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Report

Secreted frizzled-related protein 2 (SFRP2) is highly expressed in canine mammary gland tumors but not in normal mammary glands

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Summary

Canine mammary gland tumor (MGT) is the commonest tumor in female dogs and a good animal model of human breast cancer. A group of newly identified genes encoding secreted frizzled-related proteins (SFRP) have been implicated in apoptosis regulation and tumorigenesis. Canine mammary tissues from 50 spontaneous MGTs and 10 normal mammary glands (MGs) were obtained from surgically excised specimens and analyzed for expression of SFRP2, β-catenin, and cyclin D1. By RT-PCR and *in situ* hybridization, *SFRP2* gene was found abundantly expressed in neoplastic mammary tissues but not in normal mammary tissues, suggesting that SFRP2 may contribute as a tumor marker in canine MGTs. By immunohistochemical staining, the immunoreactivity of the SFRP2 protein was detected in more diverse areas than SFRP2 mRNA expression, including nuclei or/and cytoplasm and extracellular matrix of the tumor. In tumor masses, β-catenin lost its tight association with the membrane and diffused into the nucleus. The expression of β-catenin (79.4% positive) and cyclin D1 (71.4% positive) was also increased in MGTs. In the course of tumor progression, SFRP2 mRNA ($p < 0.05$) and β-catenin protein ($p < 0.01$) steadily increased but not in cyclin D1. The level of SFRP2 was linearly correlated with its downstream target β-catenin ($p < 0.05$), but not correlated with cyclin D1 ($p < 0.5$). As revealed in this study, the exclusive overexpression of SFRP2 in canine MGTs suggests that *SFRP2* is a potential candidate gene for further investigation of mammary tumorigenesis and complex etiology of the canine model of mammary neoplasms.

Introduction

Critical events in mammary tumorigenesis and metastasis include the abnormal activation of specific genes. The frizzled and secreted frizzled-related protein (SFRP) families are implicated in modulating the Wnt-Frizzled signal transduction pathway which plays an important role in normal development and oncogenesis [1]. The Wnt signaling pathway starts from interaction of Wnt proteins with receptors which have been identified as members of frizzled and SFRP families. Following Wnt proteins binding to their receptors, a cytoplasmic protein dishevelled (DSH) is phosphorylated and the activity of GSK3β is suppressed leading to accumulation of β-catenin in the nucleus of cells. β-Catenin then interacts with members of LEF-1/TCF families of HMG box transcription factors, thereby contributing to expression of specific target genes (such as cyclin D1 and c-Myc) [2, 3].

The *frizzled* gene was originally identified as a tissue polarity gene controlling tissue orientation during development in *Drosophila* [4]. Structurally, the frizzled protein is an integral membrane protein with an extracellular cysteine-rich domain (CRD) at its N-terminus followed by seven putative transmembrane segments. The conserved extracellular CRD region of the frizzled protein comprises of ∼110 amino acid residues, including 10 invariant cysteines, which is the putative binding domain for Wnt ligands [5]. A large family of genes which encode proteins with this struc-

ture has now been described in the vertebrates. SFRPs comprise a family of secreted molecules that contain a N-terminal cysteine-rich domain (CRD) homologous to the CRDs of the frizzled family of transmembrane Wnt receptors [6, 7]. In addition to the function of modulating Wnt-signaling, members of *SFRP* gene family have been implicated as having roles in control of apoptosis [8, 9], an important pathway to regulate cell proliferation and tissue homeostasis in development and oncogenesis. It has been reported that the SFRP2 possesses anti-apoptosis activity while SFRP1 induces pro-apoptosis in one breast cancer cell line [8]. However, the exact roles and mechanism of apoptosis control of all SFRPs remain to be determined.

A canine homologue of *SFRP2* cDNA was first isolated and cloned from a retina-specifically expressed gene population of a canine subtractive retinal cDNA library [10, 11]. A few recent studies have indicated that the possible involvement of the *SFRP2* in human cancers and diseases. For example, *SFRP2* is abundantly expressed in quiescent uterine stromal cells but is down-regulated by estrogens [12–15]. During disease or cancer progression, SFRP2 up-regulation in the retinas of retinitis pigmentosa may reflect an anti-apoptotic response [16]. Overexpression of transfected SFRP2 in breast adenocarcinoma cells increased their resistance to apoptotic signals, and was associated with increased intracellular levels of β-catenin [8]. SFRP2 overexpression could inhibit glioma cell motility, and was associated with down-regulation of MMP-2 activity [17]. Based on recent findings of *SFRP2* in human cancers and important roles of SFRP2 in Wnt signaling and apoptosis control, SFRP2 is a good candidate gene to be investigated in more detailed in the canine MGT model, which has importance in both veterinary and comparative medicine.

Canine MGTs are the canine counterpart and a spontaneous animal model of human breast cancer that share several similarities with it [18]. They have similar histologic origins as well as comparable regional, systemic metastases and appearances at similar relative ages [19]. The predominant malignant tumor cell type of canine MGT is adenocarcinoma (the major subgroup of tubulopapillary carcinoma) which is the same as that found in the human breast cancer. The development of mammary tumors in dogs is clearly hormone dependent. The percentages of positive expression of estrogen receptor (ER+) and progesterone receptor (PR+) are 77 and 81%, respectively, in canine MGTs [20]. In comparison, they are 63 and 74%, respectively, in human breast cancers [21]. In addition, few studies have demonstrated that canine MGTs have ER and PR profiles and corresponding prognostic implications similar to those of breast cancers [22, 23]. The complex etiology of canine MGT is mostly unknown. Here we describe the investigation of expression of SFRP2 and Wnt downstream components, β-catenin and cyclin D1, in canine MGTs to provide a basis of understanding mammary tumorigenesis and the association between the SFRP2/Wnt signaling and mammary tumors.

Materials and methods

Collection and preparation of canine MG specimens

Canine MG specimens (50 MGTs and 10 normal MG tissues) were collected and processed from freshly excised surgical specimens (Table 1). The tumor types of

Table 1. Clinical patients[a]

Patient	Breed	OHE	Age (years)	Type
T-1	Mix	N	12	TC
T-2	Poodle	Y	12	TC
T-3	Mix	N	9	TC
T-4	Maltese	N	10	TC
T-5	Chihuahua	N	10	TC
T-6	Mix	Y	10	TC
T-7	Maltese	Y	12	TC
T-8	Mix	Y	10	TC
T-9	Mix	N	10	TC
T-10	Maltese	N	12	TC
T-11	Chihuahua	Y	14	TC
T-12	Mix	N	11	TC
T-13	Mix	Y	11	TC
T-14	Mix	N	8	TC
T-15	Samoyed	Y	7	TC
T-16	Mix	N	13	TC
T-17	Mix	N	6	TC
T-18	Poodle	Y	7	TC
T-19	Cocker	Y	6	TC
T-20	Poodle	Y	16	TC
T-21	Poodle	N	7	TC
T-22	Mix	N	14	TC
T-23	Mix	N	16	SC
T-24	Mix	N	12	SC
T-25	Mix	Y	15	SC

Table 1. (continued).

Patient	Breed	OHE	Age (years)	Type
T-26	Mix	N	12	SC
T-22	Cocker	Y	10	SC
T-28	Poodle	Y	7	SC
T-29	Mix	N	14	SC
T-30	Cocker	Y	6	SC
T-31	Rottweiler	Y	13	SC
T-32	Mix	Y	10	SC
T-33	Mix	N	11	SAR
T-34	Mix	N	14	SAR
T-35	Maltese	N	8	SAR
T-36	Mix	N	13	SCC
T-37	Mix	Y	11	SCC
T-38	Mp	N	13	MMIX
T-39	Cocker	N	13	MMIX
T-40	Mix	Y	11	MMIX
T-41	Mix	N	7	AD
T-42	Mix	Y	10	AD
T-43	Maltese	Y	13	BMIX
T-44	Cocker	Y	17	BMIX
T-45	Maltese	Y	9	BMIX
T-46	Shih Tzu	N	4	BMIX
T-47	Maltese	Y	7	BMIX
T-48	Maltese	N	3	BMIX
T-49	Mix	Y	9	BMIX
T-50	Maltese	Y	9	BMIX
N-1	Maltese	N	7	NOR
N-2	Mix	N	1	NOR
N-3	Mix	N	14	NOR
N-4	Mix	Y	8	NOR
N-5	Poodle	Y	11	NOR
N-6	Mix	Y	13	NOR
N-7	Mix	N	8	NOR
N-8	Cocker	N	9	NOR
N-9	Mix	Y	4	NOR
N-10	Mix	N	12	NOR

[a] The histological classification of canine mammary tumors is based on the WHO histological classification of mammary tumors of the dog and cat. OHE: ovariohysterectomy; TC: tubulopapillary carcinoma; SC: solid carcinoma; SAR: sarcoma; SCC: squamous cell carcinoma; MMIX: malignant mixed tumors; AD: adenoma; BMIX: benign mixed tumor; NOR: normal mammary tissues.

specimens were classified based on the WHO Histological Classification of Mammary Tumors of the Dog and Cat. Among 50 MGTs, 40 tumors were considered malignant (M) and 10 tumors were benign (B). The malignant MGTs included the following histopathologies: tubulopapillary carcinoma ($n = 22$ tumors); solid carcinoma ($n = 10$ tumors); sarcoma ($n = 3$ tumors); malignant mixed tumors ($n = 3$ tumors) and squamous cell carcinoma ($n = 2$ tumors). The benign MGTs included the following histopathologies: benign mixed tumor ($n = 8$ tumors) and adenoma ($n = 2$ tumors). Taken together, all of 50 canine MGTs were separated into four subgroups: malignant tubulopapillary carcinoma (M-TC, $n = 22$ tumors); the others of malignant MGTs (M-O, $n = 18$ tumors); benign MGTs (B, $n = 10$ tumors); normal MGs (N, $n = 10$ tumors). Tissues were prepared into two major aliquots: one part was fixed in 4% paraformaldehyde (24 h) for paraffin embedding and the other part was frozen for RNA extraction. Serial sections (4 μm) were used for later *in situ* hybridization as well as immunohistochemical staining.

Primary culture from canine MG specimens

Surgically resected specimens were collected from the freshly excised specimens. Primary culture was prepared by a mechanical technique instead of enzymatic digestion as previously described [24]. After agar and dilution cloning, purified cancer cells were plated on 25T flask and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cancer cells were grown in Dulbecco's modified Eagle's (DME) culture medium supplemented with 10% fetal bovine serum (FBS). The cells were subcultured at subconfluency by harvesting with 0.05% trypsin-EDTA. The cells of primary culture were tested positive for cytokeratin 8, 18, ensuring that they were not fibroblasts.

Expression plasmids and antibodies

The canine homologue of SFRP2 cDNA was isolated and cloned from a retina-specifically expressed gene population of a canine subtractive retinal cDNA library [10, 11]. Full-length canine SFRP2 cDNA was subcloned into the mammalian expression vector pCMV-Taq4 (InVitrogen), where SFRP2 is under control of the CMV promoter and can be selected by neomycin resistance. Rabbit polyclonal anti-SFRP2 and mouse monoclonal anti-β-catenin antibodies were purchased from Santa Cruz Biotechnology. A mouse monoclonal antibody against cyclin D1 was purchased from NeoMarkers.

Cell culture and stably transfected cell lines

Primary cultures were maintained in DMEM supplemented with 10% FBS. The cells were transfected

using the Lipofectamine plus reagent (Life Technologies) according to the manufacturer's instructions. After selection in G418 (1.0 mg/ml), the transfected cells were cloned by limiting dilution. The clones expressing Flag-tagged SFRP2 were expanded and maintained in DMEM containing 10% FBS and G418 (200 μg/ml).

Total RNA extraction

Total RNA extraction from canine normal MG and MGT tissues was carried out using TRIzol (Life Technologies) according to manufacturer's instruction. The concentration and purity of RNA were determined spectrophotometrically by measuring absorbance at a wavelength of 260 and 280 nm.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR technique was used to screen *SFRP2* mRNA expression in canine normal MG and MGT tissues. RNA extraction protocols were carried out using homogenized tissues or cells using TRIzol (Life Technologies). Primers were designed based on canine retinal *SFRP2* sequence [10, 11] (GenBank accession number: AJ407833): 5′-GAC AAC GAC ATA ATG GAA ACT C-3′, 5′-CAT GCT ATG GTG GTT AAT GTG-3′. Amplification of the cDNA products was obtained in 30 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 60°C, and extension for 90 s at 72°C. Sample were kept at 72°C for 10 min after the last cycle.

In situ *RNA hybridization (ISH)*

In situ hybridization of *SFRP2* RNA in canine MG and MGT tissues was performed using digoxigenin (DIG)-labeled *SFRP2* cRNA. DIG-labeled RNA corresponding to sense and antisense probes were prepared from linearized canine retinal *SFRP2* cDNA in pBluescript by *in vitro* transcription using T7 or T3 polymerase (DIG RNA Labelling Kit, Boehringer Mannheim) based on a modified protocol described previously [11]. An amount of 1 mg/ml of DIG-labeled SFRP2 cRNA probe was added to freshly made hybridization mixture and applied to the tissue sections for 24 h at 42°C. Anti-DIG-alkaline phosphatase (Fab fragments) was added, color development was performed in a solution containing NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate). Sections were counterstained with the nuclear stain methyl green.

Immunohistochmistry (IHC)

Immunohistochemical analysis was carried out to analyze protein distribution in normal canine MG and MGT tissues. Paraffin-embedded mammary gland sections were dewaxed and rehydrated. Immunolabeling and immunodetection were carried out using the Peroxidase Substrate Kit (Vector Laboratories) using biotinylated second antibody. Color development of the slides was performed by incubation with DAB chromogen solution containing hydrogen peroxide. Sections were counterstained with the nuclear stain hematoxylin.

Scoring criteria

A semi-quantitative method for calculating positive signals was used. Positive signals in tumor and normal cells (including the acinar and ductal epithelia of mammary glands) were each counted in four fields under a light microscope at 400× magnification. The results were evaluated by two independent observers manually and expressed as the percentage of positive cells × staining intensity, according to Remmele and Schicketanz [25] with slight modification. The ISH score was obtained by multiplying the staining intensity (0 = no expression, 1+ = weak expression, 2+ = moderate expression, 3+ = strong expression, and 4+ = very strong expression) by the percentage of positive cells in the field. The maximum possible ISH score was $4 \times 100\% = 400\%$. The same scoring system was used for all immunohistochemical staining in this study and the results are referred to as IHC scores. In immunohistochemical analysis of β-catenin, the signal only localized in nucleus of the cells was determined '+'.

Proteins preparation and immunoblotting

Cells were grown to approximately 80% confluence, washed with PBS, and scraped into 100 μl lysis buffer to obtain whole cell extracts (WCE). Fractionation of cells into nuclear and cytoplasmic fractions was as described previously [26]. The details of immunoblotting were performed essentially as described [27]. The protein concentration of each sample was determined using a protein assay (Bio-Rad Laboratories). Equal amounts of proteins were resolved by SDS-polyacrylamide gel electrophoresis

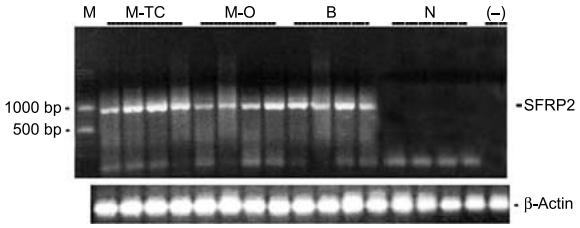


Figure 1. RT-PCR analysis of *SFRP2* in canine MGs. Transcripts of canine *SFRP2* were detected in MGT tissues by RT-PCR. Lanes 2–5 (M-TC): malignant tubulopapillary carcinomas; lanes 6–9 (M-O): the others of malignant MGTs; lanes 10–13 (B): benign MGTs; lanes 14–17 (N): normal MG; lane 18 (−): negative control; lane 1 (M): m.w. markers. Expected size of SFRP2 PCR products, 980 bp. RNA integrity was confirmed by monitoring for β-actin mRNA. Expected size of β-actin PCR products, 156 bp.

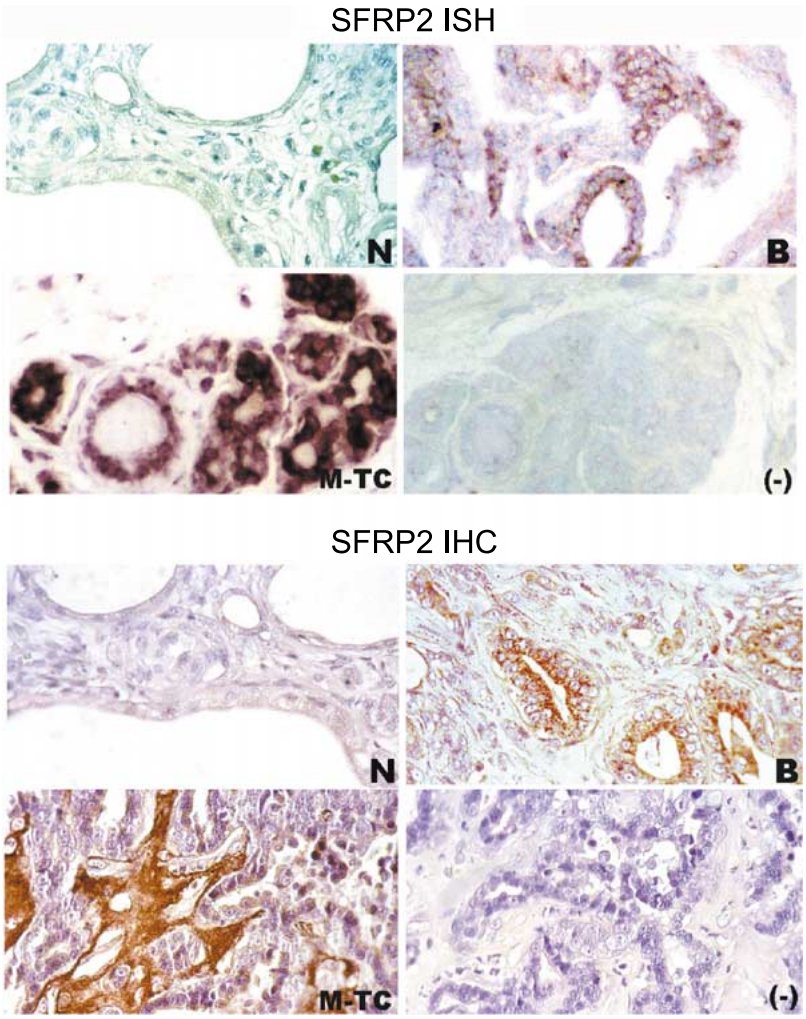


Figure 2. In situ hybridization and immunohistochemical staining for SFRP2 in canine MGs. Upper panel: *In situ* hybridization (ISH) analysis of *SFRP2*. Sections of paraffin-embedded MGs were hybridized with a digoxigenin-labeled sFRP2 antisense probe. (−): negative control, tumor tissue section hybridized to sense probe. Bottom panel: immunohistochemical (IHC) analysis of SFRP2. (−): negative control, representation of negative staining of tumor tissues with the second antibody alone. N: normal MG. B: benign MGTs. M-O: the others of malignant MGTs. M-TC: malignant tubulopapillary carcinomas. Original magnification: 400×.

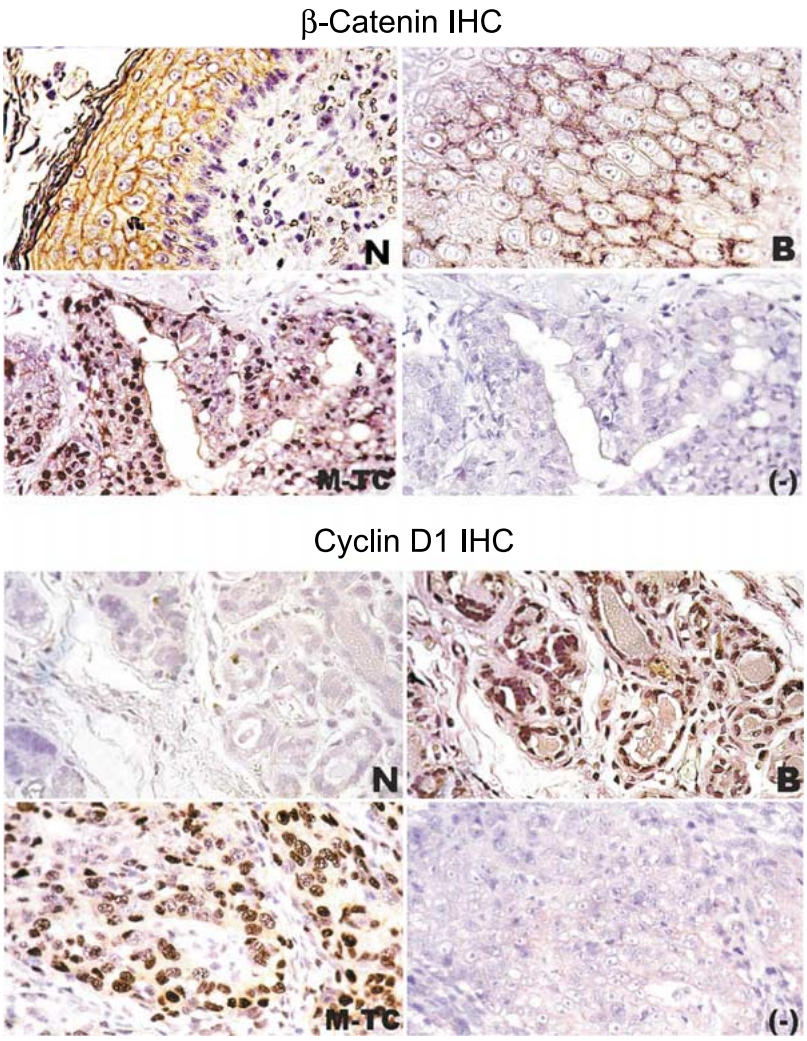


Figure 3. Immunohistochemical staining for Wnt-signaling proteins in canine MGs. Upper panel: immunohistochemical (IHC) analysis of β-catenin. Bottom panel: immunohistochemical analysis of cyclin D1. (−): negative control, representation of negative staining of tumor tissues with the second antibody alone. N: normal MGs. B: benign MGTs. M-O: the others of malignant MGTs. M-TC: malignant tubulopapillary carcinomas. Original magnification: 400×.

and analyzed by immunoblotting with anti-SFRP2, anti-β-catenin and anti-cyclin D1 using the Amersham Pharmacia Biotech chemiluminescence system for detection.

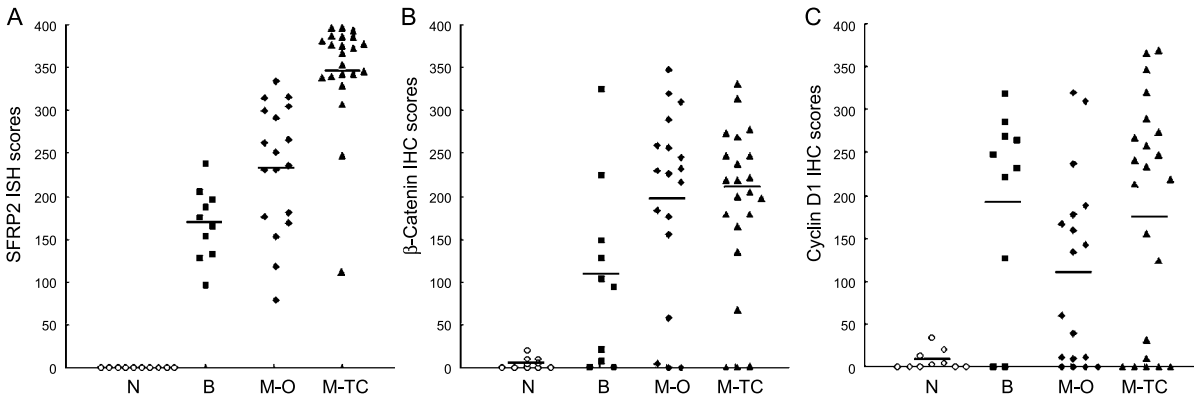
Statistical analysis

The *t*-test was used to analyze the correlations of tumor type with SFRP2 mRNA or Wnt-signaling proteins expression. The χ^2-test was used to analyze the statistical significance of the relationship between SFRP2 mRNA, β-catenin protein and cyclin D1 protein expression. The expression level was determined by ISH or IHC scores. $p < 0.05$ was considered statistically significant.

Results

RT-PCR of SFRP2

In a screen of *SFRP2* mRNA expression in 50 mammary gland tumors (MGT) and 10 normal MG tissue specimens by RT-PCR, *SFRP2* was expressed in all of the 50 MGT tissues with an expected cDNA fragment size of 980 bp regardless of their malignant or



Correlations with tumor grade

	p			
	N	B	M-O	M-TC
N		**<0.001**	**<0.001**	**<0.001**
B	**<0.001**		**<0.05**	**<0.01**
M-O	**<0.001**	**<0.05**		**<0.05**
M-TC	**<0.001**	**<0.01**	**<0.05**	

Correlations with tumor grade

	p			
	N	B	M-O	M-TC
N		**<0.001**	**<0.001**	**<0.001**
B	**<0.001**		**<0.01**	**<0.01**
M-O	**<0.001**	**<0.01**		0.5
M-TC	**<0.001**	**<0.01**	<0.5	

Correlations with tumor grade

	p			
	N	B	M-O	M-TC
N		**<0.001**	**<0.001**	**<0.001**
B	**<0.001**		**<0.05**	<0.5
M-O	**<0.001**	**<0.05**		**<0.05**
M-TC	**<0.001**	<0.5	**<0.05**	

Figure 4. Expression patterns of SFRP2 and Wnt-signaling proteins in canine MGs. Upper panel (A–C): immunohistochemical (IHC) or *in situ* hybridization (ISH) scores = % of positive cells × staining intensity. Horizontal bars indicate the average score within each group of specimens. Bottom panel: correlations of tumor type with SFRP2 or Wnt-signaling proteins. The graphs were drawn based on the scores presented in upper panel. Statistical analysis was performed by *t*-test analysis. The statistically significant *p*-values are in bold type. N: normal MG. B: benign MGTs. M-O: the others of malignant MGTs. M-TC: malignant tubulopapillary carcinomas.

benign nature. In contrast, none of the normal MG tissues expressed *SFRP2*. Despite a dramatic difference in *SFRP2* expression between normal MG and MGT tissues, there was no significant expression difference between malignant and benign MGTs by 30 cycles of RT-PCR analysis. An example of DNA gel electrophoresis results following RT-PCR is shown in Figure 1.

Expression patterns of SFRP2

To investigate the role of SFRP2 in tumor development, 50 MGT and 10 normal MG paraffin-embedded tissue specimens were subjected to ISH with DIG-labeled SFRP2-specific cRNA probe. The results showed that SFRP2 transcripts were present in cytoplasm and seemed consistently increased correlated with tumor grade ($M > B \gg N$) (Figure 2, upper panel). Consistent with the results of RT-PCR, very few positive cells were detectable in normal MGs. The positive correlation between levels of *SFRP2* mRNA and tumor grade was supported by statistical analysis ($p < 0.05$) (Figure 4(A)). Among malignant MGTs, the highest expression levels (average scores >300) were detectable in tubulopapillary carcinomas (M-TC).

A subset of these tumors were examined with an anti-SFRP2 antibody. SFRP2 mRNA levels correlated well with SFRP2 protein levels in these samples (Figure 2, bottom panel). SFRP2 protein expression was elevated in the majority of canine MGTs ($>95\%$). Immunoreactivity was found in diverse parts of the tissues, including nuclei or/and cytoplasm of the cells in the acinar cells, non-acinar parts of the tumor and extracellular matrix near the acinar cells. In contrast, normal MG tissues were negative for SFRP2 staining. In about 25% of the 50 tumor samples, the SFRP2 staining is predominantly within cells, while staining of mainly extracellular matrix was found in other 20% of samples. The rest of 55% samples were with mixture of staining both within cells and extracellular connective tissues.

Expression patterns of Wnt-signaling related proteins

To understand the interplay between SFRP2 and Wnt, and their effects on downstream signals, we examined β-catenin (Figure 3, upper panel) and cyclin D1 (Figure 3, bottom panel) expression in canine MGTs by IHC. A positive signal of β-catenin was present uniformly along the membranes of intercellular surfaces of the cells, especially in normal MGs and benign

MGTs. In malignant MGTs (including tubulopapillary carcinomas and the other types of MGTs), strong β-catenin staining was detected in the cytoplasm/nucleus of tumor cells. Consistent with β-catenin, strong cyclin D1 staining was also detected in the nucleus of tumor cells. Cyclin D1 signal was mainly located in the nucleus but expressed at extremely low levels in normal cells. As a whole, 79.4% of the cases showed positive β-catenin signals (in the nucleus) and 71.4% showed positive cyclin D1 signals in the tumor cells.

According to scoring criteria, the IHC scores of β-catenin and cyclin D1 are shown in Figure 4 (B) and (C). In the IHC scores of β-catenin, it seemed consistently increased correlated with tumor grade ($M > B \gg N$). The positive correlation was strongly supported by statistical analysis ($p < 0.01$). However, the expression level of cyclin D1 was clearly increased in all the tumors tested, irrespective of tumor grade.

Correlations between SFRP2 mRNA, β-catenin protein and cyclin D1 protein expression

Correlations between *SFRP2* mRNA, β-catenin protein and cyclin D1 protein expression are summarized in Table 2. SFRP2 was positively correlated with the level of β-catenin ($p < 0.05$). However, there was no correlation between the expression of SFRP2 ($p < 0.5$) or β-catenin ($p < 0.6$) with cyclin D1. Thus, it is very unlikely that the elevation of cyclin D1 seen in this study was due to the transcriptional activation of β-catenin.

Western blotting of SFRP2 and Wnt-signaling related proteins in primary culture model

To ensure the results of tissues screening, we examined SFRP2, β-catenin and cyclin D1 expression in primary culture from normal MGs (NMG1) and MGTs (MPG1 and MPG2) by western blotting (Figure 5). Surprisingly, SFRP2 was also expressed in MPG1 and MPG2 but not in NMG1. Additionally, β-catenin was detected in both cytoplasmic and nuclear fractions of the MPG1 and MPG2, but it was only in the cytoplasmic fractions of the NMG1. Cyclin D1 was present in the nuclear fractions of the MPG1 and MPG2. In contrast, no cyclin D1 was detected in NMG1. Consistent with the results of tissues screening, the increased expression of β-catenin in the nuclear fractions and whole cell extracts was both seen in cells overexpression of SFRP2 (MPG2-F2 > MPG2-V ∼ MPG2). Conversely, the expression of cyclin D1 in the nuclear fractions was not increased in cells overexpression of SFRP2.

Table 2. Correlations between SFRP2 mRNA, β-catenin protein and cyclin D1 protein expression[a]

	SFRP2		β-Catenin		Cyclin D1	
	r	*p*	*r*	*p*	*r*	*p*
SFRP2			0.57	**<0.05**	0.34	<0.5
β-Catenin	0.57	**<0.05**			0.28	<0.5
Cyclin D1	0.34	<0.5	0.28	<0.5		

[a] Statistical analysis was performed by the χ^2-test analysis. The statistically significant *p*-values are in bold type.

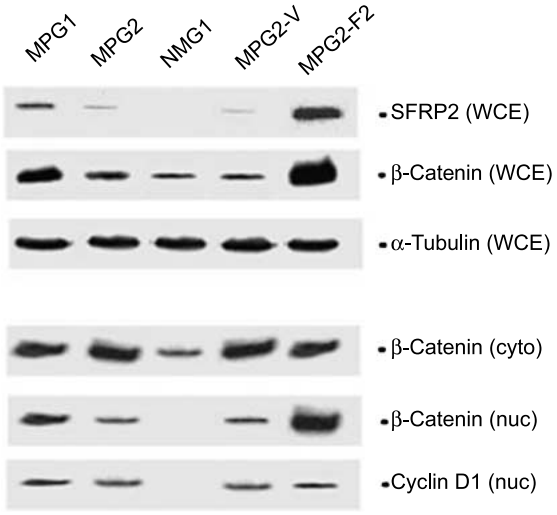


Figure 5. Western blotting analysis of SFRP2 and Wnt-signaling proteins in primary culture from canine MGs. Whole cell extracts were prepared and 50 μg of total protein were separated by SDS-PAGE (10%). Following electrophoretic transfer, a western analysis was performed with antibody against SFRP2, β-catenin and cyclin D1. To control for equal loading of whole cell extracts (WCE), a parallel western analysis was performed on the blots using antibody to α-tubulin. MPG1–2: primary culture from canine malignant tubulopapillary carcinomas. NMG1: primary culture from canine normal MGs. MPG2-V: MPG2 cells were transfected with the vector backbone. MPG2-F2: MPG2 cells were transfected with expression vectors for SFRP2 proteins. WCE: whole cell extracts. cyto: cytoplasmic cell fractions. nuc: nuclear cell fractions.

Discussion

It is well established that altered expression (down-regulation or up-regulation) or altered functions of specific genes may contribute to the pathogenesis or/and progression of tumors [28]. Several gene families that play a role during embryogenesis and differentiation are re-activated during the process of

tumorigenesis, including components of Wnt signaling pathway. The SFRPs are implicated to have dual important roles of Wnt signaling and apoptosis control that are critical in development and oncogenesis. In this paper, we have investigated the expression of the canine *SFRP2* gene in canine normal mammary and neoplastic mammary tissues. The major finding of this study is that the *SFRP2* mRNA was abundantly expressed in all neoplastic mammary gland tissues examined. The SFRP2 protein was localized in the tumor in diverse sites, including intracellular contents and extracellular matrix. In contrast, there was no *SFRP2* expression detected in normal mammary tissues.

To confirm the findings of the SFRP2 expression found in canine mammary tissues, we also studied the SFRP2 expression in primary cell lines established from tissue specimens of canine MGTs and normal MGs. The SFRP2 expression was detected in the MGT cell lines, but not in normal MG cells [24]. It was consistent with the findings in primary mammary tissues. In related studies, Wnt-1 is not expressed in normal breast tissues, but it has been directly implicated in human breast cancers. In contrast, several other Wnt family members are expressed in normal breast tissues, and some of them are overexpressed in breast tumors [29]. Wnt-1 was initially identified as an oncogene frequently involved in mouse mammary tumors [30–33]. Therefore, we hypothesized that SFRP2 may have a similar role in mammary tumorigenesis.

This is the first extensive study of the expression of SFRP2 in mammary tumors by IHC staining. In the cases of canine mammary tissues examined, we found increased and altered patterns of expression of SFRP2 mRNA and protein relative to controls, although there was variability in both levels and distribution of SFRP2 transcripts and immunoreactive protein. In previous studies, well-studied markers of breast carcinoma aggressiveness, such as ERBB2 and P53, are affected in less than 30% of breast carcinomas [34–36]. Surprisingly, SFRP2 expression is elevated in the majority of canine MGTs ($>95\%$) suggests that SFRP2 is one of the most frequent targets of genetic alteration in mammary cancer. Furthermore, SFRP2 protein localization is correlated with the mRNA pattern of expression, but it seems to be more widespread in most of our tumor tissues. It has been suggested in a retinal study by our lab that the SFRP2 protein was secreted from the synthesizing cells of neural retina and not readily detectable around the synthesizing cells or tissue sections during different disease stages. The discrepancy between *SFRP2* mRNA levels and immunoreactive SFRP2 protein in our experiment may further support this possibility. More evidence bearing on the relationship between transcription, translation, post-translational processing, and degradation of SFRP2 will be required to elucidate this. SFRP2 protein degradation during tissue processing or/and diverse stages of our sporadic tumor samples may also influence the detection of the expression levels of SFRP2 protein.

Canine MGT is the commonest tumors seen in female dogs. However, the complex etiology of the MGT is mostly unknown. In murine mammary tumor, Wnt signaling has been implicated in murine mammary tumorogenesis by MMTV, through promoter insertion [1]. Up-regulation of the *WNT1* gene in these circumstances is at least partially controlled by estrogens and leads to mammary ductal hyperplasia and neoplasia. In transgenics, expression of a *WNT1* gene driven by the mouse mammary tumor virus LTR enhancer causes extensive ductal hyperplasia early in life and mammary adenocarcinomas in approximately 50% of the female transgenic (TG) mice by 6 months of age [37]. The suggestion is that the gene is abundantly expressed in ductal epithelia (i.e., acinar cells). Consistent with this finding, the highest expression levels (average scores >300) of *SFRP2* mRNA were detectable in adenocarcinomas (the major subgroup of tubulopapillary carcinomas). Perhaps in dogs *SFRP2* elevation in acinar cells fulfills the similar role to that of *WNT1* in mice or the role is co-operative. Additionally, the consistent nature of SFRP2 elevation in all mammary neoplasms is surprising.

Wnt-mediated β-catenin/Tcf transcription not only plays a role in the promotion of cell proliferation and cell cycle progression, but also may provide an important survival function to facilitate cell transformation [38]. Cyclin D1, is a β-catenin/Tcf transcriptional target, can act as a weak oncogene on its own [39], or it can co-operate with other oncogenes such as *ras* and *myc* to increase their malignant potential [40]. In this paper, we also demonstrated that strong β-catenin staining was detected in the cytoplasm/nucleus of tumor cells and strong cyclin D1 staining was detected in the nucleus of tumor cells. In addition, SFRP2 was positively correlated with the level of β-catenin ($p < 0.05$). However, there was no correlation between the expression of SFRP2 ($p < 0.5$) or β-catenin ($p < 0.6$) with cyclin D1. Thus, it is very unlikely that the elevation of cyclin D1 seen in this study was due to the transcriptional activation of β-catenin.

Recently Zecca et al. [41] demonstrated that Wg functions as a gradient morphogen. Just as the formation of stable Wnt gradients may rely on their ability to bind proteoglycan [42], the heparin-binding property of SFRP implies that it might also function in a graded manner. In fact, gradients of interacting proteins might be a general mechanism of Wnt regulation. In a similar manner, we showed that SFRP2 exhibited extracellular staining by IHC analysis. In primary culture cells model, the presence of SFRP2 protein could be detected in the media collected from stably expressed SFRP2 cells [24]. In this study, we have for the first time presented evidence of SFRP2 up-regulated expression in sporadic canine primary mammary neoplasms. The underlying mechanism and exact roles of SFRP2 in mammary neoplasia remain to be determined. The involvement of SFRP2-modulated Wnt-signaling in canine mammary tumorigenesis remains an intriguing possibility for future investigation.

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