Frequent Expression of the *c-kit* Proto-Oncogene in Canine Malignant Mammary Tumor

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ABSTRACT. The mammary tumor is one of the popular neoplastic diseases in female dogs. In the present study, the expression of canine *c-kit* proto-oncogene in mammary tumor specimens was investigated to evaluate its potential usefulness as a tumor marker. By comparing the homology among the nucleotide sequences reported for human, mouse, rat and feline *c-kit* c-DNA, a pair of primers was synthesized for the reverse transcriptase-polymerase chain reaction (RT-PCR) method. The RT-PCR product of canine spleen total RNA was shown to have 756 bp in size and to be highly homologous to the corresponding sequences reported for the mammalian species. The expression of *c-kit* transcript was detected in 11 mammary tumors of different histopathology including adenocarcinomas, benign and malignant mixed tumors. The level of the transcription in adenocarcinomas was significantly higher than those in malignant mixed tumors. Fifteen canine tumor specimens originated from various tissues were also tested for their *c-kit* transcript. In all of the mastocytoma samples examined, high expression of the mRNA was detected. Of other 12 tumors, only low level of RT-PCR products were detected in 5 samples, whereas no apparent amplification was observed in 7 tumors. These results indicate that the high expression of *c-kit* transcript is helpful for the diagnosis of canine mammary tumors. — KEY WORDS: *c-kit*, canine, mammary tumor, RT-PCR, tumor marker.

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It has been reported that the product of proto-oncogene *c-kit*, the tyrosine kinase receptor for stem cell factor (steel factor or mast cell growth factor) is expressed in a variety of normal and malignant human tissues [2–4, 16, 20, 23, 26]. Lammie *et al*. have demonstrated that *c-kit* protein (KIT) is expressed in normal tissues such as tissue mast cells, melanocytes, glandular epitherial cells of breast, parotid, dermal sweat, esophageal glands, cerebellum, hippocampus, and dorsal horn of the spinal cord [11]. It is well known that the gene product is essential for the growth and differentiation of mast cells, and the expression is mostly persistent after malignant transformation to mastocytoma [4, 12, 26]. In small-cell type lung carcinoma (SCLC), the preferential expression of *c-kit* mRNA has been described [23, 27]. Frequent expression of both the receptor and the ligand has been also detected in non-small-cell lung carcinomas including adenocarcinomas, squamous-cell and undifferentiated carcinomas [20], as well as in most of seminoma/dysgerminoma (92%) [27]. These observations suggest that *c-kit* receptor is mainly expressed in the tumors originated from those tissues exhibiting the protein. On the other hand, Tsuura *et al*. have demonstrated that normal human breast and skin tissues displayed KIT receptor at high incidence, whereas the protein was detected in only 1 case out of 92 cases of the breast cancer examined [27]. The declined expression of KIT in malignant breast diseases has been reported as compared with those in normal tissue or benign lesions [2, 17]. Similar observation during malignant transformation and progression of melanocytes and thyrocytes has been reported [14, 18]. Because the growth regulatory role of the receptor has been suggested [19, 29], it is possible that the reduced expression is related to the tumorigenesis and/or progression of breat tumors.

The mammary tumor is one of very frequent neoplastic diseases in female dogs, and most of them (65%) are classified as benign [15]. Although London *et al*. have reported the expression of both KIT and the ligand in canine mast cell tumors [12], no report concerning the expression level of *c-kit* in canine mammary tumors is available. The present study has been carried out to investigate the status of *c-kit* mRNA expression in canine mammary tumors with the aid of reverse transcriptase polymerase chain reaction (RT-PCR), and to examine its potential significance as a tumor marker.

MATERIALS AND METHODS

Normal and tumor samples: For the positive control, a piece of spleen tissue was excised under general anesthesia from a clinically normal male beagle. Other samples were obtained from the tumor resected surgically in the Veterinary Teaching Hospital of Osaka Prefecture University. For the histological examination, a part of the tumor samples were fixed in 10% formalin and embedded in paraffin. Thin sections were, then, prepared and stained with haematoxylin-eosin. The histopathological diagnoses of these tumors are listed in Tables 1 (breast tumors) and 2 (other tumors).

Total RNA purification from normal and tumor tissues: All the tumor tissues were separated from the connective tissue and cut into small pieces in Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate buffered saline [PBS(-); 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4]

containing gentamycin sulfate at 0.8 mg/mL. The solid tumor samples were, then, washed to remove necrotic tissues in PBS (-). Total RNA from each specimen was prepared by the acid-guanidium thiocyanate-phenol-chloroform method [1], and stored at −80°C as described previously [13].

Amplification of canine c-kit cDNA from total RNAs of normal spleen and tumor tissues: Canine *c-kit* cDNA fragments were amplified by RT-PCR using total RNAs obtained from a canine normal spleen and tumor tissues as described previously with slight modifications [13]. Ten micrograms of total RNA was incubated with 200 units of Superscript II (Gibco BRL) and 500 *n*g of poly A oligonucleotide (12–18 mer, Pharmacia) at 37°C for 1 hr. The synthesized single-stranded cDNA was amplified with 2.5 units of Taq polymerase (Toyobo) and *c-kit* primers (sense: 5'-CAAATGTCACAACAAAATTG-3'; antisense: 5'-ACTTCATACATGGGTTCCTG-3') for 25 cycles (94°C for 0.5 min; 55°C for 1 min; and 72°C for 2 min) followed by incubation at 72°C for 5 min for complete elongation using a thermal cycler (Perkin-Elmer GENEAMP PCR system 2400). The sequence of RT-PCR primers were selected from the homologous region of the sequences reported for mouse, rat, human and feline *c-kit* cDNA using the Entrez search system. The expected amplified sequence includes whole transmembrane domain of human *c-kit* protein (amino acids 300–554). Alpha-tubulin primer set was used as a internal standard for the amount of mRNA included in reaction mixture (sense: 5'-TCCATCCTCACC ACCCACAC-3'; antisense: 5'-CGCTTGGTCTTGATGG TGGC-3') [13]. RT-PCR products were separated by a 1% agarose gel electrophoresis and stained with ethidium bromide.

DNA sequencing of RT-PCR product from canine spleen total RNA: The RT-PCR product from canine spleen was purified from agarose gel by Prep-A-Gene DNA purification kit (Bio Rad) and subcloned into pBluescript II KS+ (Stratagene) cleaved by Sma I. The nucleotide sequence of cloned PCR product was determined by the dideoxy-termination method [22] using Genetic analyzer A310 (Perkin-Elmer Applied Biosystems). The obtained sequence data was analyzed the homology to those reported for other mammals by a gene analysis software.

Table 1. Histological classification of canine mammary tumor samples

Sample number	Age (Year)	Histology	Amplification of *c-kit*
1	7	Benign mixed tumor	+
2	8	Adenocarcinoma	+
3	12	Adenocarcinoma	+
4	9	Malignant mixed tumor	+
5	9	Malignant mixed tumor	+
6	9	Malignant mixed tumor	+
7	6	Malignant mixed tumor	+
8	10	Malignant mixed tumor	+
9	14	Malignant mixed tumor	+
10	6	Malignant mixed tumor	+
11	7	Malignant mixed tumor	+

RESULTS

The DNA sequence of RT-PCR product: As the result of RT-PCR using the *c-kit* primer set and canine spleen total RNA, an amplified band was observed on the agarose gel. The size of the band was approximately 800 bp, corresponding to those expected from the reported mammalian *c-kit* cDNA sequences [6, 21, 25, 28]. The nucleotide sequence was detemined after cloning the amplified fragment into pBluescript II. The length of the RT-PCR product (756 bp) was same as that of corresponding mouse c-DNA region but shorter than those of human, feline

Table 2. Histological classification of various canine tumor samples

Sample number	Sex	Age (Year)	Histology	Amplification of *c-kit*
1	F	14	Adenocarcinoma of apocrine gland	–
2	MC	9	Basal cell carcinoma of skin	–
3	M	14	Perianal gland adenoma	+
4	F	9	Hemangiosarcoma	–
5	M	4	Malignant lymphoma	+
6	FS	1	Rhabdomyosarcoma of urinary bladder	+
7	F	12	Leiomyoma of vagina	–
8	F	12	Adrenal cortical carcinoma	+
9	F	8	Liposarcoma	+
10	F	8	Metastatic lymph node (Liposarcoma)	+
11	F	8	Normal Lymph node	–
12	M	8	Malignant melanoma	–
13	FS	12	Bronchiolar-alveolar carcinoma	–
14	F	8	Bronchiolar-alveolar carcinoma	–
15	M	7	Mast cell tumor	+
16	M	6	Mast cell tumor	+
17	M	11	Mast cell tumor	+

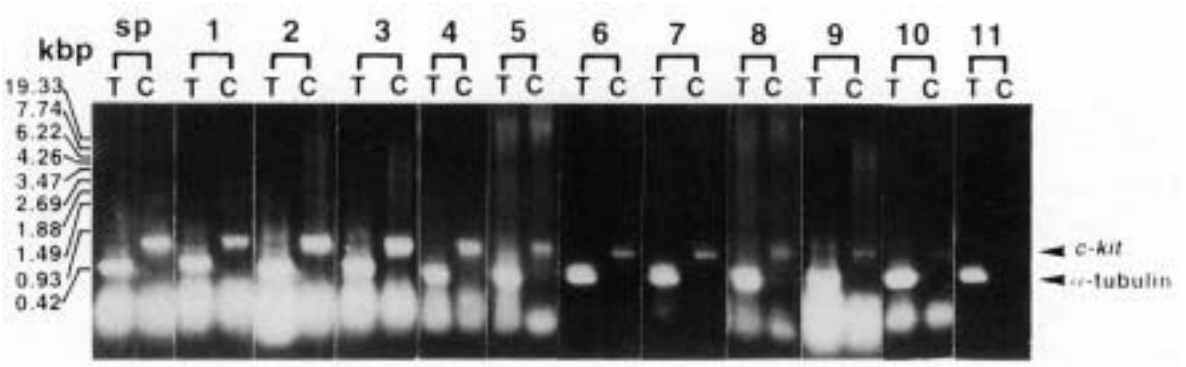

Fig. 1. Expression of *c-kit* mRNA in canine mammary tumors. The RT-PCR products were examined by 1% agarose gel electrophoreisis. The numbers of the samples are same as those in Table 1. The expression of the gene in the canine spleen is also shown (SP). C: *c-kit*, T: α-tubulin.

and rat (768 bp). The sequence is highly homologous to those reported for feline (90.4%), human (87.2%), mouse (80.3%), and rat (79.6%) *c-kit* cDNA. These results indicate that the DNA fragment amplified by the primer set is a part of canine *c-kit* cDNA.

Expression of c-kit mRNA in canine mammary tumors: Expression of *c-kit* mRNA in canine mammary tumors was examined by RT-PCR using the *c-kit* primer set. As shown in Fig. 1, the *c-kit* cDNA fragment was amplified in all the canine mammary tumor samples including one benign tumor, 2 adenocarcinomas, and 8 malignant mixed tumors (Table 1). In the normal breast samples obtained from three clinically healthy female dogs, only small amount of RT-PCR products were detected, probably because the enough number of cells were not present in these samples (data not shown). The amounts of PCR products were estimated using NIH Image ver. 1.61. Figure 2 shows the relative amount of each *c-kit* RT-PCR product to that of α-tubulin in the same tumor samples shown in Fig. 1. In the benign tumor and adenocarcinomas, the RT-PCR products were observed at the comparable level to that of the normal spleen, while the ratio was significantly depressed in maligmant mixed tumors. These results suggest the different expression level of *c-kit* mRNA among the canine breast tumors may be related to the histology, though the transcripts were positive in the canine mammary tumors examined.

Expresssion of c-kit mRNA in various tumors: The expresssion of *c-kit* mRNA in a variety of canine tumors were examined by RT-PCR as mentioned above. As reported by London *et al*. [12], intensified bands were observed after RT-PCR using the RNA samples from the three mast cell tumors (Fig. 3). The level was comparable to those of normal spleen and the mammary adenocarcinomas. As shown in Fig. 4, *c-kit* cDNA fragments were amplified at very reduced level in 5 out of 12 other canine tumor samples examined, while no appreciable amplification was obtained in 7 samples. The affected (sample no. 10) and non-affected (no. 11) lymph nodes were obtained from the patient with liposarcoma (no. 9). The RT-PCR product was detected only in the metastatic lymph node. The amplification of a-tubulin cDNA fragment was positive in all the tumor samples examined.

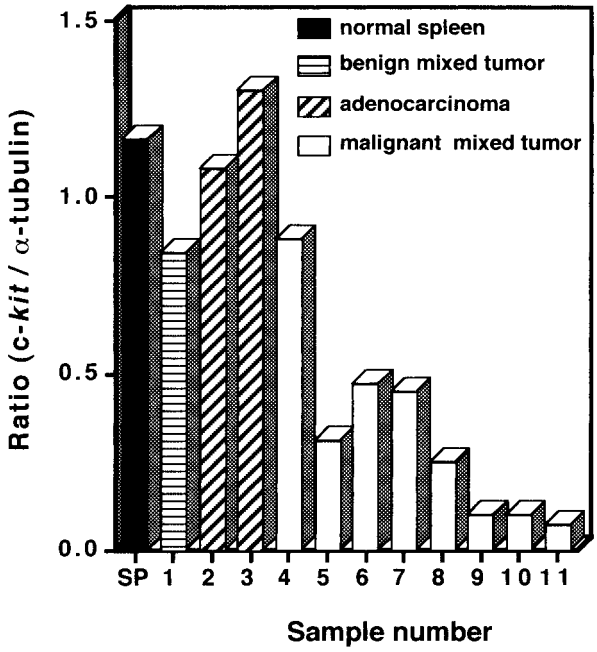

Fig. 2. Relative amount of amplified products of *c-kit* mRNA in the canine mammary tumor specimen in Table 1.

DISCUSSION

It has been reported that the administration of antisense oligodeoxynucleotides targeted to *c-kit* or the ligand mRNA inhibit the *in vitro* growth of malanoma, breast and non-small cell type lung carcinoma cells which are expressing both the receptors and the ligands [5]. Several types of point mutation in the kinase domain responsible for the ligand-independent activation of the product have been detected in a mast cell tumor cell line and gastrointestinal stromal tumors [8, 10]. These results indicate that *c-kit* plays a growth stimulatory role in these tumor cells. On the other hand, Huang *et al*. have observed apoptosis in the *c-*

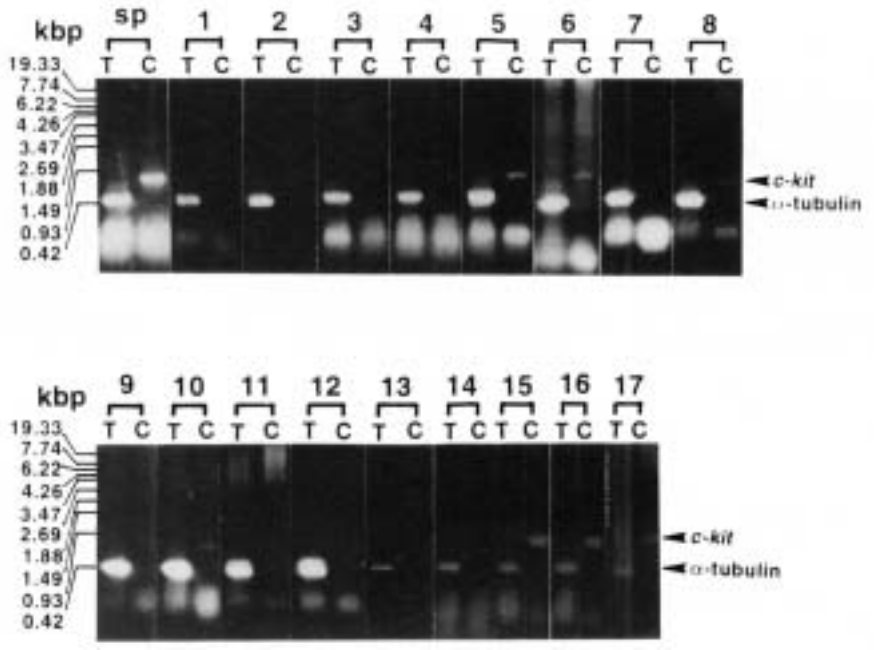

Fig. 3. Expression of *c-kit* mRNA in a variety of canine tumors. The numbers of the samples are same as those in Table 2. C: *c-kit*, T: α-tubulin.

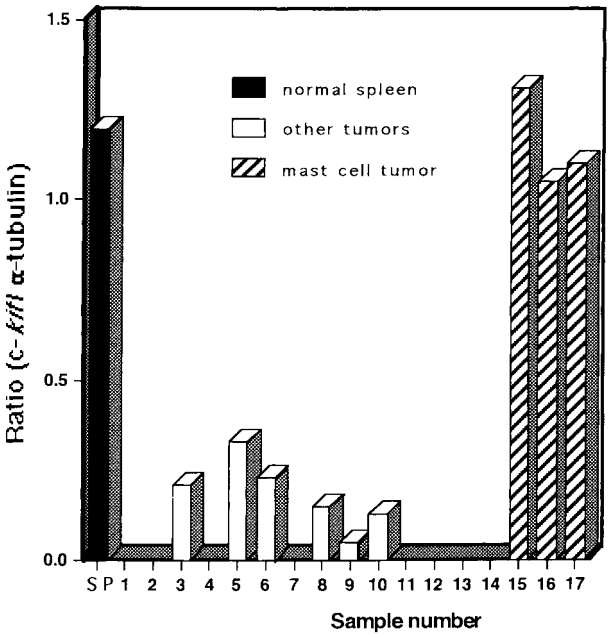

Fig. 4. Relative amplification of *c-kit* mRNA in various canine tumors listed in Table 2.

kit-transfected human melanoma cells after exposure to the ligand both *in vitro* and *in vivo* [9]. Even in the melanoma cells expressing the receptor alone, the growth inhibition by the ligand has been observed [29]. These reports suggest the role of *c-kit* gene in tumorigenesis is complicated, and still to be clarified.

There are also rather inconsistent results about the expression of *c-kit* in human mammary tumors. Hines *et al.* have observed variable but detectable level of *c-kit* mRNA in 9 tumor samples of 11 specimens from human breast carcinomas [7]. They have shown the expression of stem cell factor mRNA in all the tumor specimens, suggesting the existence of an autocrine loop. On the other hand, it has been reported that the expression of KIT protein is decreased with the increase in the malignancy of human breast tumors [16, 17, 27]. Recently, Nishida *et al.* have introduced a *c-kit* expression vector into MCF-7, a breast carcinoma cell line expressing no KIT receptor but the ligand [19]. They observed a significant growth suppression of the transfectants. The result suggests the presence of autocrine loop itself does not mean the growth stimulatory role of KIT in breast tumor cells.

In the present study, the amplification of *c-kit* mRNA in canine mammary tumor samples was observed. The declined amplification of the mRNA was, however, apparent in malignant mixed tumors (Figs. 1 and 2). The result essentially agrees with that of Hines *et al.* [7]. It is possible that most of mammary tumors express a significant level of *c-kit* mRNA, though the protein level may not be enough for the immunohistochemical detection. Alternatively, it is possible the detected transcript is not intact for the complete protein synthesis [24]. As shown in Fig. 1, the declined expression of KIT in the malignant mixed tumors is suggested. Because benign mixed tumors are the major canine mammary neoplasms and sometimes indistinguishable from the malignant mixed tumors [15], the observed low amplification of *c-kit* mRNA in the malignant tumor may serve as a tumor marker to discriminate these lesions. As shown in Fig. 3, no or greatly

reduced RT-PCR products were observed in a variety of canine tumors (Table 2) except mast cell tumors. The reduced transcription of *c-kit* in these malignant tumors, therefore, are in consistent with the observations that increasing malignancy is accompanied with depressed expression of the receptor protein.

It should be noted that the remarkable amplification of *c-kit* mRNA was observed in a particular type of canine malignant breast lesion, adenocarcinoma. The level of amplification was as high as those in mast cell tumor or normal spleen (positive control). As shown in Table 2, both the adenoma and adenocarcinoma in other tissue show no or only small amount of RT-PCR product. Because it is reasonable to expect the high level of manifestation of the receptor protein in mammary adenocarcinoma, the lesion may be categorized in the group of neoplasms such as mast cell tumor which shows persistent expression of *c-kit* protein after malignant transformation. In conclusion, *c-kit* is a potential candidate for a tumor marker in relation to the histology of mammary tumors in female dogs. However more studies are required to elucidate the exact role of the oncoprotein in tumorigenesis in the mammary gland.

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