Original Article
J Vet Sci 2017, 18(3), 359-367 · https://doi.org/10.4142/jvs.2017.18.3.359

Evaluation of expression of the Wnt signaling components in canine mammary tumors via RT^2 Profiler PCR Array and immunochemistry assays

Fang Yu[1], Roberta Rasotto[2], Hong Zhang[1], Shimin Pei[1], Bin Zhou[1], Xu Yang[1], Yipeng Jin[1], Di Zhang[1,*], Degui Lin[1,*]

[1]*Department of Veterinary Clinical Science, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China*
[2]*Clinical Pathology Department, Dick White Referrals, Cambridgeshire, CB8 0UH, UK*

The Wnt signaling pathway and its key component β-catenin have critical roles in the development of diseases such as tumors in mammals. However, little has been reported about involvement of the Wnt/β-catenin signaling pathway in canine mammary tumors (CMTs). The present study detected expression of 30 Wnt signaling pathway-related genes in CMTs; the results are potentially useful for molecular-based diagnosis of CMTs and the development of new targeted therapies. Significant upregulations of dickkopf-1 protein, secreted frizzled-related sequence protein 1 (SFRP1), frizzled 3, β-catenin, and lymphoid enhancer-binding factor 1 (LEF1) were detected in highly malignant CMTs compared to levels in normal mammary gland tissues; moreover, highly significant upregulation of WNT5A was observed in low malignancy CMTs. Downregulation was only detected for SFRP4 in malignant CMT samples. The subcellular location of β-catenin and cyclin D1 in 100 CMT samples was investigated via immunohistochemical analysis, and significantly increased expressions of β-catenin in cytoplasm and cyclin D1 in nuclei were revealed. Western blotting analysis revealed that the expression of β-catenin and LEF1 increased in in the majority of CMT samples. Taken together, the results provide important evidence of the activation status of the Wnt pathway in CMTs and valuable clues to identifying biomarkers for molecular-based diagnosis of CMT.

Keywords: RT^2 Profiler PCR Array, Wnt signaling pathway, beta-catenin, canine mammary tumor

Introduction

Mammary tumors are a common neoplastic disease in female dogs, especially in intact animals and animals spayed after the second heat. Approximately half of canine mammary tumors (CMTs) are considered malignant and these can severely compromise the quality of life of the affected dogs due to local recurrence, distant metastases, and finally death [8,22,27].

CMTs have been demonstrated to share many features with human breast cancers, including histological appearance, hormone-dependence, and biological behavior [20,25,27]. A recent genome-wide comparative analysis of human and CMTs has demonstrated a great degree of similarity in the perturbations of many cancer-related pathways, including the Wnt/β-catenin signaling pathway [28]. The Wnt/β-catenin signaling pathway is vital in embryonic development and tissue self-renewal, and it regulates various processes that are important for cancer progression [2,19]. With the activation of the Wnt/β-catenin pathway, the Wnt ligand binds to frizzled (FZD) receptors and its co-receptors low-density lipoprotein receptor-related proteins 5/6 (LRP5/6) on the cell membrane, forming a complex after recruitment of Dishevelled. Subsequent LRP6 phosphorylation and recruitment of the Axin complex to the receptors lead to the stabilization of β-catenin, which then accumulates and translocates to the nucleus to form complexes with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) and activate the expression of Wnt target genes, such as cyclin D1 and matrix metalloproteinase-7 (MMP7), to promote cell growth, proliferation, and differentiation as well as aiding in tumor cell invasion and metastasis [2,10,16].

In a recent study performed on cell lines derived from CMTs, the canonical Wnt signaling pathway was shown to be activated with aberrant expression of lymphoid enhancer-binding factor 1 (LEF1) [6]. A few immunohistochemistry studies have also documented an alteration in the expression of protein β-catenin

Received 3 Mar. 2016, Revised 4 Aug. 2016, Accepted 26 Aug. 2016
*Corresponding authors: Tel: +861062733621; Fax: +861062733621; E-mails: csama@sina.com (D Lin), dzhangdvm@cau.edu.cn (D Zhang)
pISSN 1229-845X
eISSN 1976-555X

in CMTs in parallel with the acquisition of a more invasive and undifferentiated histological phenotype, but how this affects prognosis is unclear [1,4,24]. Information about the expression profiles of other genes related to the Wnt signaling pathway in tumorigenesis of CMTs is lacking.

In this study, we employed an RT^2 Profiler PCR Array to analyze the expression of Wnt signaling components (Wnt ligands, receptors, targets and β-catenin destruction complex) and some of the genes related to Wnt signaling (such as Wnt antagonists dickkopf-1 protein [DKK1] and secreted frizzled-related sequence proteins [SFRPs]) in CMTs. We also investigated the expression of β-catenin and cyclin D1, two critical molecules in canonical Wnt signaling pathway, by using immunohistochemistry and Western blotting assays in CMT samples.

Materials and Methods

Retrospective samples and histopathology

One hundred CMT samples were collected from 100 bitches underwent regional or unilateral mastectomy and ovariohysterectomy in the Veterinary Teaching Hospital of

Table 1. Genes identified for inclusion in the Custom RT^2 Profiler PCR Array

Position	Gene	Reference sequence number	Official full name
1	APC	XM_005618034	Adenomatous polyposis coli
2	AXIN1	XM_847228	Axin 1
3	AXIN2	XM_003435186	Axin 2
4	BTRC	XM_005637636	Beta-transducin repeat containing
5	CSNK1A1	XM_005619309	Casein kinase 1, alpha 1
6	CTNNB1	NM_001137652	Catenin (cadherin-associated protein), beta 1, 88 kDa
7	DKK1	XM_005636664	Dickkopf WNT signaling pathway inhibitor 1
8	DVL1	XM_546713	Dishevelled, dsh homolog 1 (Drosophila)
9	FRZB	XM_005640381	Frizzled-related protein
10	FZD3	XM_005635664	Frizzled family receptor 3
11	FZD5	XM_545614	Frizzled family receptor 5
12	FZD7	XM_545599	Frizzled family receptor 7
13	FZD9	XM_546927	Frizzled family receptor 9
14	GSK3B	XM_535751	Glycogen synthase kinase 3 beta
15	KREMEN1	XM_005636403	Kringle containing transmembrane protein 1
16	LRP5	XM_003432415	Low density lipoprotein receptor-related protein 5
17	LRP6	XM_534886	Low density lipoprotein receptor-related protein 6
18	MMP7	NM_001242726	Matrix metallopeptidase 7 (matrilysin, uterine)
19	SFRP1	XM_003639564	Secreted Frizzled-related protein 1
20	SFRP4	XM_540377	Secreted Frizzled-related protein 4
21	TCF7	XM_003639372	Transcription factor 7 (T-cell specific, HMG-box)
22	TCF7L1	XM_849796	Transcription factor 7-like 1 (T-cell specific, HMG-box)
23	WIF1	XM_538269	WNT inhibitory factor 1
24	WNT1	XM_005636889	Wingless-type MMTV integration site family, member 1
25	WNT2	XM_849870	Wingless-type MMTV integration site family member 2
26	WNT3	XM_005624202	Wingless-type MMTV integration site family, member 3
27	WNT3A	XM_539327	Wingless-type MMTV integration site family, member 3A
28	WNT5A	NM_001287075	Wingless-type MMTV integration site family, member 5A
29	CCND1	NM_001005757	Cyclin D1
30	LEF1	XM_005639262	Lymphoid enhancer-binding factor 1
43	GAPDH	NM_001003142	Glyceraldehyde-3-phosphate dehydrogenase
44	FGDC	SA_00130	Dog Genomic DNA Contamination
45	RTC	SA_00104	Reverse Transcription Control
46	RTC	SA_00104	Reverse Transcription Control
47	PPC	SA_00103	Positive PCR Control
48	PPC	SA_00103	Positive PCR Control

Position numbers 31 to 42 are non-related genes and thus excluded from this study.

China Agricultural University from September 2012 to October 2014. Normal mammary gland tissues (NMGTs) were collected (at necropsy) from 8 intact healthy dogs that were used for general surgery practice courses for undergraduate students at the College of Veterinary Medicine. These procedures were approved by the Animal Care Committee of China Agricultural University (ID: 1114120800096). Survival of CMT patients was determined by telephoning the dog owners one year after the surgery.

After routine histologic processing and hematoxylin-eosin staining, CMT samples were evaluated by a board-certified pathologist and classified and graded as described by Goldschmidt *et al*. [5] and Peña *et al*. [18], respectively. The presence of necrosis and lymphatic invasion in the histological sections was also recorded.

RT^2 Profiler PCR Array tests

A custom RT^2 Profiler PCR Array (CAFP12858; Qiagen, Germany) that could simultaneously detect 30 genes related to the Wnt signaling pathway in canines (Table 1) was used. Each array plate contained 2 sets of 48 wells for 2 tests. Genomic DNA contamination, reverse transcription, and positive PCR controls were included in each 48-well set on each plate. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as the assay reference gene.

Thirty-five CMT samples (see Table 2) and 5 NMGT samples were used in the assays. Total RNA was isolated from each frozen tissue sample using TRIzol Reagent (Life Technologies, USA) and quantified with Nanodrop 2000 (Thermo Fisher Scientific, USA). Then total RNA (1 μg) was reverse transcribed in a final volume of 20 μL with an RT^2 First Strand Kit (catalog No. 330401; Qiagen). The RT^2 Profiler PCR Array tests were performed following the instructions of the manufacturer. Briefly, all cDNA in each sample (20 μL) was first diluted with RNase-free water to a volume of 111 μL. Then 51 μL of the diluted cDNA was mixed with 675 μL 2 × RT^2 SYBR Green ROX qPCR Mastermix (catalog No. 330523; Qiagen) and 624 μL RNase-free water. Of that mixture, 25 μL per well was added into the 48 wells of the array plate. The qPCR was carried out using the Applied Biosystems 7500 Real-Time PCR System (software ver. 2.0.6; Applied Biosystems, USA) under the following thermal cycling conditions: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The exported Ct values were input to a template Excel file provided by SABiosciences (Qiagen) and uploaded for the online analysis. After data review, qualified data from 35 CMT and 5 NMGT samples were analyzed by applying the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry (IHC) evaluation and immunohistochemical scores (IHS)

Paraffin sections of CMT and NMGT samples were dewaxed in xylene and subsequently rehydrated through an ethanol series. Antigen retrieval was carried out by microwave treatment in 0.1 M citrate buffer (pH 6.0) for β-catenin or EDTA buffer (pH 9.0) for cyclin D1. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min. Then normal goat serum was applied on the slides to block non-specific binding for 20 min. Rabbit anti-β-catenin polyclonal antibodies

Table 2. Classification and grading for canine mammary tumor samples (N = 100)

Tissue type, N (n)	Subtype group	Subtypes	N (n)	Grade			Presence of necrosis	Presence of lymphatic invasion
				I	II	III		
Benign, 37(9)	Benign	Benign mixed tumor	10 (3)	–	–	–	A	A
		Complex adenoma	22 (2)	–	–	–	A	A
		Fibroadenoma	1 (1)	–	–	–	A	A
		Simple adenoma	4 (3)	–	–	–	A	A
Malignant, 63(26)	CC	Complex carcinoma	17 (7)	13 (6)	4 (1)	0	11	0
	CMM	Carcinoma and malignant myoepithelioma	10 (6)	3 (2)	7 (4)	0	6	0
	EC	Simple tubular carcinoma	10 (4)	7 (3)	3 (1)	0	6	2
		Simple tubule-papillary carcinoma	5 (3)	2 (1)	2 (1)	1 (1)	4	1
		Solid carcinoma	6 (2)	0	3	3 (2)	5	0
		Intraductal papillary carcinoma	6 (4)	5 (3)	1 (1)	0	1	1
		Comedo carcinoma	4	0	3	1	4	2
		Adenosquamous carcinoma	2	0	2	0	2	0
		Invasive micropapillary carcinoma	3	0	1	2	2	3

In each column, the numbers of CMT samples included in the RT^2 Profiler PCR Array assays are presented in brackets. –, benign CMT samples not subjected to grading. A, absence of necrosis or lymphatic invasion. CC, complex carcinoma; CMM, carcinoma and malignant myoepithelioma; EC, epithelial carcinoma.

(1:5,000 dilution, ab6302; Abcam, USA) or rabbit anti-cyclin D1 polyclonal antibodies (1:1,000 dilution, ab185241; Abcam) were applied on the sections overnight at 4°C. The sections were visualized by using the Polink-2 HRP plus rabbit DAB detection system (Golden Bridge International, USA) and counterstained with hematoxylin. Negative controls in which PBS replaced the primary antibody were set at the same time.

The IHS of β-catenin was evaluated by determining the percentage of positive cells and the intensity of the cytoplasmic staining in accordance with the description of Khramtsov *et al.* [11]. The scores for both membrane-associated and cytoplasm-associated β-catenin ranged from 0 to 3. The IHS of cyclin D1 was assessed by determining the percentage of stained nuclei. The staining was scored as 0 when the sample had no nuclear staining or if $< 10\%$ of its nuclei were stained; whereas tumor samples with $> 10\%$ of its nuclei stained were scored as 1 [17]. The percentage of positive staining was estimated by counting no less than 500 cells in 3 randomly selected high-power fields.

Western blotting analysis

Eleven CMT samples (including 4 benign CMTs and 7 malignant CMTs) and 1 NMGT sample were selected for the

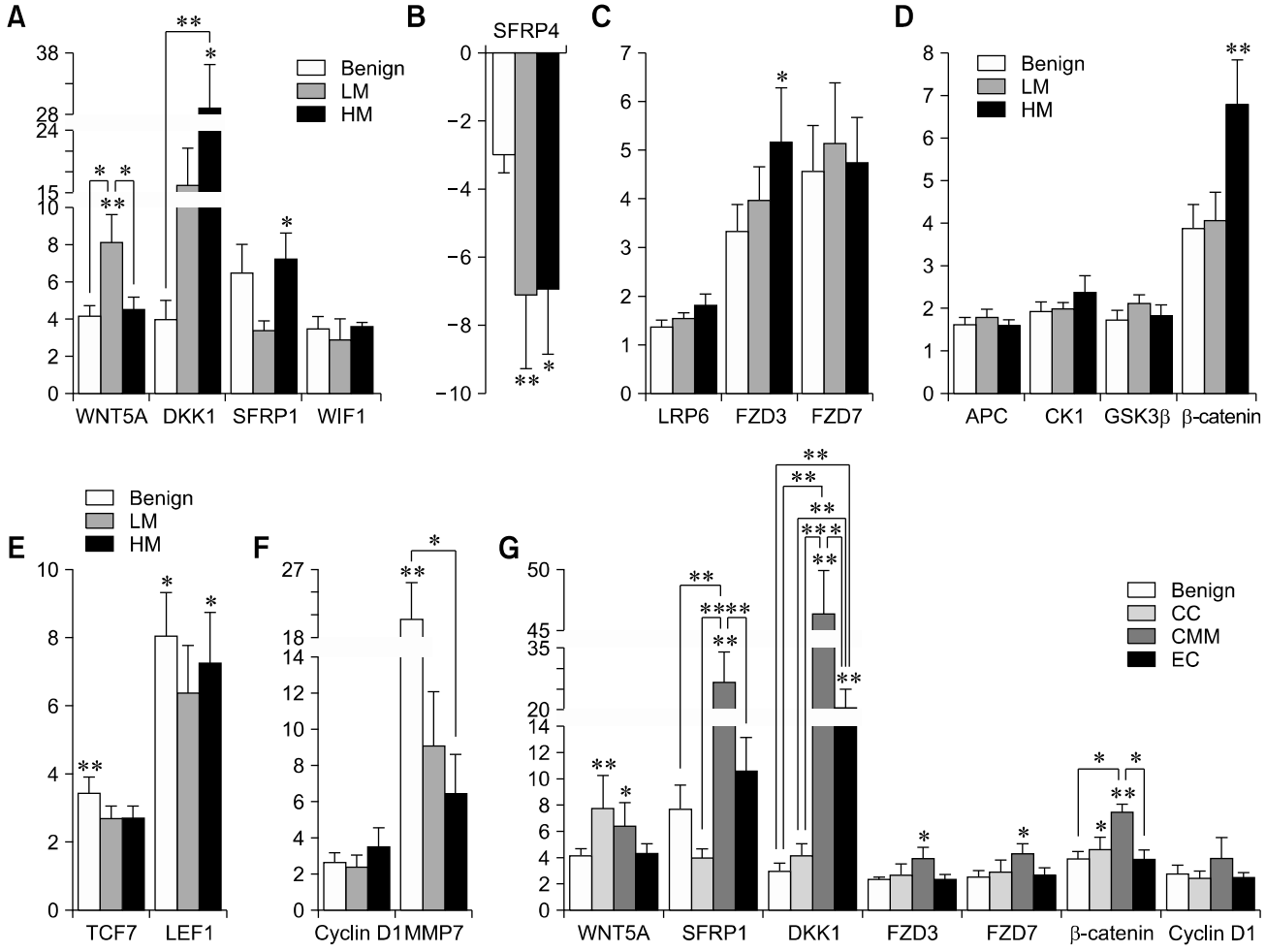

Fig. 1. Transcriptional expression profiles of Wnt signaling-related genes in canine mammary tumors (CMTs). Total RNA extracted from CMT samples or normal mammary gland tissues were reverse transcribed into cDNA for quantitative PCR analysis with an RT^2 Profiler PCR Array system. The transcriptional level of these genes are expressed as fold changes. (A and B) Wnt and Wnt antagonist genes. (C) Frizzled receptors and co-receptor. (D) β-catenin and destruction complex-related factors. (E) Transcription factors. (F) Target genes. (G) Upregulated genes in 4 groups of different CMT subtypes. Benign, benign tumor; LM, low malignant tumor; HM, high malignant tumor; CC, complex carcinoma; CMM, carcinoma and malignant myoepithelioma; EC, epithelial carcinoma (malignant tumors with generally only one component (epithelial)). Significant differences expressed as $*p < 0.05$ while highly significant differences are expressed as $**p < 0.01$. Asterisk(s) directly above the error bar of each column indicates a comparison between the indicated group and the benign group; while the Asterisk(s) above the drawn lines means the comparison between the tumor groups indicated by the lines.

detection of expressions of β-catenin, TCF7, LEF1, and cyclin D1. Proteins were extracted with RIPA lysis buffer (Macgene, China) and the soluble protein was resolved by performing electrophoresis through 12% SDS-PAGE gels followed by transferring to polyvinylidene difluorid membranes by semi-dry blotting. Non-specific binding sites were blocked by incubation for 1 h in 5% w/v milk powder in PBS, after which membranes were sliced and separately probed with rabbit polyclonal antibodies against β-catenin (diluted at 1:4,000; ab6302; Abcam), LEF1 (diluted at 1:600; ab83964, Abcam), TCF7 (diluted at 1:4,000; ab30961, Abcam), or cyclin D1 (diluted at 1:1,000; ab185241, Abcam). HRP conjugated goat anti-rabbit IgG or goat anti-mouse IgG were used as the secondary antibody. Blots were developed in ECL reagent (Macgene) and exposed to X-ray film.

Statistical analysis

For statistical purposes, Grade I tumors were assigned to the low malignant (LM) group, while Grades II and III tumors were clustered together and assigned to the highly malignant (HM) group. Similarly, some tumor subtypes were grouped together based on similarities in morphology and/or biological behavior, leading to the formation of 4 subtype groups (detailed in Table 2). SPSS (ver. 20; IBM, USA) was used for statistical analysis. A one-way ANOVA with Tukey's *post hoc* test was performed for analysis of the fold changes of genes in the RT^2 Profiler PCR Array test results. A chi-squared test and Fisher's exact test were used for analysis of the associations of β-catenin or cyclin D1 expressions with clinicopathological features. A significant difference was expressed as $*p < 0.05$ while a highly significant difference was expressed as $**p < 0.01$.

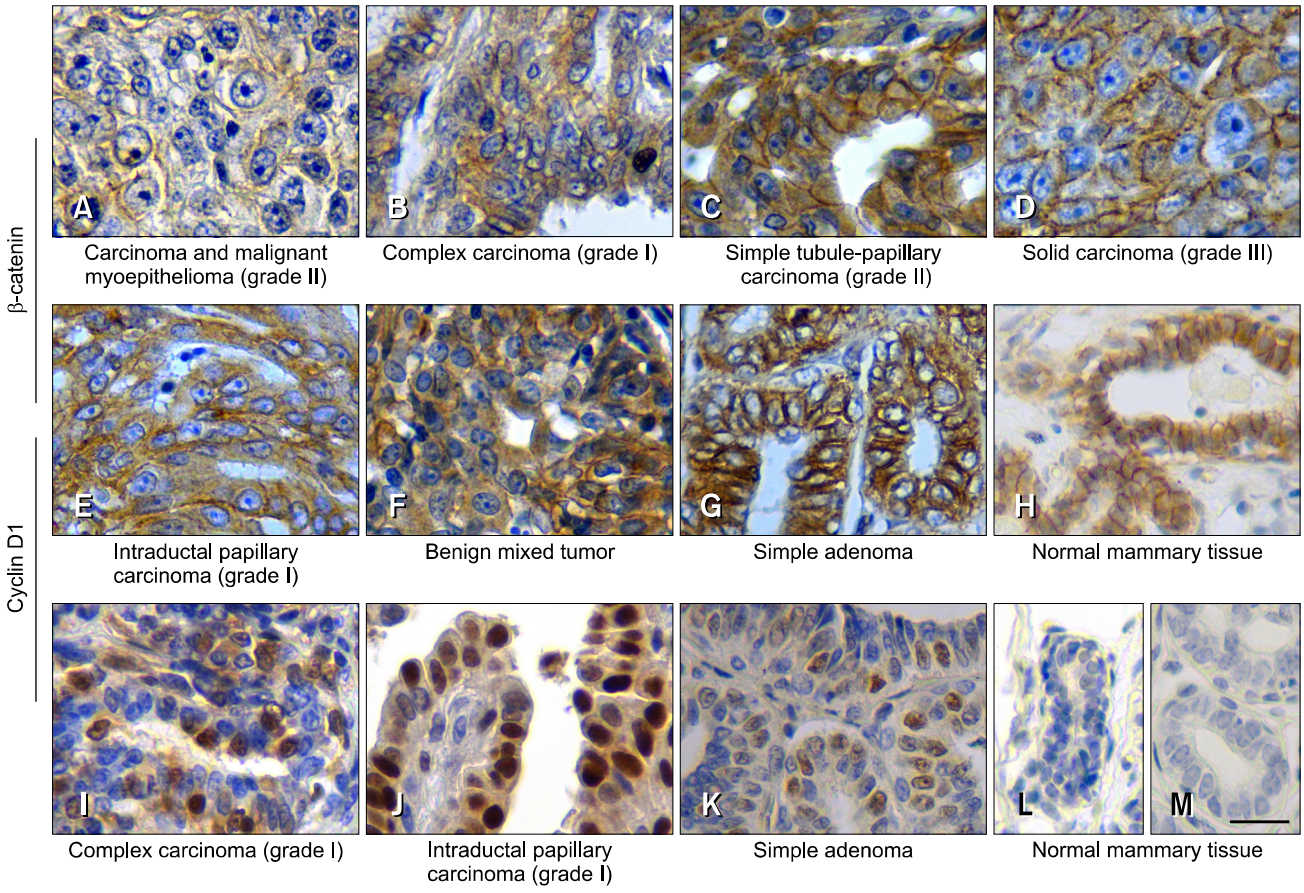

Fig. 2. The expression of β-catenin and cyclin D1 in CMTs detected via immunohistochemical staining. Tissue sections were probed with either anti-β-catenin antibodies (A–H) or anti-cyclin D1 antibodies (I–L). (A) Reduced staining of both membrane and cytoplasm; weak (D), moderate (B, E and F) and strong (C and G) staining of the cytoplasm; distinct (D), partially disappeared (C and G) or mostly disappeared (B, E and F) staining of membrane; and (H) weakly staining in cytoplasm and more than 70% of membrane staining in normal tissue section. (I–K), nuclear staining of cyclin D1. Negative control staining using PBS instead of primary antibodies was performed with normal mammary tissue (M). The red arrows indicate membrane staining of β-catenin, while the red arrowhead indicates cytoplasmic staining of β-catenin. The black arrows indicate nuclear staining of cyclin D1. The subtype and grading of the CMT samples are shown below each image. Scale bar = 20 μm.

Results

Histopathological examination of CMT samples

Histopathological examination of the 100 CMT samples revealed 37 benign tumors and 63 malignant tumors, which were distinguished in four subtypes as summarized in Table 2. Among the 100 tumors, 37 were included in subtype group 1, 17 in subtype group 2, 10 in subtype group 3, and 36 in subtype group 4. Among the 63 malignant tumors, 30 were classified as LM and 33 as HM. Necrosis and lymphatic invasion were detected in 41 and 9 malignant cases, respectively. The 8 NMGT samples were confirmed to be non-neoplastic, with only mild hyperplastic changes (lobular hyperplasia) detected in 4 samples.

Evaluation of transcriptional expression of genes related to Wnt signaling in CMTs

Based on the grading and classification of the CMT samples (Table 2), 9 benign tumors, 26 malignant tumors, and 5 NMGT samples were subjected to RT^2 Profiler PCR Array assays. Among the 30 simultaneously detected genes, 9 exhibited significant changes in expression (Fig. 1). Expression of WNT5A was significantly upregulated in LM tumors (panel A in Fig. 1). Expression of DKK1 was correlated with the degree of malignancy, showing a 16-fold change in LM tumors and a 29-fold change in HM tumors (panel A in Fig. 1). In contrast, SFRP4 was significantly downregulated in both LM and HM tumors (7.1-fold and 7.0-fold changes, respectively, panel B in Fig. 1). For β-catenin and the destruction complex-related factors investigated in this study, only β-catenin showed an obviously upregulated expression in HM tumors (6.8-fold increase, panel D in Fig. 1). A significant difference was detected in the expression of transcription factors TCF7 and LEF1 between the benign tumor and the NMGT samples (panel E in Fig. 1). Expression of the Wnt target gene MMP7 was increased in the benign CMTs (20-fold change) while it decreased in LM tumors and HM tumors (9-fold and 6.5-fold changes, respectively; panel F in Fig. 1). The data were reanalyzed after grouping the tumor samples into 4 subtypes, and the results of the analysis showed that, compared with the expressions in other subtypes and NMGTs, expressions of SFRP1 and DKK1 were highest in carcinoma and malignant myoepithelioma (CMM) subtypes. The expression pattern of β-catenin was similar to that of the SFRP1 and DKK1 (panel G in Fig. 1).

Table 3. Correlations of β-catenin or cyclin D1 expression and clinicopathological parameters in canine mammary tumors (CMTs)

Clinicopathological parameters	IHS of β-catenin			IHS of cyclin D1		
	M: 0–1 (n)	C: 2–3 (n)	*p* value	0 (n)	1 (n)	*p* value
Tissue type						
Benign (n = 37)	37	35	0.000[a]	12	25	0.000[a]
Malignant (n = 63)	63	60	0.614[b]	4	59	0.001[b]
Histological subtype group						
CC (n = 17)	17	17	0.37	1	16	0.115
CMM (n = 10)	10	9		2	8	
EC (n = 36)	36	34		1	35	
Histological grade						
I (n = 30)	30	29	0.446	2	28	0.616
II (n = 26)	26	24		2	24	
III (n = 7)	7	7		0	7	
Presence of necrosis in malignant tumor						
Present (n = 41)	41	40	0.27	2	39	0.436
Absent (n = 22)	22	20		2	20	
Lymphatic invasion in malignant tumor						
Present (n = 9)	9	8	0.375	0	9	0.531
Absent (n = 54)	54	52		4	50	
One-year survival						
Death (n = 23)	23	22	0.641	4	19	0.451
Survival (n = 52)	52	49		7	45	

M, membrane-associated; C, cytoplasm-associated. a denotes difference among CMTs and Normal mammary gland tissues, while b denotes difference among benign and malignant CMTs. Statistical analysis was performed by using chi-squared and Fisher's exact tests. IHS, immunohistochemical scores; CC, complex carcinoma; CMM, carcinoma and malignant myoepithelioma; EC, epithelial carcinoma.

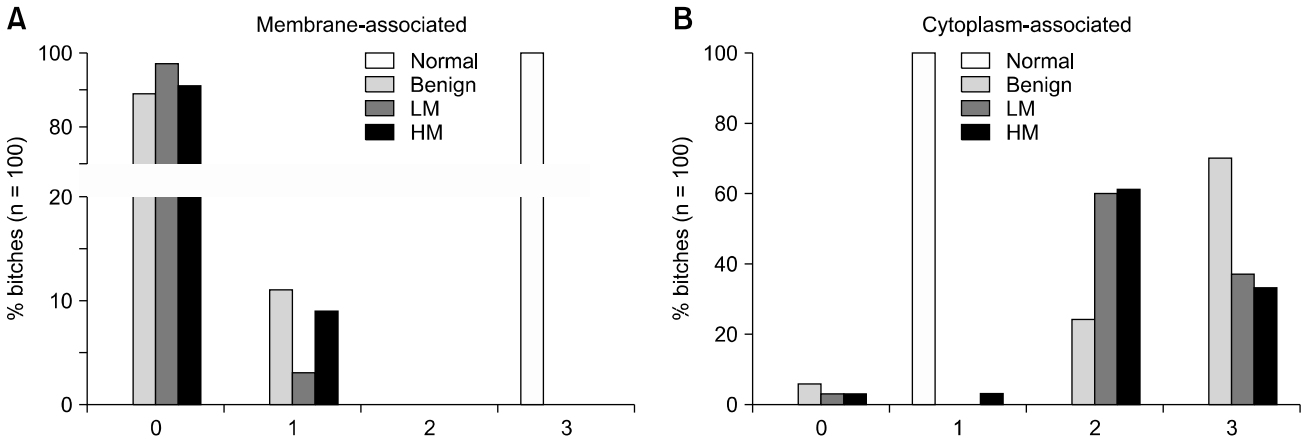

Fig. 3. Analysis of subcellular localization of β-catenin in normal mammary tissues or CMTs. The 100 CMT samples included 37 benign CMTs, 30 low malignant (LM) CMTs and 33 highly malignant (HM) CMTs, as well as 8 normal mammary tissue samples were subjected to IHS analysis. Scores of membrane-stained (A) or cytoplasm-stained (B) samples ranged from 0 (non-staining) to 3 (strongly positive stain) according to the criteria of Khramtsov *et al*. [11].

Immunohistochemical evaluation of β-catenin and cyclin D1 expression in CMTs

Expressions of β-catenin and cyclin D1 were further evaluated by performing immunohistochemical analysis. Compared to NMGTs (panel H in Fig. 2), significantly reduced membrane expression of β-catenin (IHS as 0 and 1) was observed in all tumor samples (panel A-G in Fig. 2; $p = 0.000$). Most (8/10) CMMs also showed weak to moderate intensity staining of cytoplasm (panel A in Fig. 2); while moderate to strong cytoplasm staining of β-catenin (IHS as 2 and 3) was detected in the other CMT subtypes (panel B-G in Fig. 2). Expression of β-catenin in the cytoplasm was significantly higher in CMTs than in NMGTs (Table 3). There was an obvious shift of β-catenin localization from membrane to cytosol in the comparison of CMTs with NMGTs ($p = 0.000$; Fig. 3). Nuclear expression of cyclin D1 (scored as 1) was observed in 84% (84/100) of CMTs (25 benign and 59 malignant tumors; panel I-K in Fig. 2), while its expression was absent or less than 10% (scored as 0) in the nuclei of NMGTs (panel L in Fig. 2), a statistically significant difference ($p = 0.000$; Table 3). There was significantly higher nuclear expression of cyclin D1 in malignant tumors than in benign tumors ($p = 0.001$; Table 3). With regard to the expression of β-catenin in cytoplasm and the nuclear staining of cyclin D1, there were no significant differences detected among the different histological grades, between presence/absence of necrosis or lymphatic invasion in malignant CMTs, and between death or survival at one year after surgery (Table 3).

Western blotting analysis of the expression of β-catenin, TCF7, LEF1 and cyclin D1

The expression of β-catenin increased significantly in 10 of 11 detected CMTs; the exception, a decreased expression, was

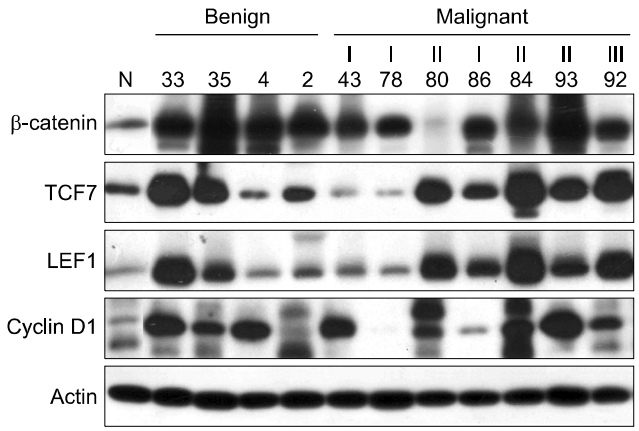

Fig. 4. Expression of β-catenin, TCF7, LEF1 and cyclin D1 in CMTs analyzed by immunoblotting. The samples include 1 normal canine mammary tissue (N), 2 complex adenomas (33 and 35), 2 simple adenomas (4 and 2), 1 complex carcinoma (43), 2 simple tubulo-papillary carcinomas (78, Grade I; 80, Grade II), 2 intraductal papillary carcinomas (86, Grade I; 84, Grade II) and 2 solid carcinomas (93, Grade II; 92, Grade III). Primary antibodies against β-catenin, TCF7, LEF1 and cyclin D1 were used for detection. β-actin was used as the reference protein. The malignant grades of the CMTs are marked as I, II, and III on the top of the image.

detected in a case with simple tubulo-papillary carcinomas. Increased LEF1 expression was detected in complex adenomas, intraductal papillary carcinomas, and solid carcinomas, while no (or slight) increases of LEF1 expression were detected in simple adenomas, complex carcinoma, and 1 simple tubulo-papillary carcinomas (Grade I). The expression levels of TCF7 were similar to those of LEF1 in CMTs. Decreased cyclin D1

expression was only detected in a case of simple tubulo-papillary carcinomas (Fig. 4).

Discussion

The RT^2 Profiler PCR Array system is a reliable tool for analyzing the expression of multiple genes and thus is extensively used for the analysis of diverse signaling pathways in human and model animals [12,15]. In the current study, we employed this tool for the analysis of transcriptional profiles of Wnt signaling components in CMTs. Among the 30 selected genes involved in the Wnt signaling pathway, remarkable expression changes at the mRNA level were observed for several Wnt-related genes (*e.g.*, the upregulation of WNT5A, FZD3, and LEF1 and the downregulation of SFRP4) in different types of CMTs. Based on previous reports [1,6], the results obtained in this study suggest that Wnt signaling components have important roles in tumorigenesis and tumor progression of CMTs.

Of the activated Wnt signaling components in CMTs, both DKK1 and SFRP1 were significantly upregulated in CMM samples, even in malignant CMTs. This is of particular interest as it may suggest a different tumorigenesis pathway for malignant myoepithelioma, as a previous study showed a significantly high Ki67 expression in such tumors [21]. Further studies using laser capture microdissection to isolate the myoepithelial component of these tumors could help to clarify the present finding. As detection of DKK1 and SFRP1 has been extensively reported following diagnostic studies of liver, pancreas, lung, prostate, and nasopharyngeal cancers of humans [7,23,26,30,31], the present result provides further evidence for the possible use of DKK1 and SFRP1 as valuable candidate biomarkers for identifying malignant CMTs.

By performing IHC assays, cytoplasm accumulation and weak membrane staining of β-catenin was detected in most CMT samples, whereas β-catenin nuclear staining was infrequently observed, indicating that Wnt/β-catenin signaling is aberrant in CMTs and, thus, could be an indicator of this kind of tumor. This phenomenon could be partially explained by the transference of β-catenin from the cell membrane to the cytoplasm along with dissociation of E-cadherin and the β-catenin complex [9,29]. In addition, we did not detect a correlation between the intensity of β-catenin expression and the survival period of CMT patients (data not shown). Concerning the inconsistencies among previous reports on the correlation between the expression and subcellular location of β-catenin and the prognosis of human breast cancer and CMTs [3,4,13,14], further studies are required to understand the roles played by β-catenin in the tumorigenesis and tumor progression of CMTs.

In conclusion, this study substantiated activation of the Wnt signaling pathway in CMTs by performing transcriptional profiling of 30 genes in RT^2 Profiler PCR Array assay results and determining ectopic subcellular distributions of β-catenin and cyclin D1 by IHC assay results. The most activated Wnt pathway-related genes, such as DKK1 and SFRP1, may have potential as predictive biomarkers of and targeted therapeutics for CMTs.

Acknowledgments

We wish to thank the graduate students in the Department of Veterinary Clinical Science for the collection of canine mammary tissue samples. This study was supported by the National Natural Science Foundation of China (31372489, 31572578 and 31402260).

Conflicts of Interest

The authors declare no conflict of interests.

References

1. **Brunetti B, Sarli G, Preziosi R, Monari I, Benazzi C.** E-cadherin and β-catenin reduction influence invasion but not proliferation and survival in canine malignant mammary tumors. Vet Pathol 2005, **42**, 781-787.
2. **Clevers H.** Wnt/β-catenin signaling in development and disease. Cell 2006, **127**, 469-480.
3. **Cuello-Carrión FD, Shortrede JE, Alvarez-Olmedo D, Cayado-Gutiérrez N, Castro GN, Zoppino FCM, Guerrero M, Martinis E, Wuilloud R, Gómez NN, Biaggio V, Orozco J, Gago FE, Ciocca LA, Fanelli MA, Ciocca DR.** HER2 and β-catenin protein location: importance in the prognosis of breast cancer patients and their correlation when breast cancer cells suffer stressful situations. Clin Exp Metastasis 2015, **32**, 151-168.
4. **Gama A, Paredes J, Gärtner F, Alves A, Schmitt F.** Expression of E-cadherin, P-cadherin and β-catenin in canine malignant mammary tumours in relation to clinicopathological parameters, proliferation and survival. Vet J 2008, **177**, 45-53.
5. **Goldschmidt M, Peña L, Rasotto R, Zappulli V.** Classification and grading of canine mammary tumors. Vet Pathol 2011, **48**, 117-131.
6. **Gracanin A, Timmermans-Sprang EPM, van Wolferen ME, Rao NAS, Grizelj J, Vince S, Hellmen E, Mol JA.** Ligand-independent canonical Wnt activity in canine mammary tumor cell lines associated with aberrant LEF1 expression. PLoS One 2014, **9**, e98698.
7. **Han SX, Zhou X, Sui X, He CC, Cai MJ, Ma JL, Zhang YY, Zhou CY, Ma CX, Varela-Ramirez A, Zhu Q.** Serum dickkopf-1 is a novel serological biomarker for the diagnosis and prognosis of pancreatic cancer. Oncotarget 2015, **6**, 19907-19917.
8. **Im KS, Kim NH, Lim HY, Kim HW, Shin JI, Sur JH.** Analysis of a new histological and molecular-based classification of canine mammary neoplasia. Vet Pathol 2014, **51**, 549-559.

9. **Kam Y, Quaranta V.** Cadherin-bound β-catenin feeds into the Wnt pathway upon adherens junctions dissociation: evidence for an intersection between β-catenin pools. PLoS One 2009, **4**, e4580.
10. **Kang CM, Kim HK, Kim H, Choi GH, Kim KS, Choi JS, Lee WJ.** Expression of Wnt target genes in solid pseudopapillary tumor of the pancreas: a pilot study. Pancreas 2009, **38**, e53-59.
11. **Khramtsov AI, Khramtsova GF, Tretiakova M, Huo D, Olopade OI, Goss KH.** Wnt/β-catenin pathway activation is enriched in basal-like breast cancers and predicts poor outcome. Am J Pathol 2010, **176**, 2911-2920.
12. **Kipp A, Banning A, van Schothorst EM, Méplan C, Schomburg L, Evelo C, Coort S, Gaj S, Keijer J, Hesketh J, Brigelius-Flohé R.** Four selenoproteins, protein biosynthesis, and Wnt signalling are particularly sensitive to limited selenium intake in mouse colon. Mol Nutr Food Res 2009, **53**, 1561-1572.
13. **Li S, Li S, Sun Y, Li L.** The expression of β-catenin in different subtypes of breast cancer and its clinical significance. Tumour Biol 2014, **35**, 7693-7698.
14. **Lim SC, Lee MS.** Significance of E-cadherin/β-catenin complex and cyclin D1 in breast cancer. Oncol Rep 2002, **9**, 915-928.
15. **Loh YN, Hedditch EL, Baker LA, Jary E, Ward RL, Ford CE.** The Wnt signalling pathway is upregulated in an *in vitro* model of acquired tamoxifen resistant breast cancer. BMC Cancer 2013, **13**, 174.
16. **MacDonald BT, Tamai K, He X.** Wnt/β-catenin signaling: components, mechanisms, and diseases. Dev Cell 2009, **17**, 9-26.
17. **Murakami Y, Tateyama S, Rungsipipat A, Uchida K, Yamaguchi R.** Immunohistochemical analysis of cyclin A, cyclin D1 and P53 in mammary tumors, squamous cell carcinomas and basal cell tumors of dogs and cats. J Vet Med Sci 2000, **62**, 743-750.
18. **Peña L, De Andrés PJ, Clemente M, Cuesta P, Pérez-Alenza MD.** Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. Vet Pathol 2013, **50**, 94-105.
19. **Polakis P.** The many ways of Wnt in cancer. Curr Opin Genet Dev 2007, **17**, 45-51.
20. **Queiroga FL, Raposo T, Carvalho MI, Prada J, Pires I.** Canine mammary tumours as a model to study human breast cancer: most recent findings. In Vivo 2011, **25**, 455-465.
21. **Rasotto R, Goldschmidt MH, Castagnaro M, Carnier P, Caliari D, Zappulli V.** The dog as a natural animal model for study of the mammary myoepithelial basal cell lineage and its role in mammary carcinogenesis. J Comp Pathol 2014, **151**, 166-180.
22. **Rasotto R, Zappulli V, Castagnaro M, Goldschmidt MH.** A retrospective study of those histopathologic parameters predictive of invasion of the lymphatic system by canine mammary carcinomas. Vet Pathol 2012, **49**, 330-340.
23. **Ren XY, Zhou GQ, Jiang W, Sun Y, Xu YF, Li YQ, Tang XR, Wen X, He QM, Yang XJ, Liu N, Ma J.** Low SFRP1 expression correlates with poor prognosis and promotes cell invasion by activating the Wnt/β-catenin signaling pathway in NPC. Cancer Prev Res (Phila) 2015, **8**, 968-977.
24. **Restucci B, Maiolino P, Martano M, Esposito G, De Filippis D, Borzacchiello G, Lo Muzio L.** Expression of β-catenin, E-cadherin and APC in canine mammary tumors. Anticancer Res 2007, **27**, 3083-3089.
25. **Rowell JL, McCarthy DO, Alvarez CE.** Dog models of naturally occurring cancer. Trends Mol Med 2011, **17**, 380-388.
26. **Shen Q, Fan J, Yang XR, Tan Y, Zhao W, Xu Y, Wang N, Niu Y, Wu Z, Zhou J, Qiu SJ, Shi YH, Yu B, Tang N, Chu W, Wang M, Wu J, Zhang Z, Yang S, Gu J, Wang H, Qin W.** Serum DKK1 as a protein biomarker for the diagnosis of hepatocellular carcinoma: a large-scale, multicentre study. Lancet Oncol 2012, **13**, 817-826.
27. **Sorenmo K.** Canine mammary gland tumors. Vet Clin North Am Small Anim Pract 2003, **33**, 573-596.
28. **Uva P, Aurisicchio L, Watters J, Loboda A, Kulkarni A, Castle J, Palombo F, Viti V, Mesiti G, Zappulli V, Marconato L, Abramo F, Ciliberto G, Lahm A, La Monica N, de Rinaldis E.** Comparative expression pathway analysis of human and canine mammary tumors. BMC Genomics 2009, **10**, 135.
29. **Valenta T, Hausmann G, Basler K.** The many faces and functions of β-catenin. EMBO J 2012, **31**, 2714-2736.
30. **Yao X, Jiang H, Zhang C, Wang H, Yang L, Yu Y, Yu J, Shi B, Shen Z, Gao H, Chen Z, Tian S, Lu S, Li Z, Gu J.** Dickkopf-1 autoantibody is a novel serological biomarker for non-small cell lung cancer. Biomarkers 2010, **15**, 128-134.
31. **Zheng L, Sun D, Fan W, Zhang Z, Li Q, Jiang T.** Diagnostic value of SFRP1 as a favorable predictive and prognostic biomarker in patients with prostate cancer. PLoS One 2015, **10**, e0118276.