J Vet Diagn Invest 16:95–100 (2004)

Detection of c-*kit* mutations in canine mast cell tumors using fluorescent polyacrylamide gel electrophoresis

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Abstract. Mutations consisting of internal tandem duplications (ITDs) in exons 11 and 12 of the proto-oncogene c-*kit* are found in 30–50% of malignant canine mast cell tumors (MCTs). Traditionally, identification of such mutations in tumor specimens has been undertaken using standard polymerase chain reaction (PCR) and agarose gel electrophoresis. This procedure is limited to the detection of insertions and deletions larger than 9 base pairs in size. The purpose of this study was to compare the efficiency and accuracy of standard agarose gel electrophoresis with fluorescent polyacrylamide gel electrophoresis (PAGE) for the detection of ITDs in canine MCTs. The results of this study demonstrate that PAGE of labeled PCR products accurately predicts the size of the ITD in each tumor. In addition, other small insertions and deletions were not identified, suggesting that if they occur in canine MCTs, they do so infrequently. Because fluorescent and polyacrylamide formats are automated and have better resolution than agarose gels, fluorescent PAGE provides a more accurate, economical, and higher throughput method for the detection of c-*kit* mutations in canine MCTs.

The proto-oncogene c-*kit* was first identified as the oncogenic component of the acutely transforming, replication-deficient Hardy-Zuckerman 4-feline sarcoma virus, which is known to induce multicentric fibrosarcoma in the domestic cat.[2] c-*Kit* encodes Kit, a transmembrane tyrosine kinase receptor that binds the ligand stem cell factor (SCF), also known as mast cell growth factor, Kit ligand, or steel factor.[9,18,29,32] Binding of SCF promotes the development of mast cells from hematopoietic precursors and can act synergistically with different cytokines to stimulate the development and proliferation of various bone marrow progenitor cells.[9,18,33] Mutations in c-*kit* leading to constitutive activation of Kit in the absence of ligand binding have been identified in malignant mast cell lines derived from humans, mice, rats, and dogs.[8,10,25–27] These mutations have also been associated with the development of malignant mast cell disease in both dogs and humans.[4,6,10,12,13,17,28,30,31]

Mast cells tumors (MCTs) are one of the most common malignant tumors of dogs, representing between 7% and 21% of all cutaneous canine tumors.[3,5,20] This is an incidence far higher than that found in humans.[11,15,16,24] In humans, point mutations in the catalytic domain of Kit are commonly associated with adult mastocytosis. In contrast, 30–50% of malignant canine MCTs possess novel internal tandem duplications (ITDs) in the negative regulatory juxtamembrane domain of Kit.[6,10,31] These mutations consist of tandem nucleotide repeats located at the 3′-end of exon 11, sometimes including portions of intron 11 and exon 12. Both types of mutation result in autophosphorylation of Kit in the absence of SCF binding.[8,10,12,13,25]

The ITDs in the juxtamembrane domain of Kit in canine MCTs are reported to have a size range from 3–79 bp.[6,10,13] Typically, screening for ITDs has involved the use of polymerase chain reaction (PCR) of genomic DNA and the analysis of the PCR products by agarose gel electrophoresis. This screening process is unlikely to detect insertions or deletions smaller than 9–10 bp in size and requires the use of 4% agarose gels, which can become costly.[6] In addition, to accurately determine the size of each ITD, the PCR product must be sequenced.

Because agarose gels have low resolution, this technique has the potential to miss a number of small deletions or insertions in Kit. Therefore, a more sensitive and efficient screening method that permits an accurate determination of duplication size was developed. The objective of the present study was to use fluorescent polyacrylamide gel electrophoresis (PAGE) to determine if canine MCT samples possessed small insertions or deletions in the juxtamembrane domain of Kit that were not detectable through agarose gel electrophoresis. Fragment analysis software developed at University of California Davis Veterinary Genetics Laboratory, Davis, California, termed STRand was used to score the product sizes.[23] An additional objective of this study was to establish whether PAGE could be used to accurately predict the size of each ITD,

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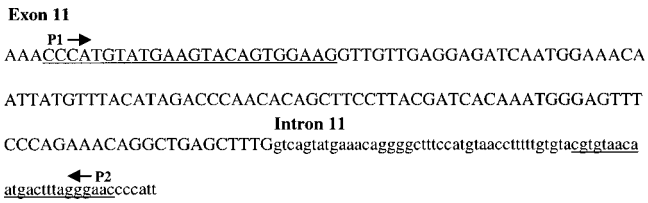

Figure 1. Location of PCR primers. The locations of the primers for canine c-*kit* used for this study are shown. P1 is placed at the 5′-end of exon 11, whereas P2 is placed in the middle of intron 11, generating an expected PCR product of 190 bp in size.

thereby avoiding the need for sequencing of each PCR product. The accuracy of mutation detection with the use of this technique has not been previously reported.

Materials and methods

Source of malignant canine mast cells. Thirty fresh MCT samples were collected from both referring veterinarians around the country and the Veterinary Medical Teaching Hospital (VMTH) of the University of California, Davis. The specimens were obtained at the time of surgical removal, placed in red top tubes, refrigerated, and shipped to the VMTH on ice for analysis. All specimens were obtained from the center of the tumor using a 4-mm punch biopsy instrument, and the location of each tumor was noted. A board-certified veterinary pathologist histologically confirmed all specimens as malignant MCTs, grade II or grade III.[19]

Preparation of genomic DNA. Tumor weight ranged from 10–25 mg, and the tumors were immediately digested in a 500 μl Proteinase K reaction buffer (20 μg/ml; 0.01 M Tris, pH 7.8; 0.005 M ethylenediaminetetraacetic acid [EDTA]; 0.5% SDS) overnight. Digested tumor samples were centrifuged, and the supernatant was transferred to 2-ml Phase Lock gel tubes.[a] DNA was isolated using standard phenol/chloroform extraction methods.[6] Isolated DNA was resuspended in 30–50 μl TE (lumM tris lmm EDTA, pH8.0), and concentrations were determined by measuring optical density. The samples were diluted in TE to 100 ng/μl for PCR. The DNA from the C2 canine MCT cell line (containing a 48-bp ITD in the juxtamembrane domain), and the BR canine MCT cell line (containing a point mutation in the juxtamembrane domain) were used as positive controls.[6] These cell lines were generously provided by Dr. Warren Gold.[b] Genomic DNA from 5×10^6 C2 or BR cells was extracted using the DNeasy tissue kit according to manufacturers instructions.[c]

Polymerase chain reaction of c-kit. The primers used for PCR amplification of the c-*kit* juxtamembrane domain were based on the 5′-end of exon 11 (PE1: 5′-CCATGTATGAAGTACAGTGGAAG-3′ sense, bp 1,657–1,680 of exon 11) and the 5′-end of intron 11 (PE2: 5′-GTTCCCTAAAGTCATTGTTACACG-3′ anti-sense, nucleotides 43–66 of intron 11) (Fig. 1). These primer pairs were expected to generate a PCR product size of 191 bp. To generate PCR products that could be observed by fluorescence detection, the PE1 (forward) primer was 5′-labeled with 6-FAM.[d] The PCR profile was for 40 cycles consisting of 1 min at 94 C denaturing, 1 min at 59 C annealing, and 1 min at 72 C for extension, with a 5 min final extension at 72 C.

Evaluation by gel electrophoresis. Approximately 20 μl of the PCR product was separated by electrophoresis on a 4% agarose gel (consisting of 50% low melting temperature agarose[e] and 50% standard agarose), stained with ethidium bromide, and "visualized" with UV light. Polymerase chain reaction products of both the C2 and BR cells lines were amplified as positive controls. An aliquot of the PCR reactions was also analyzed by PAGE. For PAGE, 0.1 μl of PCR product was denatured for 2 min at 95 C in 2 μl of loading buffer, (1 ml deionized formamide, 100 μl ROX-350 labeled[d] size standard and 200 μl loading dye). This product was electrophoresed for 2 hr on an ABI 377 DNA Sequencer[d] with a 6% polyacrylamide gel. The Genescan output was used by the STRand analysis package[f] to determine precise fragment lengths.[23]

Cloning and sequencing of c-kit from PCR products. The PCR products containing the presumed ITDs in Kit (i.e., larger products) were gel purified using the Gene Clean III kit[g] and then cloned into the p-GEMT vector[h] using JM-109 competent cells. A total of 3 μl of each purified PCR product was ligated overnight with 50 ng of p-GEMT vector, followed by the addition of 50 μl of JM-109 competent cells to 2 μl of the ligation reaction. The cells were heat shocked at 42 C for 45–50 sec and then added to 950 μl of SOC medium.[i] 100 μl of each transformation culture was plated on LB (Luna-Bertani)/ampicillin/isopropyl-L-thio-β-galactoside/X-Gal plates and incubated overnight at 37 C. Colonies were picked, grown up in LB broth overnight at 37 C, miniprepped using the Nucleospin Plasmid Miniprep kit,[j] screened by restriction digestion with NcoI and SacI,[k] and then analyzed on a 3% agarose gel. Positive clones were subsequently amplified using the Plasmid Midi Kit according to the manufacturer's instructions for high-copy plasmids.[c] These were then sequenced using the T7 and SP6 primers. Polymerase chain reaction products that showed no evidence of the presence of an ITD (no mutant band identified by agarose gel electrophoresis) were sequenced directly using the PE1 and PE2 primers after column purification.[l]

Results

Analysis of tumor specimens with known Kit ITDs. To determine whether PAGE was capable of accurately determining the size of an ITD in the juxtamembrane domain of Kit, 4 grade II and 6 grade III tumor samples were chosen for analysis from a database of MCTs previously established in the laboratory. Each tumor had previously been shown to possess an ITD, identified by standard agarose gel electrophoresis. Polymerase chain reaction was performed using both labeled and unlabeled primers, and the products were analyzed by both PAGE and agarose gel electrophoresis, respectively. Both methods of analysis demonstrated the presence of a wild-type PCR product and a mutant (larger) PCR product. The size of the ITD for each tumor sample was calculated using the soft-

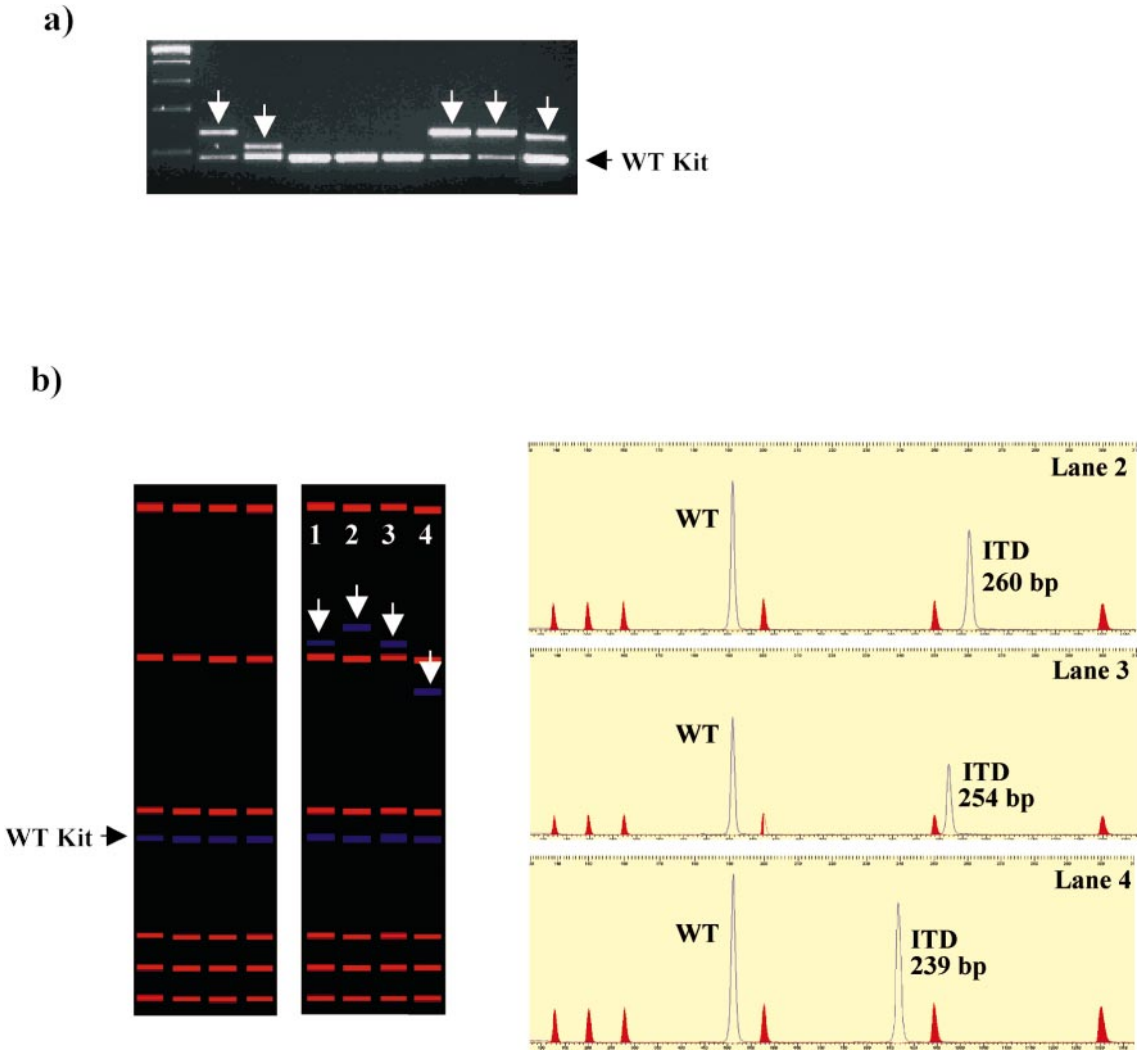

Figure 2. Agarose gel electrophoresis versus fluorescent PAGE for detection of Kit ITDs in grade II and grade III canine mast cell tumors. **a**, a representative agarose gel electrophoresis of PCR products from 8 representative grade II MCTs with and without Kit ITDs is shown. The actual ITD is denoted by the white arrow. **b**, images of a typical fluorescent PAGE gel are shown. On the left is a gel in which only MCTs with WT Kit have been analyzed. The PCR products are blue, and the molecular weight markers are red. In the center is a gel in which MCTs with KIT ITDs have been analyzed (again, the PCR products are blue). The white arrows represent the PCR products generated by the ITD. The STRand analysis of the Genescan output is shown on the right; the molecular weight markers are represented by the solid red peaks, and the PCR products are represented by the open blue peaks. The corresponding sizes of each peak are located on the top of each STRand output.

Table 1. Fluorescent PAGE of canine mast cell tumors with known Kit ITDs.

Sample	Source	Tumor grade	Product I (WT) size (bp)	Product II (ITD) size (bp)
1	outside source	II	191	251
2	UC Davis	II	191	242
3	outside source	II	191	239
4	outside source	II	191	239
5	outside source	III	191	254
6	outside source	III	191	260
7	outside source	III	191	254
8	outside source	III	191	251
9	outside source	III	191	263
10	UC Davis	III	191	260

ware package STRand for the polyacrylamide gel samples (Fig. 2). These were then compared with results of the cloned and sequenced PCR products. Table 1 demonstrates that for each tumor analyzed, the size of the ITD was accurately predicted by PAGE. The size of the insertions sizes ranged from 48 to 69 bp, all multiples of 3 and therefore not resulting in a frameshift (Fig. 3). Interestingly, 7 of the 10 samples evaluated contained insertions in which a portion of intron 11 had been incorporated. In summary, for the samples evaluated, PAGE was 100% accurate in assessing for the presence or absence of an ITD and for determining the size of the ITD.

Analysis of tumor specimens with no known muta-

Figure 3. Sequence of Kit ITDs from grade II and grade III canine MCT. The sequences for all Kit ITDs analyzed in this study are shown. Each ITD is denoted by nucleotides underlined and in bold italic; the actual sequence that is repeated is marked by the dotted underline. Several ITDs contained a portion of intron 11; this is denoted by the box at the 5′-end of the ITD.

tion in c-kit. Whereas 30–50% of grade II/grade III MCTs possess activating mutations in c-*kit* consisting of ITDs, the remaining tumors are not known to possess such mutations based on agarose gel electrophoresis of PCR products. As it has been previously reported that canine MCTs express small deletions/insertions (3–6 bp in size) or point mutations in the juxtamembrane domain, it was hypothesized that agarose gel electrophoresis would miss such mutations given its limits of resolution. Therefore, 20 grade II and 5 grade III MCTs were selected from the previously described database that were not believed to possess an ITD on the basis of previous analysis and subsequently evaluated by PAGE. In all specimens examined, there was no evidence of a larger PCR product; only a wild-type product was present suggesting that no additional deletions/insertions were present in c-*kit* that had not been detected by standard electrophoresis (Fig. 2).

To confirm that there were no small deletions/insertions, and to evaluate the specimens for the presence of point mutations, the PCR products were sequenced. Analysis of the sequencing data confirmed the length of the nonmutated samples and that they were homologous to that of the wild-type sequence. Therefore, in the population of 20 MCTs evaluated, there were no small deletions/insertions missed by PAGE. Interestingly, a polymorphism was identified at nucleotide 1,740 with 9 out of 25 samples either homozygous or heterozygous for this change. This C–T transition was in position 3 of codon 580 coding for tyrosine resulting in a silent mutation.

Discussion

Mast cell tumors are the most common skin tumor of the dog.[3,5,20] These tumors range in biologic behavior from benign to malignant.[14] In most cases, such biologic behavior can be predicted on the basis of the histologic grade, with grade I tumors more likely to be benign and grade III tumors considered to be malignant.[19] Whereas the majority of grade II tumors are cured by surgical excision (sometimes with adjuvant radiation therapy), a proportion of these exhibit aggressive behavior leading to metastatic disease and death of the affected dog.[1,7,19,21,22] During the past several years, it has become evident that approximately 30–50% of malignant grade II and grade III MCTs possess mutations in the proto-oncogene c-*kit*.[6,31] These mutations consist of ITDs in the negative regulatory juxtamembrane domain of the protein, resulting in constitutive activation in the absence of ligand binding.[6,10] A recent study found that those tumors expressing mutations were more likely to recur after surgery and more likely to metastasize.[6] Given the fact that such mutations appear to play an important role in the biology of malignant mast cell disease in the dog and that inhibitors of Kit activation are now in clinical trials, accurate and reliable detection of such mutations is essential.

Historically, mutation detection has been achieved through PCR of tumor-derived DNA using primers that flank exon 11, where all of the mutations have been detected to data.[6,10,13] Typically, the PCR products are analyzed by agarose gel electrophoresis to determine if an abnormally large PCR product is present, indicating the presence of an ITD. There are several

potential limits to this technique, including the inability to detect insertions or deletions smaller than 8–10 bp in size, the difficulty in detecting very faint abnormal PCR products (especially if only a small portion of the malignant cells possess the mutation), and the need to use high-percentage agarose gels.

The purpose of this study was to determine if another method of evaluation, fluorescent PAGE, would be more efficient and reliable for the detection of mutations in the c-*kit.* This method involves the fluorescent labeling of PCR products and subsequent electrophoresis on a polyacrylamide gel using an ABI 377 DNA Sequencer.[23] The Genescan output is then analyzed using the STRand software package. A major advantage of this system is that deletions and insertions as small as 1–2 bp in size can be readily detected. In addition, because fluorescence-based detection methods are used, very faint bands not normally visible by standard ethidium bromide imaging on an agarose gel are readily detectable. Lastly, the PAGE system permits accurate determination of PCR product size, which is not possible on an agarose gel.

Our results demonstrate that fluorescent PAGE is an extremely accurate method to detect ITDs in the juxtamembrane domain of c-*kit.* In 10/10 cases, the size of the ITD determined by PAGE was identical to that determined by sequencing of the abnormal PCR product. Given the fact that 96 samples can be run and analyzed on a single acrylamide gel in the ABI system and that the size of each ITD can be accurately determined, PAGE is clearly more efficient and sensitive than standard agarose gel electrophoresis.

Another objective of our study was to determine if the prevalence of mutations in grade II and grade III MCTs was underestimated because of the inability of agarose gel electrophoresis of PCR products to detect small deletions/insertions and point mutations. This was particularly important because another investigator did detect a point mutation and a 3- and 6-bp deletion in 3 canine MCTs.[13] The authors chose 20 grade II and 5 grade III MCTs in which an initial screening had detected no evidence of mutation and analyzed the PCR products using PAGE. No small deletions/insertions were apparent using this method, and no "faint" bands that would have been missed on an ethidium bromide–labeled agarose gel were detected. Furthermore, direct sequencing of the PCR products demonstrated no evidence of a point mutation in exon 11 of the c-*kit*. However, a polymorphism was detected in many of the samples, although this base pair change did not alter the subsequent protein sequence. Therefore, if small deletions, insertions, or point mutations do occur, it is likely they do so infrequently.

In summary, this study demonstrates that fluorescent PAGE is an extremely accurate and useful tool to analyze canine MCTs for evidence of mutation in c-*kit*, providing a more sensitive method to assess size differences in PCR products than standard agarose gel electrophoresis. Whereas PAGE is unable to determine if a single base pair change is present in a given PCR product, point mutations appear to occur infrequently in canine MCTs making it unnecessary to sequence PCR products for this application.

Sources and manufacturers

a. Eppendorf, through Fisher Scientific, Pittsburgh, PA.
b. Dr. Warren Gold, Cardiovascular Research Institute, University of California, San Francisco, CA.
c. Qiagen, Valencia, CA.
d. Applied Biosystems, Foster City, CA.
e. Continental Lab Products, San Diego, CA.
f. www.vgl.ucdavis.edu/STRand.
g. Q Biogene, Carlsbad, CA.
h. Promega, Madison, WI.
i. Invitrogen, Carlsbad, CA.
j. Becton Dickinson, Los Angeles, CA.
k. New England Biolabs, Beverly, MA.
l. Amicon Bioseparations, through Fisher Scientific, Pittsburgh, PA.

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