Expression of the Short Form of RON/STK in Feline Mammary Carcinoma

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Abstract

RON is a tyrosine kinase receptor activated by the MSP ligand that is overexpressed in human breast cancer. In humans, RON protein can be present in different isoforms, and the most studied isoform is represented by the short form of RON (sf-RON), which is generated by an alternative promoter located in intron 10 of the RON cDNA. It plays an important role in breast cancer progression. Considering the many

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similarities between feline mammary tumors and human breast cancer, the aim of this study was to investigate the expression of both RON and MSP in feline mammary carcinomas (FMCs) and to identify the presence of the sf-RON transcript. Tissue samples of spontaneous mammary tumors were collected from 60 queens (10 benign lesions and 50 carcinomas). All of the samples were tested for RON and MSP expression by immunohistochemistry; moreover, RNA was extracted from paraffin-embedded tissue samples, and the cDNA was tested by RT-PCR to identify the presence of sf-RON. IHC detected the expression of RON and MSP in 34/50 (68%) and 29/50 (58%) of FMCs, respectively. RT-PCR revealed the presence of the short-form in 18/47 (38.3%) of FMCs. This form originates, as in humans, from an alternative promoter (P2) and it codes for the proper feline short form (sf-RON). sf-RON expression was statistically associated with poorly differentiated tumors and with a shorter disease-free (DFI) period and a shorter survival (OS). These results support FMC as a suitable model in comparative oncology and identify sf-RON expression as a potential predictor of outcomes for this disease.

Keywords: feline mammary tumors, short form of RON, comparative oncology, prognosis

Introduction

Since 1980, feline mammary carcinoma (FMC) has been recognized as a suitable animal model for studying human breast cancer since it shares histological, molecular and clinical features with human breast carcinoma.[5,12,14,33]

RON/stk proto-oncogene, a member of the MET family, is a tyrosine kinase receptor that is abnormally overexpressed in a variety of human cancers including breast cancer. [20,30] The mouse homologue of human RON receptor, called stk, was identified in 1995,[15] while the feline homologue of the corresponding gene and protein, feline stk/RON, was first characterized in 2002.[11] Macrophage stimulating protein (MSP) is RON's specific ligand and it is present both in tissue and in circulating blood. Downstream signaling targets of RON activity include PI3K, Src, FAK, AKT and MAPK, which can lead to proliferation, cell survival, cell motility, cell shape changes and invasion in cancer.[9,10]

Following alternative splicing, both the human RON and the homologous mouse stk genes can generate different isoforms of the receptor lacking the extracellular domain of the receptors but conserving its catalytic activity. [28,13] The two major transcripts are the *full-length* transcript that encodes the full-size RON receptor and the *short form* of RON (*sf*-RON), also called the truncated form. In humans, the full length of RON consists of 20 exons encoding a 180 kDa protein with 1400 amino acids while the *sf*-RON is generated by an alternative promoter (5'UTR) located within intron 10 of the mRNA, regulating the transcription of *sf*-RON from the transcription initiation nucleotide represented by guanine^{2519}.[2] The presence of intron 10 in the RON transcript suggests the presence of *sf*-RON in tissues and cell lines,[3] and furthermore, the expression of both of the transcripts has been demonstrated to be associated with different methylation sites within RON promoters.[2] Figure 1 shows a schematic of the RON gene structure and transcriptional regulation of the full length and short form.

In humans, *sf*-RON was detected, at very low levels, in colon, skin and lung tissue but not in bone marrow, bone marrow-derived cells, human muscle or brain.[13,28]

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However, some reports in human medicine have demonstrated the presence of *sf-RON* in cells derived from breast cancer, lung cancer, pancreatic cancer and leukemia. It has also been correlated with invasive capability *in vitro*,[3,4] and alternatively spliced RON transcripts have been reported in other types of cancers.[17-19] In addition, it has been reported that *sf*-RON proteins are constitutively active since they are able to confer growth advantages to cells and have been involved in the epithelial-mesenchymal transition (EMT).[8]

In breast cancer, the presence of *sf*-RON is correlated with a poor prognosis and tumor recurrence.[18] Moreover, the recent development of specific RON inhibitors in combination with PI3K inhibitors with demonstrated antitumor activity suggest an important role of *sf*-RON in terms of targeted therapies. [7,32]

In consideration of the significant homologies between feline and human mammary tumors, the aim of this research was to investigate the expression of both isoforms of RON receptor and its ligand MSP in a large cohort of FMC tissues and cell lines and to identify the presence of the *sf*-RON transcript in FMC in relation to clinicopathologic findings.

Materials and methods

Cell lines

Six primary cell lines derived from FMC (FYCp, FKNp, FNNm, FMCp, FMCm and P248) and spontaneously immortalized were tested in the *in vitro* experiments[21,24,25,29]. FYCp, FKNp, FNNm, FMCp, and FMCm were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), 5000 IU/mL

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penicillin, 5 mg/mL streptomycin, and 2 mM L-glutamine; the P248 cell line was

maintained in DMEM under the same conditions but with the addition of 10 mg/ml

insulin to the medium.[22] The human GTL-16 gastric carcinoma cell line was used as

a positive control in western blot analysis and grown in DMEM as previously

described.[31]

Tissue samples and histological examination

Tissue samples from spontaneous cases of feline mammary tumors and normal

mammary tissues were collected from the 2009–2013 archives of the Diagnostic

Laboratories of the Department of Veterinary Sciences of the University of Turin

(Italy) and from the Department of Animal Pathology, University of Córdoba (Spain).

Details of age, breed, ovariectomy status and tumor size data were retrieved from the

hospital database. Each animal was surgically treated with mastectomy (partial or

total) and free surgical margins were histologically confirmed; this retrospective study

has been approved by the ethical committee of the Department of Veterinary Science

according to institutional guidelines.

Postoperative clinical, radiological and echographic examinations at 6, 12, 18 and 24

months after surgery were performed to detect the presence of distant organ

metastases or local relapse. Animals that died due to mammary carcinoma were

submitted for post-mortem examination to confirm the pathological diagnosis;

subjects that died of nontumor-related causes (confirmed by necropsy) during the

follow-up period were excluded from the study. The disease-free interval (DFI) was

considered as the number of days between surgery and tumor recurrence and/or

evidence of metastatic disease, while overall survival (OS) was considered as the

period between surgery and death. Normal mammary tissues used as a control were

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collected from necropsy (after the owners' consent). Samples were fixed in 4% neutral buffered formalin, paraffin embedded, sectioned at 4 µm and stained with hematoxylin and eosin (H&E). Samples were histologically examined and classified according to the World Health Organization (WHO) classification for tumors of domestic animals,[26] and surgical margins were evaluated to confirm that all tumor masses were completely surgically ablated. Carcinomas were graded according to Mills and colleagues (2014).[23]

Immunohistochemistry

Immunohistochemical (IHC) analysis was performed on 4 µm paraffin sections from all collected samples; immunocytochemistry was performed on all cultured cell pellets that were similarly fixed, embedded, and sectioned. After endogenous peroxidase activity blockade with 3% hydrogen peroxide for 20 min at room temperature, sections were subjected to high-temperature antigen retrieval by incubation with 98°C citric acid buffer (pH 6.0). Primary antihuman antibodies were used against RON β diluted 1:150, overnight incubation (C-20 rabbit polyclonal antibody; catalogue number sc-322, Santa Cruz Biotechnology, Dallas, Texas, USA) and against MSP diluted 1:50, overnight incubation (rabbit monoclonal antibody; catalogue number EPR6207, Abcam, Cambridge, UK). Antibodies were detected with avidin-biotin-peroxidase complexes using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, USA). Positive controls for RON and MSP were represented by lung and lymph node tissues, respectively. For the negative controls, positive control tissues were incubated with normal rabbit and mouse IgG (Santa Cruz Biotechnology, Dallas, Texas, USA). Immunostaining evaluation was performed independently by two observers (LM and SI); when there was disagreement, a

consensus was obtained using a multi-head microscope. Samples were scored semiquantitatively according to the intensity of membranous and/or cytoplasmic staining for both RON and MSP according to Ren and colleagues (2012) (0 = no staining, 1+ = weak staining, 2+ = moderate staining, 3+ = strong staining). Samples with absent or weak immunostaining (score 0 and 1+) were defined as negative, and those with moderate to strong immunostaining (score 2+ and 3+) as positive.[27] In the supplemental Figures S1–S8 examples of each score are shown.

Protein extraction and western blot analysis

Total proteins were extracted from 3 cases of feline mammary carcinomas (FMC1, FMC2 and FMC3) collected at the Veterinary Hospital of the Department of Veterinary Science of Turin and from the FYC cell line; normal feline spleen tissue from necroscopy was used as a negative control while protein lysate from GTL16 was used as a human positive control. Total proteins were extracted with fresh RIPA buffer solution (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) containing a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis Missouri, USA). Two hundred micrograms of total protein from each sample were separated on 9% SDS-polyacrylamide (PAGE) gels, transferred onto Hybond-C Extra membranes (Amersham Biosciences, Bath, UK) and incubated overnight at 4°C with a primary antibody against RON. After washing three times in PBS-Tween for 15 min and incubating with a horseradish peroxidase (HRP)-linked secondary antibody diluted 1:2000 in PBS-Tween 0.01%, the membranes were washed in PBS-Tween for 30 min and then incubated with an enhanced chemiluminescence reagent (Super Signal West Pico Rabbit IgG Detection Kit, Thermo Scientific, Waltham, USA).

DNA extraction and sequencing of genomic feline RON exon 10–exon 14

Genomic DNA was extracted from normal feline lymphoid tissue (spleen) using a QIAamp DNA mini kit (Qiagen, Hilden, Germany). PCR was performed on 100 ng of total genomic DNA using HotStart AmpliTAQ (Qiagen, Hilden, Germany), and amplification of the genomic region of the feline RON corresponding to exon 8 to exon 14 was performed. Sequences of exonic primers P1 and P2 are shown in Supplemental Table S1 and were designed on the basis of the sequence of feline RON cDNA present in the gene bank (XP_003982293). The PCR protocol for all primers was as follows: 1 cycle at 95°C for 5 min, 30 cycles (95°C 1 min, 60°C 1 min, 72°C 1 min), and 1 cycle at 72°C for 7 min. After purification of specific amplified bands from agarose by the QIAquick gel extraction kit (Qiagen, Hilden, Germany), direct sequencing of PCR products was performed using the BigDye Terminator V 3.1 Sequencing Kit and ABIPRISM 310 Genetic Analyzer (Applied Biosystem, Foster City California, USA).

c-DNA preparation, PCR and sequencing of *sf*-RON

Total RNA was extracted from paraffin-embedded tissues of FMCs, benign lesions and normal mammary glands using an RNA FFPE extraction kit (Qiagen, Hilden, Germany) and from six FMC cell lines (FYCp, P248m, FKNp, FNNm, FMCp, and FMCm) using the TRIzol method (Sigma-Aldrich, Saint Louis Missouri, USA). The quality of the RNA was evaluated using the Experion Automated Electrophoresis System (BioRad, Hercules, California, USA), and samples with degraded RNA were excluded. cDNA was synthesized from 1 μg of total RNA using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany) including genomic DNA

removal as described in the procedures of the kit. To examine the RON transcript, endpoint RT-PCR was performed with primer P3 sense and P4 antisense (Supplemental Table S1) in a final volume of 20 µl using HotStart TAQ (Qiagen, Hilden, Germany). The PCR conditions were as follows: 1 cycle at 95°C for 5 min, 30 cycles (95°C 1 min, 60°C 1 min, 72°C 1 min) and 1 cycle at 72°C for 7 min. Moreover, to exclude genomic DNA contamination, PCR of intronic sequences corresponding to intron 12 and 13 of the feline RON gene was performed on cDNA (data not shown).

Amplification products obtained from RT-PCR were extracted from the agarose by a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and directly sequenced using a BigDye Terminator V 3.1 Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystem, Foster City California, USA). Amplification of feline GAPDH was used as the housekeeping gene. The presence of CpG islands was assessed and we submitted the sequence to the online software available at

http://dbcat.cgm.ntu.edu.tw/.

Statistical analysis

IHC results, molecular data and clinicopathologic findings were grouped into contingency tables and analyzed using Fisher's exact test or Chi^2 test. Survival curves were computed using the Kaplan–Meier method and tests for differences in survival, considering all known prognostic factors for FMC, were performed using the log-rank test. OS was defined as the number of days between surgery and death, while the DFI was defined as the number of days between surgery and tumor recurrence and/or evidence of metastasis. Censored data were considered as those cases that were still alive or that did not present with tumor recurrence or metastasis

at the end of the monitoring period. Data were analyzed with MedCalc Statistical Software v.13.3 (MedCalc Software bvba, Ostend, Belgium); $P < 0.05$ was considered statistically significant.

RESULTS

Epidemiologic and clinical data

Sixty queens with mammary lesions were enrolled in this study. Their mean age was 11.3 years (range: 8–17 years) and their epidemiologic and clinical data are summarized in Table 1. Histological examination resulted in 50 FMC and 10 benign lesions. Four normal mammary tissues (from archives) were also evaluated as controls.

Regarding queens with a diagnosis of FMC, at the time of diagnosis, histologically confirmed lymph node metastases were present in 2/50 (4%) cases and no clinically or radiographically observable distant organ metastases were present; 10/50 (20%) queens did not present with tumor recurrence while 40/50 (80%) relapsed between 2 and 23 months after surgery. The median OS recorded was 180 days (range 45–730 days). Forty out of 50 cases developed pulmonary metastases during the follow-up period and died of cancer-related causes as confirmed at postmortem examination. Ten censored cases were included in this study.

Histopathology

The histopathological examination resulted in 10 benign mammary lesions and 50 carcinomas. The histopathological results are summarized in Table 2.

Immunohistochemistry

Immunoreactivity to MSP (Figure 2 and 4) and RON (Figure 3 and 5) proteins, if present, were observed in the cytoplasm of FMC cell lines and carcinomas. IgG control staining is shown in Supplemental Figure S9. Immunoreactive products were not observed in normal mammary glands (Supplemental Figure S10). Regarding the expression of RON and MSP in benign lesions, 3/10 (two adenomas and one fibroadenoma) were positive for RON, while 2/10 (one adenoma and one fibroadenoma) were positive for MSP. In FMCs, RON was positive in 34/50 (68%) of cases and MSP was positive in 29/50 (58%) of cases. A total of 52% of FMCs expressed both RON and MSP, and a statistically significant association was found between the expression of these two antibodies (P <0.05).

No statistical correlation was found between the immunohistochemical results for RON and MSP in relation to the clinicopathologic findings in the 50 FMC cases (Table 3).

Genomic Sequence of Feline RON

Sequences of DNA coding for feline RON from exon 10 to exon 14 were obtained from normal spleen and the sequences of the gene, mRNA and CDS (coding sequence) with exon-intron junctions were submitted to GenBank with the accession number KT248534.

Identification of feline sf-RON

As previously described and to examine the presence of *sf*-RON in our samples, RT-PCR was performed with P1 and P2 primers designed to amplify exon 10 or exon 11 of the feline cDNA. PCR products obtained from the amplification showed two bands, the larger containing intron 10 in the cDNA sequence and co-migrating with normal feline DNA, and a lower band, containing the full-length feline RON but not containing intron 10. Both of the bands obtained were automatically sequenced and this

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revealed that the highest band corresponded to *sf*-RON. *sf*-RON was detected in 18/47 (38.3%) of FMCs (3/50 cases were excluded from analysis because the RNA was degraded) and in 4/6 cell lines; benign tumors and normal mammary gland tissue did not express feline *sf*-RON. Figure 6 shows the rationale used to amplify *sf*-RON. In Supplemental Figure S11, RT-PCR products obtained from cDNA templates from the cell lines are shown.

Statistical analysis revealed that queens that were FMC positive for the *sf*-RON had both a shorter DFI (median 180 days vs. 635 days; HR 2.2, 95% CI, 1.04–4.6) and a shorter OS (median 365 days vs. 755 days; HR 2.2, 95% CI, 1.03–4.7) compared with queens with tumors negative for *sf*-RON (Figure 7 and Figure 8). Moreover, FMC positivity for *sf*-RON was statistically associated with poorly differentiated histological grade (Table 4).

To demonstrate if intron 10 in felines also contained typical CpG island regions acting as alternative promoter regions, the entire sequence was submitted to the software available at http://dbcat.cgm.ntu.edu.tw/. From this analysis, we found that the region corresponding to intron 10 contained more than 66% of the CpG islands (Supplemental Figure S12).

Protein Analysis

Feline mammary carcinoma samples (FMC1, FMC2, and FMC3) corresponding to poorly differentiated (FMC1 and FMC2) and moderately differentiated mammary carcinomas (FMC3) were collected. Protein analysis revealed that all FMCs examined and the FYC cell line expressed the precursor of RON at different levels resulting in a protein of 180 kDa co-migrating with the human control represented by the GTL16 cell line (Figure 9). As shown, the beta chain isoform in humans corresponds to 150 kDa while in feline samples it corresponds to 135 kDa, as

previously demonstrated by the same authors,[11] and it was present in all analyzed FMCs at different levels.

Conversely, in all samples, a specific band corresponding to 55 kDa was detected, confirming the presence of the short form in feline samples and in the FYC cell line that expresses *sf*-RON, as also shown in RT PCR (see Supplemental Figure S11); feline spleen resulted in negative findings for both isoforms (Figure 9). The presence of additional protein bands migrating at 72–90 kDa should be referred to as HMW-sfRON (high molecular weight–sfRON) as already demonstrated in human breast cancer,[18,19] which is recognized by the same antibody.

DISCUSSION

In this article, we provide evidence that the truncated form of RON kinase is expressed in feline mammary tumors and that its expression represents a prognostic factor in this type of tumor. The presence of the RON transcript in feline mammary tumors was first demonstrated in 2002 when the authors partially identified the gene coding for *full-length* RON protein.[11] In humans and mice, the gene coding for *sf*-RON is regulated by an internal promoter (P2) located in intron 10[1] as shown in Figure 1. To demonstrate if this isoform was also present in felines, the authors first determined the genomic sequence of feline RON from exon 8 to exon 14 and its intron/exon junctions; these data showed a high homology with human, canine and murine sequences and were submitted to the GenBank database. On the basis of the feline intron 10 sequence and adopting the molecular approach published by Bardella and colleagues (2004),[3] the authors evaluated the presence of both *sf*-RON by endpoint RT-PCR in cDNA from FMC cell lines and the RON protein from tissue samples from feline mammary tumors. The results obtained from RT-PCR on tissues

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demonstrated the presence of *sf*-RON in 38.3% of feline mammary carcinomas and in 4/6 of feline mammary cell line transcripts. In all of the samples, the sequence of amplicons revealed the insertion of intron 10 (P2). Moreover, in benign lesions and normal mammary glands, *sf*-RON was not detected. These results were confirmed by western blot analysis of FMCs samples and the FYC cell line. In Figure 9, all samples, at different levels, showed the mature form of the feline RON migrating at 135 kDa, as previously demonstrated by the same authors,[11] while *sf*-RON is detectable at 55 kDa in feline FMCs, FYC cell lines and human controls represented by GTL16; feline spleen was negative for both isoforms. In this experiment, the authors also detected also protein products migrating at 72–95 kDa. These proteins, as demonstrated in human breast cancer[18], can be referred to other isoforms called *sf*-RON-HMW (short form high molecular weight) derived from further different splicing of the human RON gene to be able to generate protein products of approximately 72–95 kDa.[19] The identification of sf-RON-HMW isoforms was also performed in human breast cancer tissues using the same antibody by Liu et al,[18] and this suggests that the presence of these isoforms should be investigated in the feline.

Overall, these data suggest that in feline species *sf*-RON originates, as in humans, from the alternative promoter (P2) located in intron 10. In particular, analyzing the nucleotide sequence "corresponding" to intron 10, we found a high percentage of possible CpG islands with a GC content of 66%, suggesting, as in humans, that RON gene transcription may be regulated by a promoter in the CpG islands region. Moreover, the presence of *sf*-RON was significantly associated with a poorly differentiated histological grade, with a shorter disease-free (DFI) period and a shorter survival (OS). Regarding this aspect, there is no data available as to the

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prognostic value of *sf*-RON in breast cancer patients. Otherwise, our results according to the human literature suggest that the presence of sf-RON confers aggressiveness of breast cancer in *in vitro* and *in vivo* conditions and it is able to convert slow-growing and nonmetastatic tumors into rapidly growing tumors and promotes epithelial–mesenchymal transitions via PI3K pathways[18], confirming the importance of this protein in breast cancer progression, as already demonstrated in feline mammary carcinomas.[21,22]

Immunohistochemical expression of the RON receptor and its specific ligand MSP were observed in 68% and 58% of FMCs, respectively, while 52% of cases coexpressed both proteins. However, no correlation was observed between immunohistochemical expression of the RON receptor or its ligand MSP with clinicopathologic and survival data. The antibody used in this study for the detection of RON protein (C-20) recognizes the COOH terminus of the protein (intracellular portion), which is present in both isoforms (full and short). Thus, IHC data could not differentiate between the two isoforms in tissues. These considerations could explain the lack of correlation between IHC expression of RON/MSP and the clinical outcomes compared with expression of sf RON assessed by RT-PCR.

Our IHC data confirm our previous results in feline mammary tumors [11] and are similar to the human literature where RON is expressed in 50% of primary breast carcinomas but does not represent a prognostic factor.[20]

No data are available in human breast cancer regarding the association between the presence of *sf*-RON and the clinical findings, although it is known that this isoform, through its interaction with PI3K, is able to promote metastasis.[18]

The relevance of *sf*-RON in human cancer is an emerging and interesting topic due to the development of specific molecules able to inhibit RON receptor activity[34] and

recently the potential use of molecules able to inhibit RON have been studied in cancers of the pancreas, lung, colon and breast. [6,16,17,35] As previously discussed, FMC highly resembles human breast cancer, sharing histopathological, clinical and molecular features [12,14,33]. In this study, we demonstrated that *in vitro* and *in vivo* feline mammary tumors present with the truncated form of RON and its presence is significantly associated with high grade of tumor and a worse prognosis.

As shown in Figure 7 and Figure 8, after 100 days there is no apparent effect of *sf*-RON on the DFI and OS, but its role seems to appear in a later phase. This tendency suggests that in the early stages of tumor progression *sf*-RON is not involved, but in the later stages, further additional pathways participate in tumor progression.

These results support FMC as a suitable model in comparative oncology and identify *sf*-RON expression as having potential in predicting the outcomes of this disease.

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Figure legends

Figure 1: RON/stk gene structure and transcriptional regulation of the full length and short form, respectively.

Figure 2: cell line from metastasis of mammary carcinoma, cat, case FNNm. Immunocytochemistry for MSP. Moderate positivity (score 2+) in all neoplastic cells

Figure 3: cell line from metastasis of mammary carcinoma, cat, case FNNm. Immunocytochemistry for RON. Moderate positivity (score 2+) in all neoplastic cells

Figure 4: Adenocarcinoma, mammary gland, cat. Immunohistochemistry for MSP. Moderate membrane stain for MSP (score 2+) in neoplastic cells. .

Figure 5: Adenocarcinoma, mammary gland, cat. Immunohistochemistry for RON. Moderate membrane stain for RON (score 2+) in neoplastic cells.

Figure 6: Regulation of full length and short forms of feline RON gene receptor and RT-PCR products obtained from FMCs (feline mammary carcinomas). The higher and lower bands correspond respectively to short and full-length forms of RON. K1-K7 corresponds to feline mammary carcinomas while NTC is the non-template control. GAPDH was used as the housekeeping gene control.
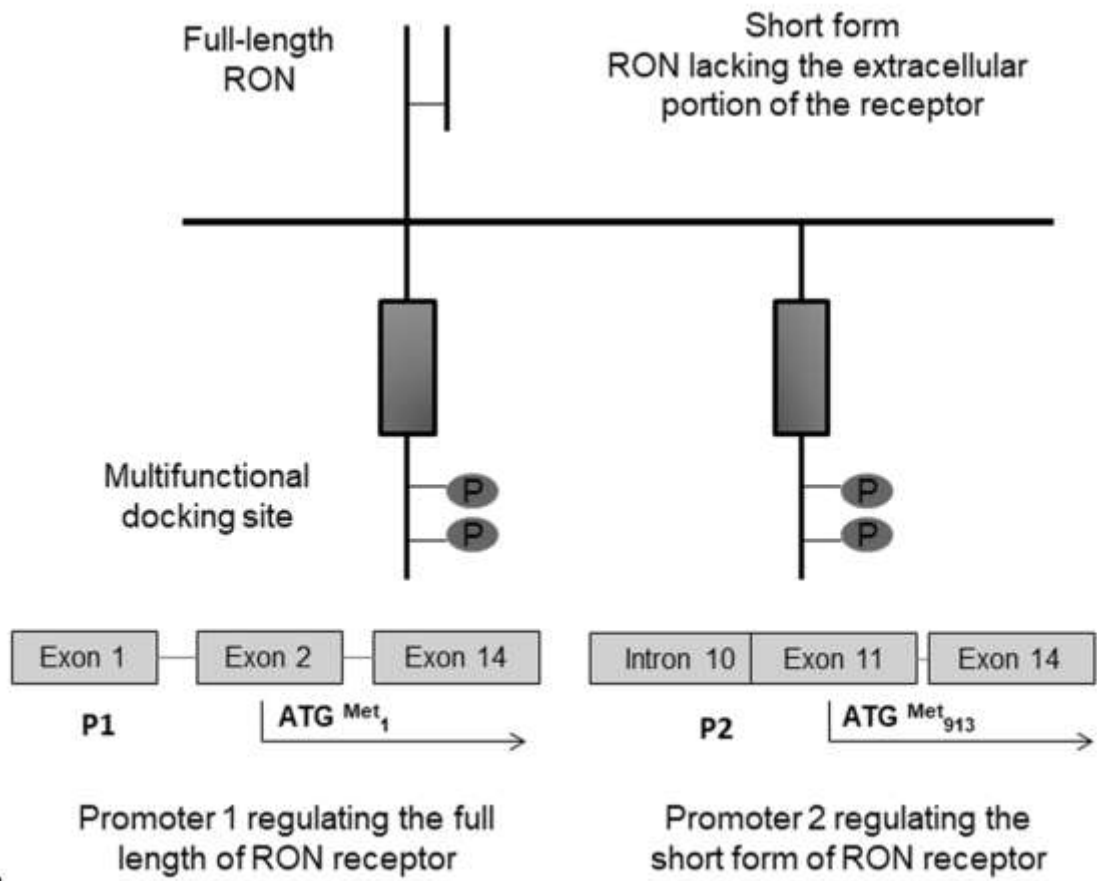
Figure 7: Kaplan–Meier survival curve of disease-free intervals (DFI) in FMCs (feline mammary carcinomas): total mean DFI: 407 days; mean sf/RON positive samples 326 days; mean sf/RON negative samples 510 days (log-rank test: $P < 0.05$). The expression of sf RON has been determined by RT-PCR.

Figure 8: Kaplan–Meier survival curve of overall survival (OS) in FMCs (feline mammary carcinomas): total mean OS: 466 days; mean sf/RON positive samples 387 days; mean sf/RON negative samples 568 days (log-rank test: $P < 0.05$). The expression of sf RON has been determined by RT-PCR.

Figure 9: Western blot against RON of protein lysates from three feline mammary carcinomas (FMC1, FMC2 and FMC3), FYC cell line, feline spleen and the human GTL16 cell line. fl-RON and sf-RON are present in all FMCs samples at different levels. Feline spleen showing non-specifc bands and GTL16 represent, respectively, negative and human positive controls.
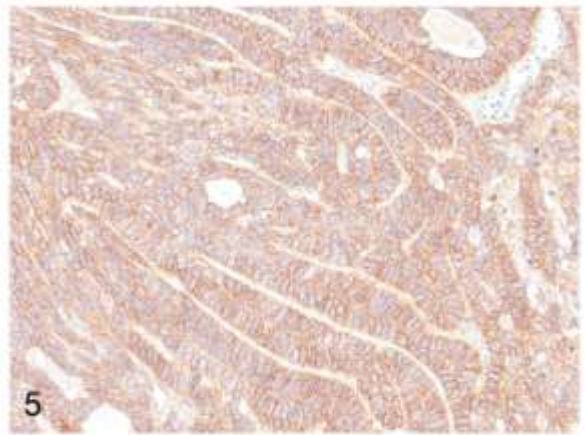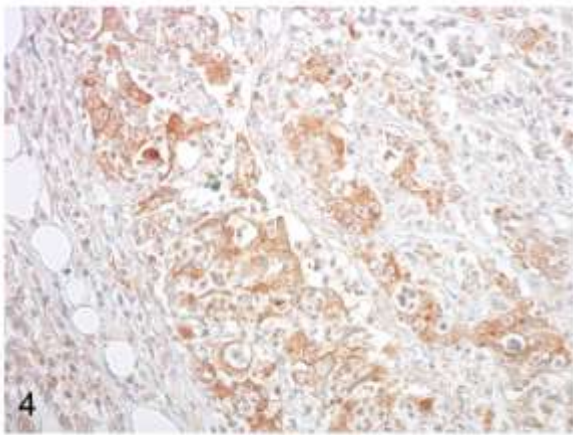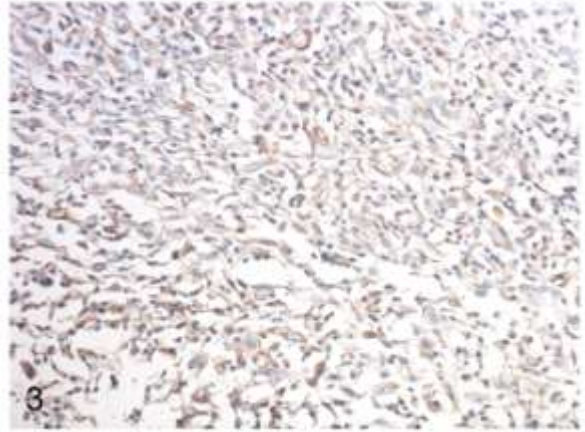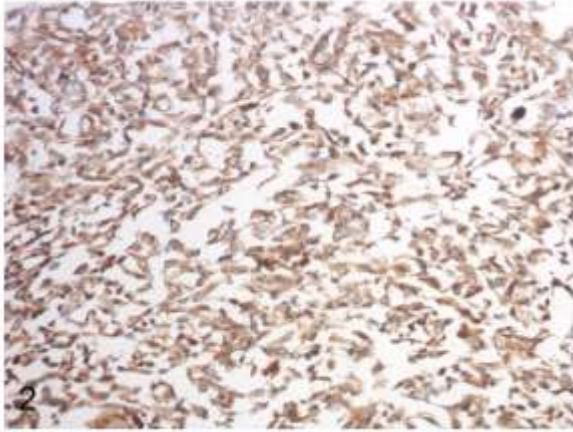
Table 1 Relation between Clinico-pathologic features and Immunohistochemical results

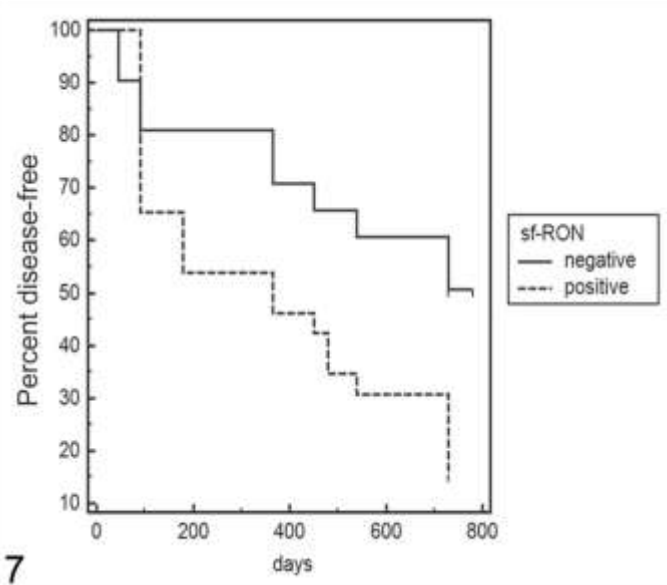| Clinicopathologic characteristics	RON+ n (%)	RON- n (%)	P	MSP+ n (%)	MSP - n (%)	P
			**			
MSP+	26 (52%)	3 (6%)	< 0.001			
MSP-	8 (16%)	13 (26%)				
Total	34	16				
>1 cm	24 (48%)	14(28%)	>0.05	21 (42%)	17 (34%)	>0.05
≤1 cm	10 (20%)	2 (4%)		8 (16%)	4 (8%)	
Total	34	16		29	21	
Grade I	8 (16%)	1 (2%)	>0.05	4 (8%)	5 (10%)	>0.05
Grade II	11 (22%)	8 (16%)		10 (20%)	9 (18%)	
Grade III	15 (30%)	7 (14%)		15(30%)	7 (14%)	
Total	34	16		29	21	
Vascular invasion	8 (16%)	5 (10%)	>0.05	9 (18%)	4 (8%)	>0.05
No vascular invasion	26 (52%)	11 (22%)		20 (40%)	17 (34%)	
Total	34	16		29	21	
Relapsed 0-6 months	13(26%)	11 (22%)	>0.05	14 (28%)	10 (20%)	>0.05
Relapsed 6-12 months	7 (14%)	3 (6%)		7 (14%)	3 (6%)	
Relapsed >12 months	5 (10%)	1 (2%)		2 (4%)	4 (8%)	
Not relapsed	9 (18%)	1 (2%)		6 (12%)	4 (8%)	
Total	34	16		29	21	

Figure 1

Figures 2-5
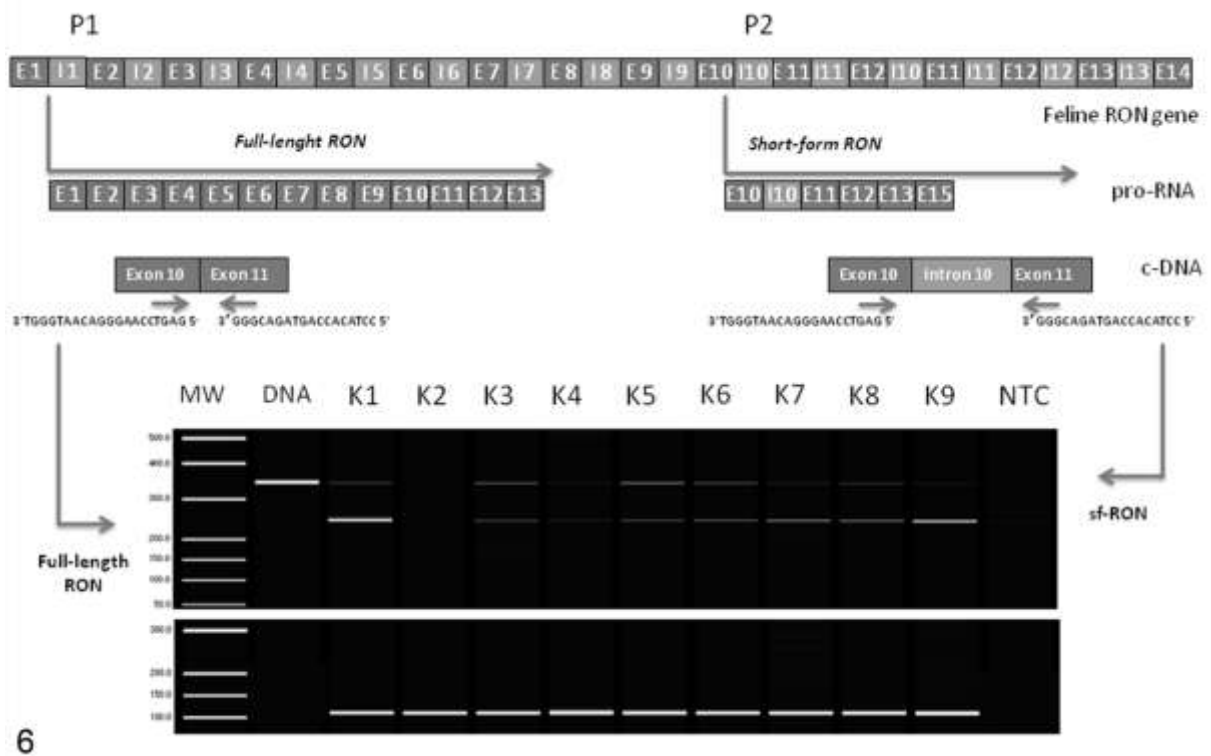
Figure 6

Figure 7

Figure 8

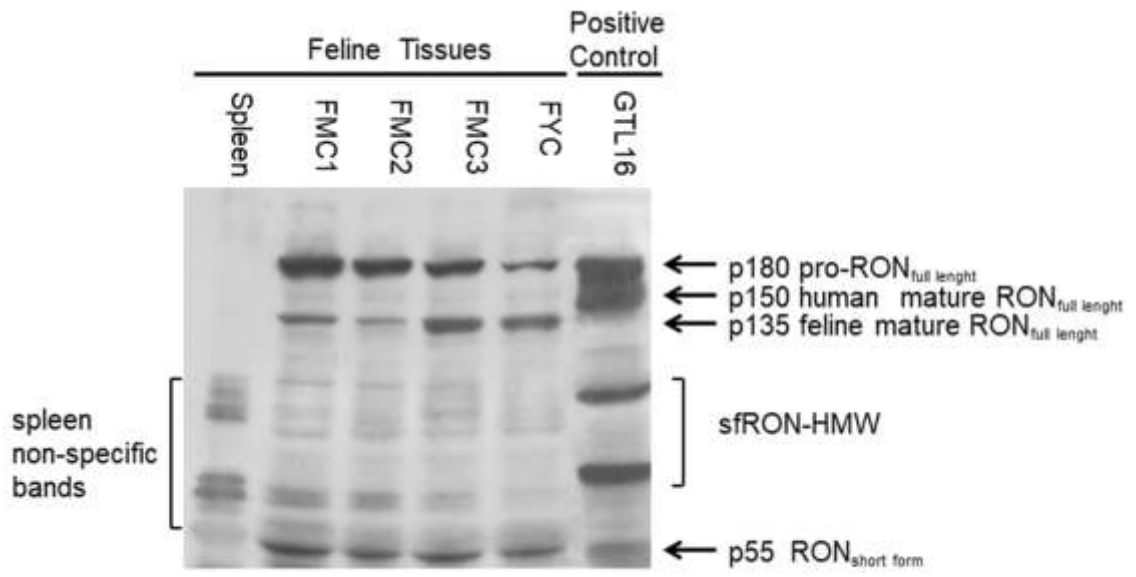Figure 9