Evaluation of an intron deletion in the *c-kit* gene of canine mast cell tumors

María José Reguera, MSc; Lluís Ferrer, DVM, PhD; Rosa M. Rabanal, PhD

Objective—To evaluate molecular abnormalities in the c-*kit* gene of canine mast cell tumors (MCT) with different grades of cellular differentiation.

Sample Population—31 normal tissue specimens from dogs and 45 canine MCT classified according to grade of cell differentiation.

Procedures—Genomic DNA extractions were made from canine MCT and normal tissues. Parts of exon 11, intron 11, and exon 12 of the c-*kit* gene were amplified by use of polymerase chain reaction. These regions were cloned, sequenced, and compared with GenBank sequences of the National Center for Biotechnology International. A statistical analysis was used to compare sequences from canine MCT and normal tissues.

Results—A significantly higher percentage of homozygous intron 11 deletion was found in canine MCT (49%) than in normal tissues (13%). This percentage was also higher in moderately and poorly differentiated MCT, compared with well-differentiated MCT. Although no mutations were detected in any of the specimens, a polymorphism at amino acid position 606 of the canine c-*kit* sequence was found in all the studied sequences.

Conclusions and Clinical Relevance—Results indicated a relationship between intron 11 deletion and MCT, and the grade of MCT differentiation. We suggest that intron 11 deletion may be implicated in the pathogenesis of MCT and could be used as a marker for diagnosis and prognosis of canine MCT. (*Am J Vet Res* 2002;63:1257–1261)

The proto-oncogene c-kit encodes for the transmembrane receptor KIT, which is a type-III tyrosine kinase receptor. A KIT receptor is a protein of 145 or 125 kd, depending on the grade of glycosylation.[1-3] The KIT receptor consists of an extracellular domain with 5 immunoglobulin-like domains, a transmembrane hydrophobic domain, and a cytoplasm domain. The intracellular domain consists of an ATP-binding region and a phosphotransferase region, which correspond to the kinase domain. The juxtamembrane region has been located in the cytoplasm domain between the transmembrane and kinase domains.[4,5]

The ligand of the KIT receptor is the **stem cell factor (SCF)**, a cytokine that binds to the extracellular domain. Stem cell factor causes receptor dimerization, which induces tyrosine autophosphorylation in the kinase domain and initiation of biological response.[6,7] The SCF is synthesized in the skin by fibroblasts, keratinocytes, and endothelial cells.[8,9] The KIT-SCF system is of major importance in the proliferation and differentiation process of mast cells, hematopoietic stem cells, germ cells, and human melanocytes.[10]

The human c-*kit* gene has been located in chromosome 4, between 4q11 and 4q21.[4] The coding sequence gene consists of 21 exons. Exon 1 encodes for the signal peptide. Exons 2 to 9 encode for the rest of extracellular domain, exon 10 codes for the transmembrane domain, and exons 11 to 20 code for the intracellular domain. Exon 11 encodes for the juxtamembrane region, which is 100% conserved in all mammals studied.[5] The ATP-binding region has been located in exon 12. Exon 21 encodes for the translation stop codon and polyadenylation site.[4,11]

Mutations in different domains of c-*kit* have been found in human patients with mastocytosis, leukemia, and gastrointestinal stromal tumors and in canine **mast cell tumors (MCT)**. Some of these mutations induce the constitutive activation of KIT receptor.[12-15]

In human patients with systemic mastocytosis, a point mutation in tyrosine kinase domain of c-*kit* was found. This mutation induces a substitution of 816-valine amino acid for aspartate (D816V). This mutation induces the constitutive phosphorylation of KIT receptor that results in ligand-binding independent KIT activation.[12,13] The same point mutation has been found in the human mast cell-1 leukemia line.[16] Furthermore, the mutation D816Y has been detected in murine P-815 and rat RBL-2H3 mast cell lines.[17,18]

Mutations in human **gastrointestinal stromal tumors (GISTs)** have been detected in the extracellular domain of c-*kit*,[15] in the juxtamembrane domain,[14, 19-22] and in the tyrosine kinase domain.[15] These mutations in c-*kit* induce the constitutive phosphorylation of KIT receptor.

Canine mast cell tumor or mastocytoma is a common skin neoplasm that represents 15% of skin and subcutaneous neoplasms in dogs.[23] The biological behavior of MCT is highly variable; some tumors have benign behavior and others have aggressive growth and metastasize.[24,25] Expression of KIT has been detected by use of immunohistochemistry in 23 canine MCT, and there exists an inverse correlation between the degree of differentiation of MCT and the expression of KIT.[26]

In spontaneous canine MCT, mutations have also been detected in the juxtamembrane region of c-*kit*. Tandem duplications affect exon 11, exon 12, and intron 11.[27] Ma et al[5] found amino acid substitutions and deletions in exon 11, which correspond to the juxtamembrane region of canine c-*kit* in canine MCT. These muta-

Received Jan 28, 2002.
Accepted Apr 17, 2002.
From the Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain.
Supported by the Dirección General de Investigación Científica y Técnica, Ministerio de Educación y Ciencia of Spain (reference No. PB96-1184).
Address correspondence to Dr. Rabanal.

tions induced constitutive phosphorylation of KIT and suggested the implication of c-*kit* mutations in the pathogenesis of canine MCT.[5] In the canine mastocytoma cell line C2, a 48-bp insertion between nucleotides 1784 and 1785 has been reported.[5, 27]

The purpose of the study reported here was to detect possible alterations in the region of the canine c-*kit* gene corresponding to the juxtamembrane and kinase domains (part of exon 11, intron 11, and exon 12) in canine MCT. We hypothesized that there could be molecular differences in c-*kit* genes between canine MCT and normal canine tissues. In addition, we expected to find molecular differences between MCT with different grades of differentiation. The molecular study of a high number of MCT could help to understand the development, biological behavior, and high incidence of MCT in dogs.

Materials and Methods

Specimens—This study was performed on 45 spontaneous canine MCT and specimens from 31 clinically normal dogs including skin, liver, kidney, and lung. Paraffin-embedded specimens were obtained from the Unit of Veterinary Pathology of the Universitat Autònoma of Barcelona. All specimens were fixed in neutral-buffered 10% formalin, processed routinely, embedded in paraffin, and sectioned at 4 µm. Sections were stained with H&E and alcian blue for mast cells. Mast cell tumors were classified according to the grading system devised by Patnaik et al[28] as grade I (well differentiated), grade II (moderately differentiated), and grade III (undifferentiated). In addition, the canine mastocytoma cell line C2[a] was used in this study.[26]

Genomic DNA extraction—Genomic DNA extraction was performed on paraffin-embedded specimens from each dog by use of a modified described method.[29–31] The MCT specimens were taken from the central part of the tumor mass, carefully avoiding margins and non-neoplastic surrounding tissue. Each specimen and block were cut and handled with new disposable blades and gloves to avoid cross-contamination with DNA among specimens. After cutting each block, the microtome blade, forceps, and entire cutting area were carefully cleaned with a 0.1*M* solution of sodium hypochlorite. Sections (10 µm thick) were prepared in an Eppendorf tube from each formalin-fixed, paraffin-embedded specimen. The specimens were deparaffinated by immersion twice for 30 minutes each at 20 C in 1 ml of xylene and were rinsed twice with 1 ml of 100% ethanol for 5 minutes. Specimens were centrifuged at 10,000 × g for 5 minutes, and the liquid was decanted between each change. To dry the specimen, 2 or 3 drops of acetone were added, and the specimen was placed in a heating block at 45 C. The sections were resuspended in 100 µl of tissue digestion solution consisting of proteinase K[b] (250 µg/ml) in 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, and 0.5 % (vol/vol) Tween 20. The Eppendorf tubes were left in a shaking water bath at 53 C overnight. Afterwards, DNA was isolated by use of double phenol-chloroform extraction.[32] The supernatants were used in a **polymerase chain reaction (PCR)** assay for DNA amplification and detection.

DNA amplification—The genomic DNA from 45 canine MCT and 31 normal canine tissues was amplified by use of PCR with *Taq* DNA polymerase[c] for 35 cycles of 1 minute at 95 C for DNA denaturation, 1 minute at 53 C for annealing, and 1 minute at 72 C for extension. The primers used for this PCR were: 5'-TAGACCCAACACAGCTTCCTTACGAT- 3' (forward primer, nt 1737–1762) and 5'-AGCATCTTAACG-GCAACAGTCA-3' (lower primer, nt 1899–1878).

The primer sequences were obtained from the published canine c-DNA c-*kit* sequence (accession No. AF044249) in the database from the GenBank of the National Center for Biotechnology International. The c-*kit* gene-amplified sequence of 443 bp included part of the juxtamembrane and kinase domains, which correspond to part of exon 11, intron 11, and exon 12.

In addition, to further determine the presence of intron 11, genomic DNA from 7 canine MCT and 2 normal canine skin specimens was amplified from intron 11 to exon 12 by use of a second PCR. It included a forward primer based in intron 11 (5'-CAGTATGAAATAGGGGCTTTCC-3'). This forward primer was chosen after DNA cloning and sequencing of the 443-bp fragment obtained as described. The same lower primer and PCR conditions were used in this PCR assay. Amplification of β-actin was used as a control for the integrity of the DNA extracted from paraffin-embedded tissues and for the PCR procedure. The primers used for β-actin amplification were: 5'-CATGTTTGACACCTTCAACACC-CC-3' and 5'-GCCATCTCTTGCTCGAAGTCCAG-3'.

The amplified PCR products were separated by use of electrophoresis in a 2% agarose gel, stained with ethidium bromide, and viewed with ultraviolet illumination.

Cloning and sequencing—The PCR products (from exon 11 to exon 12) from 16 canine MCT and 3 normal canine tissues were purified from the agarose gel by use of a gel extraction kit[d] and were cloned into pGEM-T Vector System II plasmid and JM109 competent cells,[e] following the manufacturer's instructions. The amplified products were sequenced by use of cycle sequencing.[f] The primers for the sequencing reaction were the standard T7 and SP6 of pGEM-T. The sequences were analyzed by use of a sequencer.[g] Sequences were compared in the database of GenBank by use of the BLAST program from the National Center for Biotechnology International. The DNA from canine mastocytoma cell line C2 was included as a positive control for the sequencing procedure.

Statistical analysis—χ_2 Analysis[h] was used to identify differences in c-*kit* sequences between canine MCT and normal tissues.

Results

Intron deletion in canine MCT and normal tissues—Thirty-one normal tissues and 45 canine MCT from different dogs were studied. The genomic DNA of dogs had not been sequenced yet; however, the expected PCR product size was approximately 443 bp, as suggested by results of human genomic DNA sequence analysis (GenBank accession No. L04143). In normal tissues and in MCT, the PCR products had 3 patterns of bands: homozygous dogs with a band of approximately 443 bp, homozygous dogs with a shorter band of approximately 162 bp, and heterozygous dogs with both bands (**Fig 1**). The integrity of the genomic DNA extracts was confirmed by use of PCR amplification of a fragment of 300 bp from the β-actin gene.

The sequenced PCR products had a band of 448 bp, which corresponds with DNA amplification of part of exon 11 and the complete sequences of intron 11 and exon 12 (**Fig 2**). The sequence of the shorter band had a molecular size of 165 bp, which corresponds with part of exon 11 and complete exon 12; therefore, there was a complete deletion of intron 11 in the shorter band. The canine mastocytoma cell line C2 had the expected insertion of 48 bp between nucleotides 1784 and 1785.

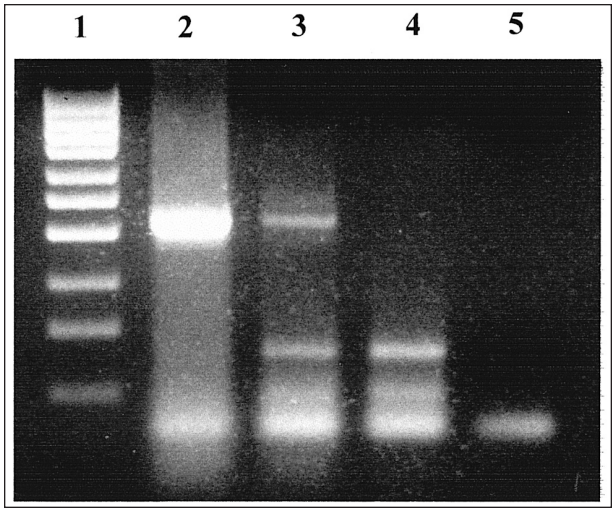

Figure 1—Ethidium bromide-stained 2% agarose gel electrophoretogram of results of polymerase chain reaction (PCR) assay for the canine c-*kit* gene in DNA extracted from canine mast cell tumors. Lane 1—Standard molecular ladder. Lane 2—Homozygous with 448- band. Lane 3—Heterozygous with 448-bp and 165-bp bands. Lane 4—Homozygous with 165-bp band. Lane 5—Negative control. The same pattern of bands was observed in DNA extracted from normal tissues.

```
423
424  tagacccaacacagcttccttacgatcacaaatgggagtttcccagaaacaggctgagc
425  tttgt[cagtatgaaataggggctttcc]atgtaacctttttgtgtacgtgtaacaatg
426  actttagggaaccccattagcttcctttgttctgttccaactgagacaataagtatttt
427  ctgtgaagtttcatcatttttgatagattcctcataaagcaccttatagagaaatgtcc
428  ttagctggatttgtccttaattccttaacaattccttgattgttgactttgaaattacc
429  cagatgctcctttggtcctatcaccacccctttactcttttcttcctttctgcagggaaa
430  actttgggtgctggtgccttcgggaaagtggttgaagccactgcatatggcctgattaa
431  gtcggatgcggcca[tgactgttgccgttaagatgct]
```

Figure 2—DNA sequence of exon 11, complete intron 11, and complete exon 12 from canine c-*kit* gene obtained after cloning and sequencing of the 448-bp band amplified by use of PCR. The forward and lower primers used in the PCR are shaded. Brackets enclose the forward and lower primers that amplify from intron 11 to exon 12. Intron 11 is underlined.

To verify the absence of intron 11 in genomic DNA extracts from canine MCT and normal tissues, the second PCR was used. A forward primer included in intron 11 was designed after the complete sequence of the canine intron was obtained (Fig 2). The amplified PCR product was a band of 383 bp in those specimens in which the first PCR had detected a band of 448 bp (specimens with intron 11). No second PCR product was obtained in those specimens in which the first PCR had revealed the band of 165 pb (sequences without intron 11).

A high percentage of canine MCT (49%) were homozygous for the deletion of intron 11. In normal tissues, this pattern was detected in 13% of specimens; the difference between these percentages was significant ($P = 0.001$). Thirty-three percent of MCT had the expected pattern of bands, and the remaining MCT were heterozygous (18%). In normal canine tissues, the normal pattern of bands was more frequent (68%) than the homozygous with deletion (13%) and heterozygous (19%; **Table 1**) patterns.

Table 1—Distribution (proportion [%]) of different patterns of bands in canine mast cell tumors (MCT) classified according to grade of cell differentiation and normal canine tissues.

	Grade of MCT				
Pattern of bands	**Grade-I MCT**	**Grade-II MCT**	**Grade-III MCT**	**Total MCT**	**Normal tissues**
Homozygous 448-bp band	8/14 (57)	5/21 (24)	2/10 (20)	15/45 (33)	21/31 (68)
Heterozygous 448-bp/165-bp bands	2/14 (14)	3/21 (14)	3/10 (30)	8/45 (18)	6/31 (19)
Homozygous 165-bp band	4/14 (29)	13/21 (62)	5/10 (50)	22/45 (49)	4/31 (13)

The canine MCT were classified according to grade of differentiation. Moderately (grade II) and poorly differentiated (grade III) tumors had a high percentage with intron 11 deletion, representing 62 and 50%, respectively. The percentage obtained for well-differentiated MCT was only 29% (Table 1). However, differences among these groups were not significant.

Nucleotide substitution—In all the studied sequences (16 canine MCT and 3 normal canine tissues), the nucleotide substitution ACC ACT in codon 606 was detected. This substitution does not change the amino acid sequence. No other nucleotide substitutions, deletions, or duplications were detected in any of the specimens investigated.

Discussion

In previous studies, London et al[27] found mutations in 5 of 11 canine MCT in the c-*kit* gene. They detected tandem duplications with exon 11, exon 12, and intron 11 involvement.[27] Also, Ma et al[5] found amino acid substitutions and deletions in 3 of 7 canine MCT. The mutations were detected in exon 11 of c-*kit* cDNA, and they induce the constitutive activation of receptor KIT. In consequence, the authors suggest that c-*kit* is implicated in tumor development.[5] Our results indicated the presence of an unexpected band of 165 bp in some specimens. We verified that this fragment corresponded with exon 11 and exon 12 of canine c-*kit* cDNA after cloning and sequencing the 165-bp PCR product and comparing it in the database of the GenBank (accession No. AF044249). This indicated that there is a loss of intron 11 in some canine MCT (49%) and in some normal canine tissues (13%). To confirm the absence of intron 11, a second PCR was performed to amplify intron 11 and exon 12, and results indicated a band of 383 bp only in those specimens in which the 448-bp band was present. All these results suggested a deletion of intron 11. However, the small band that we amplified could be an artifact generated by DNA fragmentation. It is known that DNA isolated from paraffin-embedded material can lose its integrity and often is fragmented. To eliminate this possibility, β-actin gene amplification was included as an internal control of DNA integrity and of the PCR procedure. Only specimens from which amplification of β-actin was observed were used in this study.

Another alternative explanation for the loss of intron 11 is the presence of a pseudogene. A pseudogene is a nonfunctional copy of most or all of a gene and often does not contain introns. This hypothesis

would explain the presence of the heterozygous pattern of bands (448 and 165 bp), which could correspond with the normal c-*kit* gene and the pseudogene, respectively. However, this hypothesis could not explain the presence of homozygous dogs with the intron 11 deletion, which had only a band of 165 bp. Moreover, to our knowledge, the presence of a pseudogene for the canine c-*kit* gene has not been reported. Nevertheless, to completely rule out the possibility of a pseudogene for the canine c-*kit* gene, clarification of the existence of the rest of introns in those specimens in which results indicated an intron 11 deletion must be accomplished.

Results of our study indicate that loss of intron 11 of the canine c-*kit* gene occurs in canine cells, and especially in canine MCT. A high percentage (49%) of MCT were homozygous for the intron 11 deletion. This altered sequence was also detected in normal tissues, but only in 13% of our specimens. From the statistical point of view, the relationship between the intron deletion and the tumor is clear; however, the mechanism has yet to be established. The function of intron 11 is unknown, but our findings suggest an inherent c-*kit* gene instability that facilitates the accumulation of mutations. Another possibility is that the intron deletion affects the transcription process, producing an altered mRNA product, which could be implicated in the genesis of the neoplastic growths. However, although we found the insertion of 48 bp in canine mastocytoma cell line C2, confirming previous results.[5,27] we did not find mutations in the studied region of the c-*kit* gene in any of the studied sequences from canine MCT. This is in contrast with published studies of canine MCT that were based on cDNA[5] and genomic DNA.[27]

The moderately (grade II) and poorly differentiated MCT (grade III) had higher percentages of sequences with intron 11 deletion than the well-differentiated (grade I) MCT did. Confirmation of these results will be interesting for the purpose of using the detection of intron deletion as a prognostic marker for canine MCT. The intron 11 deletion may indicate worse prognosis in canine MCT. In fact, the detection of mutations in exon 11 of the c-*kit* gene is used as a prognostic marker in human patients with gastrointestinal stromal tumors.[21,22]

These results indicate the complexity and molecular diversity of canine MCT, which explains the high variability of biological behavior of these tumors. Canine MCT probably are the result of various mutations and are genetically heterogeneous, as are most human and canine neoplasms. Each single neoplasm is, probably, the consequence of several somatic mutations, which may involve other genes, leading to abnormal cell proliferation.

[a]Provided by Dr. Caughey, University of California, San Francisco, Calif.
[b]Boehringer Mannheim GmbH, Werk Penzberg, Germany.
[c]Ecogen, SRL, Barcelona, Spain.
[d]Qiagen, Hilden, Germany.
[e]Promega Corp, Madison, Wis.
[f]DyEnamic ET Terminator Cycle Sequencing, Amersham Pharmacia Biotech Inc, Piscataway, NJ.
[g]ABI 373 Stretch DNA Sequencer, Applied Biosystems Inc, Foster City, Calif.
[h]Epi Info version 6.04 Program, CDC, Atlanta, Ga.

References

1. Majumder S, Brown K, Qui F, et al. c-kit protein, a transmembrane kinase: identification in tissues and characterization. *Mol Cell Biol* 1988;8:4896–4903.

2. Nocka K, Majumder S, Chabot B, et al. Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice: evidence for an impaired c-kit kinase in mutant mice. *Gene Dev* 1989;3:816–826.

3. Nocka K, Tan JC, Chiu E, et al. Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: W^{37}, W^{v}, W^{41} and W. *EMBO J* 1990;9:1805–1813.

4. Yarden Y, Kuang W, Yang-feng T, et al. Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J* 1987;6:3341–3351.

5. Ma Y, Longley J, Wang X, et al. Clustering of activating mutations in c-kit's juxtamembrane coding region in canine mast cell neoplasms. *J Invest Dermatol* 1999;112:165–170.

6. Rottapel R, Reedijk M, Williams D, et al. The Steel/W transduction pathway: kit autophosphorylation and its association with a unique subset of cytoplasmic signaling proteins is induced by the Steel factor. *Mol Cell Biol* 1991;11:3043–3051.

7. Kanakura Y, Ikeda H, Kitayama H, et al. Expression, function and activation of the proto-oncogene c-kit product in human leukemia cells. *Leuk Lymphoma* 1993;10:35–41.

8. Longley B Jr, Morganroth G, Tyrrell L, et al. Altered metabolism of mast-cell growth factor (c-kit ligand) in cutaneous mastocytosis. *N Engl J Med* 1993;328:1302–1307.

9. Weiss R, Whitaker D, Longley J, et al. Human dermal endothelial cells express membrane-associated mast cell growth factor. *J Invest Dermatol* 1995;104:101–106.

10. Galli S, Zsebo K, Geissler E. The kit ligand, stem cell factor. *Adv Immunol* 1994;55:1–96.

11. Vandenbark G, deCastro C, Taylor H, et al. Cloning and structural analysis of the human c-kit gene. *Oncogene* 1992;7:1259–1266.

12. Nagata H, Worobec A, Oh C, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. *Proc Natl Acad Sci U S A* 1995;92:10560–10564.

13. Longley B, Tyrrell L, Lu S, et al. Somatic c-KIT activating mutation in urticaria pigmentosa and aggressive mastocytosis: establishment of clonality in a human mast cell neoplasm. *Nat Genet* 1996;12:312–314.

14. Hirota S, Isozaki K, Moriyama Y, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* 1998;279:577–580.

15. Lux M, Rubin B, Biase T, et al. KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. *Am J Pathol* 2000;156:791–795.

16. Furitsu T, Tsujimura T, Tono T, et al. Identification of mutations in the coding sequence of the proto-oncogene c-kit in a human mast cell leukemia cell line causing ligand-independent activation of c-kit product. *J Clin Invest* 1993;92:1736–1744.

17. Tsujimura T, Furitsu T, Morimoto M, et al. Ligand-independent activation of c-kit receptor tyrosine kinase in a murine mastocytoma cell line P-815 generated by a point mutation. *Blood* 1994;83:2619–2626.

18. Tsujimura T, Furitsu T, Morimoto M, et al. Substitution of an aspartic acid results in constitutive activation of c-kit receptor tyrosine kinase in a rat tumor mast cell line RBL-2H3. *Int Arch Allergy Immunol* 1995;106:377–385.

19. Nishida T, Hirota S, Taniguchi M, et al. Familial gastrointestinal stromal tumors with germ line mutation of the KIT gene. *Nat Genet* 1998;19:323–324.

20. Nakahara M, Isozaki K, Hirota S, et al. A novel gain-of-function mutation of c-kit gene in gastrointestinal stromal tumors. *Gastroenterology* 1998;115:1090–1095.

21. Lasota J, Jasinski M, Sarlomo-Rikala M, et al. Mutations in

exon 11 of c-kit occur preferentially in malignant versus benign gastrointestinal stromal tumors and do not occur in leiomyomas or leiomyosarcomas. *Am J Pathol* 1999;154:53–60.

22. Taniguchi M, Nishida T, Hirota S, et al. Effect of c-kit mutation on prognosis of gastrointestinal stromal tumors. *Cancer Res* 1999;59:4297–4300.

23. Conroy J. Canine skin tumors. *J Am Anim Hosp Assoc* 1983; 19:91–114.

24. Bostock D. The prognosis following surgical removal of mastocytomas in dogs. *J Small Anim Pract* 1973;14:27–41.

25. Walder EJ, Gross TL. Canine mast cell tumors. In: Gross TL, Ihrke PJ, Walder EJ, eds. *Veterinary dermatopathology-a macroscopic and microscopic evaluation of canine and feline skin diseases*. St Louis: Mosby Year Book Inc, 1992;470–473.

26. Reguera MJ, Rabanal RM, Puigdemont A, et al. Canine mast cell tumors express stem cell factor receptor. *Am J Dermatopathol* 2000;22:49–54.

27. London C, Galli SJ, Yuuki T, et al. Spontaneous canine mast cell tumors express tandem duplications in the proto-oncogene c-kit. *Exp Hematol* 1999;27:689–697.

28. Patnaik A, Ehler W, MacEwen E. Canine cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. *Vet Pathol* 1984;21:469–474.

29. Shibata DK, Arnheim N, Martin WJ. Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. *J Exp Med* 1988;167:225–230.

30. Laskay T, Mikó TL, Negesse Y, et al. Detection of cutaneous *Leishmania* infection in paraffin-embedded skin biopsies using the polymerase chain reaction. *Trans R Soc Trop Med Hyg* 1995;89:273–275.

31. Roura X, Fondevila D, Sanchez A, et al. Detection of *Leishmania* infection in paraffin-embedded skin biopsies of dogs using polymerase chain reaction. *J Vet Diagn Invest* 1999;11:385–387.

32. Samrookk J, Fritsch EF, Maniatis T. Appendix E: commonly used techniques in molecular cloning. Purification of nucleic acids. In: Editors. *Molecular cloning: a laboratory manual*. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989;E3–E4.