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Research article

Detection of *c-kit* mutations in canine mast cell tumors using the polymerase chain reaction technique

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Abstract

Twenty-seven dogs with mast cell tumor conducted by the biopsy samples examined by histopathology and four samples from normal dogs which had been treated at Veterinary Teaching Animal Hospital, Kasetsart University were included. The purpose of the study was to identify the mutation pattern of *c-kit* proto-oncogene of tyrosine kinase that had internal tandem duplications (ITD) in exons 11 and 12 of tumor samples. Tumor DNA was amplified by polymerase chain reaction technique. The affected dogs were > 10 years old (50%), 6-10 years old (35%) and < 6 years old (15%). The tumor site was variable. The commonest site was cutaneous (34%). The mast cell tumor samples were divided into grade I (30 %), grade II (56 %) and grade III (15%). Mutation of the *c-kit* proto-oncogene was found in two dogs with grade II and III (7.4%) with ITD size of 43 and 52 base pairs. This result indicates *c-kit* proto-oncogene mutation is uncommon in dogs. Therefore, ITD detection is not a suitable diagnosis for mast cell tumor in dog but may be used as a predictive marker for tumor status especially in higher grade tumor. The drug response has been reported in dogs with the ITD. Therefore this test is useful in the application of the drugs therapy such as imatinib and masitinib that can inhibit the mutation of ITDs of the c-kit proto-oncogene in the dog that are not suitable for surgery.

Keywords: *c-kit* gene, Dog, Mast cell tumor, Mutation

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INTRODUCTION

Canine cutaneous mast cell tumors (MCTs) are one of the most common of all skin tumors in dogs, accounting for an estimated 7-21% of all cases (London and Seguin, 2003; Riva et al., 2005). Mast cell tumors can be highly invasive and metastatic, and frequently recurrent after having surgical removed (Misdorp, 2004; London and Thamm, 2013). MCT was diagnosed as 11-27 % of all malignant skin tumors (Riva et al., 2005). Histopathological examination of biopsy tissue has typically been required for tumor grading. Grade I is defined as a well-differentiated tumor, grade II as an intermediate-differentiated tumor, and grade III as a poorly-differentiated tumor (Patnaik et al., 1984). The etiology of canine MCTs is unclear but it is probably multifactorial. The predictors of tumor behavior are histologic grade and molecular parameters. Based on molecular abnormality, the role of the *c-kit* proto-oncogene in the development of canine MCTs has been described. Several studies have reported that the *c-kit* proto-oncogene is implicated in pathogenesis of several neoplastic diseases including gastrointestinal stromal tumors (Dagher et al., 2002), human mastocytosis (Chatterjee et al., 2015), and canine cutaneous MCTs (London et al., 1999). Researchers revealed that both Kit mRNA and *c-kit* receptor protein were expressed in canine MCTs and around 14 to 40% of canine MCTs contain the *c-kit* mutation (London et al., 1996; Letard et al., 2008; Welle et al., 2008). The *c-kit* gene encodes type III receptor protein tyrosine kinase. Kit is a cell surface receptor which, upon binding of its cognate ligand, induces a signal transduction cascade responsible for cell development, proliferation, differentiation, maturation, and survival (Nocka et al., 1990; Zsebo et al., 1990; Meininger et al., 1992; Dastych and Metcalfe, 1994; Galli et al., 1994; Yee et al., 1994; Serve et al., 1995; Broudy, 1997; Roskoski, 2005). In addition, kit is expressed normally on a variety of cells, including hematopoietic stem cells, melanocytes, and mast cells (Galli *et al*., 1994; Roskoski, 2005). Mutations in the *c-kit* proto-oncogene cause a constitutive autophosphorylation and activation of *c-kit* receptor without binding of the ligand, resulting in unregulated kit signal transduction (London et al., 1999; Ma et al., 1999; Letard et al., 2008). Several studies have demonstrated that a significant proportion of canine MCTs had mutations in *c-kit* gene involving either the juxta-membrane domain (exon 11 and 12) or extracellular domain (exons 8 and 9) (London et al., 1999; Downing et al., 2002; Jones et al., 2004; Letard et al., 2008) and consist of internal tandem duplications (ITD) and deletions (London et al., 1999; Ma et al., 1999; Zemke et al., 2001; Reguera et al.. 2002; Downing et al., 2002; Zemke et al., 2002; Pryer et al., 2003; Jones et al., 2004). ITD c-kit mutations were identified in 9% of canine cutaneous MCTs in one study that focused on the mutation status of eighty-eight randomly selected MCTs (Zemke et al., 2002). It has also been shown that *c-kit* mutations are significantly associated with histologically higher-grade canine cutaneous MCTs (Zemke et al., 2002). The activating *c-kit* mutations appear to be present in 25% to 30% of intermediate-grade to high-grade canine MCTs, and evidence suggests that they are linked to increased risk of local recurrence, metastasis, and a worse prognosis (London et al., 1999; Downing et al., 2002; Zemke et al., 2002; Webster et al., 2006; Webster et al., 2008). Identify *c-kit* mutation is the targeted therapy by

pursuing the MCT specific genes. Therefore, tyrosine kinase inhibitor drug such as imatinib or masitinib would be the choice of treatment. The goal of this study was to detect pattern of *c-kit* mutations in canine MCTs using polymerase chain reaction technique (PCR) and to consider the association between *c-kit* mutation and grading of canine MCTs.

MATERIALS and METHODS

The criteria of inclusion of samples in this study were male or female dogs of any breed and histological confirmed mast cell tumor. Tissue samples were biopsy from 4 post-mortem dog died with no clinical evidence of MCTs as the control dogs. Twenty-seven de novo MCTs from 27 client-owned dogs including; American Pit Bull (n=1), Mongrel breed (n=18), Golden Retrievers (n=2), Labrador Retriever (n=1), Pekingese (n=1), Rottweiler (n=1), Shih tzus (n=2) and Thai Bangkeaw (n=1) were included, from Veterinary Teaching Hospital (VTH) of the Kasetsart University. The data and medical treatment history of animals were followed from the outpatient department (OPD) records. The specimens were obtained at the time of surgical removal and then shipped to Kamphangsaen campus on ice for analysis. The abnormal masses were identified and detected by clinical examination. The veterinary pathologist histologically confirmed all specimens as malignant mast cell tumors and graded based on the Patnaik histologic grading system (Patnaik et al., 1984) for canine MCTs. This animal use protocol has been approved by the Kasetsart University Institutional Animal Care and Use Committee with approved protocol number ACKU61-VET-089 and found to be in accordance to the guidelines of animal care and use under the Ethical Review Board of the Office of National Research Council of Thailand (NRCT) for the conduct of the scientific research.

DNA extraction from tissue samples

The specimen from each dog was collected 2 sites of mast cell tumor for DNA analysis. The samples were obtained near the center of the tumor, avoiding the margin with normal surrounding tissue. Each tissue sample was minced into small pieces and 60 mg was used for DNA extraction by using EZNA® Tissue DNA Kit (Omega Bio-Tek, Inc) following the manufacturer's instructions. DNA concentration and purity determination were analyzed by nanodrop spectrophotometer. DNA concentration was determined by measuring the absorbance of sample at 260 nm. The purity was evaluated by spectrophotometer at A260/A280 ratio; A260/A280 ratio 1.8-2.0, < 1.8 or > 2.0 was justified as the highest and high quality DNA, respectively (Cheng et al., 2010). Isolated DNA was adjusted to 50 ng/ml and kept at -20 C until analysis.

Analysis of *c-kit* mutation by polymerase chain reaction

One hundred nanogram of DNA was amplified with specific primer of *c-kit* juxta membrane domain. The forward primer was 5'-CCATGTATGAAG-TACAGTGGAAG-3 and reverse primer was 5'-GTTCCCTAAAGTCATTGT-TACACG-3 (Cameron et al., 2004). The PCR reaction was carried out for 4 minute at 94 °C, 40 cycles of 1 minute at 94 °C for denaturation, 1 minute at 55 °C for annealing, 1 minute at 72 °C for extension and 5 minute at 72 °C for final extension. Five microliter of PCR product was separated by electrophoresis on a 1.5 % agarose gel electrophoresis to confirm the DNA amplification. The DNA fragment size was accurately identified by automated capillary gel electrophoresis (QIAGEN®, Germany). The expected DNA pattern size was 191 base pairs (bp) in wild type and > 191 bp in *c-kit* mutation with ITD.

RESULTS

Twenty-seven tumors from 27 dogs were diagnosed as canine mast cell tumor by histopathology (Figure 1). Fifty percent of these dogs were aged > 10 years, 35 % aged 6-10 years and 15 % aged < 6 years. Tumor position was most commonly the trunk (34 %), hind limb (23 %) and skin (23 %). The nose, neck, tail, perianal, scrotal were less frequently affected (Figure 2).

Figure 1 Representative figure of some canine mast cell tumor in this study. **A**; a 7-year-old dog, mixed breed with MCT grade II at inguinal area and medial thigh of left hind limb. **B**; a 9-year-old dog, mixed breed with MCT grade III (red arrow). **C**; a 10-year-old dog, mixed breed with MCT grade II at rear part of skin. **D**; a 8-year-old dog, mixed breed with MCT grade I at skin throughout the body.

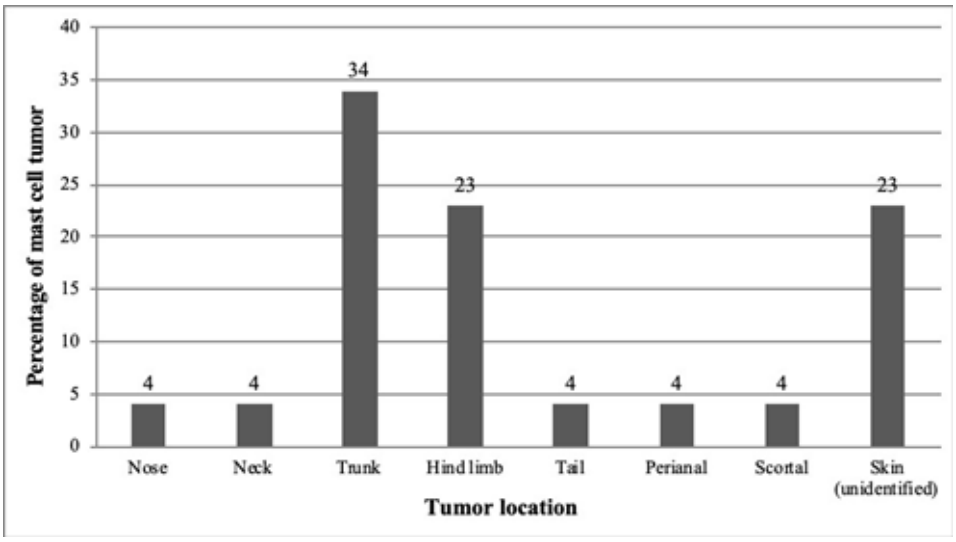

Figure 2 The percentage of mast cell tumor in each body location (n = 27).

In order to investigate the effect of sample collection, 2 sites of tissue sample were collected and DNA analysis performed. No *c-kit* mutations were detected in tissue samples of 4 control dogs. The *c-kit* mutation was found in 2 dogs (7.4 %) of 27 canine mast cell tumor samples. The DNA fragments with ITD were 243 bp and 234 bp compared to 191 bp of wild type DNA fragment

Two dogs (MCT #3 and MCT #29) were identified with *c-kit* ITDs. MCT #3 and MCT #29 dogs had heterozygous genotype 191, 243 and 191, 234, respectively (Table 1). MCT #3 had recurrent disease and died one year after the second surgical removal, at which time no tumor was evident. The clinical signs before death were generalized pain, vocalisation, high limb rigidity, and non-weight bearing hind limb lameness bilaterally. MCT #29 had tumor recurrence at right popliteal lymph node.

Histopathological examination of these 27 MCTs showed that 8 (29.63 %) were grade I, 15 (55.56 %) were grade II and 4 (14.81 %) were grade III. To assess the recurrence of the MCTs, OPD and telephone records were used. Out of these MCT dogs, 15 dogs (3 dogs with MCT grade I, 9 dogs of MCT grade II, 3 dogs of MCT grade III) had recurrence of tumor at the same location at least 30 days after surgical removal (Figure 3).

Mast cell tumors were most commonly found on the body and skin (cutaneous and subcutaneous), which agreed with other reports indicating the common area of tumor (Riva et al., 2005). The incidence of *c-kit* mutation in this study was 7.40 %. However, several studies reported higher incidence of *c-kit* ITD varied from 9 to 46 % (London et al., 1999; Downing et al., 2002; Zemke et al., 2002; Pryer et al., 2003; Jones et al., 2004; Webster et al., 2006; Isotani et al., 2008; Takeuchi et al., 2013).

Table 1 Tumor location, tumor grade and result of *c-kit* ITD mutation in 27 mast cell tumor dog samples

Dog ID.	Tumor location	Tumor grade	*c-kit* ITD mutation	DNA fragment size (bp)	
				WT	ITD
#1	Left hind limb	I	No	191	
#2	Left axillary	II	No	191	
#3	Inguinal area & inner thigh	II	Yes	191	243
#5	Tail	II	No	191	
#6	1st digital pad	I	No	191	
#7	Right inguinal	II	No	191	
#8	Left thorax	I	No	191	
#9	Neck	II	No	191	
#10	Muzzle	III	No	191	
#12	Trunk	II	No	191	
#13	Right trunk between mammary gland	II	No	191	
#14	Skin	II	No	191	
#15	Skin	II	No	191	
#16	Skin at scrotal sac	III	No	191	
#17	Left digit of hind limb	II	No	191	
#18	Skin at right ear	III	No	191	
#19	Right trunk	I	No	191	
#20	Perianal	II	No	191	
#22	Left trunk	I	No	191	
#23	Skin	II	No	191	
#26	Left stifle	I	No	191	
#27	Left trunk	II	No	191	
#28	Lateral of right hind limb	I	No	191	
#29	Right hind limb (interdigit 4^{th} & 5^{th})	III	Yes	191	234
#30	Skin	I	No	191	
#31	Left hind limb	II	No	191	
#32	Sternum	II	No	191	

Note : WT = wild type, ITD = internal tandem duplication

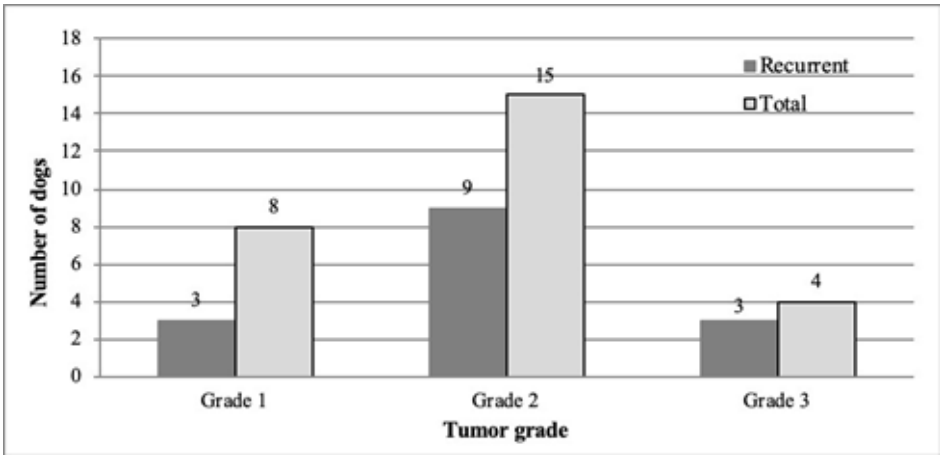

Figure 3 Tumor grade and recurrent of mast cell tumor (n = 27).

The size of ITDs in this study was 43 bp and 52 bp, and were in the same range (15-69 bp) as other reports (London et al., 1999; Jones et al., 2004; Webster et al., 2006; Isotani et al., 2008) (Table 2).

Table 2 The incidence of *c-kit* ITD mutation, size of ITD and tumor grade

MCT samples	ITD mutation (%)	ITD size (bp)	Tumor grade	Reference
27	2 (7.4)	43, 52	II, III	This study
11	5 (45.5)	45-70	ND	London et al., 1999
157	52 (33.0)	ND	I, II, III	Downing et al., 2002
88	8 (9.1)	ND	II, III	Zemke et al., 2002
14	5 (36.0)	ND	II, III	Pryer et al., 2003
30	10 (33.3)	48-69	II, III	Jones et al., 2004
60	9 (15.0)	15-60	II, III	Webster et al., 2006
21	5 (23.8)	27-69	ND	Isotani et al., 2008
47	8 (17.0)	ND	I, II, III	Takeuchi et al., 2013

ND = no data

Many patient factors such as germ-line genetic factors or kinds of gene mutation have a major role in the development of MCTs in different dog breeds that may affect the result of each study (Arendt et al., 2015). Boxers, Golden retrievers, Labrador retrievers, and Chinese shar-pei, had a high incidence of MCTs (White et al., 2011). Most of our samples came from crossbred dogs whereas previous studies used purebred dogs. The remaining tumors may have mutations in other genes or the same gene in the different position. Mutation in

the Tet methylcytosine dioxygenase 2 (*TET2*) (Zorzan et al., 2015) and G Protein Subunit Alpha I2 gene (*GNAI2*) (Arendt et al., 2015) was identified as an increased risk of MCT in dogs. Histological grade I MCTs are more likely to be benign; grade II and grade III are considered to be malignant. In this study, recurrence occurred with every grade of MCT. Some dog breeds are reported to be the report of predisposed to MCT (such as Boxer and Boston Terrier), whereas, Golden Retriever, Labrador Retriever and crossbred dog are not supposed to be predisposed (Zemke et al., 2002). This is in contrast to our study in which sixty-six percent of cases were crossbred dogs. The influence of breed on the occurrence and aggressive of the tumor may need more investigation.

In our study, we only found *c-kit* proto-oncogene mutation in two MCTs, one of grade II and one of grade III. These 2 grades exhibit aggressive behavior and more likely to metastasis. Based on our study, these 2 dogs with *c-kit* mutation had recurrence of MCTs. Medical, surgical excision or radiotherapy is the therapeutic choice in most MCTs with a good responsive is seen in 47 % of cases (Thamm et al., 1999). However, recent evidence found that MCTs possessing *c-kit* mutation are predisposed to recurrence after surgical removal (Downing et al., 2002). Our finding of *c-kit* proto-oncogene mutation in grade II and grade III is in agreement with the study of Zemke and colleague in 2002. This indicated the relationship of *c-kit* ITD and higher grades of mast cell tumor. Therefore, *c-kit* may be used as a predictive marker for tumor status especially in higher grade tumors (Zemke et al., 2002)

There are reports of drugs that can inhibit the effect of *c-kit* mutation. These drugs specifically target the *c-kit* mutation and include imatinib and masitinib. These drugs inhibit *c-kit* mutation with ITD in exons 11 and 12 of *c-kit* proto-oncogene (Isotani et al., 2008), even if there is a low incident of *c-kit* proto-oncogene mutation. Detection of *c-kit* proto-oncogene mutation is often considered essential if these drugs are to be used as an alternative to surgery.

CONCLUSION

Our results indicate that mast cell tumors have the mutation of the *c-kit* proto-oncogene. Thus, ITD detection is not a suitable diagnosis for mast cell tumor in dog. Histopathology is a useful diagnostic method because it helps to identify the tumor margins after excision and is useful for determining the prognosis for recurrence or metastasis especially after surgical removal of the MCT tumor. For practitioners, histopathology with *c-kit* identification has been suggested especially in the grade II and grade III MCTs. PCR technique and automated polyacrylamide gel electrophoresis have the potential to screen ITD of *c-kit* mutation. The findings of canine MCTs with *c-kit* mutations may be useful for assessing the efficacy of therapeutic agents in the dog that are not suitable for surgery

CONFLICT of INTEREST

There is no conflict of interest.

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