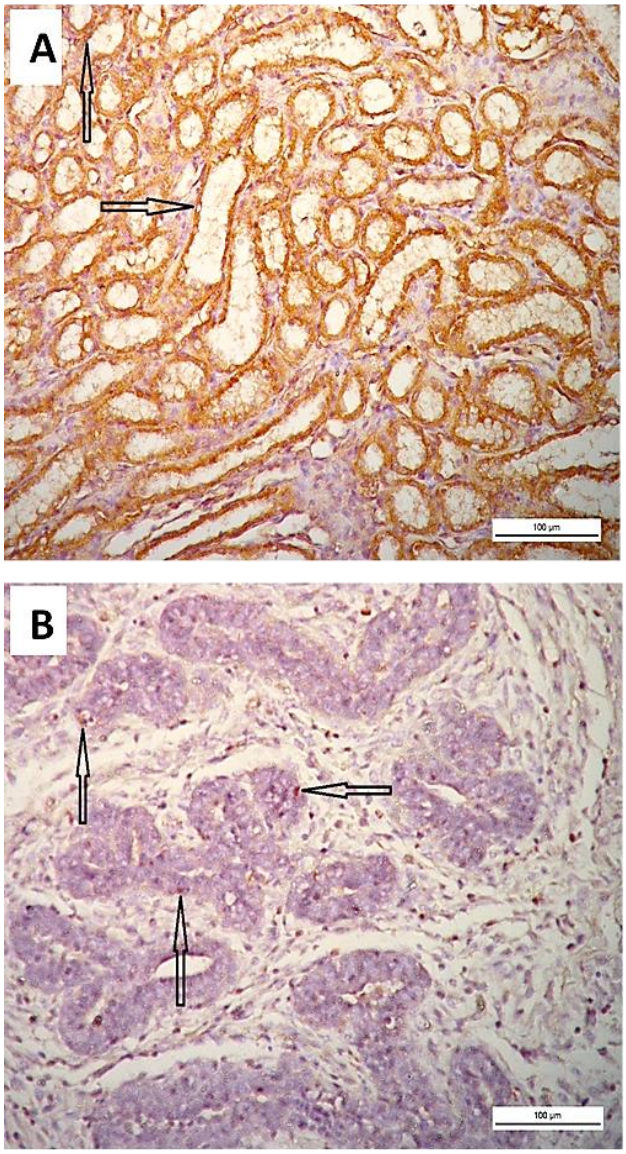

Fig. 3: Fibroblast growth factor receptor 2 expression and localization in canine kidney epithelial cells and normal canine mammary gland; **A)** showing cytoplasmic FGFR2 localization of the protein in the cells (arrows) **B)** Very weak expression of FGFR2 can be observed in the normal mammary gland (arrows).

better understanding and more precise therapeutic targeting. The FGFR protein localization in the cytoplasm and nucleus observed in CMT was already described in human breast cancer (Kuroso et al. 2010; Krook et al. 2021). The nuclear localization of the FGFRs has been explained to be due to internalization of vesicles containing activated FGFRs to peri-nuclear areas in the cell, where they are involved in cell proliferation (Zammit et al. 2001). In this study, 10 CMT tissues had FGFR3 localized in the nucleus, but the localization was not associated with any of the clinical or pathological parameters, which could be as a result of small number of tumors in this study. In human breast cancer, a splice variant of FGFR3 lacking transmembrane domain has been reported to predominantly localize in the nucleus (Johnston et al. 1995). This FGFR3 splice variant is expressed in both malignant and non-malignant breast tissues, but localized to the nucleus only in the malignant breast tissues indicating a possible role of the relocalization in cancer progression. It will be very

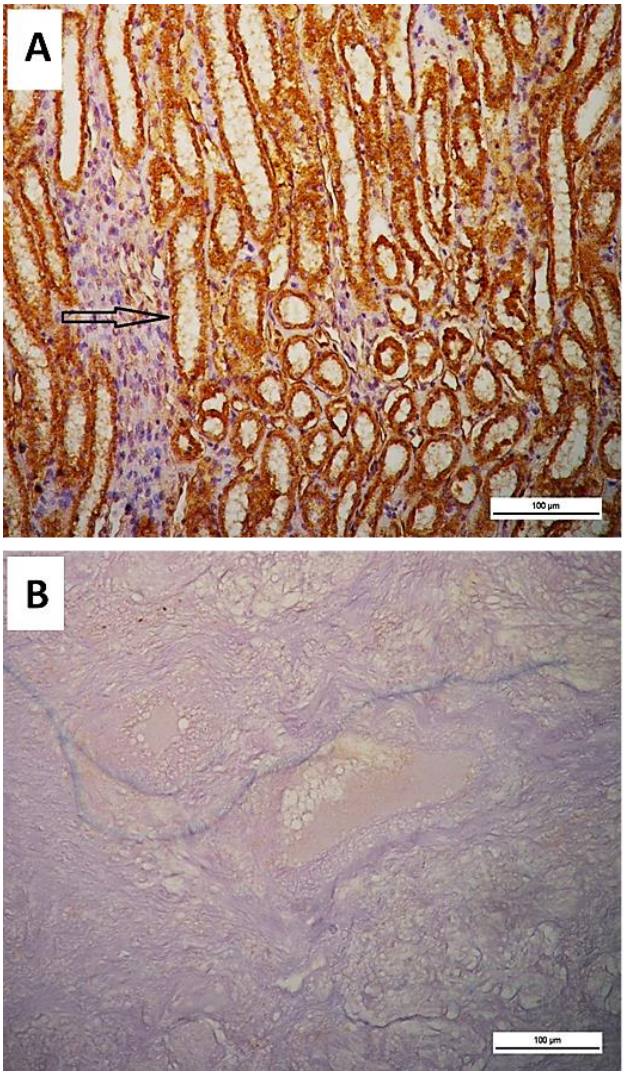

Fig. 4: Fibroblast growth factor receptor 3 expression and localization in canine kidney epithelial cells and normal mammary gland; **A)** showing cytoplasmic FGFR3 protein localization in the cells **B)** FGFR3 expression in normal canine mammary gland showing weak staining.

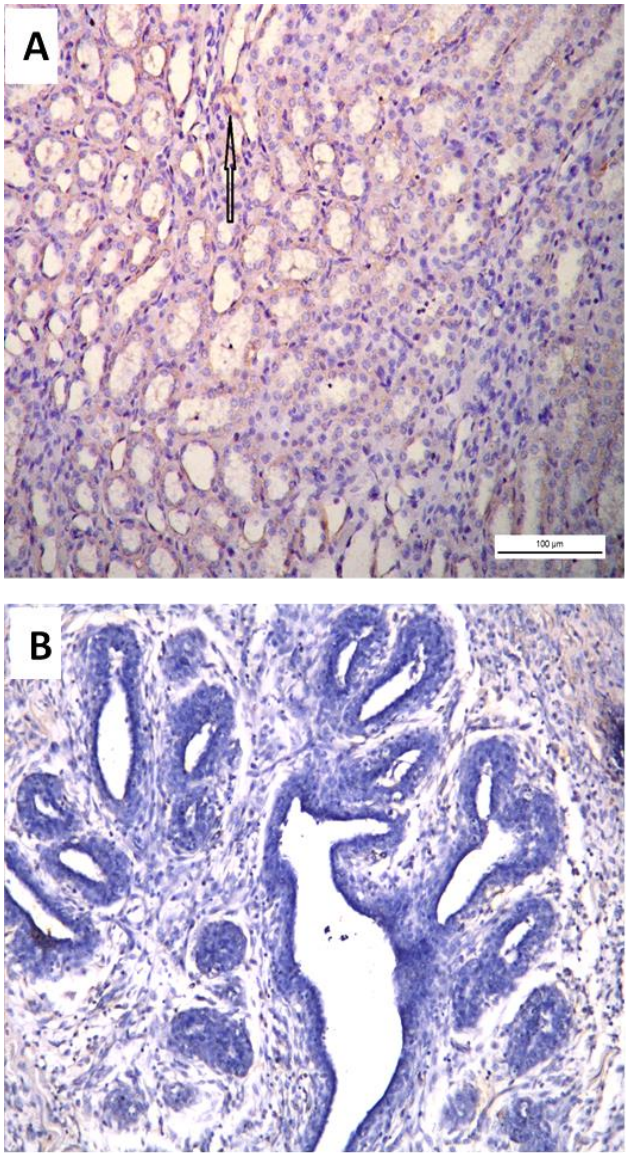

Fig. 5: Fibroblast growth factor receptor 4 expression and localization in canine kidney epithelial cells and normal canine mammary gland; **A)** showing weak cytoplasmic FGFR4 expression and localization **B)** FGFR4 expression in normal canine mammary gland showing weak staining.

interesting to explore further, the reason for the nuclear localization of FGFR3 in these tissues through gene sequencing to establish whether or not, it is as a result of the previously reported splice variant in human breast cancer lacking transmembrane domain (Johnston et al. 1995) been expressed in these particular CMT tissues.

The expression of FGFR4 in the tissues is found to be lower than FGFR2 and FGFR3, despite high FGFR4 expression been associated with large breed dogs and large tumor size. In human breast cancer, mutations and SNPs in FGFRs have been associated with tumorigenesis and progression of the disease, without necessarily increasing the expression in the tumor tissues (Thussbas et al. 2006; Fearon et al. 2013; Wimmer et al. 2019). This could explain why despite having lower expression levels compared to the other proteins, FGFR4 expression was able to influence the size of the tumors. Although this study did not look for any mutations or SNPs, in the tumor tissues, it could not be ruled out as absent. Another reason for lowered FGFR4 expression could be that FGFR4 expression in the normal tissues is lower than those of FGFR2 and FGFR3. This is supported by the fact that FGFR4 expression in 7 of the tumor tissues is more than the expression in the kidney (positive control tissue), which should have the highest expression in the normal tissues. Only one CMT tissue showed negative FGFR2 and FGFR3 expressions. This finding is similar to human breast cancer where possible explanation for this scenario is that the expressions of FGFR2 and FGFR3 are somewhat closely associated or molecularly linked to each other (Cerliani et al. 2012). Since both of these receptors are possibly absent in this particular CMT (grade 1 tumor with very low PCNA and Vimentin expressions), it may suggest that different pathways are involved in tumorigenesis and is not solely dependent on FGFRs signalling and that the expression of these 2 receptors is important for tumor progression that could be measured by looking at tumor grade and the expressions of PCNA and Vimentin (all of which are low in this particular tissue). Absence of FGFR2 and FGFR3 in this tissue could be due to deactivating mutations in a shared or central FGFRs mRNA translation factor,

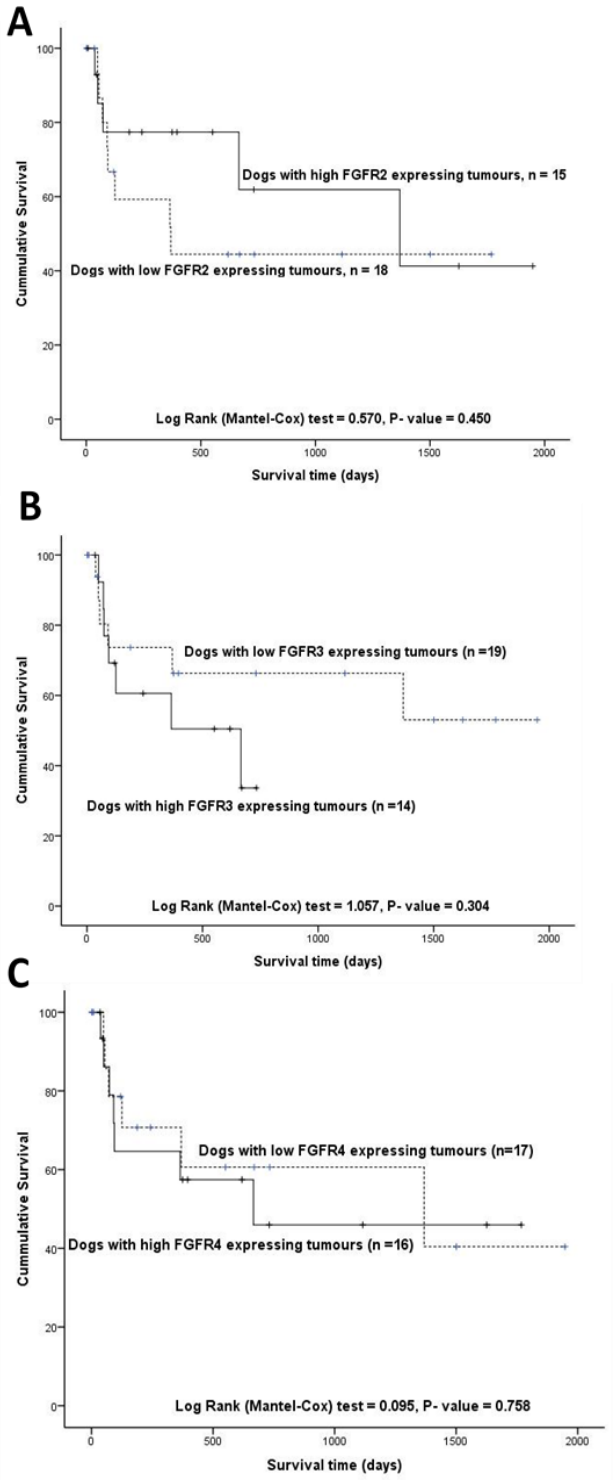

Fig. 6: Survival curves of canine mammary tumor cases in relation to fibroblast growth factor receptors expression. None of the fibroblast growth factor receptors expression in the CMT tissues could predict postsurgical survival of the dogs.

which could stop protein translation from mRNA to matured FGFRs proteins as previously proposed (Cerliani et al. 2012). It would be interesting to further investigate the reason behind absence of both these receptors in this particular CMT where additional mRNA profiling and gene sequencing could be explored.

The result of this study revealed a relationship between FGFR2 and high histopathology grade of tumors, similar to findings reported in human breast cancer (Blanckaert et al. 1998; Haugsten et al. 2010; Kuroso et al. 2010). The relationship shows that FGFR2 signalling enhances the proliferation of cancer cells and progression of tumors and indicates the usefulness of FGFR2 expression in CMT as an indicator of increased malignancy. The FGFR2 gene amplification, which could explain why the protein is over expressed in the CMT tissues studied, has been reported in triple-negative (ER^-, PR^- and $HER2^-$) breast cancer, which is considered to be the most advanced form of human breast cancer due to its poorest prognosis (Turner et al. 2010). The FGFRs genes amplification were not investigated in the present study on CMT and for that it is not possible to confidently relate the over expression of FGFR2 protein to gene amplification in CMT. Although FGFR2 expression did not associate with the other clinical and histopathological parameters in the present study, it has been reported to be associated with tumor size in human breast cancer (Sun et al. 2012).

The FGFR3 expression in the tumors was not associated with any of the clinical or histopathological parameters investigated, similar to a report in human breast cancer (Kuroso et al. 2010). However, although not statistically significant, FGFR3 expression demonstrated its potential as a prognostic marker in CMT, with dogs having high FGFR3 expressing tumors surviving only 1/3 the survival time of dogs with low FGFR3 expressing tumors. It could be expected that in a larger study, FGFR3 will demonstrate its real value as a prognostic marker in CMT. Large tumors have shown significantly higher FGFR4 expression. Tumor size is one of the criteria for tumor staging (TNM), which placed large tumors on stages 2 and above (Goldschmidt et al. 2011). Tumor size has also been reported to be an independent prognostic factor in canine mammary gland tumors (Philibert et al. 2003; Moon et al. 2022). Moreover, increasing tumor size was reported to be associated with decreasing 5-year survival in a study involving about 25,000 breast cancer patients (Carter et al. 1989). Taken together, this finding suggests that FGFR4 expression in tumors is an important driver of tumor progression, and by extension, influence prognosis in dogs with CMT, as reported in patients with oral cancer (Gu et al. 2023). Large breed dogs have a significantly higher FGFR4 expression in this study, but FGFR4 expression in the tissues did not predict postsurgical survival in the dogs. However, in another study (Itoh et al. 2005), large breed dogs were reported to have shorter postsurgical survival time compared to small breed dogs. When these results are considered together, it could be deduced that FGFR4 expression in CMT is a useful marker of advanced tumor stage and possibly a prognostic marker in large breed dogs.

Although this study did not find significant association between FGFRs and Vimentin expressions, the leading role of FGFRs in the activation of epithelial-mesenchymal transition (EMT) in mouse mammary tumor virus-Neu epithelium (Qian et al. 2004) and human breast cancer, with Vimentin expression been associated with FGFR1 expression in human breast cancer, have been documented (Cheng et al. 2013). Moreover, FGFR2 expression is also reported to be associated with EMT in endometrial cancer (Adamczyk-Gruszka et al. 2022). Beside their roles in EMT, the roles of FGFRs signalling extend to the next step in the establishment of metastasis, which is mesenchymal epithelial transition (MET). The MET in tumors allows

circulating tumor cells to settle in a tissue and acquire epithelial characters to establish secondary tumor (Chaffer et al.2006), as well as drug resistance (Hu et al. 2022). The splice variant of FGFR2 (FGFR2 IIIC) has been shown to be involved in MET, following the reversal of the process after the inhibition of the receptor in bladder cancer cell lines (Chaffer et al. 2006). The expression of FGFRs was not associated with PCNA expression in this study. The FGFRs are required for several key cellular processes such as cell proliferation (Powers et al. 2000), which could be quantified by the PCNA labelling index (Peña et al. 1998). Furthermore, it has been reported that FGFRs expression is associated with high cancer cell proliferation (Suh et al. 2020), PCNA labelling index in human fibroadenoma of the breast, with tumors having high FGFRs expression having high PCNA index (Hasebe et al. 1999). Taken together, it could be explained that these variations in associations between key proteins involved in tumor proliferation and progression pathways indicate the intricate nature of gene expression regulation, with possible redundancy in the activity of seemingly indispensable proteins and pathways that drive tumor development and progression.

Conclusion

Based on these findings, FGFR2 and FGFR4 can be used as markers for advanced and aggressive CMT, which warrant further studies to evaluate for targeted therapy, so that dogs with CMT can benefit from the FGFR inhibitors that are currently used to specifically target the FGF receptors in several human cancers.

Some limitations of this study include small number of CMT tissues for evaluation with the FGFRs especially the fact that this is the first study on the markers in CMT. In the future, it is recommended that *in-vitro* and *in-vivo* studies as well as gene sequencing analysis be conducted to further investigate genetic changes in the FGFRs genes responsible for the observed phenotype and to further understand the influence of FGFRs in tumorigenesis, progression and outcome of CMT.

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Authors' Contributions

S.K took part in the design, carried out the experiments and drafted the manuscript. S.G.T conceptualized and designed the research, and took part in some of the experiments and revised the manuscript. G.K.D took part in data acquisition and revised the manuscript. R.S.S supported some of the experiments and revised the manuscript. N.M.M took part in data analysis and revised the manuscript. All authors read and approved the submitted version of the manuscript.

Conflict of Interest: The authors declare that there is no conflict of interest.

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