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Evaluation of tyrosine-kinase receptor c-KIT (c-KIT) mutations, mRNA and protein expression in canine leukemia: Might c-KIT represent a therapeutic target?

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A B S T R A C T

The tyrosine-kinase receptor c-KIT (c-KIT) plays an important role in proliferation, survival and differentiation of progenitor cells in normal hematopoietic cells. In human hematological malignancies, c-KIT is mostly expressed by progenitor cell neoplasia and seldom by those involving mature cells. Tyrosine kinase inhibitors (TKIs) are actually licensed for the first-and second-line treatment of human hematologic disorders. Aim of the present study was to evaluate c-KIT mRNA and protein expression and complementary DNA (cDNA) mutations in canine leukemia. Eleven acute lymphoblastic leukemia (ALL) and acute undifferentiated leukemia (AUL) and 12 chronic lymphocytic leukemia (CLL) were enrolled in this study. The amounts of c-KIT mRNA and protein were determined, in peripheral blood samples, by using quantitative real time RT-PCR, flow cytometry and immunocytochemistry, respectively. The presence of mutations on c-KIT exons 8-11 and 17 were investigated by cDNA sequencing. Higher amounts of c-KIT mRNA were found in ALL/AUL compared to CLL, and this latter showed a lower pattern of gene expression. Transcriptional data were confirmed at the protein level. No significant gain-of-function mutations were ever observed in both ALL/AUL and CLL. Among canine hematological malignancies, ALL/AUL typically show a very aggressive biological behavior, partly being attributable to the lack of efficacious therapeutic options. The high level of c-KIT expression found in canine ALL/AUL might represent the rationale for using TKIs in future clinical trials.

Abbreviations: AL, acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AU, arbitrary units; AUL, acute undifferentiated leukemia; c-KIT, proto-oncogene c-KIT; CLL, chronic lymphocytic leukemia; CTRL, control; FLT3, fins-like tyrosine-kinase 3; ITDs, internai tandem duplications; PDGFR, platelet-derived growth factor receptor; qPCR, quantitative real-time RT-PCR; RQ, relative quantification; TKIs, tyrosine kinase inhibitors.

1. Introduction

Lymphohaematopoietic malignancies are common spontaneous canine diseases, and their clinical presentation and biologic behavior closely resemble those of humans (Valli et al., 2002; Breen and Modiano, 2008). Among these ones, the acute leukemia (AL) is a rapidly progressive disease associated with proliferation of malignant and undifferentiated blasts cells. It typically shows a very aggressive biological behavior, owing to the rapid outcome and the lack of efficacious therapeutic options (Usher et al., 2009). On the contrary, the chronic lymphocytic leukemia (CLL), characterized by abnormal proliferation of small lymphocytes (Leifer and Matus, 1985), is usually associated with longer survival time; nevertheless, it remains incurable with standard therapies. Affected dogs eventually relapse, become refractory to treatment or undergo disease transformation (Comazzi et al., 2011).

The tyrosine-kinase receptor c-KIT (c-KIT), also referred as stem cell factor receptor or CD117, is a trans-membrane glycoprotein that includes 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 (Edling and Hallberg, 2007). The ligand (the stem cell factor) binding to c-KIT leads to receptor dimerization and trans-phosphorylation of tyrosine residues, an event followed by the interaction and phosphorylation of the c-KIT receptor with specific cytoplasmic signaling proteins (Advani, 2006). In normal hematopoietic cells, c-KIT plays an important role in proliferation, survival and differentiation of progenitor cells (Advani, 2006). It is expressed in hematopoietic stem cells, myeloid progenitor cells, dendritic cells, mast cells, pro-B and pro-T cells. In many cell types, like B and T cells, the expression of c-KIT is lost with cell differentiation; on the contrary, mast cells, natural killer cells and immune System dendritic cells retain c-KIT expression (Ray et al., 2008).

In humans, previous studies have shown that the constitutive activation of tyrosine-kinases such as c-KIT, the platelet-derived growth factor receptor (PDGFR) and the fins-like tyrosine kinase 3 (FLT3), play an important role in the development of hematopoietic tumors (Mizuki et al., 2003). In particular, a number of reports have shown that c-KIT was expressed in human AL cells, frequently in acute myeloid leukemia (AML), and in few cases of early T-acute lymphoblastic leukemia (ALL) and B-cell lineage ALL (Sperling et al., 1997; Bene et al., 1998; Tsao et al., 2004). Furthermore, several missense point mutations and sequence insertion/deletion, for the most part affecting c-KIT exons 7 and 17, and less common internal tandem duplications (ITDs) in exon 11 were detected in human AML (Beghini et al., 2004; Goemans et al., 2005; Shimada et al., 2006). In light of these evidences, tyrosine kinase inhibitors (TKIs) were tested in human AML clinical trials to find new treatment strategies (Mizuki et al., 2003; Advani, 2006; Masson and Ronnstrand, 2009; Stirewalt and Meshinchi, 2010;Amrein, 2011).

Despite the advances in morphological diagnostics and immunophenotyping of hematologic malignancies no reports about c-KIT expression in canine leukemia have been published so far, except for a recent study on the frequency of RAS, FLT3 and c-KIT mutations in canine AML and ALL (Usher et al., 2009). Thus, the aim of the present study was to measure c-KIT gene and protein expression as well as the presence of sequence mutations in canine AL and CLL.

2. Materials and methods

2.1. Case load and classification

Peripheral blood samples were collected in EDTA-containing tubes from dogs with hematopoietic neoplasia and sent by the referring veterinarians, for diagnostic purposes, either to the Department of Veterinary Pathology, Hygiene and Health at the University of Milan or the Department of Veterinary Sciences at the University of Turin.

Each blood sample was analyzed by using the Sysmex XT-2000iV (Sysmex Corporation, Kobe, Japan). Peripheral blood and bone marrow smears were stained with May-Grunwald-Giemsa from Merck KGaA (Frankfurt, Germany) and used to obtain leukocyte differential counts and morphologic evaluations of the blast cells. To determine the tumor immunophenotype, flow cytometric investigations were performed on peripheral blood samples as previously described (Comazzi et al., 2011). To this purpose, the following monoclonal antibodies were used: CD45-PEb (clone YKIX716.13, Serotec, Oxford, UK, leukocytes), CD3-FITC (clone CA17.2A12, Serotec, T cells), CD4-FITC (clone YKIX302.9, Serotec, T-helper cells and neutrophils), CD8-PE (clone YCATE55.9, Serotec, T-cytotoxic/suppressors), CD5 (clone YKIX322.3, T-cells), CD21-PE (clone CA21D6 Serotec, mature B cells), CD34-PE (clone 1H6 Pharmingen, Becton Dickinson, San Jose, CA, precursor cells), CD79a (clone HM57, Dako, Atlanta, GA, all stages of B-cells), CD14 (clone TUK4, Serotec, monocytes) and CD11b (clone CA163E10, Serotec, granulocytes, monocytes). Data acquisition was performed by using a FACSCalibur (Becton Dickinson, Buccinasco, Milan, Italy), and the analysis executed by using commercially available software (Cell Quest, Becton Dickinson, Buccinasco, Milan, Italy).

Clinical, clinic-pathological and immunophenotypic data were used to classify leukemia samples as previously described (Gelain et al., 2010). In particular, AL was diagnosed by the presence of:

moderate to severe anemia and/or thrombocytopenia, a leukocyte morphology suggestive of immature or blast cells, the presence of more than 20% of blast cells in the bone marrow, CD34 positivity, and either CD3, CD5, CD4, and CD8 positivity (T-cell ALL) or CD21 and CD79a positivity (B-cell ALL). Samples were classified as acute undifferentiated leukemia (AUL) when atypical cells expressed only CD34 and CD45. Chronic lymphocytic leukemia was diagnosed by the presence of: severe lymphocytosis, monomorphic population of mature lymphocytes, negative serologic titer for *Ehrlichia, Leishmania* or any other identifiable cause of lymphocytosis, and either CD3, CD5, CD4 and CD8 positivity (T-cell CLL) or CD21 and CD79a positivity (B-cell CLL).

Besides pathological samples, peripheral blood samples obtained from six healthy dogs matched according to age, breed and gender were used as controls (CTRL) for flow cytometric, immunocytochemical and quantitative real-time RT-PCR (qPCR) investigations. Ali samples were obtained during an annual general health visit, and informed consent was obtained from all the owners.

2.2. Sampling procedure

Peripheral blood samples were at first submitted to erythroid lysis with 2mL of an erythrocyte lysis buffer containing 8% ammonium chloride (Sigma-Aldrich, Munich, Germany). Cells were then suspended in RPMI 1640 medium containing 5% fetal bovine serum and 0.2% sodium azide (Sigma-Aldrich, Munich, Germany). Part of each sample was used for routine flow cytometric and immunocytochemical analyses, while the remainder was put in polypropylene tubes with 10 parts of RNAlater® solution (Life Technologies, Carlsbad, CA) for total RNA isolation.

2.3. Quantitative real-time RT-PCR

Total RNA was isolated from both cell pellet and RNAlater® suspension, as recommended by Dunmire et al. (2002), by using the RNeasy Mini Kit (Qiagen, Milan, Italy) and according to the manufacturer's instructions. To avoid genomic DNA contaminations, an on-column DNase digestion with RNase-free DNase set (Qiagen, Milan, Italy) was performed. Total RNA concentration and quality were measured by means of a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) 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, Milan, Italy) and according to the manufacturer's protocol. The complementary DNA was used as a template for qPCR analysis in a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland), for which standard PCR conditions were used. Primer pairs and the human UPL probe for c-KIT amplification were those reported in Giantin et al. (2012). The qPCR reaction (10 uL final volume) consisted of IX LightCycler 480 Probe Master (Roche Diagnostics, Milan, Italy), 600 nM forward and reverse primers (Eurofins MWG Operon, Ebersberg, Germany), 100nM human Universal Probe Library probe (UPL, Roche Diagnostics, Milan, Italy) and 2.5 pi of a 40-fold diluted cDNA. Calibration curves, made by using a 2-fold serial dilution of a cDNA pool, revealed a PCR efficiency of 1.923 and an error value of 0.034. Canine Golgin Al (GOLGA1) was chosen as internal control gene. Its amplification efficiency was approximately equal to that of the target gene; moreover, no statistically significant differences in its gene expression profile were never observed either between healthy and pathological samples than among AL and CLL samples. Data were analyzed with the LightCycler480 software release 1.5.0 (Roche Diagnostics, Milan, Italy) by using the fit point method. Messenger RNA relative quantification was performed using the AACt method (Livak and Schmittgen, 2001).

2.4. Flow cytometric analysis of CD117

Fifty *u*L of cell suspensions were used for surface staining of $CD117_{PE}$ (ACK45 Pharmingen, San Diego, CA, USA) and $CD45_{FITC}$ (YKIX716.13, AibD 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 and CD45 staining, by using either the forward scatter (FSC) versus side scatter (SS) dot plot than the $CD45_{FITC}$ versus SS dot plot, respectively (Fig. 1). For each sample, CD117

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 CD117 positive events was determined, too.

2.5. Immunocytochemistry

The cellular suspension designated for immunocytochemical analysis was prepared on cytospin and fixed with 3/4 part of acetone and 1/4 part of methanol. After fixation, a primary antibody incubation step (anti-human CD117, c-KIT, A4502, Dako Italia SpA, Milan, Italy) was performed by means of an automated System (Ventana Medical Systems, Tucson, AZ, USA). The remainder of the staining procedure, including the incubation with a biotinylated anti-mouse secondary antibody, diaminobenzidine substrate and hematoxylin counterstain was performed by using a Ventana ES automated immunohistochemistry System. Negative-control slides were incubated with isotype-matched immunoglobulin in parallel with each batch of staining, to confirm the antibody specificity. Each slide was scanned with a 400x power objective to identify positive immunostaining.

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

Exons 8-11 and 17 were chosen to discover c-KIT mutations. Two uL of a 5-fold diluted (AL) or undiluted cDNA (CLL) were used as a template for PCR amplification. For exons 8-11 PCR amplification, previously published primers were used (Giantin et al., 2012); on the contrary, for the exon 17 the following oligonucleotides were designed *ex novo* by using Primer3 software (http://frodo.wi.mit.edu/primer3/): F: 5'-GATGTG ACTCCT GCC ATC AT-3'; R: 5'-GGC CAT CCA CTT CAC AGG TA-3'. The following PCR conditions were used: an activation step at 95 °C for 3 min, 35 cycles of 30 s at 95 °C, 45 s at 63 °C or 60 °C (exons 8-11 and 17, respectively), 45 s at 72 °C, and a final extension step of 5 min at 72 °C. These reactions were carried out in a TPersonal thermocycler (Biometra GmbH, Gòttingen, Germany) according to Giantin et al. (2012). 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 distinct analyses.

2.7. Statistical analysis

The statistical analysis of gene expression and flow cytometry data (MFI and percentage of CD117 positive cells) was performed by using the Kruskal-Wallis test followed by the Dunn's post test. The GraphPad Prism 5 software (San Diego, CA, USA) was used for statistical evaluations. The level of statistical significance was set at $P< 0.05$.

3. Results

3.1. Clinical results

Eleven dogs with AL and twelve with T-cell CLL were enrolled in the study. Among AL cases, two of them were classified as T-cell ALL (CD34+CD5+), two as B-cell ALL (CD34+CD79a+) and seven as AUL (CD34+CD45+). The mean age of dogs was 8 years (range, 2-12 years) and they were all females. Ali dogs showed anemia, thrombocytopenia and leucocytosis. As regards CLL cases (all T-cell CLL), eight were CD8+, one CD4+, one CD21 + and two CD5+CD4-CD8-. Dogs mean age was 10 years (range 5-13 years), and 10 were males and 2 were females. Ali dogs showed leucocytosis, and six of them were anemic, too. Mean percentage values (range) of neoplastic cells in blood were 98.1% (72-100%) and 88.2% (72-96%) for AL and CLL, respectively. A complete list of control and leukemia cases enrolled in the present study (including tumor identity, diagnosis and global c-KIT results) is reported in Table 1.

3.2. Quantitative real-time RT-PCR

Measurable and amplifiable amounts of c-KIT mRNA were noticed in all dogs with AL (12/12; see

Table 1); by contrast, the c-KIT gene was detectable but not quantifiable in the 50% (6/12) of dogs with CLL (RQ.= 0 arbitrary units, AU). Dogs suffering of AL showed about 200-fold higher c-KIT mRNA amounts than those with CLL, with mean RQ values of 11.32 ± 7.44 AU and 0.05 ±0.12 AU, respectively ($P< 0.001$). Although c-KIT gene expression profile in CTRL samples was comparable with CLL, statistically significant differences were observed between CTRL and AL ($P<0.05$: see Fig. 2A).

3.3. Flow cytometry

Blood samples from CTRL dogs showed a very low MFI level (1.09±0.35, 1.0. and 0.77-1.70, which represent the mean value±S.D., the median and min-max values, respectively) and few positive cells (0.20 ±0.08, 0.18 and 0.12-0.31; see Table 1). In AL, MFI levels were 9.96 ± 19.22, 4.66 and 1.58-67.64, while in CLL they were 1.46 ±0.38, 1.50 and 0.80-1.90. The percentage of positive cells in AL samples was 41.17±25.30, 41.12 and 5.20-83.97, while in CLL it was 2.61 ±2.10, 1.75 and 0.43-6.20. Both MFI levels and the percentage of CD117 positive cells were higher in leukemic samples compared to CTRL dogs, but such a difference was statistically significant only between CTRL and AL ($P< 0.001$). A higher ($P<0.01$) CD117 positivity was observed in AL compared to CLL (see Fig. 2B and C). No statistically significant differences were ever noticed between ALL and AUL (data not shown).

3.4. Immunocytochemistry

In Fig. 3, representative images of cytoplasmic c-KIT protein staining in AL (A) and CLL cells (B) are shown. In AL, all dogs showed lymphoid leukemia cells with an intense and diffuse immunostaining for c-KIT, whereas only 3 out of 12 CLL dogs showed c-KIT positive immunolabelling.

3.5. Sequencing

The molecular screening of the c-KIT coding region (exons 8-11 and 17) was performed by PCR amplification and direct sequencing. In all samples, the agarose gel electrophoresis showed no evidence of visible internal tandem duplications (ITDs) nor deletions in exons 10-11, and a wild type c-KIT sequence was evidenced. No point mutations, small ITDs or deletions were revealed by direct sequencing of selected exons.

4. Discussion

Growth, survival and differentiation of hematopoietic cells are regulated by the interaction between hematopoietic growth factors and their receptors. While defects in their interaction result in an insufficient hematopoiesis, aberrantly elevated activations lead to the neoplastic trans-formation of hematopoietic cells (Mizuki et al., 2003). The constitutive activation of tyrosine kinase receptors like c-KIT, PDGFR or FLT3 plays a key role in the development of hematopoietic malignancies (Mizuki et al., 2003). Among these latter ones, c-KIT has gained an increasing importance for the assignment of cell lineage as well for the diagnosis of AML in the World Health Organization classification (Advani et al., 2008); in fact, a high c-KIT expression has been shown to be associated with shorter progression-free survival and overall survival in AML (Advani et al., 2008). As a consequence, TKIs have been actually tested in clinical trials for the treatment of human hematologic malignancies (Mizuki et al., 2003; Advani, 2006; Masson and Ronnstrand, 2009; Stirewalt and Meshinchi, 2010; Amrein, 2011).

In veterinary medicine, even though morphological diagnostics and immunophenotyping techniques have consistently improved in the past decade, not many progresses have been recorded in treatment options of canine acute and chronic leukemia (Usher et al., 2009). The aim of the present study was to evaluate c-KIT mRNA and protein expression as well as cDNA mutations in canine leukemia. To be successful in such an objective, an integrated approach (qPCR, flow cytometry and immunocytochemistry) was used.

Significant higher amounts of c-KIT mRNA and protein were observed in AL dogs compared to CTRL

blood samples. Such a behavior might be explained by the fact that canine acute leukemia cells might not retain the aptitude to properly differentiate into mature cells, thereby maintaining c-KIT expression (Adam et al., 2009). Similar results have been reported in human literature: both AML and AUL express c-KIT, and this latter is considered as a specific marker for the myeloid lineage, too (Bene et al., 1998). On the contrary, the detection of c-KIT in ALL lymphoblasts is definitely rare and restricted to T-lineage diseases (Bene et al., 1998; Paietta et al., 2004). In the present study, no differences were ever noticed between ALL and AUL.

In CLL, lower c-KIT mRNA and protein amounts were observed, likewise to CTRL samples. Few information about c-KIT expression in human CLL are actually available; a CD117 positivity has been described in unusual cases of association of B-CLL and AML as well as CLL and systemic mastocytosis, where myeloid lineage and mast cell components might have influenced to a certain extent the level of c-KIT expression (Carulli et al., 2007; Horny et al., 2006).

Presented data showed a 200-fold higher amounts of c-KIT mRNA in AL compared to CLL. Protein levels, expressed as MFI index and the percentage of positive cells, were about 7- and 16-fold higher in AL than in CLL, respectively. This finding was confirmed by immunocytochemistry, too; in fact, AL cells showed an intense and diffuse immunoreactivity to c-KIT protein, while a reduced number of cells in CLL were immunoassayed. Despite the different order of magnitude noticed at the transcriptional and post-translational level, correspondent c-KIT results were obtained at both mRNA and protein level and according to Kern et al. (2006), where a high correlation between microarray and flow cytometry data was pointed out in acute and chronic leukemias.

In the present study, c-KIT expression was evaluated in ALL and AUL, but not in AML. Data obtained, albeit of limited relevance for the low number of cases here considered, substantially differ from those reported in humans. In fact, canine ALL and AUL showed an equivalent elevated expression of c-KIT. It should be underlined that some cases were classified as AUL merely assuming the lack of expression of lymphoid and myeloid markers as a criterion; nevertheless, the myeloid origin of blast cells cannot be excluded a priori, owing to the limited number of available canine specific antibodies raised against myeloid antigens.

High c-KIT MFI values have been associated with a poor prognosis in human AML (Advani et al., 2008). A number of reasons have been offered to justify the high level of c-KIT expression noticed in some hematological malignancies (Advani et al., 2008), and the present difference in c-KIT expression between AL and CLL might at least partly explain the very dissimilar clinical behavior and outcome observed in dogs with AL or CLL (Comazzi et al., 2011).

The high pattern of c-KIT expression noticed in AL samples might represent the rationale to use c-KIT as a molecular target for a new therapeutic strategy. Tyrosine-kinase inhibitors have already been considered for the treatment of other canine spontaneous malignancies (London et al., 2003; Isotani et al., 2008; London et al., 2009) as well as in human clinical trials for the chemotherapy of hematological malignancies (Wang et al., 2005; Advani, 2006). In addition, c-KIT sequencing should be considered as an useful, specific, and precise tool to phenotype the cancer patient and, consequently, better address the ensuing chemotherapy. Several c-KIT missense point mutations, sometimes found in combination with sequence insertion/deletion, have been detected in human AML. These mutations for the most part affect the kinase domain, but sometimes also the ligand-binding domain (Beghini et al., 2004; Care et al., 2005; Goemans et al., 2005; Lennartsson et al., 2005; Shimada et al., 2006). Internal tandem duplications have been found to a lower extent in the juxtamembrane domain (Beghini et al., 2004; Corbacioglu et al., 2006). In a recent work, 41 samples obtained from dogs suffering with acute leukemia were analyzed for the occurrence of c-KIT mutations. Single nucleotide polymorphisms were detected in a total of 3 cases out of the 41 taken into account (7.3%), and all these dogs were suffering of AML (Usher et al., 2009). In the present study, no c-KIT mutations were ever noticed in both AL and CLL samples. Apart from the aforementioned low incidence, the present result might also be due to the poor number of AL cases enrolled in the present study.

In conclusion, this is the first study in which c-KIT gene and protein expression was investigated in canine AL and CLL by using an integrated approach. A high pattern of c-KIT gene/protein expression was observed in AL samples compared to CTRL and CLL ones, according to human data. Therefore, the measurement of c-KIT expression and the identification of its mutations might be potentially useful for

canine hematopoietic malignancies therapy and outcome. In particular, presented data would suggest the use of TKIs for canine AL chemotherapy, whose clinical relevance needs to be investigated more in depth in further clinical trials.

Conflict of interest statement

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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References

Adam, F., Villiers, E., Watson, S., Coyne, K., Blackwood, L, 2009. Clinical pathological and epidemiological assessment of morphologically and immunologically confirmed canine leukaemia. Vet. Comp. Oncol. 3, 181-195.

Advani, A.S., 2006. Targeting the c-kit receptor in the treatment of acute myelogenous leucemia. Curr. Hematol. Malig. Rep. 1,101-107.

Advani, A.S., Rodriguez, C, Jin, T., Jawde, R.A., Saber, W., Baz, R., Kalay-cio, M., Sobecks, R., Sekeres, M., Tripp, B., His, E., 2008. Increased c-kit intensity is a poor prognostic factor for progression-free and over-all survival in patients with newly diagnosed AML. Leuk. Res. 32, 913-918.

Amrein, P.C., 2011. The potential for dasatinib in treating chronic lymphocytic leukaemia, acute myeloid leukaemia, and myeloproliferative neoplasms. Leuk. Lymphoma 52 (5), 754-763.

Beghini, A., Ripamonti, C.B., Cairoli, R., Cazzaniga, G., Colapietro, P., Elice, F., Nadali, G., Grillo, G., Haas, O.A., Biondi, A., Morrà, E., Larizza, L., 2004. KIT activating mutations: incidence in adult and pediatric acute myeloid leucemia, and identification of an internal tandem duplication. Haematologica 89, 920-925.

Bene, M.C., Bernier, M., Casanovas, R.O., Castaldi, G., Knapp, W., Lanza, F., Ludwig, W.D., Matutes, E., Orfao, A., Sperling, C, van't Veer, M.B., 1998. The reliability and specificity of c-kit for the diagnosis of acute myeloid leukemias and undifferentiated leukemias. Blood 92, 596-599.

Breen, M., Modiano, J.F., 2008. Evolutionarily conserved cytogenetic changes in hematological malignancies of dogs and humans - man and his best friend share more than companionship. Chromosome Res. 16,145-154.

Care, R.S., Valk, P.J., Goodeve, A.C., Abu-Duhier, F.M., Geertsma-Kleinekoort, W.M., Wilson, G.A., Gari, M.A., Peake, I.R., Lòwenberg, B., Reilly, J.T., 2005. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. Br. J. Haematol. 121,775-777.

Carulli, G., Marini, A., Baccelli, E., Lambelet, P., Lari, T., Azzarà, A., 2007. Association of B-chronic lymphocytic leukemia and acute myeloid leukemia. J. Exp. Clin. Cancer Res. 26, 421-424.

Comazzi, S., Gelain, M.E., Martini, V., Riondato, F., Miniscalco, B., Marconato, L., Stefanello, D., Mortasino, M., 2011. Immunophenotype predicts survival time in dogs with chronic lymphocitic leukemia. J. Vet. Intern. Med. 25,100-106.

Corbacioglu, S., Kilic, M., Westhoff, M.A., Reinhardt, D., Fulda, S., Debatin, K.M., 2006. Newly identified c-KIT receptor tyrosine kinase ITD in childhood AML induces ligand-independent growth and is responsive to a synergistic effect of imatinib and rapamycin. Blood 108, 3504-3513.

Dunmire, V., Wu, C, Symmans, W.F., Zhang, W., 2002. Increased yield of total RNA from fine-needle aspirates for use in expression microarray analysis. Biotechniques 33, 890-896.

Edling, C.E., Hallberg, B., 2007. c-KIT - a hematopietic cell essential receptor tyrosine kinase. Int. J.

Biochem. Cell. Biol. 39,1995-1998.
Gelain, M.E., Rossi, G., Giori, L., Comazzi, S., Paltrinieri, S., 2010. Identification of neoplastic cells in blood using the Sysmex XT-2000iV: a preliminary step in the diagnosis of canine leukemia. Vet Clin Pathol. 39,169-179.
Giantin, M., Vascellari, M., Morello, E.M., Capello, K., Vercelli, A., Granato, A., Lopparelli, R.M., Nassuato, C, Carminato, A., Martano, M., Mulinelli, F., Da casto, M., 2012. c-KIT messenger RNA and protein expression and mutations in canine cutaneous mast cell tumors: correlations with post-surgical prognosis. J. Vet. Diagn. Invest. 24,116-126.
Goemans, B.F., Zwaan, C.M., Miller, M., Zimmermann, M., Harlow, A., Meshinchi, S., Loonen, A.H., Hàhlen, K., Reinhardt, D., Creutzig, U., Kaspers, G.J., Heinrich, M.C., 2005. Mutations in KIT and RAS are frequent events in pediatric core-binding factor acute myeloid leukemia. Leukemia 19,1536-1542.
Horny, H.P., Sotlar, K., Stellmacher, F., Valent, P., Grabbe, J., 2006. An unusual case of systemic mastocytosis associated with chronic lymphocytic leukaemia (SM-CLL). J. Clin. Pathol. 59, 264-268.
Isotani, M., Ishida, N., Tominaga, M., Tamura, K., Yagihara, H., Ochi, S., Kato, R., Kobayashi, T., Fujita, M., Fujino, Y., Setoguchi, A., Ono, K., Washizu, T., Bonkobara, M., 2008. Effect of tyrosine kinase inhibition by imatinib mesylate on mast cell tumors in dogs. J. Vet. Intern. Med. 22, 985-988.
Kern, W., Kohlmann, A., Schoch, C, Schnittger, S., Haferlach, T., 2006. Comparison of mRNA abundance quantified by gene expression profiling and percentage of positive cells using immunophenotyping for diagnostic antigens in acute and chronic leukemias. Cancer 107, 2401-2407.
Leifer, C.E., Matus, R.E., 1985. Lymphoid leukemia in the dog. Acute lymphoblastic leukemia and chronic lymphocytic leukemia. Vet. Clin. North Am. Small Anim. Pract. 15, 723-739.
Lennartsson, J., Jelacic, T., Linneldn, D., Shivakrupa, R., 2005. Normal and oncogenic forms of the receptor tyrosine kinase kit. Stem Cells 23,16-43.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-AACt} method. Methods 25, 402-408.
London, C.A., Hannah, A.L., Zadovoskaya, R., Chien, M.B., Kollias-Baker, C, Rosenberg, M., Downing, S., Post, G., Boucher, J., Shenoy, N., Mendel, D.B., McMahon, G., Cherrington, J.M., 2003. Phase I dose-escalating study of SUI 1654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. Clin. Cancer Res. 9, 2755-2768.
London, C.A., Malpas, P.B., Wood-Follis, S.L., Boucher, J.F., Rusk, A.W., Rosenberg, M.P., Henry, C.J., Mitchener, K.L., Klein, M.K., Hintermeister, J.G., Bergman, P.J., Couto, G.C., Mauldin, G.N., Michels, G.M., 2009. Multicenter, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SUI 1654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. Clin. Cancer Res. 15, 3856-3865.
Masson, K., Ronnstrand, L, 2009. Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3. Cell. Signal. 21, 1717-1726.
Mizuki, M., Ueda, S., Matsumura, I., Ishiko, J., Schwable, J., Serve, H., Kanakura, Y., 2003. Oncogenic receptor tyrosine kinase in leukemia. Cell Mol. Biol. (Noisy-le-grand) 49, 907-922.
Paietta, E., Ferrando, A.A., Neuberg, D., Bennett, J.M., Racevskis, J., Lazarus, H., Dewald, G., RoweJ.M., Wiernik, P.H., Tallman, M.S., Look, A.T., 2004. Activating FLT3 mutations in CD117/KIT(+) T-cell acute lymphoblastic leukemias. Blood 104, 558-560.
Ray, P., Krishnamoorthy, N., Ray, A., 2008. Emerging functions of c-kit and its ligand stem cell factor in dendritic cells. Cell Cycle 7, 2826-2832.
Shimada, A., Taki, T., Tabuchi, K., Tawa, A., Horibe, K., Tsuchida, M., Hanada, R., Tsukimoto, I., Hayashi, Y., 2006. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukaemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood 107, 1806-1809.
Sperling, C, Schwartz, S., Buchner, T., Thiel, E., Ludwig, W.D., 1997. Expression of the stem cell factor receptor C-KIT (CD117) in acute leukemias. Haematologica 82, 617-621.
Stirewalt, D.L., Meshinchi, S., 2010. Receptor tyrosine kinase alterations in AML - biology and therapy. Cancer Treat. Res. 145, 85-108.

Tsao, A.S., Kantarjian, H., Thomas, D., Giles, F., Cortes, J., Garcia-Manero, G., Huh, Y., Yang, Y., Shen, Y., Albitar, M., Estey, E., 2004. C-kit receptor expression in acute leukaemia - association with patient and disease characteristics and with outcome. Leuk. Res. 28, 373-378.

Usher, S.G., Radford, A.D., Villiers, E.J., Blackwood, L, 2009. RAS, FLT3, and C-KIT mutations in immunophenotyped canine leukemias. Exp. Hematol. 37, 65-77.

Valli, V.E., Jacobs, R.M., Parodi, A.L., Vernau, W., Moore, P.F., 2002. Histological Classification of Haematopoietic Tumors of Domestic Animals. World Health Organization, Second Series, vol. III. Armed Forces Institute of Pathology, Washington, DC.

Wang, Y.Y., Zhou, G.B., Yin, T., Chen, B., Shi, J.Y., Liang, W.X., Jin, X.L., You, J.H., Yang, G., Shen, Z.X., Chen, J., Xiong, S.M., Chen, G.Q,, Xu, F., Liu, Y.W., Chen, Z., Chen, S.J., 2005. AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec. Proc. Natl. Acad. Sci. U.S.A. 102,1104-1109.

Table 1
Complete list of control and leukemia cases considered in the present study. For each sample the identity (ID), diagnosis and c-KIT results (mRNA expression, CD117 mean fluorescent intensity, and the percentage of CD117 positive cells) are reported.

ID	Diagnosis	c-KIT results		
		mRNA (RQ)	MFI	Cell positivity (%)
c514	CTRL	0.04	0.78	0.17
c515	CTRL	0.04	0.87	0.31
c533	CTRL	0.02	0.90	0.12
c534	CTRL	0.07	1.10	0.17
c543	CTRL	0.09	1.16	0.19
c532	CTRL	0.07	1.75	0.29
M100-c38/11	B-cell ALL CD79a+	15.30	2.90	41.12
M102-c58/11	B-cell ALL CD79a+	22.63	3.16	45.25
M145-c347/11	T-cell ALL CD5+	1.93	2.16	5.20
M160-c458/11	T-cell ALL CD5+	5.70	67.64	83.97
M127-c224/11	AUL	13.88	1.59	9.21
M147-c357/11	AUL	8.79	2.69	25.61
M148-c371/11	AUL	1.61	4.60	17.54
M131-c237/11	AUL	4.24	4.96	39.19
M93-c04/11	AUL	13.22	5.84	55.12
M113-c151/11	AUL	22.01	6.85	72.49
M111-c128/11	AUL	15.24	7.17	58.22
M137-c310/11	T-cell CLL CD3+CD5+CD4−CD8−	0.02	1.69	1.56
M96-c12/11	B-cell CLL CD21+CD79a+	0.13	1.50	5.76
M94-c08/11	T-cell CLL CD3+CD5+CD8+	0.00	1.40	6.20
M120-c208/11	T-cell CLL CD3+CD5+CD8+	0.00	1.12	0.98
M125-c221/11	T-cell CLL CD3+CD5+CD8+	n.a.	1.29	1.34
M76-c415/10	T-cell CLL CD3+CD5+CD4+	0.42	n.a.	n.a.
M157-c416/11	T-cell CLL CD3+CD5+CD8+	0.06	0.89	3.28
M152-c397/11	T-cell CLL CD3+CD5+CD8+	0.00	1.51	1.48
M104-c76/11	T-cell CLL CD3+CD5+CD8+	0.00	1.95	5.14
M143-c326/11	T-cell CLL CD5+CD4−CD8−	0.01	1.89	3.31
M136-c284/11	T-cell CLL CD3+CD5+CD8+	0.00	0.91	0.43
M133-c244/11	T-cell CLL CD3+CD5+CD8+	0.00	1.98	1.94

CTRL, control; RQ, relative quantification value; MFI, mean fluorescence intensity (measured as the neoplastic cells MFI/unstained cells MFI ratio); n.a., not available.

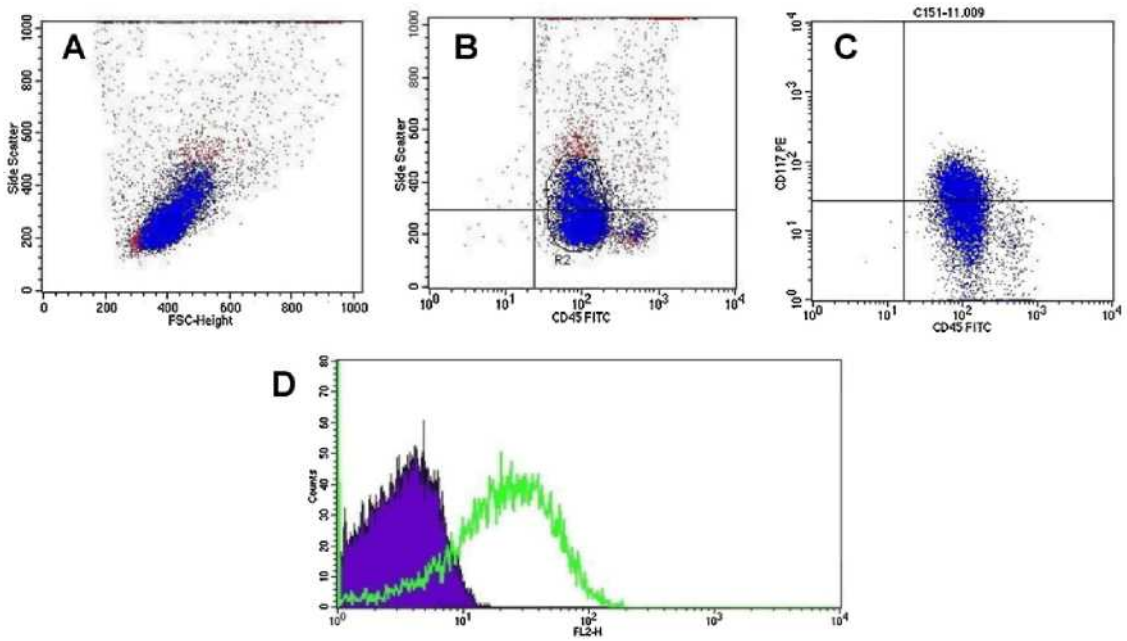

Fig. 1. Flow cytometric analysis of an acute lymphoblastic leukemia (ALL) sample. Panel A: morphological scatter plot with forward scatter (FSC) versus side scatter (SSC). Panel B: gating strategy. Blasts were identified based on normal to diminished CD45 expression and side scatter properties. Panel C: percentage of positive cells double stained for CD45 and CD117. Panel D: blasts CD117 (c-KIT) mean fluorescence intensity (MFI). The autofluorescence of unstained cells is indicated by the violet curve (on the left), while the fluorescence of stained cells is indicated by the green curve (on the right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

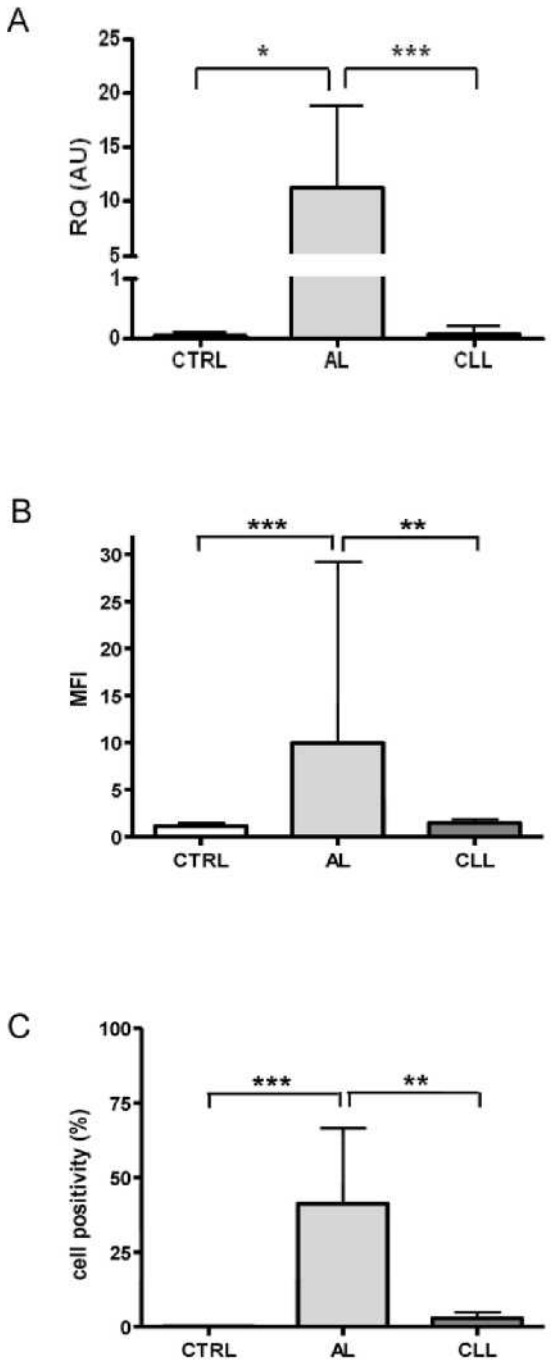

Fig.2. Messenger RNA (A) and protein (B and C) amounts of c-KIT (CD117) in peripheral blood samples of control (CTRL), acute leukemic (AL) and chronic lymphocytic leukemic (CLL) dogs. The amounts of c-KIT mRNA, measured by using a qPCR assay, are expressed as relative quantification values (RQ and arbitrary units, AU); protein data, obtained through a flow cytometric analysis, are presented either as the mean fluorescence intensity index (MFI, B) then as the percentage of CD117 positive cells (C). All data are expressed as mean± SD. Statistical analysis: Kruskal-Wallis test plus Dunn's post test. "P<0.01 ; "*P< 0.001.

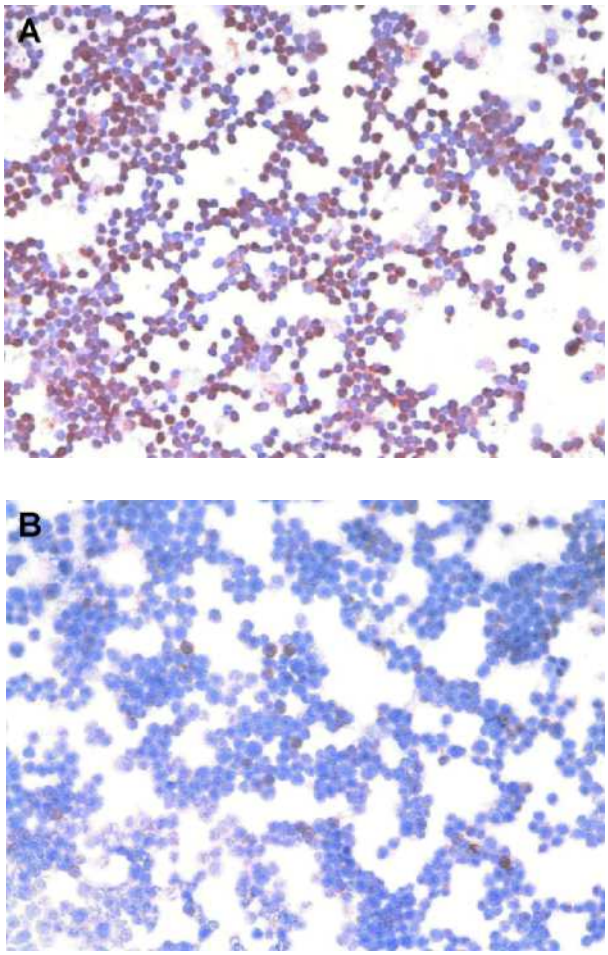

Fig. 3. Representative images of cytoplasmic c-KIT protein staining for AL (A) and CLL cells (B). An intense and diffuse immunostaining of lymphoid leukemia cells was observed in AL, whereas a rare positive immunolabelling was noticed in CLL.