1 **Immunohistochemical and quantitative RT-PCR methods to assess *RANK* expression in**

2 **normal and neoplastic canine mammary gland**

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

16

17   **Abstract.** The receptor activator of nuclear factor-kB (*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 ($\geq$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.

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35   **Key words:** Canine; cell line; immunohistochemistry; mammary; p63; quantitative RT-PCR;

36   RANK; tissue samples; tumors.

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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

61   routinely. Age of dog, tumor size, histologic classification,[7] and histologic grade of malignant

62   tumors[13] 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 IgG_2 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 classification[7] 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 IgG_1 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 group[15] 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   ACTCCTTATTTACACTTAGG-3' (reverse); GAPDH, 5'-GGCACAGTCAAGGCTGAG-3'

123   (forward), 5'-CCAGCATCACCCCATTTGAT-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) $- \Delta Ct$ (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 (≥67%). Sixty-five

191   percent of RANK+ cells were reported in breast cancer[16] 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 ($\geq$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**

226 **Funding**

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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.