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Immunohistochemical and quantitative RT-PCR methods to assess *RANK* expression in normal and neoplastic canine mammary gland

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Running title: *RANK* expression in canine mammary gland

Abstract. The receptor activator of nuclear factor-kB (*RANK*) gene is found in both human and murine mammary epithelial cells and in human cancer cell lines. We analyzed *RANK* expression in normal and proliferative canine mammary tissue samples (n = 47) and cell lines (n = 10), and identified its expression in epithelial cell populations. The correlation of RANK protein with clinicopathologic parameters was also studied. A double immunohistochemical method using RANK and p63 antibodies was applied to 33 tissue samples to analyze RANK protein expression and its possible co-expression with p63 protein, the latter used to identify myoepithelial (ME) cells (p63-positive) or luminal epithelial (LE) cells (p63-negative). RANK protein expression was found in ~75% of the tissue samples analyzed, at a similar level in all of the histologic types studied: dysplasias (4 of 4, 100%), malignant tumors (13 of 17, 76%), normal glands (12 of 17, 70%), and benign tumors (6 of 9, 67%). ME and LE cells expressed RANK protein at a similar level. A higher level of RANK protein expression was found in older animals (≥10 y, p = 0.027). Quantitative RT-PCR was applied to 6 ME (1 normal and 5 neoplastic) and 4 LE (1 normal and 3 neoplastic) primary cell lines. The *RANK* gene was found at similar expression levels in all canine mammary ME and LE cell lines studied. We found *RANK* expression in normal, dysplastic, and neoplastic canine mammary tissues and cell lines, in both ME and LE cell populations.

Key words: Canine; cell line; immunohistochemistry; mammary; p63; quantitative RT-PCR; RANK; tissue samples; tumors.

The receptor activator of nuclear factor-kB (RANK) is a receptor of the tumor necrosis factor (TNF) family of cytokines, which upon binding to its ligand (RANKL) transduces a variety of survival, proliferation, differentiation, and migration signals.[12] RANK and RANKL play key roles in bone remodeling and bone-related lesions.[20] RANK is expressed primarily on the surface of osteoclasts,[20] in dendritic cells,[19] in T-cells,[19] and in mammary epithelial cells.[4] Furthermore, RANK protein is critical for mammary gland development.[4] *RANK* gene expression has been analyzed in both normal and neoplastic mammary gland specimens and their metastases in humans and murine species,[2,9,16] and in several human breast cancer cell lines.[2,9] At the time of writing, we found no studies on RANK expression in the canine mammary gland.

Mammary gland tumors are the most common neoplasms in female dogs (25–50% of all tumors in intact female dogs).[10] Ducts and alveoli of normal glands are composed of 2 cell layers, an inner or luminal epithelial (LE) cell layer and an outer layer of myoepithelial (ME) cells.[6] Although frequently presented as a spontaneous model of breast cancer, mammary carcinomas in the female dog have lower biological aggressiveness than those in women. This fact has been linked, at least in part, to the higher participation of ME cells in canine mammary tumors, which are considered to be natural paracrine suppressors of invasion and metastasis.[18]

We analyzed RANK protein expression in normal, hyperplastic, and neoplastic canine mammary tissue samples by immunohistochemistry, and *RANK* gene expression in canine cell lines by quantitative reverse transcription PCR (RT-qPCR). In addition, we determined RANK expression in the ME and/or LE cell populations specifically. Thirty-three mammary gland biopsies or mastectomy specimens from 26 female dogs were collected from the archives of the Department of Comparative Pathology of the University of Córdoba (Spain). Tissue samples had been fixed in 10% neutral-buffered formalin for 24–72 h, embedded in paraffin, and processed

routinely. Age of dog, tumor size, histologic classification,[7] and histologic grade of malignant tumors[13] were evaluated. The 33 specimens comprised 3 normal glands, 4 dysplastic glands (including ductal hyperplasia, lobular hyperplasia, and duct ectasia), 9 benign tumors, and 17 malignant tumors. The latter had been classified into histologic grade 1 (n = 9), grade 2 (n = 7), and grade 3 (n = 1). Normal tissue comprised the 3 normal mammary gland specimens, plus unaltered, normal mammary gland tissue surrounding tumor specimens in 14 of the cases. For immunohistochemistry (IHC), all cases were analyzed using a double-immunostaining method according to the manufacturer's protocol (EnVision doublestain system, Dako, Glostrup, Denmark). Two primary antibodies were used: 1) anti-RANK (Polyclonal IgG antibody, Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:90, and 2) anti-p63 (monoclonal [clone 4A4] isotype IgG_2 antibody, Santa Cruz Biotechnology) diluted 1:100 and selected as the marker of ME cells.[6] A commercial antibody diluent (Dako) was used throughout. RANK immunostaining was developed in fast red (Permanent red substrate-chromogen, liquid, Dako), and p63 immunostaining was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) brown (Dako). As negative control, primary antibodies were replaced by the immunoglobulin fraction of serum from non-immunized rabbits and mouse IgG2 (Dako), respectively, diluted as for the primary antibodies. As positive controls, canine lymph node and normal skin were used for RANK and p63 antibodies, respectively. Furthermore, tissue-associated macrophages were used as internal positive controls for RANK antibody.

Immunolabeled slides were randomized and masked for blind examination, which was performed independently by 2 observers (R Sánchez-Céspedes, J García-Macías). When there was disagreement (<5% of slides), a consensus between the 2 observers was reached using a multi-head microscope. RANK scoring was rated by comparing labeling intensity with that of

the internal positive control (tissue-associated macrophages) as follows: absent (RANK0), positive but less intense than internal control tissue (RANK1+), positive and equal to the internal control tissue (RANK2+), and positive but more intense than the internal control tissue (RANK3+). Cells were considered to be p63+ when they displayed brown nuclear labeling and p63-negative (p63–) when they lacked brown nuclear labeling. For quantification, images were captured (40× microscope objective) from 10 randomly selected neighboring, non-overlapping fields. A sample was considered to be RANK+ when immunostaining intensity was RANK2+ or RANK3+ in >50% of cells.[16] The co-expression of RANK and p63 antigens was classified as follows: p63+/RANK–, p63+/RANK+, p63–/RANK–, and p63–/RANK+. The number of cells belonging to each group was determined by 2 independent observers (R Sánchez-Céspedes, J García-Macías) with a digital pen tablet (Volito 2, Wacom Europe, Germany), and the percentages were calculated using Image-Pro Plus 4.5 (Media Cybernetics, Rockville, MD).

Three fresh samples of mammary tumors and 1 of normal mammary gland (Table 1) were collected from 3 female dogs during surgery at the Department of Veterinary Sciences, University of Turin, Italy (cases 1–3). These fresh samples were processed to obtain primary ME and LE cell lines according to our method proposed previously.[15] Thus, the magnetic-activated cell sorting (MACS) technique based on the binding of antibody-coated magnetic microspheres to Thy1 (ME cell–specific surface antigen) using an anti-Thy1 antibody was used to purify and isolate canine mammary ME cells (positive selection) or LE cells (negative selection).[3,15] Afterward, immunocytochemistry using typical ME or LE lineage markers was carried out to confirm the phenotype of the cells in primary culture.[15] All 4 tissues were also processed routinely and stained for histologic classification[7] and immunophenotyping using the ABC method (Avidin–biotin–complex, Vector Laboratories, Orton Southgate, Peterborough, UK),

with anti-cytokeratin (CK)14 polyclonal rabbit antibody (Covance Research, Munich, Germany; diluted 1:500) for ME cells and anti-CK8/18 antibody (clone NCL-5D3, isotype IgG_1 antibody, Euro-Diagnostica, Malmö, Sweden; diluted 1:20) for LE cells.[15] Furthermore, in order to increase the number of cell lines studied, 2 ME cell lines characterized previously by our research group[15] were also used: CmME-K1 (complex carcinoma) and CmME-K2 (simple tubulopapillary carcinoma).

For RT-qPCR expression analysis, total RNA was obtained from ME and LE cell lines, and 1 µg of total RNA was reverse-transcribed using commercially available reagent sets (QiantiTec reverse transcription kit, Qiagen, Hilden, Germany). Quantitative RT-PCR was used to measure the quantity of RANK relative to the quantity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyl transferase (HPRT) messenger (m)RNA using commercially available reagent sets (IQ SYBR Green supermix and IQ 5 detection system, Bio-Rad, München, Germany). GAPDH and HPRT were used as housekeeping genes. Primer sequences were designed using Primer Express v.2.5 (Thermo Fisher Scientific, Waltham, MA): RANK, 5'-ATGTGGTTTGTAGTTCTTCTC-3' (forward), 5'-ACTCCTTATTTACACTTAGG-3' (reverse); GAPDH, 5'-GGCACAGTCAAGGCTGAG-3' (forward), 5'-CCAGCATCACCCCATTTGAT-3' (reverse); and HPRT, 5'-CACTGGGAAAACAATGCAGA-3' (forward), 5'-ACAAAGTCAGGTTTATAGCCAACA-3' (reverse). Real-time PCR parameters were: cycle 1, 95°C for 30 s; cycle 2, 95°C for 10 s, 60°C for 30 s for 40 cycles. The level of gene expression was calculated using a relative quantification assay corresponding to the comparative threshold cycle (Ct) method: the amount of target, normalized to the endogenous housekeeping genes and relative to the calibrator (control sample),

was then transformed by $2^{-\Delta\Delta Ct}$ (fold increase), where $\Delta\Delta Ct = \Delta Ct$ (sample) – ΔCt (control); ΔCt is the Ct of the target gene subtracted from the Ct of the housekeeping genes.

Immunohistochemical and clinicopathologic results were grouped into contingency tables and analyzed using the Fisher exact test; $p \leq 0.05$ was considered statistically significant. Data were analyzed with GraphPad Prism v.4.0 (GraphPad Software, San Diego, CA).

RANK labeling was seen in the cytoplasm of epithelial ductal and alveolar cells of normal, dysplastic, and neoplastic glands, osteoclasts of mixed tumors, and tissue-associated macrophages within and around the tumors. The latter 2 cell types were used as internal positive controls of RANK labeling. Cytoplasmic staining was diffuse and an apical/luminal RANK labeling pattern was also observed in some ductal and alveolar cells.

RANK expression varied with histologic classification, although differences were not statistically significant (Table 2). Thus, 12 of 17 (70%) normal, all (4 of 4, 100%) dysplastic, and 19 of 26 (73%) tumorous mammary glands were classified as RANK+ cases (Table 2). The single simple adenoma studied (composed of LE cells exclusively) was classified as RANK– (Fig. 1), whereas 1 of 2 (50%) complex adenomas was negative and 5 of 6 (83%) benign mixed tumors were considered RANK+ cases (Fig. 2). The majority of simple and complex carcinomas (80% and 89%, respectively) and a single (1 of 3, 33%) mixed carcinoma were classified as RANK+ cases.

The median percentage of RANK+ cells found in RANK+ cases was similarly high in all groups (93% in normal and 80% in dysplastic glands; 76% in benign and 71% in malignant tumors; Table 2). The median percentage of both ME and LE cells expressing RANK was similar in the different histologic types of samples studied (Table 2).

In both normal and dysplastic glands, RANK+ cells were found in the LE cells of the ductal and lobular system with both diffuse and apical/luminal staining patterns (Fig. 3). Furthermore, RANK+ cells were also found in the single flattened or spindle ME cell layer located around normal ducts and alveoli with a diffuse staining pattern (Fig. 3). In RANK+ benign tumors, most LE and ME cells located in the inner and the outer cell layers, respectively, of neoplastic tubules were RANK+ cells showing a diffuse staining pattern. However, the apical/luminal staining pattern was also occasionally seen. Fusiform, polygonal, or round RANK+ ME cells formed fascicles without atypia in all RANK+ complex adenomas, and were also embedded in lacunae of cartilaginous matrix in 2 of 5 RANK+ benign mixed tumors (Fig. 2). In malignant tumors, 4 staining patterns were observed. First, RANK+ ME cells were seen forming a single complete or incomplete layer of flattened or spindle cells located around neoplastic nodules, tubules, and papillae (Fig. 4). Second, RANK+ fusiform ME cells forming nests or fascicles were also seen in complex and mixed carcinomas. Third, RANK+ LE cells forming 1–3 layers of proliferating cells into the lumen of neoplastic tubules were observed in malignant tumors with either diffuse or apical/luminal RANK staining patterns (Fig. 4). And fourth, rounded cells of the cartilage nests observed in the mixed carcinoma were RANK0 and p63–.

RANK protein expression was higher in animals ≥10 y old ($p = 0.027$; Table 3). RANK expression was not related to tumor size or histologic grade of the malignant tumors (Table 3).

RANK gene expression level was similar in both normal ME and LE cell lines (CmME-N1 and CmLE-N1, respectively). The tumor ME (CmME-T2, CmME-T3, CmME-K1, CmME-K2) and LE (CmLE-T2, CmLE-T3) cell lines expressed *RANK* gene at levels similar to their respective controls from normal ME (CmME-N1; Fig. 5) and LE (CmLE-N1; Fig. 6) cell lines,

except for the CmLE-T1 cell line (from case 1, complex carcinoma) that expressed twice as much RANK as normal cells (Fig. 6). *RANK* expression was detected in most of the tissue samples and in all cell lines studied. ME and LE cells expressed RANK at a similar level in normal, dysplastic, and neoplastic canine mammary tissues and in primary cell lines. RANK protein labeling was found in ~75% of the tissue samples analyzed. We found no statistically significant differences in RANK protein expression between the histologic types: dysplasias (100%), malignant tumors (76%), normal glands (70%), and benign tumors (67%). This could be because of the high Ki67 proliferation index found in dysplasia (data not shown). In human breast tissue, a positive correlation between RANK expression and Ki67 labeling index has been reported.[1] *RANK*+ malignant tumors are more common in dogs (76%) than are breast carcinomas in women (57% reported by some authors and 6% from others).[8,16] Different methodologies to evaluate IHC findings could contribute to discrepancies among studies. When grouped by histologic subtypes, all tumor subtypes expressed *RANK* at a similar level. To our knowledge, there are no published reports of a correlation of *RANK* gene expression with histologic subtype (simple, complex, mixed) in breast cancer; however, there is one study in which RANK expression was independent of neoplasm subtype (ductal vs. lobular).[17] All RANK+ cases, regardless of their histologic subtype, had a high percentage of RANK+ cells (≥67%). Sixty-five percent of RANK+ cells were reported in breast cancer[16] according to our results (71% of RANK+ cells in malignant tumors), but there are no published data concerning other histologic types of samples.

Double-labeling IHC was performed to analyze RANK labeling in the 2 epithelial cell populations of the mammary gland: ME and/or LE cells. After observing the cytoplasmic and/or apical/luminal RANK labeling pattern, we selected p63 as the marker of ME cells because of its

nuclear staining pattern.[5] RANK protein expression was similar in both ME (57%) and LE (56%) cells, which corresponds with the observation of RANK protein in both compartments of murine mammary epithelial cells.[8] A higher level of RANK protein expression was found in older animals (≥10 y, $p = 0.027$). Statistically significant differences between RANK protein expression and tumor size or histologic grade of malignancy were not observed in canine mammary glands. In human breast cancer, increased RANK expression was correlated with higher histologic grade of malignancy by IHC,[14] and a higher *RANK* gene expression was observed in bigger tumors by microarray analysis.[17] However, microarray analysis showed no correlation between age and RANK expression.[17] Comparison between results from 2 different methodologies (IHC and microarray) may have intrinsic limitations. It is important to note that in human and murine mammary gland tumors, most authors report that high RANK level in primary tumors is predictive of poorer prognosis.[17] Unfortunately, we do not possess available data concerning the biological behavior of the tumors included in our study to support this hypothesis.

Transcript levels of RANK were shown by RT-qPCR to be similar between canine mammary normal ME versus LE cell lines, and between normal versus neoplastic cell lines, in accordance with IHC results. Only the CmLE-T1 cell line had higher RANK levels than the normal counterpart, which could be the result of the fact that the tumor had been classified as grade 3 malignancy, whereas the rest of the malignant tumors had been classified as grades 1 and 2 (data not shown). In humans, studies on *RANK* gene expression by RT-qPCR in ME and/or LE cell lines from the breast have not been found, and those studies in neoplastic cell lines are contradictory. Thus, some authors have shown that higher RANK expression in breast cancer cells correlated with greater metastatic rates in bone,[2,20] whereas other authors have shown that

transcript levels of *RANK* gene were reduced in tumor samples when compared with normal tissue, and that reduced *RANK* expression was associated with poor clinical outcomes, disseminated metastasis, bone metastasis, and death.[11]

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 1. Clinical and pathologic features of dogs with mammary tumors used for isolation of myoepithelial (ME) and luminal epithelial (LE) cells.

Case	Breed	Age (y)	Sex	Location of tumor	Size of tumor (cm)	Histologic classification of tumor	ME cell line	LE cell line
1*	Poodle	14	Female	II right	0.9	Complex carcinoma	CmME-T1	CmLE-T1
2	Rottweiler	8	Female	III left	0.4	Simple tubulopapillary carcinoma	CmME-T2	CmLE-T2
3	Shih Tzu	8	Female	IV right	1	Benign mixed tumor	CmME-T3	CmLE-T3

* Fresh tissue sample from normal mammary gland (V right) of case 1 was also collected, named CmME-N1 and CmLE-N1 for the ME and LE cell lines obtained, respectively.

Table 2. RANK protein expression in cases under study and the median percentage of myoepithelial (ME; p63+) and luminal epithelial (LE; p63–) cells expressing RANK antigen in different mammary tissues.

Sample type	No. of cases	No. of RANK+ cases	% of RANK+ cells in RANK+ cases	% RANK+ ME cells	% RANK+ LE cells
Normal mammary tissue	17	12 (70)	93	54	66
Dysplasia	4	4 (100)	80	59	60
Benign tumor	9	6 (67)	76	46	42
Simple adenoma	1	0	0	0	0
Complex adenoma	2	1 (50)	67	42	60
Benign mixed tumor	6	5 (83)	77	77	70
Malignant tumor	17	13 (76)	71	67	57
Simple carcinoma	5	4 (80)	68	76	58
Complex carcinoma	9	8 (89)	69	68	66
Mixed carcinoma	3	1 (33)	91	56	48
Total	47	35 (74)	80	57	56

Numbers in parentheses are percentages.

Table 3. RANK protein expression and clinicopathologic parameters of the 26 dogs.

Parameter/range	No. of total cases	No. of RANK+ cases
Age*		
<10 y	6	1 (17)
≥10 y	20	18 (90)
Tumor size		
<2 cm	15	10 (67)
≥2 cm	11	9 (82)
Histologic grade of carcinoma		
1	9	6 (67)
2	7	6 (86)
3	1	1 (100)

Numbers in parentheses are percentages.

* $p = 0.027$

Figure 1. Simple adenoma in a canine mammary gland; p63+ cells form a single complete or incomplete layer of flattened cells around neoplastic ducts and alveoli in a RANK– case. Double immunohistochemical labeling for RANK (red) and p63 (brown) (EnVision doublestain system, Dako). Bar = 20 µm.

Figure 2. Benign mixed tumor in a canine mammary gland. In the lacunae of cartilaginous matrix, both RANK+/p63– cells (black arrows) and co-expression of RANK and p63 antigens are present in some cells (red arrow). Double immunohistochemical labeling for RANK (red) and p63 (brown; EnVision doublestain system, Dako). Bar = 20 µm.

Figure 3. Dysplasia in a canine mammary gland. RANK labeling was observed in both p63– and p63+ cells. RANK+/p63– cells are present in the outer, proliferative, and luminal layers of neoplastic tubules (black arrows). Co-expression of RANK and p63 proteins is present in all 3 cell layers of neoplastic tubules (red arrows). Double immunohistochemical labeling for RANK (red) and p63 (brown; EnVision doublestain system, Dako). Bar = 20 µm.

Figure 4. Simple carcinoma in a canine mammary gland. Round-to-oval cells form the neoplastic nodules that histologically appeared to be of only one type. Double immunohistochemical labeling revealed 4 different cell types: 1) RANK+/p63– cells (black arrows); 2) RANK+/p63+ cells (red arrows); 3) RANK–/p63+ cells (black stars); and 4) RANK–/p63– cells (red stars). Double immunohistochemical labeling for RANK (red) and p63 (brown; EnVision doublestain system, Dako). Bar = 20 µm.

Figure 5. *RANK* gene expression by RT-qPCR in canine mammary myoepithelial (CmME) cell lines. The fold increase of each specific mRNA was normalized with the normal ME cell line (CmME-N1), and the error bars indicate one standard deviation of experimental triplicates.

313 *RANK* gene expression level was similar in the neoplastic ME cell lines compared to the
314 normal ME cell line.
315 **Figure 6.** *RANK* gene expression by RT-qPCR in canine mammary luminal epithelial (CmLE)
316 cell lines. The fold increase of each specific mRNA was normalized with the normal LE cell
317 line (CmLE-N1), and the error bars indicate one standard deviation of experimental
318 triplicates. The neoplastic LE cell lines expressed *RANK* at levels similar to the normal LE
319 cell line; only the neoplastic CmLE-T1 cell line showed a 2-fold increase in *RANK* expression
320 compared to the normal LE cell line.