Short communication

Evaluation of tyrosine-kinase receptor c-kit mutations, mRNA and protein expression in canine lymphoma: Might c-kit represent a therapeutic target?

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Abbreviations: AML, acute myeloid leukemia; AU, arbitrary units; CGI-119, transmembrane BAX inhibitor motif containing 4; c-kit, tyrosine kinase receptor c-kit; FNA, fine needle aspirate; FSC, forward scatter; HG, high grade; HL, Hodgkin lymphoma; ITDs, internal tandem duplications; LG, low grade; MFI, mean fluorescence intensity; nHL, non-Hodgkin lymphoma; qPCR, quantitative real-time RT-PCR; RQ, relative quantification; SEM, standard error medium; SS, side scatter; TKIs, tyrosine kinase inhibitors; VEGF, vascular endothelial growth factor.

Abstract
c-kit plays an important role in proliferation, survival and differentiation of hematopoietic progenitor cells. In human hematopoietic malignancies, c-kit is mostly expressed by progenitor cell neoplasms and seldom by mature cell neoplasms. Aim of this study was to evaluate c-kit expression in canine lymphoma. Twenty-five B-cell lymphomas and 21 T-cell lymphomas were enrolled in the study, c-kit mRNA and protein expression was measured in lymph node fine needle aspirates by quantitative real-time RT-PCR, flow cytometry and immunocytochemistry, while the occurrence of KIT mutations on exons 8-11 and 17 was investigated by direct cDNA sequencing. KIT mRNA was amplifiable but below the limit of quantification in 76% of B-cell lymphomas and 33% of T-cell lymphomas. Remaining samples showed a very low expression of KIT, except for some high grade (HG) T-cell lymphomas where a comparatively higher mRNA amount was observed. Transcriptional data were confirmed at the protein level. No gain-of-function mutations were observed. Among canine lymphomas, T-cell lymphoma typically shows an aggressive biological behavior, partly being attributable to the lack of efficacious treatment options, and the evidence of c-kit expression in HG T-cell lymphomas might represent the rationale for its routinely diagnostic evaluation and the use of tyrosine kinase inhibitors in future clinical trials.

Keyword: c-kit, Dog B-cell lymphoma T-cell lymphoma Tyrosine kinase inhibitors
1. Introduction

Lymphoma is the most frequent hematopoietic neoplasm in dog, and it shares some clinical, cytological, histopathological and molecular similarities with human non-Hodgkin lymphoma (nHL, Valli et al., 2002; Breen and Modiano, 2008; Adam et al., 2009). The understanding of lymphoma biology has significantly progressed in the last decade, and new data on the heterogeneity of clinical presentation, behavior, response to treatment and prognosis are now described in literature (Marconato, 2011). In this context, the dog has been proposed as a model for preclinical evaluation of new therapeutic approaches (Marconato et al., 2012).

The tyrosine kinase receptor c-kit, also referred to as stem cell factor receptor, proto-oncogene KIT or CD117, is a transmembrane glycoprotein that shows structural similarities with the platelet-derived growth factor and macrophage colony-stimulating factor-1 receptors (Edling and Hallberg, 2007). The receptor consists of a glycosylated extracellular domain containing five immunoglobulin-like repeats, a single hydrophobic transmembrane domain and a cytoplasmic domain containing a split tyrosine kinase catalytic domain. The stem cell factor binding to c-kit leads to receptor dimerization and trans-phosphorylation of tyrosine residues, followed by interaction and phosphorylation of specific cytoplasmic signaling proteins (Advani, 2006).

The proto-oncogene KIT is constitutively expressed in myeloid progenitor cells, dendritic cells, mast cells, pro-B and pro-T cells, and plays an important role in proliferation, survival and differentiation of hematopoietic progenitor cells (Advani, 2006). The B and T cells lose the c-kit expression upon cell differentiation, whereas mast cells, natural killer cells and dendritic cells retain expression of c-kit, lending a crucial role for this factor (Ray et al., 2008).

In humans, c-kit expression has been well documented in different hematopoietic neoplasms, like acute myeloid leukemia (AML, Escribano et al., 1998), granulocytic sarcoma (Chen et al., 2001), systemic mastocytosis (Natkanem and Rouse, 2000), T-cell acute lymphoblastic leukemia (Sykora et al., 1997) and multiple myeloma (Ocqueteau et al., 1996); contradictory results have been reported in lymphoma (Pinto et al., 1994; Aldinucci et al., 2002; Rassidakis et al., 2004). c-kit itself is considered as a prognostic factor in several hematopoietic neoplasms (Lyman and Jacobsen, 1998). Furthermore, KIT sequence mutations have been detected in AML and nHL. These mutations mostly affect the ligand binding and tyrosine kinase domains (insertion/deletion), but internal tandem duplications (ITDs) have been found in the juxtamembrane domain (Beghini et al., 2004; Zimpfer et al., 2004; Goemans et al., 2005; Shimada et al., 2006).

Despite this, in veterinary medicine no reports about the diagnostic/prognostic relevance of c-kit in canine hematological malignancies are actually available, except for a recent study where the frequency of KIT mutations were evaluated in acute myeloid and lymphoid leukemia (Usher et al., 2009) and two previous ones describing the c-kit expression in epitheliotrophic and intestinal lymphoma, both of T-cell origin (Shiomitsu et al., 2012; Ozaki et al., 2006). Thus, the present study analyzed c-kit mRNA and protein expression as well as the occurrence of KIT mutations in canine lymphoma, by using an integrated approach (qPCR, flow cytometry and immunocytochemistry and cDNA sequencing, respectively).

2. Materials and methods

2.1. Caseload and classification

Fine-needle aspirates (FNAs) of enlarged lymph nodes from dogs suspected of lymphoma were collected and sent to the Department of Veterinary Pathology, Hygiene and Health, University of Milan and to the Department of Veterinary Sciences, University of Turin. An informed consent was obtained from all the owners, according to the regulations of each institutional animal care committee.

All dogs were submitted to a complete staging, including physical examination, complete blood count, serum biochemistry panel, thoracic radiographs, abdominal
ultrasound examination, and bone marrow cytology. Lymphoma subtypes were classified based on the Kiel-updated cytological classification (Fournel-Fleury et al., 1997). The flow cytometric analysis was performed on lymph node FNAs by using the following monoclonal antibodies: CD45-PEb (clone YKIX716.13, Serotec, Oxford, UK), CD3-FITC (clone CA17.2A12, Serotec, Oxford, UK, T-cells), CD4-FITC (clone YKIX302.9, Serotec, Oxford, UK, T-helper cells and neutrophils), CD8-PE (clone YCATE55.9, Serotec, Oxford, UK, T-cytotoxic/suppressors), CD5 (clone YKIX322.3, T-cells), CD21-PE (clone CA21D6, Serotec, Oxford, UK, mature B-cells), CD34-PE (clone 1H6 Pharmingen, Becton Dickinson, San Jose, CA, precursor cells), and CD79a (clone HM57, Dako, Atlanta, GA, all stages of B-cells). Acquisition was performed with FACSCalibur (Becton Dickinson, Buccinasco, Milan, Italy) and the analysis was conducted by using a commercially available software (Cell Quest, Becton Dickinson, Buccinasco, Milan, Italy). Lymphoma subtype lineage was defined by the expression of specific lineage markers of at least 20% of the gated cells in the lymph node: CD3, CD5, CD4, CD8 for T-cells and CD21, CD79a for B-cells. Samples leading to ambiguous diagnosis, or characterized by low cellularity or viability, were excluded from the present study.

2.2. Sampling procedure

A part of the cell suspension obtained from FNA was used for routine flow cytometry analysis and immunocytochemistry, while the remainder was put in polypropylene tubes filled with 10 part of RNAlater® solution (Life Technologies, Carlsbad, CA) for total RNA isolation. Only samples containing at least 2 x 10^6 cell/mL were considered.

2.3. Quantitative real-time RT-PCR (qPCR)

Total RNA was isolated from both cell pellet and RNAlater suspension, as recommended by Dunmire et al. (2002), using the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. To avoid genomic DNA contaminations, on-column DNase digestion with the RNase-free DNase set (Qiagen, Milan, Italy) was performed. Total RNA concentration and quality were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Germany) and by denaturing gel electrophoresis.

First-strand cDNA was synthesized from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol and stored at -20°C until use.

The cDNA was used as a template for qPCR analysis. The KIT primer pairs and the appropriate human Universal Probe Library (UPL) probe were selected from the extracellular domain (exon 6, which does not represent a hot spot for mutations) of the canine KIT mRNA sequence NM_001003181.1, by using UPL Assay Design Center web service default parameters. The canine KIT transcript was amplified with 5'-CCTTGGAAGTAGTAGATAAAGGATTCA-3' (forward primer), 5'-CCCCAATTGCAACCTTGAAC-3' (reverse primer), and number #60 human Universal Probe Library (UPL, Roche Applied Science, Indianapolis, IN) probe. Primers specificity was evaluated in silico by means of BLAST tool and also experimentally by Power SYBR Green I (Life Technologies, Carlsbad, CA) amplification and melting curve analysis. The amplicon size was 89 base pairs. The qPCR reactions (10 µL final volume) consisted of IX LightCycler 480 Probe Master (Roche Applied Science, Indianapolis, IN), 600 nM forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany), 100 nM human UPL probe and 2.5 µL of 40-fold diluted cDNA.

qPCR analysis was performed in duplicate in a LightCycler 480 Instrument (Roche Applied Science, Indianapolis, IN) using standard PCR conditions (an activation step at 95 °C for 10 min; 45 cycles at 95 °C for 10 s and at 60°C for 30 s; a cooling step at 40 °C for 30 s) and LightCycler 480 clear plates (Roche Applied Science, Indianapolis, IN). No template controls and no reverse transcription controls were included on each plate. Calibration curves, using a 2-fold serial dilution of a cDNA pool, revealed a PCR efficiency of 92.3%, an error value of
0.034 and a dynamic range of about 7 cycles (27.59-34.84).

Canine transmembrane BAX inhibitor motif containing 4 (TMBIM4 or CGI-119) was chosen as reference gene. Its amplification efficiency (90.2%) and error value (0.003) were comprised in the range of acceptability (90-110%, error <0.2). Reference gene assay parameters were comparable to that of the target gene; moreover, no statistically significant differences were observed in its expression profile between healthy and pathological samples, as well as between B-cell and T-cell lymphoma samples.

Data were analyzed with the LightCycler480 software release 1.5.0 (Roche Applied Science, Indianapolis, IN) by using the fit point method. Messenger RNA relative quantification was performed using the AACt method (Livak and Schmittgen, 2001) and a cDNA pool comprehending both healthy and pathologic samples as calibrator.

In accordance with MIQE guidelines, in the future work it will be necessary to improve the validity of the assay evaluating RNA Integrity Number (R.I.N.), the limit of detection (LOD) and considering more than one reference gene for the normalization step.

2.4. c-kit (CD117) analysis by flow cytometry

Fifty microliters of cells suspension were used for surface staining for CD117<sub>PE</sub> (ACK45 Pharmingen, San Diego, CA, USA) and CD45<sub>FITC</sub> (YKIX716.13, AbD Serotec, Raleigh, NC, USA). Unstained cells with the proper isotype control were used as control. For each sample, the neoplastic population was gated based on morphological properties using forward scatter (FSC) versus side scatter (SS) dot plot. For each sample, the c-kit mean fluorescent intensity (MFI) was calculated as the ratio of MFI of neoplastic cells/MFI of unstained cells (Advani et al., 2008). The percentage of c-kit positive events was also assessed.

2.5. Immunocytochemistry

The cellular suspension designated for immunocytochemical analysis was prepared on cytopsin and fixed with 9% part of acetone and 14 part of methanol. After fixation, a primary antibody incubation step (anti-human c-kit, A4502, Dako Italia S.p.A., Milan, Italy) was performed by means of an automated system (Ventana Medical Systems, Tucson, AZ, USA). The remainder of the staining procedure, which includes an incubation with a biotinylated anti-mouse secondary antibody, the diaminobenzidine substrate and a counterstain with hematoxylin, was performed by using the Ventana ES automated immunohistochemistry system. Negative-control slides were incubated with isotype-matched immunoglobulin in parallel with each batch of staining to confirm the specificity of the antibody. Each slide was scanned with a 400 x power objective to identify positive immunostaining.

2.6. Amplification and sequencing of KIT exons 8-11 and 17

Exons 8-11 and 17 were sequenced to identify possible KIT mutations. Two microliters of undiluted cDNA were used as template for PCR amplification. Details of PCR amplification are reported in Giantin et al. (2013). Amplicons were visualized in a 1% agarose gel electrophoresis. Finally, sequencing and sequence analysis were performed as previously reported (Giantin et al., 2012). Abnormal sequencing results were confirmed by at least two repeated analyses.

2.7. Statistical analysis

The statistical analysis of gene expression and flow cytometry data (MFI and percentage of c-kit positive cells) was performed by using the Mann-Whitney test (Graph-Pad Prism 5 software, San Diego, CA, USA). Statistical significance was set at P< 0.05.
3. Results and discussion

A total of 46 canine lymphomas were enrolled in the study. Twenty-five were of B-cell immunophenotype: twenty-one high grade (HG, centroblastic polymorphic) and 4 low grade (LG, lymphocytic lymphoma). Twenty-one lymphomas were of T-cell immunophenotype: thirteen HG (pleomorphic mixed) and 8 LG (small clear cells).

The KIT mRNA was amplifiable but below the limit of quantification in 76% of B-cell lymphomas and 33% of T-cell lymphomas. Single values (expressed in terms of relative quantification, RQ) are reported in Fig. 1. Mean KIT mRNA values were significantly higher (P<0.01) in T-cell lymphomas with respect to B-cell lymphomas (0.14 ± 0.22 and 0.01 ±0.02 arbitrary units, AU, respectively: see Fig. 2A). Highest KIT mRNA levels were noticed in five HG and three LG T-cell lymphomas, but the difference between HG and LG T-cell lymphomas was not statistically significant (see Fig. 3A). The low number of LG B-cell lymphomas did not permit such a comparison with HG B-cell lymphomas.

As regards c-kit protein expression, flow cytometry analysis revealed that the MFI index was 1.45 ±0.43 (standard error medium, SEM: 0.09; median: 1.33; min-max: 0.70-2.10) and the percentage of positive cells was 3.91 ±4.39 (SEM: 0.91; median: 2.47; min-max: 0.49-19.45) in B-cell lymphomas, while the MFI index was 1.58 ±0.55 (SEM: 0.12; median: 1.4; min-max: 0.95-3.10) and the percentage of positive cells was 4.08 ±4.67 (SEM: 1.02; median: 2.36; min-max: 0.13-16.08) in T-cell lymphomas. Differences between B-cell and T-cell lymphomas were not statistically significant for both MFI and the percentage of positive cells (see Fig. 2B-C). On the contrary a higher MFI index, but not significant, was recorded in HG versus LG T-cell lymphomas (P= 0.06: see Fig. 3B), whereas a statistically significant higher percentage of c-kit positive cells was observed in HG versus LG T-cell lymphomas (P= 0.01: see Fig. 3C).

In B-cell lymphomas, immunocytochemical investigations showed that c-kit was expressed in the cytoplasm of lymphoid cells in six dogs, whereas eight cases were considered negative. In T-cell lymphomas, seven cases showed a cytoplasmic immunostaining for c-kit, whereas four samples were considered negative. A representative image of c-kit immunolabelled cells for B-cell and T-cell lymphomas is reported in Fig. 4.

As a whole, presented data demonstrated that KIT transcript is poorly expressed in B-cell lymphomas, whereas T-cell lymphomas showed a variable pattern of mRNA expression. Moreover, KIT mRNA amount was higher in HG T-cell lymphomas compared to LG T-cell lymphomas. The c-kit immunocytochemistry confirmed qPCR data for both lymphoma subtypes. The flow cytometry showed a low positivity for c-kit in both B-cell and T-cell lymphomas; nevertheless, a higher c-kit expression was noticed in HG compared to LG T-cell lymphomas.

Few and contrasting results have been published about c-kit expression in human lymphomas. Pinto et al. (1994) and Aldinucci et al. (2002) reported a c-kit protein positivity in classical Hodgkin lymphoma (HL), HL-derived cell lines as well as in anaplastic large cell lymphoma, but not in nHL. However, recent studies demonstrated that c-kit expression is extremely rare in CD30+ lymphomas (Rassidakis et al., 2004). Zimpfer et al. (2004) profiled, by using the tissue-microarray technology, a large number of samples (824 nHL and 342 HL) and observed a general low pattern of c-kit expression: in particular, none HL and only two out of the 747 nHL cases were positive for c-kit, displaying a cytoplasmic and membranous staining pattern. Finally, c-kit protein was detected in one case of B-cell nHL carrying the t(14;18) translocation and in only 2 out of 17 patients affected by mantle cell lymphoma (Bravo et al., 2000; Potti et al., 2002). Altogether, the human literature corroborates the present rare c-kit positivity, particularly in B-cell lymphomas.

The most interesting data in our work was the significant difference observed between HG and LG T-cell lymphomas. The HG T-cell lymphoma represents one of the most aggressive lymphoma subtype. The backbone of current therapy for canine B-cell lymphoma is a systemic multi-agent chemotherapy, in which drugs such as vincristine, cyclophosphamide
and doxorubicin, with or without L-asparaginase, are used for short treatment cycles (Marconato, 2011). On the contrary, there is no consensus about a "first-line" therapeutic approach in T-cell lymphomas (Simon et al., 2006), and in many cases the response to chemotherapy is often suboptimal (Marconato, 2011). Recently, exciting advances in human oncology have focused on the use of alternative treatments, such as monoclonal antibodies and molecular-targeted therapies (e.g., tyrosine kinase inhibitors, TKIs), acting synergistically with chemotherapy. Present results might represent the rationale for the setting up of a new protocol for T-cell lymphomas chemotherapy based on c-kit positivity and consequent case selection, as already suggested by some authors (Marconato, 2011; Marconato et al., 2012).

Actually, TKIs represent a therapeutic molecular target option for the chemotherapy of other spontaneous canine tumors, but their use has never been considered in lymphoma (London et al., 2003, 2009; Isotani et al., 2008). Moreover, it should be emphasized that among TKIs licensed for veterinary use, toceranib phosphate is considered possess anti-angiogenic properties, as it blocks the activity of vascular endothelial growth factor (VEGF) receptor (London, 2009). In this respect, we have recently described higher VEGF-A mRNA and protein expression in HG T-cell lymphomas with respect to HG B-cell lymphomas and LG T-cell lymphomas (Arico et al., 2013).

As far as KIT mutations are concerned, few studies investigated the occurrence of sequence alterations in human lymphomas: Nakatsuoka et al. (2002) detected 21 mutations in KIT gene (exons 11 or 17) in 10 out of 14 nHLs, while Hongyo et al. (2000) identified several c-KIT mutations in exons 11 and 17 in a sinonasal natural killer/T-cell lymphoma in Chinese and Japanese populations. In dogs, KIT mutations have been only described and characterized in canine acute leukemia (Usher et al., 2009; Giantin et al., 2013), whereas no data are actually available for lymphoma. In the present study, a molecular screening of KIT exons 8-11 and 17 was performed in all B-cell and T-cell lymphomas. No visible ITDs nor deletions in exons 10-11 were ever noticed with agarose gel electrophoresis; likewise, no point mutations, small ITDs nor deletions were noticed with direct cDNA sequencing of selected exons. Altogether, our samples were considered to have a wild type KIT sequence. The lack of detectable mutations in c-kit positive lymphomas has been previously described in human (Zimpfer et al., 2004). Therefore, present data suggest that KIT mutations in canine lymphoma are occasional and, on a second instance, that c-kit aberrant expression is not strictly related to the presence of a KIT mutation (Zimpfer et al., 2004); rather, it may represent a separate event, as already noticed in canine mast cell tumors (Giantin et al., 2012).

In conclusion, a low pattern of c-kit expression was generally recorded, at both mRNA and protein level, in canine lymphomas, with the merely exception of HG T-cell lymphomas. One limit of this study is the absence of correlations with follow-up data. Thus, more in depth studies, aiming to characterize c-kit expression profile and its role in canine lymphoma molecular biology, are actually needed.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Fig. 1. Single KIT relative quantification (RQ) values of each B-cell and T-cell lymphoma sample. mRNA amounts obtained by quantitative real-time RT-PCR are expressed in arbitrary units (AU). Each single data corresponds to the mean RQ value of technical duplicates. Light gray columns correspond to B-cell lymphomas, while dark gray columns to T-cell lymphomas. Low grade (LG) and high grade (HG) samples are also indicated.
Fig. 2. c-kit mRNA (A) and protein (B-C) expression in B-cell (B) and T-cell (T) lymphoma samples. mRNA amounts, obtained by quantitative real-time RT-PCR, are expressed as relative quantification values (RQ, AU), while protein results obtained through a flow cytometry analysis are presented either as the mean fluorescence intensity (MFI) index (B) than the percentage of c-kit positive cells (C). Data are expressed as mean±SD. Statistical analysis: Mann-Whitney test (**P<0.01).
Fig. 3. c-kit mRNA (A) and protein (B-C) expression in low grade (LG) and high grade (HG) T-cell lymphoma samples. mRNA amounts, obtained by quantitative real-time RT-PCR are expressed as relative quantification values (RQ, AU), while protein results obtained through flow cytometry analysis are presented either as the mean fluorescence intensity (MFI) index (B) than the percentage of c-kit positive cells (C). Data are expressed as mean ± SD. Statistical analysis: Mann-Whitney test (*P< 0.05).
Fig. 4. Representative images of c-kit cytoplasmic pattern of protein staining in a B-cell (A) and T-cell lymphoma (B). A rare positive immunolabelling was noticed in B-cell lymphoma, whereas a more intense and diffuse immunostaining of tumor cells was observed in T-cell lymphoma.