Kit receptor tyrosine kinase dysregulations in feline splenic mast cell tumours

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Abstract (150 w)

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Introduction

Visceral mast cell tumours (MCTs) are much more common in cats than in dogs, with more than 50% of cases occurring in extracutaneous sites, and account for 15–26% of feline splenic disease. (Spangler and Culbertson, 1992; Henry and Herrera, 2013)

Cats with splenic mast cell tumours (SMCTs) may have signs of systemic illness, including lethargy, anorexia, weight loss, and intermittent vomiting. Palpation of the abdomen reveals massive splenomegaly in most affected cats and, occasionally, peritoneal effusion. (Liska et al., 1979; Withrow, 2013; Henry and Herrera, 2013)

One third of cats with SMCT are anaemic, and up to 50% have evidence of bone marrow and peripheral blood involvement. (Carpenter, 1987; Withrow, 2013) Metastatic spread to the liver is also frequent. (Henry and Herrera, 2013)

Furthermore, the coexistence of splenic and cutaneous disease has been increasingly reported, especially in cats presenting with multiple skin lesions, and it is not clear in those cases which primary form of the tumour is more prevalent. (Litster and Sorenmo, Henry and Herrera, 2013) Some authors have hypothesised an origin of feline visceral MCT from haematopoietic cell precursors, supporting a multicentre, "liquid" growth. (Garner and Ligeman, 1970)

Splenectomy is the treatment of choice, even in the presence of multi-organic disease, resulting in a reported median survival time of 19 months. (Liska et al., 1979; Henry and Herrera, 2013) The role of chemotherapy for the adjuvant treatment of feline MCT has not been clearly established. Similarly, there is no proven utility for corticosteroid administration. (Henry and Herrera, 2013)

The c-KIT proto-oncogene encodes the tyrosine kinase receptor Kit, which regulates mast cell survival, proliferation, differentiation, and migration. (Columbo et al., 1992; Meininger et al., 1992) In canine MCT, juxtamembrane domain c-KIT mutations and aberrant cytoplasmic Kit protein localization have been associated with increased cellular proliferation and with both

reduced progression-free and overall survival. (Webster et al., 2006; Webster et al., 2007) Consequently, Kit receptor tyrosine kinase inhibitors (TKIs) have entered as an active part of MCT treatment in dogs. (Hahn et al., 2008; London et al., 2009)

Aberrant cytoplasmic Kit immunohistochemical expression has been reported in 29% to 67% of feline cutaneous MCTs, and a higher frequency was observed in tumours with unfavourable outcome. (Rodriguez-Cariño et al., 2009; Sabattini et al., 2010; Mallett et al., 2013) Furthermore, between 62% and 68% harbour c-KIT mutations in exons 8 and 9, and there are anecdotal reports of beneficial response to molecular targeted therapy. (Isotani et al., 2009; Sabattini et al., 2013) These findings suggest that TKIs may offer a new treatment avenue for selected cases of feline mast cell neoplasia.

In a report by Dank *et al*. (2002) no KIT mutation was identified in 10 cats with splenic MCT; (Dank et al., 2002) however, exons 8 and 9 had not been investigated in that study and, since then, no specific research has been carried out to assess the true frequency of Kit receptor dysregulations in feline splenic MCTs. A better understanding of the role that Kit plays in the development of feline SMCTs may possibly provide help in the clinical approach and treatment of this disease.

The present study was aimed at characterizing Kit receptor dysregulations (*i.e.,* cytoplasmic immunohistochemical expression and/or *c-Kit* mutations) in a series of feline splenic mast cell tumours. Specifically, our goals were: (1) to assess whether aberrant cytoplasmic expression of Kit could be regarded as indicative of c-KIT mutations, and (2) to evaluate the relationship between Kit dysregulation and tumour morphology, proliferation, and overall survival.

Materials and Methods

Inclusion criteria and histological parameters

Formalin-fixed and paraffin-embedded (FFPE) surgical samples of feline splenic mast cell tumours were retrieved from the archives of the Department of Veterinary Medical Sciences (University of Bologna, Italy) and Laboratoire IDEXX Alfort (Alfortville, France). Four micrometers histological sections stained with haematoxylin and eosin were reviewed and a metachromatic stain (toluidine blue) was applied in all cases to confirm the diagnosis and to evaluate the amount of cytoplasmic granules. Table 1 summarizes the quantitative and qualitative histological parameters evaluated.

Immunohistochemical analysis

Kit protein expression pattern was evaluated immunohistochemically. Tumour sections were immunolabelled by using a commercial anti-human antibody (CD117, rabbit polyclonal, Dako, Glostrup, Denmark) with validated reactivity in feline tissues. (Sabattini et al., 2013; Sabattini et al., 2015)

Endogenous peroxidase activity was blocked by incubation for 30 mins with 3% hydrogen peroxide in distilled water. For antigen retrieval, slides were microwaved in citrate buffer (pH 6.0), for two cycles of 5 mins, at 750 W. Following incubation in a blocking solution (10% goat serum) for 30 minutes at room temperature, slides were incubated overnight at 4°C in a humid chamber with the primary antibody; CD117 was diluted 1:100 in phosphate-buffered saline.

Sites of primary antibody binding were identified using a secondary biotinylated antibody (1:200 in blocking solution, Dako) for 30 minutes at room temperature. Sections were then incubated with a commercial streptavidin-biotin-peroxidase kit (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, California, USA) and 3,3'-diaminobenzidine (DAB tablets, Diagnostic BioSystems, Pleasanton, California, USA) was used as chromogen. Sections were counterstained with Papanicolaou's haematoxylin, dehydrated, and mounted.

A feline cutaneous MCT was used as positive control. Negative controls were obtained by omitting the primary antibody. Table 1 reports the methods for the evaluation of immunopositivity.

Mutation analysis

Genomic DNA was purified from 10 µm sections (3 to 5) of FFPE tumour samples by using a commercial kit (AllPrep DNA/RNA FFPE kit, Qiagen, Hilden, Germany) following manufacturer's instructions. The extracted DNA concentration and purity were measured using Nanodrop ND-1000 spectrophotometer.

The entire sequence of exons 8, 9, 11 and 17 of feline KIT were PCR amplified using the oligonucleotides already described.(Isotani et al., 2010) Furthermore, for exons 8 and 9, whenever the full length amplicon could not be obtained using those primers, the target was amplified using different combinations of the same outer and novel inner primers, as schematically indicated in Figure 1. Detailed primer sequence is reported in Table 2.

Two microliters of diluted DNA (up to 50 ng/µl) were used as a template for the PCR amplification. Reactions were carried out in a T-Personal thermocycler (Biometra GmbH, Göttingen, Germania) by using 0.5 µM of each primer (Tema Ricerca, Castenaso, Italy), 0.02 U/µl of Q5 High Fidelity DNA polymerase (EuroClone, Pero, Italy), 200 µM deoxyribonucleotide triphosphate mix and 1× Q5 buffer (final concentrations). The following PCR conditions were used: an activation step at 98°C for 45 s, 35 cycles of 15 s at 98°C, 15 s (exons 8 and 9 shorter fragments) or 30 s (full length amplicons) at 56°C (full length exons 9, 11, 17) or 58°C (for all the remaining amplicons), 20 or 40 s at 72°C (for exons 8 and 9 shorter fragments and full length amplicons, respectively), and a final extension step of 2 mins at 72°C. Amplicons were visualized in a 1% agarose gel electrophoresis. All the PCR products were sequenced on an automated sequencer by BMR Genomics (Padua, Italy) and Macrogen Europe (Amsterdam, The Netherlands). Amplicons were sequenced starting from

both forward and reverse primers used for the PCR amplification and from at least two different PCR products.

Obtained electropherograms were analyzed by FinchTV tool (Geospiza, Seattle, WA, USA). The alignment of the wild type sequence available in the database Ensembl Genome Browser with sequences obtained in this study was performed using Multalin interface page (http://multalin.toulouse.inra.fr/multalin/).

Medical records

Patient records were reviewed to collect demographic information.

Referring veterinarians and/or owners were contacted for additional information, including history, clinical stage, laboratory findings, ultrasound/surgical findings, treatment details and outcome. The availability of these data was not considered among inclusion criteria.

Statistical analysis

When appropriate, data sets were tested for normality by use of the D'Agostino and Pearson omnibus normality test. Values were expressed as mean ± standard deviation in case of normal distribution, or as median with a range in case of non-normal distribution. Differences in qualitative and quantitative variables were assessed by Fisher's exact test and analysis of variance, respectively.

Overall survival (OS) was defined as the time (days) from the date of diagnosis to the last reported date that the patient was seen alive. The patient status was recorded as alive, dead because of non MCT-related causes or dead because of MCT-related causes. The log-rank test was used to compare survival distributions.

Data were analysed by use of a commercial software program (SPSS Statistics v 19, IBM); P values ≤ 0.05 were considered significant.

Results

Medical records

Twenty-two cats with a histologically confirmed splenic mast cell tumour were included in the study. There were 21 domestic shorthair and one domestic longhair. Age ranged from 6 to 16 years, with a mean of 11.6 ± 2.42 years. There were 8 males (36.4%) and 14 females (66.7%). Vomiting, dysorexia and weight loss were the most common clinical signs at presentation. Four patients had liver metastases. Mastocytemia was investigated in only 5 cases and was reported in 2. Bone marrow cytology, performed in 3 cases, was positive for MCT infiltration in 2. Two cats had also a single cutaneous nodule at presentation and four later developed disseminated cutaneous MCTs.

One cat with massive neoplastic diffusion was euthanized immediately following diagnosis, one cat only received corticosteroids. The remaining 20 underwent splenectomy: 12 were treated with splenectomy alone, 7 received adjuvant corticosteroids, and one cat received adjuvant lomustine and a tyrosine kinase inhibitor (imatinib).

Follow-up information was available in 16 cases. Median survival time was overall 780 days (range, 1-1219). The presence of an exclusive splenic involvement (negative staging) was significantly (P = 0.042) associated with longer survival times (807 days versus 120 days). There was no significant difference between cats treated with surgery alone or surgery plus medical therapy.

Histological examination

In 18 cases (81.8%) the mastocytic infiltrate was diffuse, whereas 4 MCTs (18.2%) showed a lower cellularity and splenic infiltration was multifocal.

Eight tumours (36.4%) were classified as well-differentiated and were composed by sheets of round to oval cells, 10-15 µm in diameter, with distinct cell borders. Cytoplasms were moderate to abundant, faintly granular, basophilic or pale eosinophilic. Nuclei were round to

oval, with finely dispersed chromatin and inconspicuous nucleoli. The size of cells and nuclei within the neoplasm was rather uniform (variations < 50%). (Figure 1).

Nine tumours (40.9%) were classified as moderately differentiated and were characterized by sheets of cells, 15-20 µm in diameter, with mostly distinct cell borders and low amounts of eosinophilic cytoplasm. Nuclei were round or occasionally indented, with irregularly stippled chromatin and a small single central nucleolus visible in most cells. A variation of 50-100% in nuclear size was recorded (Figure 1).

Five tumours (22.7%), classified as poorly differentiated, were composed by polygonal cells, 15-20 µm in diameter, with mostly indistinct borders and scant, amphophilic cytoplasm. Nuclei were large, vesicular, often indented or bizarre, with prominent nucleoli. A variation of up to 100% in nuclear size was present (Figure 1).

Mitotic index (MI) ranged from 0 to 6 (mean, 2.2 ± 1.94). MI was significantly higher in moderately or poorly differentiated SMCTs (mean MI, 2.8) compared with well-differentiated SMCTs (mean MI, 1.1; $P = 0.05$).

Eosinophilic infiltrate was moderate to high in 2 cases (9.1%); in the remaining 20 cases (90.9%), eosinophils were low to absent. Necrosis was observed in 4 cases (18.2%). Cytoplasmic granules were evaluated with toluidine blue stain and rated low in 3 cases (13.6%), moderate in 8 (36.4%) and high in 11 (50%). There was no statistical relationship among histological parameters, nor between histopathological parameters and survival.

Kit pattern

Twenty-one cases were immunoreactive for Kit protein. Three tumours (14.3%) revealed a prevalent membranous expression (Kit pattern 1), 11 (52.4%) had a focal cytoplasmic expression (Kit pattern 2) and 7 (33.3%) a diffuse cytoplasmic expression (Kit pattern 3; Figure 2). There was no statistical relationship between Kit pattern and tumour differentiation, mitotic activity or survival.

Mutation analysis

Mutation analysis was carried out on all samples. In two cases (9.1%) amplification was not successful due to the poor quality of extracted DNA, so the following information will refer to a total of 20 cases.

Missense mutations (altering the amino acid composition of Kit) were detected in 13 out of 20 tumours (65%). Eleven (19%) were located in exon 8, and 3 (71%) in exon 9; one tumour had two concurrent mutations in exons 8 and 9. No mutations were detected in exons 11 and 17. The location of the observed mutations is illustrated in Figure 3. The mutations found in exon 8 were three internal tandem duplications (ITD) and one deletion-insertion. Seven cases (35%) presented the same ITD, consisting in the duplication of amino acids in position 415-418 and "QILT" insertion (c.1245_1256dup; p.418T_419H ins QILT). The other two ITDs, observed in 2 and one case, respectively, were c.1246_1257dup (p.419H_420E ins ILTH) and c.1247_1261dup (p.420E_421S ins ILTHE). The remaining mutation, resulting in the introduction of a stop codon (c.1254_1263 delinsGTAA), was observed in one case (Figure 3).

In exon 9, two types of single nucleotide polymorphisms (SNPs) were detected: c.1430 G>T (p.477S>I), occurring in two cases and c.1426 C>A (p.476Q>K), occurring in one case (Figure 3). Silent mutations were observed in other 3 cases.

All the mutated tumours displayed an aberrant (focal or diffuse cytoplasmic) Kit pattern (P = 0.031), but a cytoplasmic expression was also encountered in 4 out of 7 (57.1%) non mutated tumours.

The mutational status was not apparently associated with tumour morphology or mitotic activity. There were no significant differences in survival time between mutated or non mutated SMCTs, nor between tumours harbouring an exon 8 ITD and the other cases (Table 2).

Discussion

The goal of this study was to investigate the nature and incidence of Kit dysregulations in feline splenic mast cell tumours.

Given the infrequency of this disease, the analysis was performed on a relatively small number of cases, which may have resulted in loss of statistical power. The retrospective nature of the study is a further limitation, as cases lacked uniformity in staging and therapy protocols.

We confirm a greater frequency of this disease in geriatric patients, with an age of onset (mean, 12 years) of some years higher than that of cutaneous MCT (mean, 8-9 years), and a clinical presentation characterized by nonspecific gastrointestinal symptoms, with the exception of the presence, in several patients, of a palpable abdominal mass. In more than 20% of cases there were concurrent skin nodules. In no case, however, it was possible to establish with certainty the primitive location.

Compared with dogs, in feline MCT staging procedures are far less consolidated. This is probably due to the lower frequency of MCT in cats and to the fact that the biological behaviour of these tumours is considered less aggressive. While this is generally true for the localized cutaneous forms, it does not apply to visceral MCTs or systemic mastocytosis; in these cases, accurate staging and close monitoring can drastically affect prognosis. In fact, the presence of a disease limited to the spleen was the only factor significantly affecting survival in this study.

At present, there are no available studies describing the histological features of FSMCT. The observation of our cases has not led to the identification of histological types similar to those reported in cutaneous MCTs. The evidence, in a limited number of cases, of a multifocal pattern, as opposed to the more frequent diffuse pattern, would likely be attributable to an early stage neoplastic infiltration. Nevertheless, we have identified different degrees of

differentiation and, as previosuly observed in cutaneous and intestinal MCTs, a poor cell differentiation appears to match a high mitotic activity, the only recognized histological prognostic factor in feline MCT. (Sabattini et al., 2013; Sabattini et al., 2015) It is important to point out that the attribution of the degree of differentiation was based in this study solely on the morphological appearance; the distribution and density of granules have been evaluated separately and were not correlated to the degree of differentiation. This also confirms previous studies on feline cutaneous and intestinal MCTs. (Sabattini et al., 2013; Sabattini et al., 2015)

So far, c-KIT mutations of have been detected with a frequency of 56% in cutaneous MCTs, whereas they have not been identified in intestinal MCTs. (Sabattini et al., 2013; Sabattini et al., 2015) Although studies have been published evaluating mutations in feline MCT with unspecified location, (Isotani et al., 2010) this is the first time that c-KIT mutation analysis is performed specifically on a cohort of FSMCT.

One possible limitation derives from the fact that the starting material for mutational analysis were tissue samples fixed in formalin and embedded in paraffin. This can lead to an underestimation of the mutations due to the potential presence of PCR inhibitors (solvents, paraffin), or because of poor DNA quality (excessive fragmentation following formalin fixation). In order to improve the results of sequencing, for exons 8 and 9 we adopted the strategy of the amplification of shorter fragments,. When unsuccessful, the phases of extraction and amplification were repeated several times and it was ultimately possible to carry out mutation analysis in 20 cases out of 22.

The mutations identified in this study are very similar for frequency and type to those previously reported in feline cutaneous MCTs. (Isotani et al., 2010; Sabattini et al., 2013) This can represent a further confirmation of the intimate connection between the splenic and cutaneous forms, as opposed to the intestinal form. All the mutations were located at the level

of exons 8 and 9, corresponding to the fifth immunoglobulin-like domain (IgD5) of Kit receptor. As in cutaneous MCTs, two hot spots were identified at the level of residues 415-421 (exon 8) and 476-477 (exon 9), but while in cutaneous MCTs exon 9 mutations clearly prevailed, mutations in SMCT seem to occur much more frequently at the level of exon 8. These mutations were located in a neighbouring residue of mutations that have been reported in patients with pediatric mastocytosis and have been uncommonly described in canine mast cell tumour. (Lanternier et al., 2008; Letard et al., 2008) Mutations at this level were considered to affect the affinity of the hemophilic dimerization of Kit, resulting in the constitutive activation of the kinase domain without ligand binding. In particular, the duplication c.1244_1255dup, consisting of the insertion of 4 amino acids (glutamine, isoleucine, leucine, and threonine) between residues 418 and 419, was the most frequent mutation observed in previous studies. (Hadzijusufovic et al., 2009; Isotani et al., 2010; Sabattini et al., 2013) The insertion reported here (AAATCCTGACTC) is shifted to a base compared with that previously reported (AATCCTGACTCA), but results in the same protein change.

Exon 9 mutations were also previously reported and mutations at that level were demonstrated to cause ligand-independent activation of Kit. (Isotani et al., 2010; Takanosu et al., 2012) Mutations around this residue are not observed in human tumours; however, identical substitutions were reported on the corresponding codon of Ser479 in canine MCT as an activating mutation. (…)

In the dog a good degree of correlation is observed between the presence of genomic mutations and aberrant protein localization, although mutational analysis remains the gold standard for the selection of candidates for targeted therapies. Previous studies on feline cutaneous MCT failed to demonstrate a statistically significant association between the presence of mutations and Kit pattern. (Sabattini et al., 2013) In this study, the association

was statistically significant, although we observed a very short incidence of membranous pattern compared with the aberrant pattern, even in non-mutated cases, so we can conclude that immunohistochemistry cannot be considered as a surrogate marker for mutation analysis in feline MCTs. Moreover, as in the previous studies, we did not evidence a correlation between mutations and survival time, mitotic activity or degree of differentiation; suggesting a secondary role of c-KIT mutations in affecting tumour biological behaviour As a possible explanation, these mutations may simply reflect a genomic instability (passenger mutations) and do not provide any substantial advantage to the neoplastic population.

In conclusion, mutations of KIT are present in more than 60% of feline splenic MCTs and are mostly represented by internal tandem duplications at the level of exon 8. However, their assessment for prognostic purposes appears to be of limited value in this species.

The predictive role of Kit receptor remains to be investigated by evaluating the response to tyrosine kinase inhibitors of FSMCT in relation to the mutational status.

References

Figure legends

Figure 1. Feline splenic mast cell tumour (FSMCT), representative examples of tumour degree of differentiation. (A) Well differentiated FSMCT, (B) moderately differentiated FSMCT and (C) poorly differentiated FSMCT. Haematoxylin and eosin; x400 magnification.

Figure 2. Photomicrographs of the different patterns of Kit immunostaining. (A) Kit pattern 1, (B) Kit pattern 2 and (C) Kit pattern 3. CD117 immunohistochemistry; haematoxylin counterstain; x1000 magnification.

Figure 3. Alterations in the nucleotidic and aminoacidic sequences of feline c-kit (exons 8 and 9) observed in 20 splenic mast cell tumours.