AUSTRALIAN VETERINARY JOURNAL
AUSTRALIA'S PREMIER VETERINARY SCIENCE TEXT
SMALL ANIMALS

Prevalence of exon 11 internal tandem duplications in the *C-KIT* proto-oncogene in Australian canine mast cell tumours

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Objective To measure the prevalence of internal tandem duplications (ITDs) in exon 11 of the proto-oncogene *C-KIT* in a sample of Australian cutaneous canine mast cell tumours (MCTs) drawn from general practice and to evaluate relationships between tumour mutation status and prognostic factors including signalment, tumour histological grade, tumour anatomical location and tumour size.

Methods *C-KIT* exon 11 ITDs were detected by PCR in DNA extracted from formalin-fixed, paraffin-embedded canine MCTs sourced from three veterinary diagnostic laboratories in Adelaide and Melbourne. Tumours were graded according to two different systems (Patnaik and Kiupel systems) by board-certified anatomical pathologists blinded to the PCR results. Relationships between tumour mutation status and prognostic factors were evaluated using a generalised binary logistic regression analysis.

Results ITDs were identified in 13 of 74 cutaneous canine MCT samples, giving an overall prevalence of 17.6% (95% confidence interval: 8.9–26.2%). ITDs were detected in 10 of 18 Patnaik grade III MCTs (55.6%) and 11 of 22 Kiupel high-grade MCTs (50%). Wald chi-square analysis revealed that detection of tumour ITDs was significantly associated with both Patnaik's and Kiupel's histologic grading systems (each: $P < 0.001$). The presence of the ITDs in MCTs was not associated with signalment, tumour anatomical location or tumour size.

Conclusion The prevalence of *C-KIT* exon 11 ITDs in Australian canine MCTs is similar to the prevalence in overseas canine populations (overall prevalence in Australia approximately 18%). ITDs were more frequently identified in higher grade MCTs.

Keywords *C-KIT*; diagnostic pathology; dogs; mast cell tumours

Abbreviations bp, base pair; CAHC, Companion Animal Health Centre; FFPE, formalin-fixed paraffin-embedded; ITD, internal tandem duplication; MCT, mast cell tumour; SCF, stem cell factor; WT, wild type

Aust Vet J 2017;95:386–391 doi: 10.1111/avj.12636

Spontaneous cutaneous mast cell tumours (MCTs) are the most common type of skin cancer in dogs, accounting for up to 21% of all cutaneous neoplasms.[1–4] They commonly appear in the dermis as a single lesion, but it is not unusual to find them in the subcutis or presenting as multiple tumours.[5] MCTs have highly variable biological behaviour, which can range from benign tumours to fatal metastatic cancers. A three-tier grading system proposed by Patnaik et al.[6] grades cutaneous tumours based on tumour cellular and nuclear morphology, position and mitotic rate and has been used for many years to predict tumour prognosis. Grade I tumours are generally relatively benign and have predictably good long-term prognosis, whereas grade III tumours are typically highly metastatic and have predictably poor prognosis.[6] Limitations to the applicability of the Patnaik grading system are apparent with intermediate grade II MCTs. Approximately 60% of cases fall into the grade II category with biological behaviour that is difficult to reliably predict and as a result, optimal treatments are unclear.[7] Additionally, studies have shown interobserver variation between pathologists applying the Patnaik system.[7,8] A two-tier system proposed by Kiupel et al. was developed to overcome these limitations.[8] Grading tumours as either high grade or low grade increases consistency between pathologists and the Kiupel system has been widely adopted by most veterinary pathologists and oncologists.

Molecular information can be used to supplement histological grading for refining prognosis. Markers of cell proliferation such as the expression levels of AgNORs (argyrophilic nucleolar organiser region-associated proteins) and Ki-67 have been found to be prognostic indicators; however, measurement of these markers is not readily available in a commercial setting. Another useful molecular marker in predicting MCT prognosis is the mutational status of the *C-KIT* proto-oncogene.[9–11] *C-KIT* encodes a transmembrane tyrosine kinase receptor protein, KIT, activated by haematopoietic stem cell factor (SCF).[12] KIT activation leads to upregulation of DNA replication and subsequent mast cell differentiation, maturation and proliferation.[13] The juxtamembrane domain of KIT, encoded by exon 11, plays an important role in the auto-inhibition of the KIT protein. In the absence of SCF, the juxtamembrane domain interacts directly with the catalytic region of KIT to repress its activity.[14]

Activating (gain-of-function) mutations that have been found in *C-KIT* disrupt the normal function of the KIT protein, resulting in ligand-independent KIT activation and leading to uncontrolled mast cell proliferation with consequent tumour development.[15] Somatic mutations are present in *C-KIT* from many canine MCTs. Although not common, substitution mutations, insertions and deletions have been identified in exons 8, 9, 11 and 17.[16,17] The most frequently identified mutations in *C-KIT* in canine MCTs are internal tandem duplications (ITDs) in exon 11, with reported prevalence of up to almost 46%.[16] Several studies have shown that ITDs correlate with higher grade, more aggressive MCTs.[9,10,18]

Management of MCTs routinely includes surgical removal, and in some cases, adjuvant chemotherapy or radiation therapy. Several different chemotherapeutic agents have shown efficacy for MCT

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treatment, but none is entirely effective. Toceranib, a relatively new chemotherapeutic agent, is a tyrosine kinase inhibitor and a useful treatment option for some MCT patients.[13] Toceranib has been approved for use in Australia as a second-line chemotherapeutic agent for treating grades II and III MCTs that are recurrent or unresponsive to first-line treatments.[19] Toceranib has been shown to decrease tumour size, decrease tumour recurrence and increase survival time of dogs with MCTs.[20] Dogs with ITDs in *C-KIT* are more likely to respond to toceranib treatment compared with those without these mutations.[20,21]

Given that ITDs in exon 11 of *C-KIT* are more frequently identified in higher grade MCTs, and tumours with these mutations more effectively respond to toceranib, knowledge of each tumour's *C-KIT* exon 11 mutation status would provide useful information to veterinary practitioners and help guide management decisions. A useful substitute for individual tumour testing would be knowledge of the prevalence of exon 11 *C-KIT* ITDs in each of the different tumour histological grades.

International studies report the ITD prevalence ranges between 0% and 46% in canine MCTs.[16,22] This wide range provides limited useful information to clinicians. The validity of these studies is also restricted because of the small sample sizes and sample selection bias confounding the findings. Moreover, the prevalence of MCT *C-KIT* exon 11 ITDs in Australian dog populations is unknown. The primary aim of the present study, therefore, was to determine the prevalence of *C-KIT* exon 11 ITDs in MCTs from dogs seen in Australian general practice. A second aim was to investigate relationships between tumour mutation status and tumour size, tumour histological grade and signalment (patient breed, age, sex and neuter status).

It was hypothesised that the ITD prevalence in MCTs from Australian canine patients would be similar to the prevalence (15%) reported in a study conducted in the USA on tumour samples selected primarily on the basis of histopathological diagnosis of MCT.[10] It was predicted that there would be no significant relationships between tumour ITD presence and signalment, tumour anatomical location or tumour size. However, the ITD prevalence was predicted to be greater in grade III (Patnaik) and high-grade (Kiupel) MCTs, compared with grade I (Patnaik) and low-grade (Kiupel) MCTs.

Materials and methods

Study design

The prevalence of ITDs in exon 11 of *C-KIT* was determined by dividing the number of cases with detected mutations by the number of tumours analysed in this study. To assess whether there was an association between tumour ITD mutation with tumour histological grade and patient signalment, an analytic cross-sectional study was performed.

Tumour selection

This study included 123 formalin-fixed paraffin-embedded (FFPE) spontaneous cutaneous canine MCTs sequentially submitted to the Companion Animal Health Centre (CAHC) at the University of Adelaide between December 2011 and December 2012 (n = 15) and to Gribbles Veterinary Pathology laboratories in Adelaide and Melbourne (n = 108) between 2014 and 2015. Tumours were eligible for inclusion in this study if there was a histopathological diagnosis of MCT. Tumours were graded histologically and, where available, information was collected on patient signalment, tumour anatomical location and tumour size. Tumour size was determined according to the largest diameter of the fixed tumour sample. Clinical outcomes of patients were not followed. Testes discarded after routine surgical neutering procedures in the CAHC served as a source for normal canine DNA.

Pathological grading

A single haematoxylin and eosin-stained section of each tumour was graded by two pathologists (AK, ED). Tumour histological grade was determined using both the Patnaik scheme[6] and Kiupel scheme.[8] For the grading, both pathologists were blinded without knowledge of the other pathologist's grades or the results of the molecular analysis. Where the grades were discordant, the pathologists re-examined and discussed the same sections until they came to an agreement. Where an agreement still could not be made, the higher grade was assigned to the tumour to account for the worst case scenario.

Genomic DNA extraction

Genomic DNA was extracted from 20-µm thick sections of FFPE tumour blocks using a QIAamp AllPrep FFPE Tissue Kit (Qiagen, VIC, Aust) according to the manufacturer's instructions. DNA was eluted in 60 µL of Buffer ATE (Qiagen) and stored at 4°C for subsequent mutation detection. DNA quantity and quality were measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Genomic DNA was extracted from testes using an AllPrep DNA/RNA Mini Kit (Qiagen) according to manufacturer's instructions.

Amplification of C-KIT *exon 11*

Previously described primers that flank exon 11 and the 5′ end of intron 11, PE1 and PE2,[18] were used to amplify exon 11 of the *C-KIT* proto-oncogene for detection of ITDs. Hypoxanthine-guanine phosphoribosyltransferase gene primers, HPRT2 FRD2 (5′-GCG AGA GAG AAC CTT GTG TG-3′) and HPRT2 REV2 (5′-GGG ACT TTG GGG AAC TGA C-3′) were used as an independent control to confirm DNA quality. PCR conditions were identical for both sets of primers. PCR reactions were prepared to final concentrations of approximately 0.5 ng/µL of target DNA, 250 nmol/L of each primer, 5 units/µL of Abgene *Taq* polymerase (Thermo Scientific), 250 µmol/L of dNTP mix (Fisher Biotech Australia, WA, Aust), 1.5 mmol/L $MgCl_2$ and 2 µL 10× Reaction Buffer IV (Thermo Scientific) in a total volume of 20 µL. The PCR cycling conditions were 2 min at 95°C; 35 cycles of 15 s at 95°C, 10 s at 60°C and 1 min at 72°C; and a final extension of 10 min at 72°C. Resulting PCR products were analysed by gel electrophoresis on 2% agarose gel. If no *C-KIT* band was detected (163 bp), the PCR was repeated using a range of template quantities. If the DNA still failed to amplify, the samples were not included in this study.

Sequencing amplified C-KIT *fragments*

Two samples in which mutant exon 11 *C-KIT* DNA was detected were chosen for sequencing. The ITDs and wild-type (WT) DNA

were amplified by PCR using PE1 and PE2 primers.[18] Mutant and WT bands were purified from the gel using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions, and the purified bands were submitted to the Australian Genome Research Facility (Urrbrae, SA, Aust) for sequencing.

Statistical analysis

To account for non-normally distributed data and binomial variables, a binomial logistic regression model was fitted to the tumour mutation status data. Wald chi-square analysis was used to test for significance ($P < 0.05$) between tumour mutation status and histological grade, breed, age, sex, neuter status, tumour anatomical location and tumour size. Pairwise comparisons were used to test for significant differences of mutation frequencies between different histological grades. All data were analysed in IBM SPSS version 21 (Armonk, NY, USA).

Results

Evaluable tumours

A total of 123 cutaneous MCTs from 104 dogs were submitted for this study; 6 of the dogs had 2 MCTs and 1 dog had 4 MCTs, all other dogs had a single tumour. The DNA extracted from tumour blocks varied from 1.1 to 1255 ng/μL and the 260 : 280 absorbance ratio ranged from 0.3 to 3.3. *C-KIT* exon 11 could be amplified from DNA extracted from 60% of these tumours. Therefore, the final analyses were restricted to 74 independent tumours from 65 dogs.

Population demographics

The mean age of the 65 dogs in this study was 8.22 years (range, 3–13 years, 2 dogs: unknown ages), and they were arbitrarily grouped into young (≤ 7 years, n = 27) or old (> 7 years, n = 36) for analysis. There were 28 males (11 intact, 17 neutered) and 33 females (10 intact, 23 neutered). There were 4 dogs with unknown sex and neuter status.

Dogs were grouped into one of eight breed groups according to the Australian National Kennel Council's breed grouping. Breed groups included 'cross bred' (n = 13), 'Gun dog' (n = 12) and 'Terrier' (n = 8). Staffordshire Bull Terriers (n = 9) and boxers (n = 8) were grouped independently because of the the high numbers of these breeds in this study. Dogs of unknown breeds or of a single breed not related to another group were grouped as 'Other' (n = 15) and included 4 or fewer dogs from the Hounds, Non Sporting, Toy, Utility and Working breed groups and a single dog with an unrecorded breed status.

Tumour characteristics

Tumours were categorised as small (≤ 20 mm; n = 48), medium (> 20 mm ≤ 50 mm; n = 10), large (> 50 mm; n = 5) or unknown size (n = 11). Small tumours outnumbered the combined numbers of medium and large tumours, accounting for more than 75% of tumours with known size. Tumour anatomical locations included genitalia (n = 5), head and neck (n = 7), limb (n = 19), torso (n = 31) and unknown (n = 12).

Table 1. Number of mast cell tumours without (wild type) or with (mutant) *C-KIT* exon 11 ITDs by histological grading system

	Wild type	Mutant	Total (% of each grade)
Patnaik histological grade			
Grade I	6	0	6 (8.1)
Grade II	47	3	50 (67.6)
Grade III	8	10	18 (24.3)
Kiupel histological grade			
Low grade	50	2	52 (70.3)
High grade	11	11	22 (29.7)
Total	61	13	74

Pathological grading

According to the Patnaik system, grade I, II and III tumours comprised 8%, 68% and 24%, respectively, of the 74 tumours (Table 1). According to the Kiupel system, 70% of tumours were low grade (Table 1). All Patnaik grade I tumours were graded as Kiupel low grade and all Patnaik grade III tumours were Kiupel high grade. Of the Patnaik grade II tumours, 92% were graded as Kiupel low grade (n = 46) and the other 8% were graded as Kiupel high grade (n = 4).

All of the MCTs located on the genitalia were Patnaik grade II or III or Kiupel high grade (Figure 1A). According to the Patnaik grading scheme, all tumours on the head or neck were grade II or III, but in Kiupel's grading scheme, 3/7 tumours on the head or neck were low grade (Figure 1B). There was no statistically significant relationship between any tumour location and histological grade in either system.

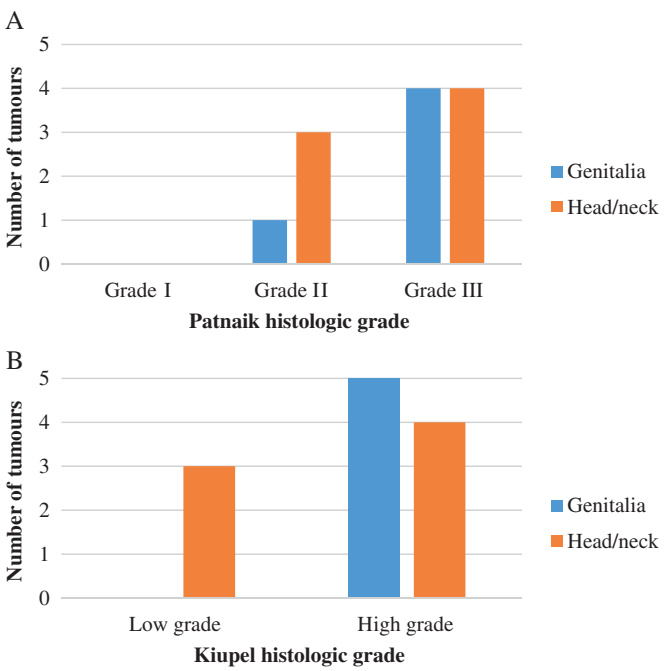

Figure 1. (A, B) Numbers of mast cell tumours located on the genitalia or head and neck of Australian dogs according to histological grading system.

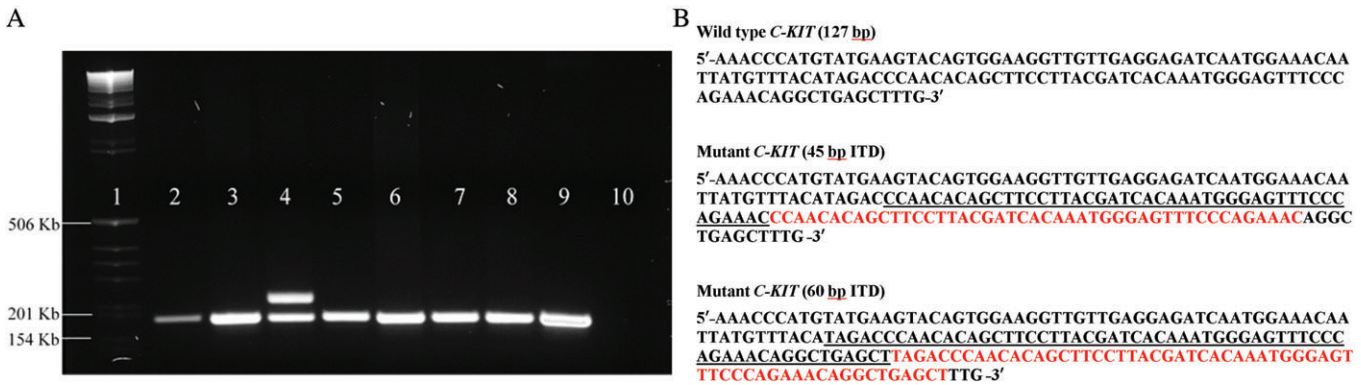

Figure 2. Detection of internal tandem duplications (ITD) in Australian canine mast cell tumours. (A) Representative agarose gel electrophoresis results for PCR amplification of exon 11 of the *C-KIT* gene. Wild-type (WT) PCR product is 190 base pairs (bp), ITD PCR products are larger. Lane 1: 1 Kb DNA ladder. Lanes 2–8: tumour samples. Lane 9: positive control (WT testis DNA). Lane 10: negative control (water). Lane 4 shows the wild-type (lower) and mutant (upper) bands. (B) Nucleotide sequence of exon 11 of the *C-KIT* gene from a wild-type sample and two samples with ITD mutations. The region of the DNA that is duplicated is underlined and the duplication is in red lettering. Intron 11 sequence that formed part of the 190-bp PCR amplicon is not shown.

PCR and agarose gel electrophoresis for the detection of ITDs

To determine the prevalence of *C-KIT* ITDs in exon 11 of this population of canine MCTs, genomic DNA was isolated from FFPE tumour blocks and a PCR assay was performed using primers PE1 and PE2.[18] ITDs were readily detected by agarose gel electrophoresis (Figure 2A). The expected amplicon size for WT *C-KIT* DNA is 190 bp, represented by the lower bands on the gel, while the upper bands represented DNA sequences with an ITD. The size of the upper fragment varies according to the size of the ITD it contains.

Sequencing results of WT and mutant DNA

To confirm the identity of the PCR bands, the purified upper bands of two different samples with an ITD, and the single WT band from a separate sample were sequenced using primers PE1 and PE2.[18] The WT band sequence was an exact match with previously published data.[18] The two different duplication mutations, respectively 45 bp and 60 bp, occurred in the 3′ end of exon 11 (Figure 2B).

C-KIT *mutation*

C-KIT exon 11 ITDs were identified in 13 of 74 MCTs (17.6%, 95% CI: 8.9–26.2%). None of the Patnaik grade I MCTs possessed an ITD. ITDs were identified in 3 of the grade II MCTs and in 10 of the grade III MCTs (Table 1). Wald chi-squared analysis revealed a significant relationship between the presence of an ITD and Patnaik histological grade ($P < 0.001$, Table 2). Pairwise comparisons showed that the ITD frequencies in Patnaik grade I and II tumours were significantly different from those of grade III tumours (Table 3). The ITD frequencies of Patnaik grade I and II tumours were not significantly different. There were no significant correlations between the presence of an ITD and patient's breed, age, sex, neuter status, tumour anatomical location or tumour size (Table 2).

ITDs were identified in 2 of the Kiupel low-grade MCTs (these were Patnaik grade II tumours) and 11 of the high-grade MCTs (one of these was Patnaik grade II) (Table 1). Wald chi-squared analysis revealed a significant relationship between ITD frequency and Kiupel histological grade ($P < 0.001$, Table 2). The mutation frequencies of Kiupel low- and high-grade tumours were significantly different from each other as determined by pairwise comparison ($P < 0.001$).

The dog that had 4 MCTs had 3 tumours in which only WT *C-KIT* was detected, but an ITD was detected in the 4th tumour. In dogs with 2 tumours, WT DNA only was detected in both tumours of 4 dogs. In the remaining 2 dogs, a *C-KIT* exon 11 ITD was detected in both tumours from 1 dog, and only 1 tumour from the other dog. There was a high prevalence of ITDs detected in tumours from the genital region (40%, n = 5) and the head and neck (43%, n = 7) of the dogs compared with the ITD prevalence on the torso (16%, n = 31), limbs (11%, n = 19) or unknown areas (8%, n = 12). However, mutation frequency differences between different anatomical locations were not statistically significant.

Discussion

The primary finding of this study was that, in a sample of MCTs from dogs in south-east Australia, the overall prevalence of ITDs in exon 11 of the *C-KIT* proto-oncogene was approximately 18%. Previous publications have shown that the most frequently identified mutations in *C-KIT* in canine MCTs are exon 11 ITDs, but the prevalence of these mutations has been unclear. For example, the mutation prevalence has been reported as 0% (Italy),[22] 15% (USA)[10] and 46% (USA).[16] The variability of the results could potentially reflect biological differences in the regional canine populations. However, in the majority of these studies, various biases including sample selection and small sample size confound the results. The sole criterion for tumour inclusion in the current study was a histopathological diagnosis of MCT. Tumours included 108 submitted sequentially over an 18-month period by general practitioners to a commercial pathology laboratory for routine diagnosis and 15 samples accrued sequentially at the CAHC in a study pilot phase establishing the molecular methods. The overall *C-KIT* exon 11 ITD prevalence found by this study was very similar to that (15%) of the one other study in which there was also no selection bias towards breed or histological grade.[10] A secondary aim was to determine the correlations, if any, that exist between detection of *C-KIT* exon 11 ITDs in

Table 2. Wald chi-square analysis for determination of the significance of relationships between PCR detection of a *C-KIT* exon 11 ITD in canine mast cell tumours and patient signalment and tumour characteristics

Patient or tumour feature	Statistical significance (P value)
Age	0.996
Breed	0.980
Sex	0.249
Neuter status	0.624
Tumour anatomical location	0.244
Tumour size	0.791
Patnaik histological grade	< 0.001*
Kiupel histological grade	< 0.001*

* P < 0.05 considered to be statistically significant.
ITD, internal tandem duplication.

tumours and patient factors that have been related to disease outcomes. Our findings are in agreement with those of others, namely that the detection of a *C-KIT* exon 11 ITD did not correlate with breed, age, sex, neuter status, tumour anatomical location or tumour size, but did correlate with higher histological grade.[9–11] Patient sex and neuter status were investigated in this study because gonadectomy in both male and female dogs has been reported to increase the risk of MCT development.[23] The relationship between *C-KIT* exon 11 ITD detection and neuter status had not been previously evaluated. The present study found no significant correlations (Table 2).

The tumours in this study were initially graded independently according to both Patnaik's scheme (1986) and Kiupel's scheme (2011), resulting in 80% and 93% agreement, respectively, between pathologists. After tumour re-evaluation, there was 86.5% agreement between pathologists when using Patnaik's scheme compared with 96% agreement when using Kiupel's scheme. This is consistent with previous studies that report increased agreement among pathologists when using Kiupel's grading system.[7,8]

When graded using the Patnaik system, ITDs in *C-KIT* were found exclusively in the grade II and III MCTs, with 56% of the grade III tumours possessing an ITD, similar to results reported by others.[10,17,18] Pairwise comparisons indicated that the ITD frequency in grade III MCTs differed significantly from that in grade I and grade II MCTs. No significant difference in ITD prevalence was detected between grade I and II MCTs (P = 0.074), perhaps reflecting the low number of grade I tumours. As might be expected, the association of *C-KIT* ITDs with high histological grade tumours was maintained when tumours were graded using the Kiupel system. The *C-KIT* exon 11 ITD was detected in two Kiupel low-grade tumours, both of which were Patnaik grade II. In two retrospective studies directly comparing the two grading systems, tumour-related mortality over 4–6 years from the date of surgery was zero for animals with Patnaik grade I tumours, but approximately 10% for Kiupel low-grade tumours.[7,8] It is tempting to speculate that the presence or absence of an ITD might be an explanatory variable for this difference. To investigate the clinical significance of ITD mutations in Patnaik grade I and Kiupel low-grade tumours will require expanding this study to include a larger number of Patnaik grade I tumours and analysis of patient survival data. This was beyond the scope of this project, but is currently in the planning stage for future research.

Table 3. Pairwise comparison of mutation frequencies between Patnaik histological grades

Patnaik histological grade		Statistical significance (P value)
Grade I	Grade II	0.074
	Grade III	< 0.001*
Grade II	Grade I	0.074
	Grade III	< 0.001*
Grade III	Grade I	< 0.001*
	Grade II	< 0.001*

* P < 0.05 considered to be statistically significant.

A total of 123 spontaneous cutaneous canine MCTs were submitted for this study, but PCR products were only obtained from 74 of the samples. The 60% success rate of the PCR may be related to the poor quality of DNA extracted from some FFPE tumour blocks. Formalin fixation causes DNA–protein cross-linking that must be reversed during DNA extraction. Prolonged formalin fixation, particularly in low pH unbuffered fixatives, results in DNA degradation and consequent poor PCR results.[24,25] However, neutral buffered formalin is the standard fixative used in veterinary general practice and assuming a mid-week excision and same-day courier transport to the laboratory, we estimate the majority of samples were processed within 24–36 h of fixation. Using a different method to extract DNA from FFPE tissues, Weiss et al.[25] PCR-amplified bands of 80, 161, and 245 nucleotides from 100% of feline lymphoma, feline myocardium and canine cutaneous histiocytoma samples, but only 60%, 38%, and 45% of MCT samples, and from only 43% of DNA extracted from frozen MCT samples. DNA gel electrophoresis showed no significant fragmentation differences between successfully and unsuccessfully amplified DNA samples from frozen or FFPE samples and the authors suggested that tissue-specific PCR inhibitors may be retained during DNA extraction from MCTs. Others have also reported variable PCR results for DNA from FFPE MCTs.[26] PCR success rates using DNA from FFPE material may be higher for shorter PCR amplicons and this could be one avenue to explore for further PCR optimisation.[25,26]

Sequencing of two mutant DNA samples showed two unique ITD sequences (Figure 2B). The 45-bp duplication has been previously reported,[18] but the 60-bp duplication has not been reported, although it is similar to other reported mutations.[9,16–18] Previous studies have reported exon 11 3′ ITD sizes ranging from 27 to > 60 residues.[9,16–18] Other mutations detected in our study were within this approximate size range based on their gel electrophoretic appearance and are therefore presumed to be ITDs.

Activating mutations are most common at the 3′ end of exon 11 of *C-KIT*.[9,16–18] Other mutations involving ITDs, insertions, deletions or single nucleotide substitutions have also been recorded in exons 8, 9 and 11 of *C-KIT* in canine MCTs, the majority of which have been confirmed as activating.[15,17,22,27] An uncommon mutation in exon 17 has also been identified and in human mastocytosis this

mutation confers resistance to tyrosine kinase inhibitors.[28] In one large study, mutations in exons 8 and 9 and 17 comprised almost half of the mutations detected in Patnaik grade II and grade III recurrent or unresectable tumours.[17] If the same proportion held true for the dogs in the current study, the overall prevalence of mutant, constitutively active *C-KIT* in tumours from Australian dogs could be as high as approximately 36%, rising to nearly 100% of all dogs with Patnaik grade 3 or Kiupel high-grade tumours. This would be surprising and further investigation is required to determine the true frequency and biological significance of these mutations for dogs.

To the authors' knowledge, the current study is the first survey of *C-KIT* mutations to be conducted in Australia. We conclude that ITDs in the 3′ region of exon 11 of *C-KIT* occur in approximately 18% (95% CI: 9–26%) of Australian canine MCTs, but this is likely to be an underestimate of the proportion of MCTs harbouring activating *C-KIT* mutations. The exon 11 ITD mutations can be readily detected using a procedure to isolate and PCR-amplify DNA from approximately 60% of routinely processed samples. The mutation was not detected in Patnaik grade I tumours, but this result is qualified by the small number of grade I tumours in the study. An ITD was detected in approximately 55% of the Patnaik grade III MCTs and 50% of Kiupel high-grade MCTs. The chemotherapeutic tyrosine kinase inhibitor toceranib reportedly induces greater responses in canine MCTs with *C-KIT* exon 11 ITDs compared with MCTs lacking these.[21] Until *C-KIT* mutation testing becomes routine and readily available, knowledge of the prevalence of exon 11 ITDs in Patnaik grade III or Kiupel high-grade MCTs may assist clinicians faced with difficult therapeutic situations to make more informed medical management decisions.

Acknowledgments

The authors thank the veterinary clinics participating in the pilot phase of this study, Gribbles Veterinary Pathology for their material and personnel support, and Dr Cynthia Bottema for her assistance in the laboratory.

Conflicts of interest and sources of funding

The authors declare no conflict of interest for the work presented here.

This work was supported in part by a grant from the Australian Companion Animal Health Foundation.

References

1. Brodey RS. Canine and feline neoplasia. *Adv Vet Sci Comp Med* 1970;14:309–354.
2. Er J, Sutton R. A survey of skin neoplasms in dogs from the Brisbane region. *Aust Vet J* 1989;66:225–227.
3. Finnie J, Bostock D. Skin neoplasia in dogs. *Aust Vet J* 1979;55:602–604.
4. Rothwell T, Howlett C, Middleton D et al. Skin neoplasms of dogs in Sydney. *Aust Vet J* 1987;64:161–164.
5. Bostock D. The prognosis following surgical removal of mastocytomas in dogs. *J Small Anim Pract* 1973;14:27–40.
6. Patnaik A, Ehler W, MacEwen E. Canine cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. *Vet Pathol Online* 1984;21:469–474.
7. Sabattini S, Scarpa F, Berlato D et al. Histologic grading of canine mast cell tumor. Is 2 better than 3? *Vet Pathol* 2015;52:70–73.
8. Kiupel M, Webster J, Bailey K et al. Proposal of a 2-tier histologic grading system for canine cutaneous mast cell tumors to more accurately predict biological behavior. *Vet Pathol Online* 2011;48:147–155.
9. Zemke D, Yamini B, Yuzbasiyan-Gurkan V. Mutations in the juxtamembrane domain of c-KIT are associated with higher grade mast cell tumors in dogs. *Vet Pathol Online* 2002;39:529–535.
10. Webster JD, Yuzbasiyan-Gurkan V, Kaneene JB et al. The role of c-KIT in tumorigenesis: evaluation in canine cutaneous mast cell tumors. *Neoplasia* 2006;8:104–111.
11. Webster JD, Yuzbasiyan-Gurkan V, Thamm DH et al. Evaluation of prognostic markers for canine mast cell tumors treated with vinblastine and prednisone. *BMC Vet Res* 2008;4:32.
12. Williams DE, Eisenman J, Baird A et al. Identification of a ligand for the c-kit protooncogene. *Cell* 1990;63:167–174.
13. London CA, Thamm DH. Mast cell tumors. In: Withrow SJ, MacEwen EG, editors. *Small animal clinical oncology*. 5th edn. Saunders Elsevier, St Louis, MO, 2013:335–355.
14. Chang PM, Ilangumaran S, La Rose J et al. Autoinhibition of the Kit receptor tyrosine kinase by the cytosolic juxtamembrane region. *Mol Cell Biol* 2003;23:3067–3078.
15. Ma YS, Longley BJ, Wang XM 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.
16. 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.
17. Letard S, Yang Y, Hanssens K et al. Gain-of-function mutations in the extracellular domain of KIT are common in canine mast cell tumors. *Mol Cancer Res* 2008;6:1137–1145.
18. Downing S, Chien MB, Kass PH et al. Prevalence and importance of internal tandem duplications in exons 11 and 12 of c-kit in mast cell tumors of dogs. *Am J Vet Res* 2002;63:1718–1723.
19. Australian Pesticides and Veterinary Medicines Authority. *Agricultural and veterinary chemicals*. APVMA, Kingston, 2011:17–20.
20. London CA, Hannah AL, Zadovoskaya R et al. Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. *Clin Cancer Res* 2003;9:2755–2768.
21. London CA, Malpas PB, Wood-Follis SL et al. Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), 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* 2009;15:3856–3865.
22. Riva F, Brizzola S, Stefanello D et al. A study of mutations in the c-kit gene of 32 dogs with mastocytoma. *J Vet Diagn Invest* 2005;17:385–388.
23. White CR, Hohenhaus AE, Kelsey J et al. Cutaneous MCTs: associations with spay/neuter status, breed, body size, and phylogenetic cluster. *J Am Anim Hosp Assoc* 2011;47:210–216.
24. Nam SK, Im J, Kwak Y et al. Effects of fixation and storage of human tissue samples on nucleic acid preservation. *Korean J Pathol* 2014;48:36.
25. Weiss ATA, Delcour N, Meyer A et al. Efficient and cost-effective extraction of genomic DNA from formalin-fixed and paraffin-embedded tissues. *Vet Pathol Online* 2011;48:834–838.
26. Granato A, Giantin M, Ariani P et al. DNA and RNA isolation from canine oncologic formalin-fixed, paraffin-embedded tissues for downstream "-omic" analyses: possible or not? *J Vet Diagn Invest* 2014;26:117–124.
27. Giantin M, Vascellari M, Morello EM et al. c-KIT messenger RNA and protein expression and mutations in canine cutaneous mast cell tumours: correlations with post-surgical prognosis. *J Vet Diagn Invest* 2012;24:116–126.
28. Akin C, Brockow K, D'Ambrosio C et al. Effects of tyrosine kinase inhibitor STI571 on human mast cells bearing wild-type or mutated c-kit. *Exp Hematol* 2003;31:686–692.

(Accepted for publication 11 December 2016)