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

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14

15 Running title: *RANK* expression in canine mammary gland

16

17 **Abstract.** The receptor activator of nuclear factor- κ B (*RANK*) gene is found in both human and
18 murine mammary epithelial cells and in human cancer cell lines. We analyzed *RANK* expression
19 in normal and proliferative canine mammary tissue samples ($n = 47$) and cell lines ($n = 10$), and
20 identified its expression in epithelial cell populations. The correlation of RANK protein with
21 clinicopathologic parameters was also studied. A double immunohistochemical method using
22 RANK and p63 antibodies was applied to 33 tissue samples to analyze RANK protein expression
23 and its possible co-expression with p63 protein, the latter used to identify myoepithelial (ME)
24 cells (p63-positive) or luminal epithelial (LE) cells (p63-negative). RANK protein expression
25 was found in ~75% of the tissue samples analyzed, at a similar level in all of the histologic types
26 studied: dysplasias (4 of 4, 100%), malignant tumors (13 of 17, 76%), normal glands (12 of 17,
27 70%), and benign tumors (6 of 9, 67%). ME and LE cells expressed RANK protein at a similar
28 level. A higher level of RANK protein expression was found in older animals (≥ 10 y, $p = 0.027$).
29 Quantitative RT-PCR was applied to 6 ME (1 normal and 5 neoplastic) and 4 LE (1 normal and 3
30 neoplastic) primary cell lines. The *RANK* gene was found at similar expression levels in all
31 canine mammary ME and LE cell lines studied. We found *RANK* expression in normal,
32 dysplastic, and neoplastic canine mammary tissues and cell lines, in both ME and LE cell
33 populations.

34

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

37

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

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

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

61 routinely. Age of dog, tumor size, histologic classification,⁷ and histologic grade of malignant
62 tumors¹³ were evaluated. The 33 specimens comprised 3 normal glands, 4 dysplastic glands
63 (including ductal hyperplasia, lobular hyperplasia, and duct ectasia), 9 benign tumors, and 17
64 malignant tumors. The latter had been classified into histologic grade 1 ($n = 9$), grade 2 ($n = 7$),
65 and grade 3 ($n = 1$). Normal tissue comprised the 3 normal mammary gland specimens, plus
66 unaltered, normal mammary gland tissue surrounding tumor specimens in 14 of the cases. For
67 immunohistochemistry (IHC), all cases were analyzed using a double-immunostaining method
68 according to the manufacturer's protocol (EnVision doublestain system, Dako, Glostrup,
69 Denmark). Two primary antibodies were used: 1) anti-RANK (Polyclonal IgG antibody, Santa
70 Cruz Biotechnology, Heidelberg, Germany) diluted 1:90, and 2) anti-p63 (monoclonal [clone
71 4A4] isotype IgG₂ antibody, Santa Cruz Biotechnology) diluted 1:100 and selected as the marker
72 of ME cells.⁶ A commercial antibody diluent (Dako) was used throughout. RANK
73 immunostaining was developed in fast red (Permanent red substrate-chromogen, liquid, Dako),
74 and p63 immunostaining was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB)
75 brown (Dako). As negative control, primary antibodies were replaced by the immunoglobulin
76 fraction of serum from non-immunized rabbits and mouse IgG2 (Dako), respectively, diluted as
77 for the primary antibodies. As positive controls, canine lymph node and normal skin were used
78 for RANK and p63 antibodies, respectively. Furthermore, tissue-associated macrophages were
79 used as internal positive controls for RANK antibody.

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

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

96 Three fresh samples of mammary tumors and 1 of normal mammary gland (Table 1) were
97 collected from 3 female dogs during surgery at the Department of Veterinary Sciences,
98 University of Turin, Italy (cases 1–3). These fresh samples were processed to obtain primary ME
99 and LE cell lines according to our method proposed previously.¹⁵ Thus, the magnetic-activated
100 cell sorting (MACS) technique based on the binding of antibody-coated magnetic microspheres
101 to Thy1 (ME cell-specific surface antigen) using an anti-Thy1 antibody was used to purify and
102 isolate canine mammary ME cells (positive selection) or LE cells (negative selection).^{3,15}
103 Afterward, immunocytochemistry using typical ME or LE lineage markers was carried out to
104 confirm the phenotype of the cells in primary culture.¹⁵ All 4 tissues were also processed
105 routinely and stained for histologic classification⁷ and immunophenotyping using the ABC
106 method (Avidin-biotin-complex, Vector Laboratories, Orton Southgate, Peterborough, UK),

107 with anti-cytokeratin (CK)14 polyclonal rabbit antibody (Covance Research, Munich, Germany;
108 diluted 1:500) for ME cells and anti-CK8/18 antibody (clone NCL-5D3, isotype IgG₁ antibody,
109 Euro-Diagnostica, Malmö, Sweden; diluted 1:20) for LE cells.¹⁵ Furthermore, in order to
110 increase the number of cell lines studied, 2 ME cell lines characterized previously by our
111 research group¹⁵ were also used: CmME-K1 (complex carcinoma) and CmME-K2 (simple
112 tubulopapillary carcinoma).

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

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

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

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

139 RANK expression varied with histologic classification, although differences were not
140 statistically significant (Table 2). Thus, 12 of 17 (70%) normal, all (4 of 4, 100%) dysplastic, and
141 19 of 26 (73%) tumorous mammary glands were classified as RANK+ cases (Table 2). The
142 single simple adenoma studied (composed of LE cells exclusively) was classified as RANK–
143 (Fig. 1), whereas 1 of 2 (50%) complex adenomas was negative and 5 of 6 (83%) benign mixed
144 tumors were considered RANK+ cases (Fig. 2). The majority of simple and complex carcinomas
145 (80% and 89%, respectively) and a single (1 of 3, 33%) mixed carcinoma were classified as
146 RANK+ cases.

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

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

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

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

174 except for the CmLE-T1 cell line (from case 1, complex carcinoma) that expressed twice as
175 much RANK as normal cells (Fig. 6). *RANK* expression was detected in most of the tissue
176 samples and in all cell lines studied. ME and LE cells expressed RANK at a similar level in
177 normal, dysplastic, and neoplastic canine mammary tissues and in primary cell lines. RANK
178 protein labeling was found in ~75% of the tissue samples analyzed. We found no statistically
179 significant differences in RANK protein expression between the histologic types: dysplasias
180 (100%), malignant tumors (76%), normal glands (70%), and benign tumors (67%). This could be
181 because of the high Ki67 proliferation index found in dysplasia (data not shown). In human
182 breast tissue, a positive correlation between RANK expression and Ki67 labeling index has been
183 reported.¹ *RANK*+ malignant tumors are more common in dogs (76%) than are breast carcinomas
184 in women (57% reported by some authors and 6% from others).^{8,16} Different methodologies to
185 evaluate IHC findings could contribute to discrepancies among studies. When grouped by
186 histologic subtypes, all tumor subtypes expressed *RANK* at a similar level. To our knowledge,
187 there are no published reports of a correlation of *RANK* gene expression with histologic subtype
188 (simple, complex, mixed) in breast cancer; however, there is one study in which RANK
189 expression was independent of neoplasm subtype (ductal vs. lobular).¹⁷ All *RANK*+ cases,
190 regardless of their histologic subtype, had a high percentage of *RANK*+ cells ($\geq 67\%$). Sixty-five
191 percent of *RANK*+ cells were reported in breast cancer¹⁶ according to our results (71% of
192 *RANK*+ cells in malignant tumors), but there are no published data concerning other histologic
193 types of samples.

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

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

211 Transcript levels of RANK were shown by RT-qPCR to be similar between canine
212 mammary normal ME versus LE cell lines, and between normal versus neoplastic cell lines, in
213 accordance with IHC results. Only the CmLE-T1 cell line had higher RANK levels than the
214 normal counterpart, which could be the result of the fact that the tumor had been classified as
215 grade 3 malignancy, whereas the rest of the malignant tumors had been classified as grades 1 and
216 2 (data not shown). In humans, studies on *RANK* gene expression by RT-qPCR in ME and/or LE
217 cell lines from the breast have not been found, and those studies in neoplastic cell lines are
218 contradictory. Thus, some authors have shown that higher RANK expression in breast cancer
219 cells correlated with greater metastatic rates in bone,^{2,20} whereas other authors have shown that

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

223 **Declaration of conflicting interests**

224 The authors declared no potential conflicts of interest with respect to the research, authorship,
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- 276

277 **Table 1.** Clinical and pathologic features of dogs with mammary tumors used for isolation of
 278 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

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

281

282 **Table 2.** RANK protein expression in cases under study and the median percentage of
 283 myoepithelial (ME; p63+) and luminal epithelial (LE; p63-) cells expressing RANK antigen in
 284 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

285 Numbers in parentheses are percentages.

286

287 **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)

288 Numbers in parentheses are percentages.

289 * $p = 0.027$

290

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

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

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

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

310 **Figure 5.** *RANK* gene expression by RT-qPCR in canine mammary myoepithelial (CmME) cell
311 lines. The fold increase of each specific mRNA was normalized with the normal ME cell line
312 (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.