

A novel, minimally invasive diagnostic test for *KIT* exon 11 internal tandem duplications in canine cutaneous mast cell tumours II: proof-of-principle applications

Liam S. Hill · Jacob O. Evans · Giusy Tornillo · Melanie Dobromylskyj · Sam Beck · Grace L. Edmunds · Matthew J. Smalley

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Abstract

Background The clinical management of canine cutaneous mast cell tumour (cMCT) presents challenges. Treatment decisions are often informed by histopathological and molecular analysis of excisional biopsy samples, however, these may not always be available. Furthermore, current gold standard assays for the most informative of molecular biomarkers, *KIT* receptor tyrosine kinase exon 11 internal tandem duplications (ITDs), are not quantitative. Here, we have tested our new qPCR-based assay for *KIT* exon 11 ITDs, the minA assay, on a cohort of canine cMCT formalin-fixed paraffin-embedded (FFPE) tissue. We have also assessed its suitability for use with fine needle aspirates (FNAs) and ‘liquid biopsy’ (blood/plasma samples).

Results DNA was extracted from nineteen archival FFPE samples. DNA extraction failed in one sample. Two additional samples consistently failed to give interpretable PCR results. Of the remaining sixteen, the minA assay correctly identified nine samples as wild type, seven with ITDs (confirmed by exon 11 sequencing; 100% sensitivity and specificity of interpretable samples). The ITD sequence could be diluted to 4% of the overall sample and still be detected in a quantitative manner, providing each reaction contained at least 5ng of sample DNA. The minA assay was tested in seven FNA cytology samples; in one of these (from a lymph node containing metastatic tumour cells) an ITD was detected. The minA assay was also able to detect the exon 11 ITD in cell-free DNA isolated from plasma of dogs with cMCTs (nine samples from eight animals). However, while some plasma samples gave a strong signal, other cases gave weak or equivocal signals, or, in one known ITD-positive case, no signal at all.

Conclusions The minA assay is a rapid, sensitive, specific, quantitative approach to determining *KIT* exon 11 status in canine cMCT. It is suitable for use in formalin-fixed material and fine needle aspirates, making it ideal for situations where excision biopsy of a mass is not possible. However, for liquid biopsy, better standardisation of sample collection and longitudinal studies are required to understand the clinical significance of detecting (or not detecting) an ITD. This assay represents a significant advance in the management of canine cMCT.

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Background

The cutaneous form of mast cell tumour (cMCT) is among the most common cutaneous tumours of dogs, accounting for 7–21% of all canine skin tumours [1–8]. Mast cell tumours can also occur less commonly in the intestines, viscera or other sites [9]. Although any dog may develop a cMCT, there are clear breed predispositions. A multivariable, case-control analysis of dogs from the VetCompass database for MCT risk factors showed that Boxers, Pugs and Staffordshire Bull Terriers are at increased risk of being diagnosed with MCT relative to crossbreed animals, while Cocker Spaniels, Springer Spaniels, West Highland White Terriers, Border Collies and German Shepherd Dogs were at lower risk [10]. However, this retrospective study did not distinguish between the cutaneous and subcutaneous forms, which may have different clinical behaviour and prognosis [11], although this remains uncertain [12, 13]. Other studies have suggested that Golden Retrievers, Labrador Retrievers and Weimaraners are specifically at higher risk of cutaneous MCTs [14–19]. Older dogs tend to be at higher risk of MCT, as are larger dogs, but there is no sex bias [10]. While Boxers, Staffordshire Bull Terriers and Pugs are at highest risk of developing MCTs in general, their tumours are more likely to be benign [1, 20].

The challenges associated with management of cutaneous mast cell tumours are summarised in Fig. 1. A diagnosis of canine cutaneous or subcutaneous MCT is usually based on clinical examination and microscopic assessment of a fine needle aspirate (FNA) taken from the mass lesion and stained with Romanowsky-type, Wright-Giemsa or toluidine blue stains [9, 21]. Cytologic grading may aid in pre-surgical decision making [22]. Ideally, the tumour is surgically excised with suitable margins, followed by histopathological assessment of excised tissue. Local lymph nodes and liver and spleen may also be aspirated or biopsied for staging. Diagnostic histopathology carried out on formalin fixed tissue allows assessment of margins, categorisation of the tumour as a cutaneous or subcutaneous tumour, grading of cutaneous lesions, determination of mitotic index, labelling for Ki67 as a marker of cell proliferation, labelling patterns of KIT receptor expression and evaluation of the mutation status of the KIT receptor tyrosine kinase (typically using semi-quantitative PCR and gel-based size fractionation of amplicons to determine whether or not internal tandem duplications are present in either exons 8 or 11 of the *KIT* gene) [23]. Treatment of a cMCT, including whether or not to use systemic therapy, will depend on post-surgical evaluation of any or all of these parameters together with clinical staging, taking into account an owner's wishes and financial circumstances [23, 24]. If a tumour is not suitable for excision, then a treatment plan must be made with more limited information, because many of the above tests require evaluation of biopsied tissue obtained at surgery.

Deregulated activation of the KIT receptor tyrosine kinase is thought to be a key driver of mast cell neoplasia [7]. Activating mutations in the *KIT* gene have been reported in the extracellular (exons 8 and 9) and juxta-membrane (exons 11 and 12) domains [7, 25–31]. Internal tandem duplications (ITDs) of *KIT* exon 11 are reported in 9 to 45% of canine MCTs, depending on the study [25, 26, 28, 31–33]. The clinical relevance of *KIT* exon 11 ITDs in canine MCTs has been investigated in particular detail, including their overall prognostic value, and their utility as a predictor of treatment response in patients receiving tyrosine kinase inhibitors. In several studies, *KIT* exon 11 ITDs in cMCT were shown to predict progression-free survival but in multivariable analysis this result was likely correlated with histologic grade, rather than KIT being an independent determinant of survival [34–36]. *KIT* exon 11 status can nevertheless provide useful information for prognosis as it is more objective than assessment of grade [23].

The evidence for exon 11 ITDs as a predictor of response to the broad-spectrum tyrosine kinase inhibitor (TKI), toceranib, suggests that MCTs with exon 11 ITDs have a better objective response [37], but no difference in time to progression [37]. Furthermore, amongst dogs treated with toceranib, those with a *KIT* exon 11 ITD exhibited a shorter progression-free survival [38] but no difference in overall survival [38]. In contrast, overall survival was significantly improved amongst canine MCT patients carrying a *KIT* exon 11 ITD treated with the TKI masitinib mesylate, compared to patients with tumours carrying a wild type exon 11 [39]. Indeed, masitinib mesylate is specifically licenced for use in canine MCTs carrying a *KIT* exon 11 ITD (Masivet™; European

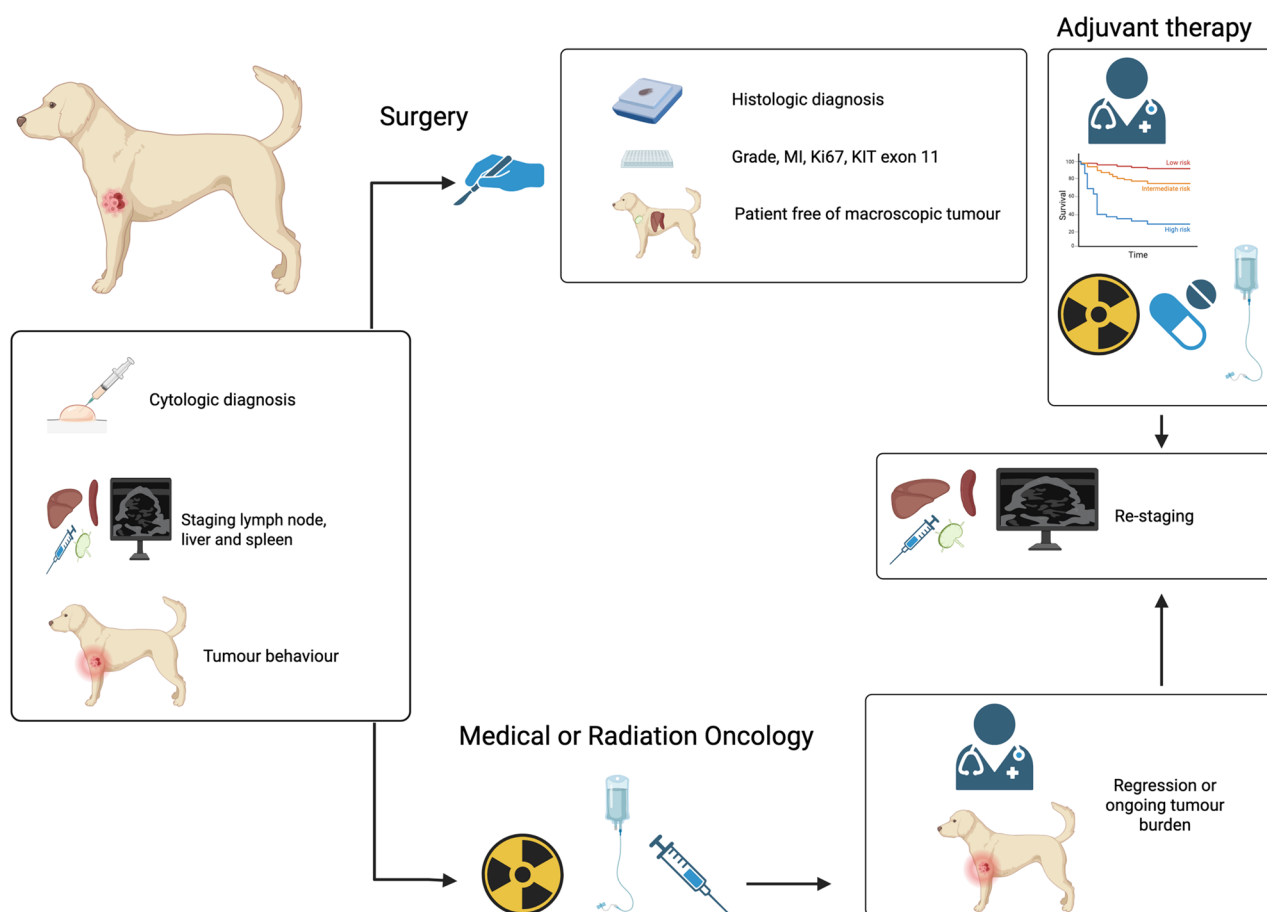


Fig. 1 Concepts and challenges in the management of canine cutaneous mast cell tumours. When a patient presents with a suspected mast cell tumour, an initial diagnosis is often made by cytology. Subsequent clinical decisions are based on tumour size, location and behaviour. For example, ulceration, rapid growth, inflammation, redness and itching may be associated with aggressiveness, and tumours in locations such as the distal limb or the head may not be resectable with appropriate margins. Mast cell tumours most commonly metastasise to the lymph nodes, spleen and liver. Thus, staging involves fine needle aspiration of those organs, often requiring sedation or general anaesthetic. Fine needle aspirates may miss early metastatic disease if it is heterogeneously distributed within the liver and spleen. In those animals with resectable tumours of appropriate stage, surgery is carried out. This enables the tumour burden to be reduced or eradicated. FFPE tissue can also be analysed histologically to confirm a diagnosis of mast cell tumour, and used to determine parameters which have been associated with prognosis or treatment response e.g. mitotic index, Ki67 proliferative index and *KIT* exon 11 mutation status. Veterinary clinicians will integrate such information to make an ongoing treatment plan. Monitoring of the surgical site allows assessment of recurrence. Restaging (again requiring procedures under general anaesthetic) may be carried out to identify the development of subsequent metastatic disease. Non-resectable tumours are typically managed with chemotherapy, tyrosine kinase inhibitors (e.g. masitinib, licensed specifically in those tumours with a *KIT* genetic mutation), radiotherapy or intralesional injection of tigilanol tiglate. Clinical decision making is more challenging in cases of non-resectable mast cell tumour, as the clinician lacks access to FFPE tissue and as such, prognostic information including histologic grade, mitotic index, Ki67 and *KIT* mutation status. Notably, use of masitinib in these cases may be off-license, as *KIT* mutation status is unknown. Created in BioRender. Edmunds, G. (2026) <https://BioRender.com/aon0xx0>

Medicines Agency product number EMEA/V/C/000128). As TKIs are expensive medicines with the potential to cause side effects, testing for exon 11 ITDs can be an important part of the clinical management of a dog with cMCT, as part of deciding whether or not a dog should receive systemic therapy, including a TKI, instead of or alongside other treatment modalities such as chemotherapy, radiotherapy or intralesional tigilanol tiglate [23, 24] (Fig. 1). However, as all of the current literature used surgical biopsies to determine the *KIT* exon 11 mutation status, the existing body of evidence is biased towards those patients whose tumours were resected. Therefore,

further studies including both resectable and non-resectable canine MCTs are needed to determine the overall prevalence and utility of *KIT* exon 11 ITD as (i) a predictor of prognosis in canine MCT and (ii) a predictor of treatment response.

We have developed a novel qPCR-based assay for *KIT* exon 11 ITDs in canine cMCTs [40], termed the ‘min’ assay. The assay is based on identification of a minimally commonly amplified region (the ‘min’ region) in MCTs with *KIT* exon 11 ITDs and the analysis of qPCR melt curves of the amplification products of primer sets, minA/minA-ALT and minB/minB-ALT which anneal to this region (the ‘ALT’ versions have sequences which vary by a single nucleotide to correspond to allelic variations at polymorphisms in primer binding sites). The presence of a *KIT* exon 11 ITD is determined from inspection of the melt curves [40]. We categorised tumours containing *KIT* exon 11 ITDs into Type I, Type II and Type III, depending on the size and structure of the duplicated segment detected by the ‘min’ assay. The assay primer configuration and products are cartooned in Additional File 1.

In the development of the assay [40], we analysed samples from fifteen formalin-fixed paraffin-embedded (FFPE) cMCTs with an exon 11 mutation status that had already been externally validated (five wild-type, ten with ITDs). With this sample set, we showed that using the minA/minA-ALT primer sets, the assay had an analytical sensitivity and specificity of 100% and 94% respectively, to detect a *KIT* exon 11 ITD given an input of 25ng DNA per reaction. We also showed that the strength of the minA/minA-ALT ITD signal was quantitatively related to the proportion of cells containing an ITD in the sample [40]. The minB/minB-ALT primer sets could also robustly distinguish between wild type samples and samples containing an ITD if the latter were homogenous. However, they performed less well in samples which were a heterogenous mix of wild type and ITD-containing cells and we proposed the minA/minA-ALT primers as most suitable for routine use [40].

Here, we have explored the utility of the min assay in research and clinical settings, testing its performance in an independent set of archival FFPE samples, as well as on genomic DNA extracted from fine needle aspirates (FNA) and cell-free DNA (cfDNA) isolated from plasma samples (so called ‘liquid biopsy’). We demonstrate that using the assay, MCT cells containing a *KIT* exon 11 ITD can be detected in an FNA sample from a mast cell tumour-draining lymph node containing metastatic cells. We also show that a *KIT* exon 11 ITD can be detected in blood via ‘liquid biopsy’.

The wide range of sample options suitable for our min A assay contrasts with currently available methods for determining *KIT* exon 11 status, which require surgically resected tissue. Although experimental deep learning models have the potential to distinguish between exon 11 ITD positive and exon 11 wild type mast cell tumours using H&E stained whole slide images, performance of the models is laboratory specific, affected by variability resulting from different H&E staining protocols and use of different slide scanners [41]. They are not yet, therefore, suitable for general application or indeed for use with FNAs. Our assay provides flexibility for determination of *KIT* exon 11 status in any given patient, including the possibility of indirect sampling of non-resectable lesions, for example by direct FNA, FNA of a sentinel lymph node, or blood sampling and liquid biopsy.

Furthermore, whereas staging and monitoring of systemic disease burden in canine MCT currently involves fine needle aspiration of spleen and liver, necessitating sedation or anaesthetic, the min assay opens the door to the use of serial blood sampling during follow-up, although further work is required before the significance of detection of a *KIT* exon 11 ITD in plasma from a dog under treatment, and its relationship to clinical outcomes, can be determined. Notably, the rapid turnaround time of our new assay means that *KIT* exon 11 ITD status could be available within hours, which could assist in rapidly determining a treatment plan, in advance of surgery, intralesional injection or radiotherapy. Thus, the min assay provides a research tool which can be used to evaluate the clinical significance of *KIT* exon 11 ITDs in populations of dogs with resectable and non-resectable cutaneous mast cell tumours, and its relevance to prognosis and treatment response. Furthermore, its potential to be used as a minimally invasive test which enables rapid clinical decision making, staging and assessment of recurrence makes it a major contributor to the development of personalised care in companion animal oncology.

Methods

Aim

To provide proof of principle demonstrations of the utility of the min assay for KIT exon 11 ITDs in a range of clinical samples from dogs with cutaneous mast cell tumours.

Ethical approval

This study was approved by the Bristol University Animal Welfare and Ethics Review Body (VIN number 22–041). All samples had been taken during routine diagnosis as part of standard clinical veterinary care. Informed consent was provided by owners prior to the use of any non-archival samples.

Clinical samples

Cut sections from nineteen high Patnaik and Kiupel grade canine cMCTs of unknown KIT exon 11 status were provided as archival FFPE scrolls for DNA extraction by AML Laboratory (Antwerp, Belgium). As the archival samples had been previously collected as part of routine clinical management of patients from multiple referring practices, no information was available on time until fixation, fixation procedure or fixation duration. Sample quality was therefore unknown prior to DNA extraction. One sample (AM17) was excluded due to a failure of DNA extraction and is not further discussed. Surplus tissue from fine needle aspirates was collected from four dogs with histologically confirmed canine cutaneous MCT being managed at Langford Vets. Matched EDTA plasma and whole blood samples were available for two of these dogs. Surplus whole blood and EDTA plasma were also available from an additional six dogs with histologically confirmed canine cutaneous MCT but without matched FNA samples, being managed at Langford Vets. Blood and plasma samples were maintained at -80 °C from collection until DNA isolation. Sample details are provided in Additional File 2.

Histology

Haematoxylin and eosin (H&E) staining was carried out using standard approaches. For KIT and Ki67 immunohistochemistry, samples underwent heat-induced epitope retrieval with Envision Flex TRS high pH buffer (Agilent Laboratories). KIT labelling used a polyclonal rabbit anti-CD117 (A4502; 1:200) with the Envision FLEX+ system for visualisation. Ki67 labelling used mouse monoclonal anti-Ki67 (clone MIB-1; GA626; provided ready-to-use) with the Envision FLEX system. One author (SB) assessed each sample to provide Ki67 indices and KIT expression patterns as previously described [42].

Measurement of area of tumour relative to the total area of tissue within a section was carried out using QuPath [43] on whole slide images.

DNA extraction from FFPE sections

DNA was extracted from 5 µm FFPE sections provided as scrolls in 1.5 ml Eppendorf tubes (3 or 4 scrolls per sample). Scrolls were washed twice in xylene for 5 min each and then twice in 100% ethanol for 5 min each. Samples were centrifuged between washes to avoid loss of material. After the last ethanol wash, samples were air-dried. DNA was then extracted using the QIAamp DNA FFPE tissue kit (Qiagen), including the RNaseA step. DNA was eluted in 100ul nuclease-free water.

DNA extraction from Fine Needle Aspirates (FNAs)

FNAs were scanned using the Olympus VS200 whole slide scanner at 20x. After scanning, coverslips were removed by soaking overnight in xylene. Slides were then washed once in fresh xylene for 5 min and then twice in 100% ethanol for 5 min each before being allowed to air dry.

Cellular material was removed from the washed and dried slides using a single-use disposable swab. The swab was dipped in 300ul PBS in a 1.5 ml Eppendorf tube. Excess PBS was removed by pressing the swab against the side of the tube and the damp swab was then wiped over the sample on the slide until the cellular material was fully transferred to the swab. The swab was then returned to the 1.5 ml Eppendorf tube and the tip cut off so that the tube could be closed. The tube was vortexed for 1 min and briefly spun down to collect the liquid at the bottom on the tube. The swab tip was removed, allowing as much liquid as possible to drain off before the tip was discarded. The volume of PBS remaining was then measured. If below 200ul, it was adjusted to 200ul. If above 200ul, the volume above 200ul was removed to a second clean tube and the volume of this second tube adjusted to 200ul.

The tube or tubes were then processed for genomic DNA extraction using the DNeasy Blood and Tissue kit (Qiagen, Manchester, UK). 20ul proteinase K was added to each 200ul volume of PBS, followed by 200ul buffer AL. This was mixed thoroughly by vortexing and incubated at 56 °C for ten minutes, after which the manufacturer's protocol was followed. If two tubes needed to be prepared then they were combined at the column purification stage by passing both through the same spin column sequentially. The column was washed according to the manufacturer's protocol and DNA was eluted in 50ul nuclease-free water.

cf/ctDNA extraction from plasma

The Thermofisher Applied Biosystems MagMAX Cell-Free DNA isolation kit (#A29319) was used to isolate cell-free DNA/circulating tumour DNA (cf/ctDNA) from plasma. The manual protocol was used with 1.5 ml Eppendorf tubes and the DynaMag Magnet starting from step 3 (samples were provided as pre-separated plasma and buffy coat layers; the proteinase K step was not required). Starting plasma volumes were recorded (typically between 50 and 250ul). At the final stage, cf/ctDNA was eluted in 30–50ul of elution solution.

PCR

See Additional File 1 for a cartoon of the principle behind the assay. See Additional File 3 for details of primers and PCR conditions used in the study [40]. Primers were ordered from Merck-Sigma-Aldrich (Gillingham, Dorset, UK) as 'Pure and Simple Primers' (<https://www.sigmaaldrich.com/GB/en/configurators/tube?%20product=puresimple>), cartridge purified and lyophilised. Primers were resuspended in nuclease-free water as 100uM stocks and then diluted to 10uM working aliquots before use.

All PCR reactions were set up in a room separate from areas where template was prepared, in a designated pre-PCR area in a Class II laminar flow hood. Template was not handled until the prepared PCR reactions were taken out of the pre-PCR room. Template was added to reactions in a designated PCR laminar flow hood in a separate area.

Standard semi-quantitative PCR reactions were carried out using the GoTaq Green PCR system (Promega, Chilworth, Hampshire, UK) as described [40]. Amplified products were resolved on 2% agarose TBE (Tris base 90mM, Boric acid 90mM, EDTA 2mM) gel (all reagents from Thermofisher) with a 100 bp DNA ladder (Promega).

Quantitative real time PCR on genomic DNA was carried out using a Quantstudio 7 system (Thermofisher) as described [40]. If the concentration of the DNA in a sample to be tested was too low to be measured, then the amount of sample available was divided between the reactions. In some cases, after the program had finished, the contents of representative wells were resolved on 2% agarose TBE gels to confirm product sizes.

In most cases, C_t (the amplification cycle at which the product crossed an automatically determined threshold) and T_m (the temperature at which the melt curve reached its peak value) [40] values for each reaction were determined automatically by the Quantstudio 7 software. In some cases where the melting curve had two distinct peaks, the T_m for both peaks could be determined automatically. However, in some cases where one peak was small, the T_m for that peak had to be determined by manual inspection of fluorescence reporter derivative data (i.e. the measured values for the melt curve).

For all melt curve plots, the X-axis is the temperature ($^{\circ}\text{C}$) and the Y-axis is the fluorescence reporter derivative. The fluorescence reporter derivative values across the melt curve temperature range were imported into GraphPad Prism 10.2.3 (GraphPad Software LLC, Boston MA, USA) for plotting.

Sequencing

KIT exon 11 genomic DNA sequencing was carried out either by (i) PCR amplification of exon 11 using the whole exon 11 primers (Additional File 3) and GoTaq PCR conditions, with 50ng template in a reaction, or (ii) qPCR amplification of ITDs using minB primers, with very low levels of template input (5pg per reaction or 1:20 dilutions of cf/ctDNA eluates).

Low template input minB sequencing takes advantage of the minB/minB-ALT qPCR reaction producing only single product from a duplicated *KIT* exon 11, with a melt curve T_m of approximately 80°C . The product of a WT exon 11 with these primers has a melt curve T_m of approximately 77°C . These can be difficult to distinguish in a heterogeneous template mix, but if the template is a pure ITD sequence then a pure ITD amplicon is produced, which is of sufficient size for Sanger sequencing (in contrast, the minA/minA-ALT primers always produce both WT and ITD amplicons, even if the template is a pure ITD, so these can never be used to create a pure product for sequencing). By dilution of the template going into the reaction to approximately 5pg (the amount of DNA in one canine diploid cell, therefore using 5pg of template in a reaction is equivalent to adding only one or two copies of the template) the DNA targets for amplification can be partitioned into reactions containing only WT templates, reactions containing a mix of WT and ITD templates and reactions containing only ITD templates. The latter can clearly be distinguished by the melt curves at the end of the qPCR amplification process, recovered from the plate and purified. An example of this approach is provided in Additional File 4.

The amount of DNA in one canine diploid cell was calculated as follows. Taking the haploid length of the canine genome as $\sim 2.4\text{ Gb}$ [44], and 978 Mb of DNA as having a mass of 1pg [45], then the DNA content for the average diploid canine cell is $\sim 5\text{pg}$.

Whole exon 11 amplicons were purified either by QIAquick PCR purification spins columns (Qiagen) if only a single WT product was produced or by QIAex gel extraction kits (Qiagen) if both WT and ITD products were produced and required gel size separation. minB qPCR amplicons were purified by QIAquick columns directly from 96-well plates. Purified amplicons were sent for Sanger sequencing by Eurofins (Eurofins GmbH, Ebersburg, Germany) using the whole exon 11 or minB primers as appropriate. Where possible, both the genotype of the wild-type and ITD bands at the chr13:47940619 (C/T; in the minA forward primer binding site) and chr13:47940672 (T/C; in the minB reverse primer binding site) SNPs, as well as the overall structure of the ITD in each sample, were determined. Sequences were analysed in DNASTar 18.0.3.2 (DNASTar Inc.).

Statistics

Statistical analysis (linear regression) was carried out using GraphPad Prism 10.2.3.

Results

Confirmation of diagnosis of high grade cMCT in a cohort of archival diagnostic FFPE samples

We (SB) first confirmed the histological diagnosis of high grade cMCT in a cohort of archival diagnostic samples by review of H&E images using current gold standard guidelines [46, 47] (low power whole slide images are shown in Additional File 5). Ki67 labelling to assess proliferation and assessment of KIT protein labelling patterns was also carried out [23, 42, 48, 49]. Results are summarised in Table 1.

Next, using the current standard of care approach, genomic DNA isolated from these samples was tested for *KIT* exon 11 ITDs by semi-quantitative PCR amplification with primers that amplified the whole exon, followed by resolving products on an agarose gel. Results are summarised in Table 1. All samples except AM14 (in which amplification failed) produced a band at the expected size for a WT product (~190 bp) (Fig. 2A). In addition, AM3, 5, 6, 9, 15 and 16 produced one or more additional bands in the range 220 to 300 bp, consistent with the presence of an ITD. These results were consistent with samples AM3, 5, 6, 9, 15 and 16 containing a mixture of WT and mutated cells.

To confirm that the 220–230 bp bands did indeed represent *KIT* exon 11 ITDs, they were cut out, gel extracted and Sanger sequenced. Extraction and sequencing of AM3 and 5 failed due to poor recovery from the gel but sequence was obtained from AM6, 9, 15 and 16. The results confirmed that these bands contained exon 11 ITDs. On the basis of our previous categorisation [40] (Additional File 1), AM9 and 16 were Type I/III ITDs; AM6 and 15 were Type II.

Quantitative qPCR detection of *KIT* exon 11 ITDs in FFPE sections from cMCTs using the novel min assay

Next, the samples were tested by our novel minA qPCR assay (Fig. 2B). Three samples (AM8, 13, 14) were considered to be non-interpretable in this assay due to poor amplification/melt curve quality. Eight samples produced only a single WT peak on the melt curves, while seven samples (AM3, 5, 6, 9, 15, 16, 19) produced melt curve peaks consistent with the presence of an exon 11 ITD (Fig. 2B, red arrows; Additional File 6). While the results of the standard of care PCR approach and the minA assay were consistent for the majority of samples, AM19 produced discordant results (results are summarised in Table 2).

The min assay exhibited superior performance in detecting a *KIT* exon 11 ITD when results were discordant, compared with standard PCR

For sample AM19, either the standard PCR approach had resulted in a false negative or the minA assay had resulted in a false positive. To address this, the standard semi-quantitative PCR assay with gel-based resolving of amplicon sizes was repeated using whole exon 11 primers, as well as with the minA primers and the minB primer set [40]. In semi-quantitative PCR from *KIT* exon 11 WT samples, the minA primers amplify a 44 bp fragment, while the minB primers amplify an 88 bp fragment. In contrast, from *KIT* exon ITD samples, minA amplifies both a 44 bp and a > 90 bp band, sometimes with a 'ladder' or 'smear' of larger amplification products on the gel, while minB amplifies a fragment of > 130 bp, depending on the ITD [40] (Additional File 1).

The results of this repeat on AM19 (Additional File 7 A) demonstrated amplification by the whole exon 11 primers of fragments of a size consistent with the presence of both WT and ITD exon 11 templates, as expected from a mixture of cells with WT exon 11 and cells carrying an ITD. Similarly, the minA primers produced both a small product consistent with the expected 44 bp WT product and a larger band/smear consistent with the ITD, while the main minB amplification product was ~150 bp, again consistent with an ITD, although there was also a faint WT band. Therefore, this repeat suggested that the minA qPCR analysis was correct in classifying AM19

Table 1 Histological features of archival cMCT FFPE samples used in this study

Sample	Histo- logical Diagnosis / Location	Kiupel grading criteria				Kiupel grade (High/Low)	Patnaik grade (I/II/III)	² c-KIT staining pattern	² % Ki67 + ve nuclei (number counted)
		¹ Mitotic figures	¹ Aniso-karyosis	¹ Multi- nucle- ated cells	¹ Pleo- mor- phic nuclei				
AM1	Mast cell tumour, haired skin from nose	11	Y	Y	Y	High	III	II	4.82 (933)
AM2	Mast cell tumour, right axilla	19	Y	N	N	High	III	III	11.65 (901)
AM3	Mast cell tumour, right ear	11	Y	N	Y	High	III	III	7.48 (856)
AM4	Mast cell tumour, haired skin from right hind	8	Y	Y	Y	High	III	III	4.39 (887)
AM5	Mast cell tumour, tail base mass	10	Y	N	Y	High	III	III	14.01 (714)
AM6	Mast cell tumour, right tarsus	12	Y	Y	Y	High	III	III	10.3 (573)
AM7	Mast cell tumour, haired skin	71	Y	N	Y	High	III	III	48.91 (920)
AM8	Mast cell tumour, haired skin from left fore	17	N	N	N	High	III	ND	ND
AM9	Mast cell tumour, right hind interdigital skin	9	Y	N	N	High	III	III	7.78 (913)
AM10	Mast cell tumour, left mammary glands	12	Y	N	N	High	III	III	6.44 (652)
AM11	Mast cell tumour, haired skin from right flank	11	Y	Y	Y	High	III	III	8.47 (874)
AM12	Mast cell tumour, left knee	11	N	N	N	High	III	II	6.6 (576)
AM13	Mast cell tumour, left thorax	15	Y	N	N	High	III	ND	ND
AM14	Mast cell tumour, digit	9	Y	Y	N	High	III	ND	ND
AM15	Mast cell tumour, haired skin	3	Y	N	Y	High	III	III	4.29 (746)

Table 1 (continued)

Sample	Histo-logical Diagnosis / Location	Kiupel grading criteria		¹ Multi-nucle-ated cells	¹ Pleo-morphic nuclei	Kiupel grade (High/Low)	Patnaik grade (I/II/III)	² c-KIT staining pattern	² % Ki67 + ve nuclei (number counted)
		¹ Mitotic figures	¹ Aniso-karyosis						
AM16	Mast cell tumour, haired skin from knee	9	Y	Y	Y	High	III	III	8.08 (755)
AM18	Mast cell tumour, haired skin from right hind	16	Y	N	N	High	III	III	10.2 (1020)
AM19	Mast cell tumour, haired skin	18	Y	N	N	High	III	III	26.32 (1208)

¹Number of mitotic figures, and presence/absence of anisokaryosis, multinucleated cells and pleomorphic nuclei were determined according to [48]

²Ki67 and KIT staining were determined according to [41, 50, 51]

as having a *KIT* exon 11 ITD, and the result of the original semi-quantitative PCR analysis (as used in the current standard of care) was a false negative.

To further test this, AM19 genomic DNA was analysed by minB qPCR melt curve analysis. Although less sensitive than minA qPCR analysis of *KIT* exon 11 ITDs, minB qPCR melt curves can distinguish between WT and ITD exon 11 targets in some cases [40]. The AM19 sample was analysed together with a confirmed WT sample, AM2 (Additional File 7B). The latter produced a single melt curve peak with a T_m of 77.08 ± 0.01 (mean $\pm$ SD, $n = 3$). The AM19 sample, in contrast, produced two peaks, one with a T_m of 77.77 ± 0.00 (WT) and one with a T_m of 81.41 ± 0.06 (ITD). Therefore, we can be confident that the original minA result was correct, not a false positive, and that sample AM19 was carrying a *KIT* exon 11 ITD.

Finally, the low input minB sequencing approach (see Methods) was used to sequence AM19 ITD as well as the two samples, AM3 and AM5, in which gel extraction of the ITD band after standard PCR had not been successful. The results (all results are summarised in Table 2) demonstrated that AM3 was a Type I duplication while AM5 and AM19 were Type II duplications [40]. The aligned sequences, against a WT reference, are shown for all seven samples with an ITD in Additional File 8 and the sequences are provided as FASTA files in Additional File 9.

Determination of exon 11 status by the minA assay is not confounded by allelic variants

The *KIT* exon 11 sequence, at the primer binding site for the forward minA primer, contains a polymorphic locus, *C.fam* 47,940,619 C/T, meaning that the genomic sequence here could incorporate either a cytosine (C) nucleotide or a thymine (T) nucleotide. However, the minA primer sequence contains a C at that position and we have demonstrated that the assay performs less well in *KIT* exon 11 ITD detection if the genetic sequence within the duplication contains a T at that locus [40]. Although unlikely, considering the agreement between the results of the standard semi-quantitative PCR analysis and the minA qPCR assay, it was considered possible that if any of the AM samples were of the 'T' genotype, the minA assay would perform suboptimally and potentially result in false negatives.

We tested the likelihood of false negatives in the minA assay by Sanger sequencing the WT products of whole exon 11 amplification for seventeen samples. Thirteen samples (including all the samples with an ITD) were homozygous C/C, three were heterozygous C/T, one was homozygous T/T at *C.fam* 47,940,619 (Additional File 10). If samples which are germline C/C have a duplication, they must contain a C at *C.fam* 47,940,619 in the

ITD, and for these samples minA is the optimal primer set. However, heterozygous C/T (AM7, AM8, AM10) or homozygous T/T (AM1) samples should also be tested with the alternative primer set, minA-ALT, which has a T at the equivalent position to *C.fam* 47,940,619.

Therefore, we directly compared the performance of minA and minA-ALT primers on AM1, 7, 8 and 10 (Additional File 11). Only the WT *KIT* exon 11 signal was detected with both the minA and minA-ALT primer sets in all samples. There were no obvious qualitative or quantitative differences in AM1 or AM7. The minA-ALT signal was weaker in AM8 and AM10 but still gave only a single peak at a T_m consistent with a WT exon 11. Therefore, this analysis confirmed that these samples were WT. Notably, in this analysis the minA assay was successful with AM8, in contrast to the analysis shown in Fig. 2B (see Discussion), giving a total of sixteen tumours with a successful minA assay.

In summary, this sample set contains nine tumours with a wild-type *KIT* exon 11 and seven tumours with a *KIT* exon 11 ITD, with two tumours that could not be analysed. For those samples which were interpretable, the minA assay distinguished between the WT and ITD tumours with 100% accuracy.

The ratio of the ITD peak height to the WT peak height in the min assay correlates with the amount of tumour in the analysis sample

We had previously demonstrated [40] a correlation between the ratio of the height of the ITD peak to the height of the WT peak (PdITD/PdWT) and the ratio of the amount of tumour material in a sample to the total amount of tissue. The PdITD/PdWT value could therefore be used to quantify the amount of (exon 11 duplicated) tumour material in a potentially heterogeneous sample.

To test whether this held true in an independent sample set, we determined the relative amounts of tumour to total tissue in the samples using QuPath analysis of whole slide images (Additional File 6; Additional File 12 A). Then we plotted the PdITD/PdWT values against the tumour: tissue ratios for the seven ITDs samples and carried out a simple linear regression analysis (Additional File 12B). This demonstrated the slope of the PdITD/PdWT values and tumour: tissue ratios was significantly non-zero (simple linear regression, $P = 0.0299$; equation of line $Y = 1.761 \cdot X + 0.4178$; goodness of fit $R^2 = 0.6435$; runs test P value = 0.2000 indicating deviation from linearity non-significant), supporting the PdITD/PdWT value as a quantitative measure of the amount of *KIT* exon 11 ITD in a sample. Addition of the PdITD/PdWT values and tumour: tissue ratios from our previous analysis (the 'FP' sample set) [40] demonstrated that the effect was consistent across the combined sample sets (simple linear regression, slope of line significantly non-zero, $P = 0.0046$; equation of line $Y = 1.775 \cdot X + 0.4479$; goodness of fit $R^2 = 0.4246$; runs test P value = 0.8235 indicating deviation from linearity non-significant) (Additional File 12 C).

Defining the detection thresholds of the minA assay

We have previously calculated the analytical specificity and sensitivity of the assay as 96% ($n = 33$ individual qPCR reactions) and 100% ($n = 37$ individual qPCR reactions), respectively [40], with an input of 25ng of template in each reaction. However, the potential for false negative results is not only dependent on the technical robustness of the assay but also on tumour heterogeneity and the extent to which a minA ITD signal from tumour cells in a sample can be diluted out by DNA from other cell types and still be detectable (i.e. the assay minimum detection threshold).

To test this, DNA from AM16 (a Type I ITD tumour) or AM15 (a Type II ITD tumour) were mixed with DNA from a non-transformed canine epithelial cell line (MDCK cells) at ratios of 1 in 2, 1 in 5 and 1 in 25 (four replicates in each reaction), with a constant amount of template in each replicate of 5ng (equal to approximately 1000 cells-worth of DNA, based on an average amount of DNA in a canine diploid cell as 5pg; see Methods), and the ability of the minA assay to detect the ITD assessed. Clear positive detections were seen with 5ng of both 1 in 2 and 1 in 5 ratios of tumour DNA to total, for both the Type I and Type II (mean PdITD/PdWT values $\pm$ SD from

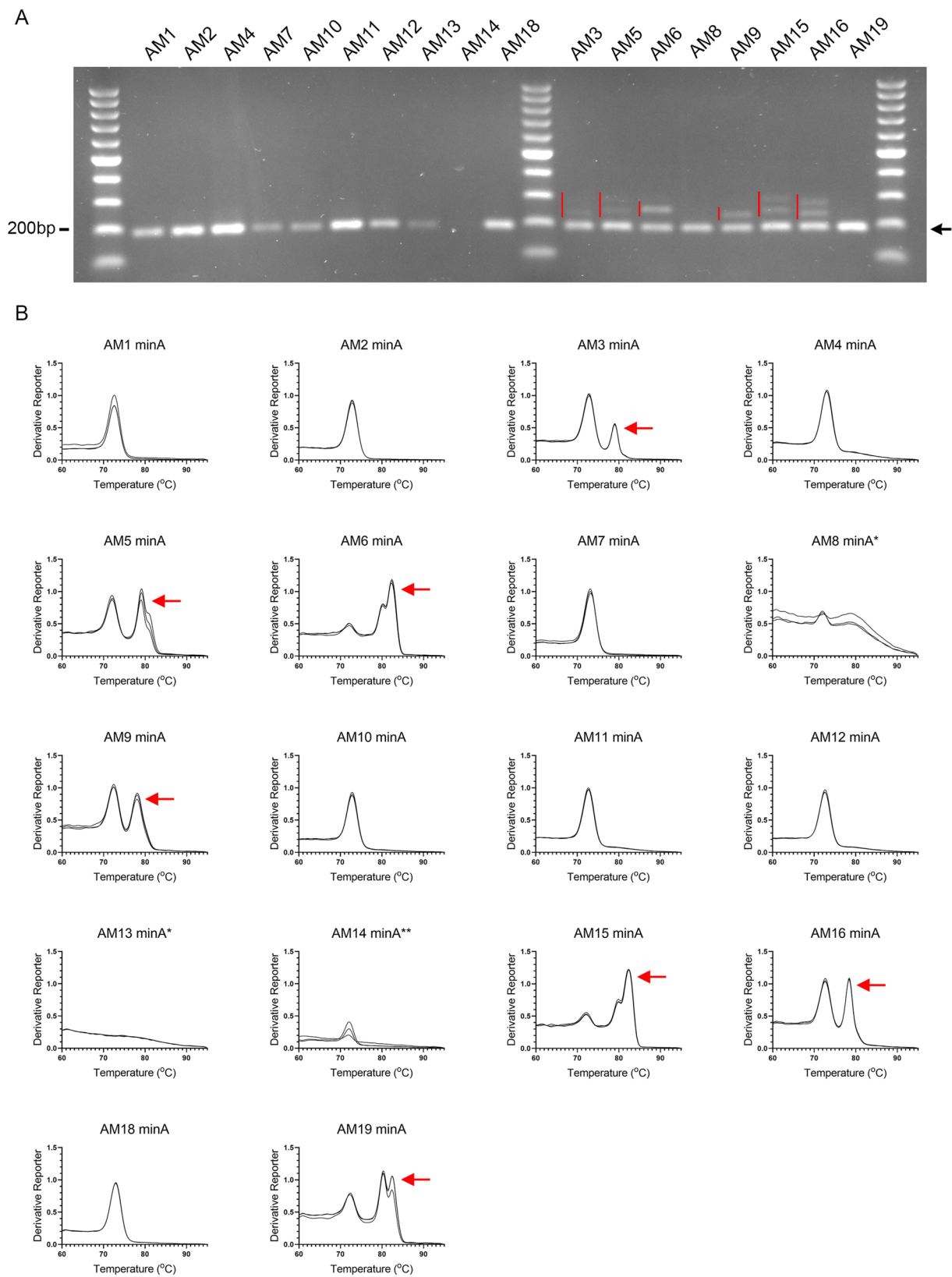


Fig. 2 *KIT* exon 11 ITDs detection in FFPE samples using current standard approaches and minA assay. **A** Semi-quantitative PCR analysis using whole exon 11 primers of AM samples. Samples were initially processed in several batches and run on multiple separate gels. Once all samples had been processed, they were re-run together on a single gel (shown here) to enable comparison. The 200 bp band on the ladder is indicated. The WT PCR product is indicated with an arrow; the products resulting from the ITD with a red line. AM14 failed to amplify; ITDs were detected in AM3, AM5, AM6, AM9, AM15 and AM16. **B** minA qPCR melt curves for clinical samples. ITD peaks (AM3, AM5, AM6, AM9, AM15, AM16, AM19) indicated by red arrows. *None of the three replicates from AM8 or AM13 reached the amplification detection threshold. **Only two of three AM14 replicates reached the amplification detection threshold. An uncropped gel image is shown in Additional File 22

0.930 ± 0.081 to 0.447 ± 0.051 ; Fig. 3A-C). With 1 in 25, the ITD peaks were small but consistent across replicates (mean PdITD/PdWT values $\pm$ SD 0.129 ± 0.019 for the Type I mix and 0.126 ± 0.054 for the Type II mix; Fig. 3A-C). Plotting the PdITD/PdWT ratios against the proportion of tumour DNA in each mix confirmed that the ratios were a quantitative measure of the amount of ITD DNA in a sample (Fig. 3D). The change in the PdITD/PdWT ratios as the proportion of tumour DNA changed fitted a semi-log regression model (Fig. 2D) which enabled the point at which the PdITD/PdWT ratio regressed to zero (i.e. when the ITD would have been undetectable) to be determined for both the Type I and Type II tumour dilutions. These were 1 in 37 (2.7% tumour DNA in the sample or 27 cells in a 1000) and 1 in 43 (2.3% tumour DNA in the sample or 23 cells in a 1000) respectively.

A dilution of 1:25, or 4% tumour DNA in the sample, in a total amount of 5ng approximately equates to 40 in 1000 cells-worth of DNA. To further test the minimum threshold for detection, the minA assay was next carried out with template input of 25ng, 5ng, 1ng or 0.5ng per replicate, with either Type I or Type II tumour DNA added in a constant ratio of 1 in 25 (Additional File 13). This approximately equates to 200 in 5000, 40 in 1000, 8 in 200, and 4 in 100 cells-worth of DNA (4% of total input in all cases).

For the Type I dilutions, ITD peaks were present with 25ng (mean PdITD/PdWT $\pm$ SD 0.074 ± 0.012), 5ng (mean PdITD/PdWT $\pm$ SD 0.030 ± 0.035 ; two of four reactions only) and 1ng (mean PdITD/PdWT $\pm$ SD 0.064 ± 0.020) input but not in 0.5pg. For Type II dilutions, ITD peaks were present with 25ng (mean PdITD/PdWT $\pm$ SD 0.059 ± 0.014) and 5ng (mean PdITD/PdWT $\pm$ SD 0.033 ± 0.006) but not 1ng. A single replicate in the 0.5ng Type II reactions had a peak (PdITD/PdWT 0.123; overall mean PdITD/PdWT $\pm$ SD 0.031 ± 0.062).

The minA assay is suitable for use in fine needle aspirates

The minA assay has high sensitivity (100%) and specificity (96%) when 25ng of tumour DNA are used for reaction templates [40], however, it can also detect *KIT* exon 11 ITDs with smaller amounts of template (5ng or below), especially when tumour DNA forms a high proportion of the sample (Fig. 3). We therefore reasoned that the assay would be suitable for use on DNA isolated from diagnostic FNAs taken from MCTs, which would likely consist of small sample amounts with a high proportion of tumour cells.

To test this, we isolated DNA from FNA tissue from histologically confirmed cutaneous mast cell tumours from three dogs (Additional File 2; Methods). One had a single tumour, one had two tumours and one had three tumours sampled (Fig. 4 and Additional File 14). The samples showed varying amounts of material on each slide, with nucleated cells with a cytologic appearance consistent with mast cell tumour (Fig. 4). In all six samples, the amount of DNA recovered was too low to be quantified by the methods available to us.

The Ct values for the minA reactions (a measure of the amount of starting template in each reaction) varied from 29.71 ± 0.11 (mean $\pm$ SD, $n=4$) to 38.13 ± 1.16 ($n=3$). In five out of six samples, all replicates showed WT only peaks with both minA and minA-ALT primer sets. In one sample (Fig. 4C, FNA3 chin, asterisk), there was a small ITD peak in one minA-ALT replicate. Considering the other WT-only replicate reactions from this sample, and the WT-only results from the other two samples from this dog, this is most likely a false positive.

Therefore, although the assay can be used on FNAs taken directly from MCTs, all the samples tested here were WT.

Next, we tested the performance of the assay on an FNA from a popliteal lymph node draining a canine MCT on the patient's hock (Fig. 5). The tumour was not suitable for surgical excision and had not been directly sampled

Table 2 Overview of *KIT* exon 11 ITD analysis from archival cMCT FFPE samples

Sample	ITD status by standard PCR	ITD status by minA assay	ITD type*	Size of duplicated segment
AM1	WT	WT		
AM2	WT	WT		
AM3	ITD	ITD	Type I	42 bp+42 bp
AM4	WT	WT		
AM5	ITD	ITD	Type II	54 bp+54 bp
AM6	ITD	ITD	Type II	63 bp+63 bp
AM7	WT	WT		
AM8	WT	WT**		
AM9	ITD	ITD	Type III	45 bp+45 bp
AM10	WT	WT		
AM11	WT	WT		
AM12	WT	WT		
AM15	ITD	ITD	Type II	57 bp+57 bp
AM16	ITD	ITD	Type I	45 bp+45 bp
AM18	WT	WT		
AM19	WT***	ITD***	Type II	57 bp+57 bp

Assay results indicating presence of an ITD are shaded. WT results are unshaded. Sample AM13 excluded as minA assay failed to amplify. Sample AM14 excluded as amplification failed in all assays. The size of the duplicated segment is given as the sizes of the 5' and 3' arms of the tandem duplication. For example, for AM3, a 42 bp region was duplicated, given rise to an 84 bp duplication consisting of two identical 42 bp sequences. *See Additional File 1 for an overview of ITD type classification. **After re-analysis shown in Additional File 6. ***Note discordant results between standard semi-quantitative PCR and the minA assay for AM19. Re-analysis and sequencing confirmed that the result of the standard semi-quantitative PCR was a false negative

by other methods. The sample had been analysed cytologically by a boarded specialist to confirm a diagnosis of mast cell tumour metastatic to the popliteal lymph node (Fig. 5A). A substantial amount of DNA was recovered from the sample (concentration of 54ng/ul) and the sample was tested by minA assay with two different amounts of starting template, 270ng (Fig. 5B) and 25ng (Fig. 5C). The minA-ALT assay was also carried out with 270ng input. With both the 270ng and 25ng inputs, ITD peaks were detected in all four replicates from the minA reactions. The PdITD/PdWT ratios were similar (0.105 ± 0.15 and 0.102 ± 0.14 for 270ng and 25ng respectively; mean $\pm$ SD, $n=4$). ITD peaks were also detected in the 270ng minA-ALT reactions, although with a weaker signal (PdITD/PdWT ratio 0.064 ± 0.009 ; mean $\pm$ SD, $n=4$), consistent with a C/C genotype at SNP 619.

To characterise the ITD, minB-ALT qPCRs were carried out using a series of limiting dilutions from 25ng to 5pg to obtain a pure ITD amplicon (Fig. 5D, E). At higher input levels (25ng, 100pg) only WT minB-ALT products were amplified. However, at 5pg, an ITD amplicon was generated (Fig. 5D, E) which could be purified and sequenced. The sequence (Fig. 5F) confirmed the presence of a Type II duplication. Thus, the *KIT* mutation status of the primary tumour could be inferred utilising metastatic cells present within the draining lymph node.

Detection of the *KIT* exon 11 ITD in cfDNA from plasma

We next assessed whether the minA assay could detect *KIT* exon 11 ITDs in circulating tumour DNA within plasma from dogs with cMCT ('liquid biopsy'). We processed nine plasma samples for cfDNA isolation (plasma volumes ranging from 73ul to 333ul) from eight animals (Additional File 2) undergoing treatment for cMCT. For two dogs (FNA1 and FNA4), we had previously determined *KIT* ITD status of the tumours (Figs. 4 and 5). However, for the remaining six (LVS1–6) *KIT* ITD status was not known a priori as biopsy samples were not available. For these samples, to maximise the likelihood of detection, and to test the reproducibility of the findings, the eluates were split between two independent repeats of the assay, with triplicate samples in each independent repeat. The assays and results are summarised in Fig. 6A; examples of minA melt curves from these assays are shown in Fig. 6B. Full details of the results are provided in Additional File 15 and all melt curves are shown in Additional Files 16 and 17.

In four samples (FNA1, FNA4, LVS1, LVS4) only WT signals were detected in all replicates from both repeats. One sample (LVS5) gave an ITD peak in one replicate only which was likely a false positive.

Two samples (LVS3 and LVS6) gave equivocal results which were inconsistent between replicates and repeats, suggesting an ITD may be present but at the limits of detection of the assay. The samples were further investigated using the low template input minB/minB-ALT (as appropriate) sequencing approach. This was unable to detect an ITD in LVS3 (Additional File 18 A) but did detect an ITD in LVS6 which was confirmed by Sanger sequencing (Type I ITD; Additional File 18B-D).

However, in the case in which two samples, taken one month apart, were available from the same dog (LVS2), while only WT signals were detected in both repeats of the first sample (LVS2A), a strong ITD peak was observed in all replicates from both repeats in the second sample (LVS2B; this was taken one month after LVS2A). The PdITD/PdWT ratios were 0.843 ± 0.149 for the first repeat and 0.946 ± 0.028 for the second repeat (mean $\pm$ SD; $n=3$). This was a clear positive detection of the *KIT* exon 11 ITD in plasma and was followed up with low template input minB-ALT sequencing. This confirmed the presence of a Type I duplication with a 6 bp insert between the 5' and 3' duplicated segments (Additional File 19).

Taken as a whole, these results demonstrate that the *KIT* exon 11 ITD can be detected in liquid biopsy of dogs with a cMCT, but that there is not a straightforward relationship between liquid biopsy detection and whether or not the tumour carries an ITD.

Discussion

Cutaneous mast cell tumours are the most common cutaneous tumours of dogs [1–8] and their clinical management can offer a number of challenges (Fig. 1) in both first opinion and referral settings. While a diagnosis may be made by cytology, an initial decision between surgical or medical management, or a combination of the two, will depend on the location of the lesion and staging. If the lesion is suitable for surgery, histopathology will provide a confirmation of diagnosis as well as additional important prognostic information. Molecular analysis is also available following surgical excision. However, if the lesion is not suitable for surgery, either due to its location or because staging indicates that surgery will offer little benefit, then histopathology and molecular analysis are not possible.

In the accompanying paper [40] we describe the development of a novel qPCR-based assay for the detection of *KIT* exon 11 internal tandem duplications in cMCT, a molecular biomarker with both prognostic and predictive value in this disease. Here, we have applied the assay to a range of sample types to explore both the research and clinical applications of the test and to better understand its strengths and limitations.

Supporting our previous findings on FFPE archival samples, in a new FFPE sample cohort the minA assay identified *KIT* exon 11 status with 100% sensitivity and specificity in those samples with interpretable PCR results ($n=16$ out of 18 samples, excluding AM13 and AM14 as amplification in those samples failed in multiple attempts), when considering the overall interpretation of each assay and with an input amount of 25ng of template to each reaction. When considering the results on a replicate by replicate basis ($n=48$ reactions), then the sensitivity and specificity was also 100%. In contrast, one of the eleven samples originally scored as WT by standard of care semi-quantitative PCR was a false negative, so the sensitivity of the existing approach in our hands was 91%. The superior performance of the minA assay may reflect its shorter amplification target, which is better suited to fragmented FFPE-derived DNA.

The performance of the minA assay is dependent on two inter-related parameters – the absolute amount of template and the ratio of *KIT* exon 11 genomic sequences which are WT compared to those which have ITDs (i.e. the ratio of *KIT* mutant to *KIT* WT cells).

The absolute amount of DNA affects the *specificity* of the reaction – the false positive rate. This is because as the amount of template is reduced, the ratio of minA primers to primer binding sites increases. This increases the likelihood of high affinity (genuine) minA primer sites being saturated and therefore the possibility that excess

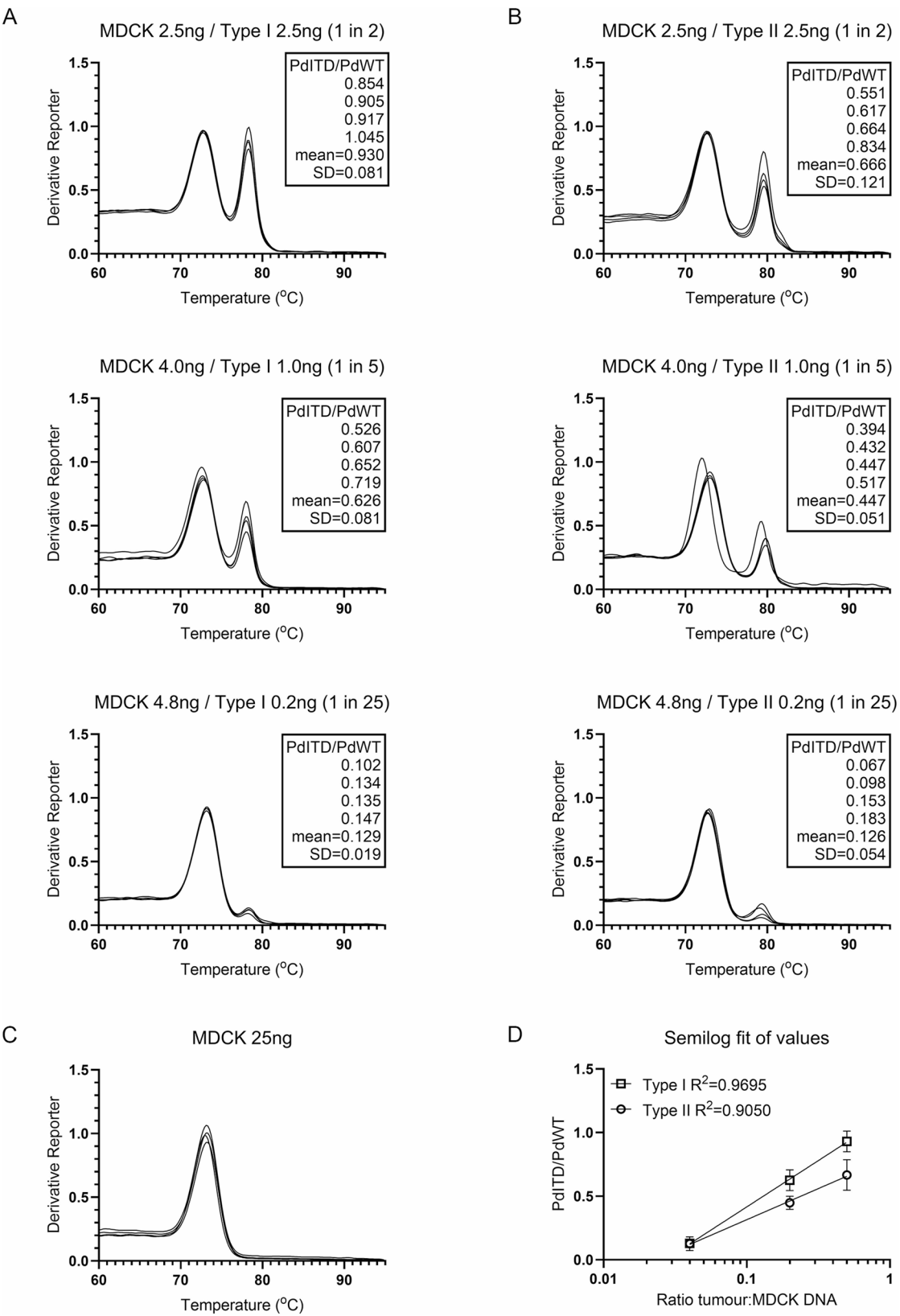


Fig. 3 Detection of *KIT* exon 11 ITD positive DNA diluted 1:25 in normal DNA. **A, B** qPCR melt curves for a series of quadruplicate minA reactions in which MDCK template was spiked with DNA from a Type I (AM16) (**A**) or Type II (AM15) (**B**) tumour. The total input in the DNA mixes was kept constant (5ng per reaction) but the amount of tumour DNA changed from 1/2 to 1/5 to 1/25 of the total. The PdITD/PdWT ratio for each replicate, mean and SD are shown on each curve. **C** Melt curves from quadruplicate pure MDCK template 25ng minA reactions for comparison. **D** Correlation of PdITD/PdWT ratios with ratio of tumour: MDCK DNA. Both the Type I and Type II dilution series correspond to a semilog fit with high accuracy (Type I R^2 0.9695; Type II R^2 0.9050) enabling the point at which the regression line crosses the X-axis (i.e. the limits of detection) to be determined. For the Type I tumours, the limit is a dilution of approximately 1/37 tumour to normal DNA, and for the Type II it is approximately 1/43

minA primers will bind to low affinity (off target) binding sites. This is likely the mechanism behind the false positive result in one minA-ALT replicate of the FNA3, chin, sample (Fig. 4C). Note that the off-target amplification is also possible even at higher levels of template input, but the likelihood will be much reduced.

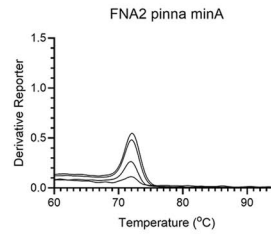
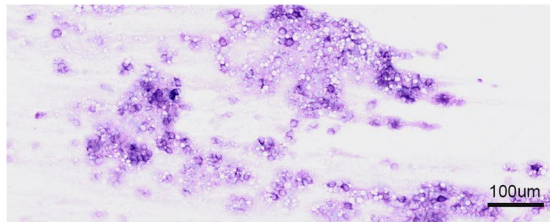
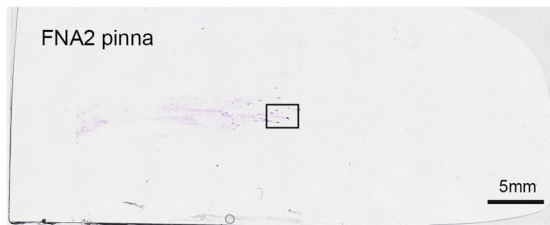
The height of the ITD melt curve peak is not a guide to whether or not that reaction is a false positive, as although large peaks are likely to be genuine detections, small peaks are not necessarily false positives. Rather, the consistency of the signal across replicates and repeats indicates whether or not a signal is likely to be a false positive. We use, if possible, 25ng of input per replicate and a minimum of three replicates for each sample, preferably four. We then determine either the mean height $\pm$ SD of the ITD peak or the mean ratio $\pm$ SD of the ITD peak to the WT peak as a guide to assessing whether or not an ITD detection is genuine. If the mean minus the SD is greater than 0, then the detection can be considered genuine. If the mean minus the SD is less than 0, then the detection is likely a false positive. However, with low total amounts of input DNA containing a low percentage of ITD targets, results should be interpreted cautiously.

The ratio of *KIT* exon 11 genomic sequences which are WT compared to those which have ITDs determines the *sensitivity* of the minA reaction – the false negative rate. The fewer the ITD sequences (i.e. DNA derived from *KIT* mutant cells) in a sample, the less likely that the minA primers will bind to them, resulting in preferential amplification of WT sequences. We calculate that the limit of reliable detection as 4% tumour DNA in normal DNA, with 2–3% tumour-in-normal as potentially detectable. Again, however, the interpretation of results from samples with low total input of DNA template may be confounded by trying to distinguish false positive signals from genuine positive signals detecting very rare ITD targets at such low abundance they are not equally partitioned across all replicates (i.e. present in some reactions but not in others). Low template input (< 5 ng) and low ITD abundance (< 1 in 25 WT sequences) increase the risk of false negatives. Although this situation is unlikely to arise in tissue biopsy samples, it may occur in FNAs or in cf/ctDNA from plasma (Additional File 20).

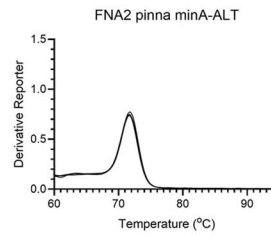
The relative amounts of WT to ITD DNA sequences (i.e. normal to mutant cells) in a sample can be determined from the relative heights of the WT and ITD peaks in a replicate (the PdITD/PdWT ratio). In sample FNA4, similar PdITD/PdWT ratios at 25 ng and 270 ng input confirm that the ratio reflects the relative, rather than absolute, abundance of ITD sequences. In *KIT* exon 11 mutant tumours carrying an ITD, there is a positive association between the ITD peak/WT peak ratio and the relative area of tumour compared to the total area of normal tissue in a section. We have demonstrated this independently in the two data sets analysed [40]; combining the results from the two sets makes the association stronger. However, the area of tumour tissue is not an exact surrogate for the number of neoplastic mast cells carrying an ITD in the sample. Every sample taken from a mast cell tumour contains neoplastic tumour cells as well as stroma (e.g. fibroblasts) and inflammatory cells (e.g. eosinophils). These elements are observed to vary histologically with densely and paucicellular tumours, accompanied by varying severities of inflammation secondary to degranulation and varying release of chemokines / cytokines. For this reason, the calculations of the limits of detection of the minA assay as 2–4% ITD-carrying cells in a population of WT cells is likely to be an underestimate of the sensitivity of the assay, as the tumour material will not itself be a pure ITD-carrying population.

Nevertheless, the strong correlation between tumour area and ITD/WT peak ratios for most ITD-positive tumours suggests the proportion of WT (non-transformed) to ITD (neoplastic *KIT* mutant) cells within each tumour is relatively consistent. However, there are some samples in which either the ITD/WT ratio is higher

A

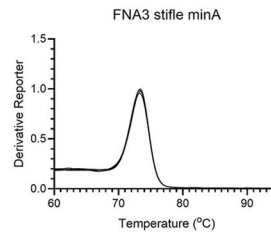
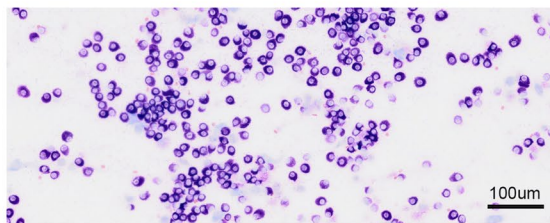
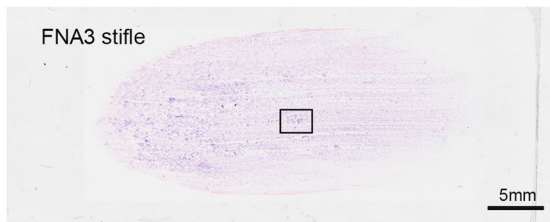


FNA2 pinna minA
Mean Ct=38.13
SD=1.16
n=3*

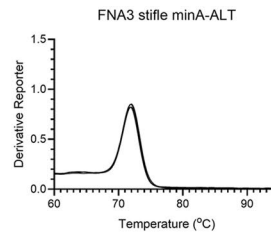


FNA2 pinna minA-ALT
Mean Ct=32.47
SD=0.36
n=4

B

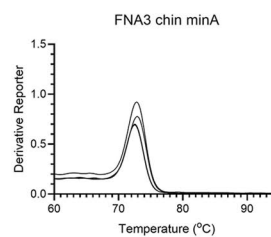
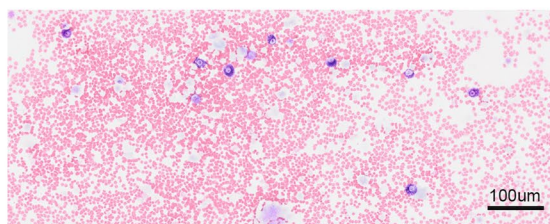


FNA3 stifle minA
Mean Ct=29.71
SD=0.11
n=4

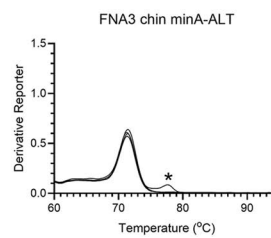


FNA3 stifle minA-ALT
Mean Ct=30.44
SD=0.16
n=4

C



FNA3 chin minA
Mean Ct=35.36
SD=0.79
n=4



FNA3 chin minA-ALT
Mean Ct=35.24
SD=0.25
n=4

Fig. 4 DNA isolated from stained Fine Needle Aspirates is suitable for the minA assay. Results from three FNA samples taken from MCTs after surgical excision, each showing a low power whole slide image (upper; bar = 5 mm) and a high-power image (lower, corresponds to box in upper panel; bar = 100 μ m) of the stained sample. The corresponding melt curves for minA/minA-ALT assays carried out on DNA isolated from the slide are shown adjacent. Eluted DNA was made up to 135 μ l and then 5 μ l used as input into each reaction. Mean Ct $\pm$ SD values are provided on each curve. **A** Case FNA2, MCT on the pinna (unspecified). **B** Case FNA3, MCT on the right stifle. **C** Case FNA3, MCT on the chin. Note the single peak with a Tm consistent with an ITD is likely to be a false positive signal as it is seen in only one of four replicates, and none of the other two MCT samples from this patient (see also Additional File 14) show any evidence of an ITD

than expected for the amount of tumour material (FP6 and FP8) and some in which the ITD/WT ratio is lower than expected (AM3, AM9, FP12). In the former case, this may be because these tumours are less inflamed or are relatively paucicellular, thus the tumours contain more ITD-carrying transformed cells than other tumours of comparative size. In the latter case, when the ITD/WT ratio is lower than expected, the tumour contains fewer ITD-carrying transformed cells than other tumours of comparative size. An intriguing possibility is that some tumours may contain a mixture of *KIT* exon 11 WT and ITD-positive clones, reflecting the acquisition of ITDs during tumour evolution. This heterogeneity could influence response to TKI therapy and contribute to therapeutic resistance. The ITD/WT ratio may therefore provide additional information to support clinical decision-making.

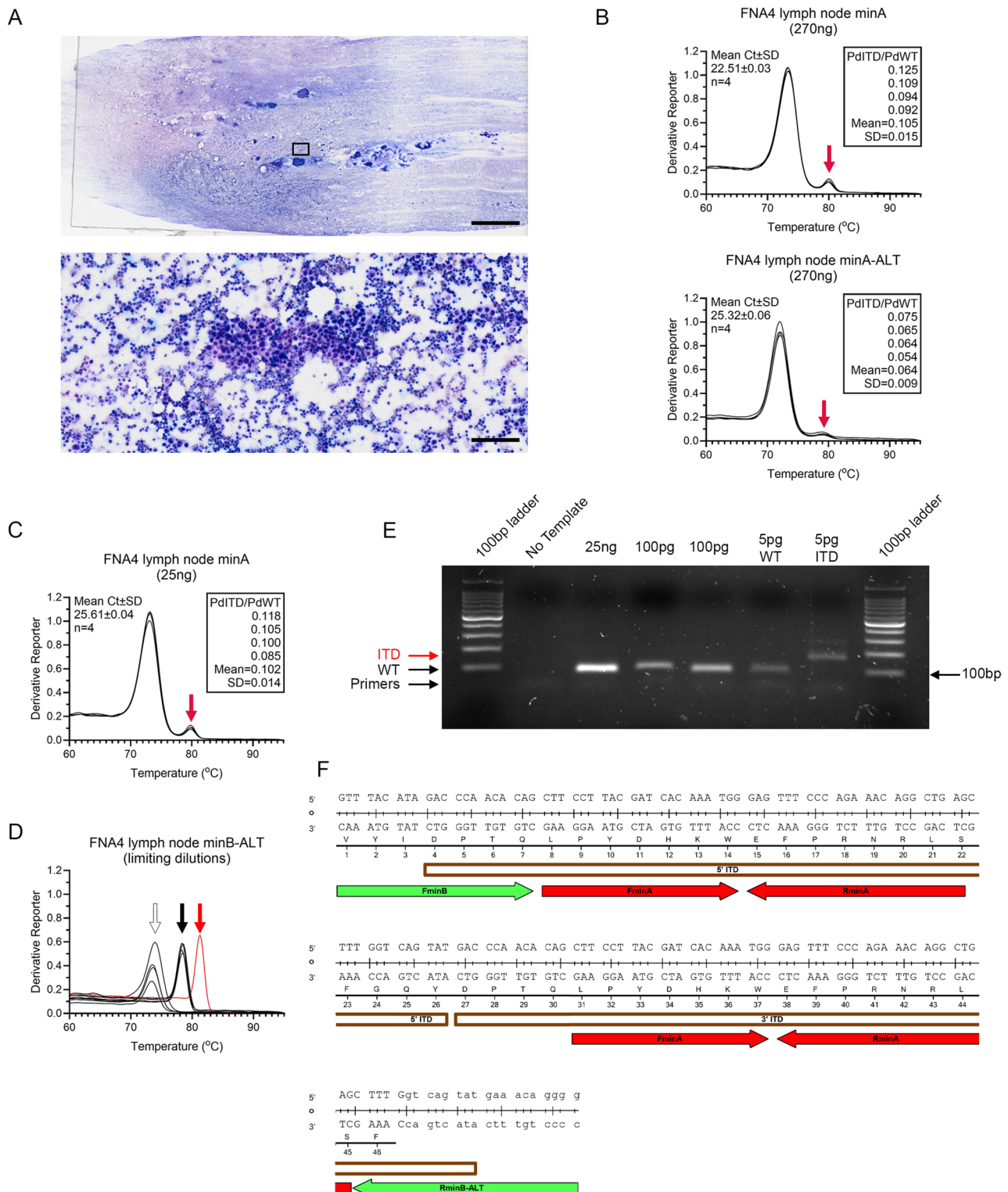
The high sensitivity and specificity of the minA assay in FFPE samples when the amount of template which can be used in a reaction is not a limiting factor, together with its rapidity, ease of interpretation and quantitative nature represent an advance over the current gold standard approach of semi-quantitative PCR followed by electrophoresis-based analysis of amplicon size. However, FFPE samples still require an excisional biopsy, which is not always available.

In contrast, cytology of a suspected cMCT will be available in most cases, irrespective of suitability for surgery. Even if cytology of the primary mass is not available, samples may be taken from sentinel lymph nodes or other tissues as part of staging. Therefore, our demonstration that the minA assay works in such material will allow molecular analysis of *KIT* exon 11 status to be extended to a wider range of dogs to support clinical decision making. Notably, we attempted to use the DNA isolated from the FNA samples for a standard semi-quantitative PCR approach using the whole exon 11 primers but we were unable to reliably generate amplicons, suggesting that current clinical standard assays are not suitable for use with FNAs.

As with all FNA-based testing, however, tumour heterogeneity may produce an incomplete representation of the lesion. Although cytological review improves sample quality assessment, if the tumour contains a mixture of transformed mast cell clones which includes some clones WT for *KIT* exon 11 and some clones with ITDs, sampling bias may still give an incomplete picture of the *KIT* status. Notably, the dog from which the FNA4 lymph node sample was taken was not suitable for surgery and was instead treated medically with masitinib. The dog was in full remission at the time of writing (GE, pers. comm.). Although only a single case, it is encouraging that the clinical picture is consistent with the assay findings.

We have also tested the applicability of the minA assay in a cf/ctDNA liquid biopsy setting. Liquid biopsy offers the potential for remote sampling of the cellular and genetic features of tumours, and even their transcriptional profiles, by the isolation of circulating tumour cells (CTCs), circulating tumour DNA fragments (ctDNA), cell-free RNAs, tumour-educated platelets or exosomes from plasma [50]. Circulating cell-free DNA is normally found in the plasma of healthy individuals and originates primarily from haematopoietic cells, but also other tissues [51] and in individuals with cancer there is a substantial release of tumour-derived DNA into the circulation (likely due to apoptosis, necrosis or active secretion in exosomes), resulting in a rise in the amount of cf/ctDNA in plasma which can amount to an order of magnitude [51, 53–55].

Different approaches have been taken to use of cf/ctDNA as a tumour biomarker in the human setting. Quantitation of overall cf/ctDNA levels have concluded that higher levels of cf/ctDNA either at presentation or following therapy is a poor prognostic marker [56–60]. To examine the mutational landscape of tumours to predict and monitor response to therapy, two strategies are generally used: tumour-informed and tumour-agnostic analyses [61, 62]. The tumour-informed approach depends on sequencing of a biopsy of the primary tumour to identify



mutations which can be subsequently detected in liquid biopsy using targeted sequencing to monitor response to disease. In contrast, the tumour-agnostic approach assumes the primary tumour has not been sequenced, and either uses a whole genome NGS sequencing approach to identify mutations solely from the sampled cf/ctDNA [63] or relies on a panel of previously validated mutations of known predictive value to inform therapeutic choice [64]. Tumour-informed assays are more sensitive and can predict therapy efficacy better than tumour-agnostic

Fig. 5 *KIT* exon 11 ITD detection in a lymph node Fine Needle Aspirate. **A** Whole slide image (upper panel; bar = 5 mm) and high-power view showing mass of adherent mast cells (lower panel, corresponds to box in upper panel; bar = 100 μ m) of a popliteal lymph node FNA from case FNA4 (MCT on right hock). **B, C** Melt curves for minA/minA-ALT assays carried out on FNA4 DNA. 270ng (**B**) or 25ng (**C**) template used in each reaction (minA-ALT assay only carried out on 270ng input). Mean Ct $\pm$ SD values, PdITD/PdWT ratios for each replicate with mean and SD are provided. Red arrows indicate the ITD peaks detected in all four replicates in all assays. Note the close correspondence between the mean PdITD/PdWT ratios in both the 270ng and the 25ng template input reactions. **D** Melt curves from nine low-input (5pg) FNA4 qPCR minB-ALT reactions for ITD sequencing. Results of the nine curves are superimposed for comparison. Four of the wells shown gave only a peak consistent with primer dimers (open arrow). Four of the wells gave only a single peak consistent with the WT product (black arrow). However, one well gave a single peak with a T_m consistent with the ITD (red curve, red arrow). **E** Gel electrophoresis analysis of FNA4 minB-ALT qPCR reaction products with differing amounts of template input (No Template, 25ng, 100pg, 5pg). The approx. 100 bp band is consistent with the minB-ALT WT band. However, in the final lane, one 5pg input reaction has generated a larger band, consistent with a pure ITD product. This reaction was the same one which gave the ITD melt curve trace in red in panel (**D**). **F** Sanger sequencing results of the pure ITD product shown in (**D**) and (**E**). The ITD was determined to be Type II (ITD size 69 bp + 69 bp). Uncropped gel image shown in Additional File 22

assays [57, 61] but the latter have a shorter turn-around time and are better suited to monitor tumour heterogeneity [57, 65]. However, they also have the shortcoming of requiring approaches which enable ‘normal’ cfDNA to be distinguished from ctDNA. Approaches to this include analysis of cfDNA fragment size, shown to be shorter in tumour-derived DNA fragments [51, 66–68] and analysis of patterns of cf/ctDNA methylation [69]. As a hybrid model which exploits the benefits of both tumour-informed and tumour-agnostic approaches, the use of panels of previously validated mutations to interrogate cf/ctDNA for predictive biomarkers in situations when samples cannot be taken from the tumour mass, is now recommended in a variety of indications as detailed in recent ESMO guidelines [64].

In a veterinary context, whole genome NGS sequencing of cfDNA is currently the most widely reported use of liquid biopsy. This may be tumour-agnostic and used as a cancer screening tool [70] or, if matched sequencing from primary tumours is available for a tumour-informed approach, it is used to train or validate cfDNA analysis approaches for the development of screening tools [71, 72]. Analysis of overall mutational landscapes of veterinary tumours by tumour-informed liquid biopsy is currently limited to a research approach [73, 74].

Similar to humans, dogs with more advanced, malignant tumours have higher levels of tumour-derived cfDNA in the plasma [68, 70, 71, 74–76], the ctDNA is shorter or more fragmented than normal cell-derived cfDNA [68, 72, 75, 77, 78] and cf/ctDNA levels fall following surgery [71]. Specific mutations found in primary tumours can be detected in cf/ctDNA [74] although detectability of copy number variations (CNVs) in plasma depends on depth of NGS seq coverage and, importantly, the fraction of tumour ctDNA in the plasma [73]. This demonstrates that cf/ctDNA sequencing is a feasible approach to monitoring changes in intra- and inter-tumour heterogeneity in response to therapy. However, a study in which concordance between sequencing of tumour biopsies and cf/ctDNA from both breast cancer patients and dogs with mammary tumours was examined, demonstrated that more variants were found in the tumour samples than the cf/ctDNA from the human patients, but the reverse was true for the canine patients [73]. It is unclear whether this represents unrepresentative tumour biopsies in the canine patients, low sensitivity of cf/ctDNA sequencing in the human patients or other systematic errors.

A tumour-agnostic cf/ctDNA NGS sequencing approach is the basis for the CANDiD cancer screening tool for dogs. The original multicenter study of > 1300 cases identified cancer-associated variants in cfDNA using a training cohort to develop a screening tool which reported as either ‘cancer detected’ or ‘cancer not detected’ [63]. The study reported an overall sensitivity of 54.7% and overall specificity of 98.5% with better sensitivity (85.4%) across three particularly aggressive tumour types (lymphoma, hemangiosarcoma, osteosarcoma). The assay is currently used both for cancer screening in healthy dogs as well as to aid diagnosis [79, 80] and can also detect recurrence of a tumour prior to clinical signs during long-term post-surgical follow-up [81], although it cannot distinguish between a recurrence and the development of a new tumour in this setting, a limitation of all tumour agnostic NGS approaches. Additional limitations of all NGS approaches include cost, the volume of

A

Sample	Plasma volume (ul)	Eluate volume (ul)	First repeat			Second repeat			Sequencing	Interpretation
			Eluate per reaction (ul)	% of circulating plasma sampled per reaction*	Result	Eluate per reaction (ul)	% of circulating plasma sampled per reaction*	Result		
FNA1	170	45	5	0.00131%	Wild Type	N/A	N/A	N/A	N/A	Wild Type
FNA4	90	45	2.5	0.00032%	Wild Type	N/A	N/A	N/A	N/A	Wild Type
LVS1	135	30	5	0.00145%	Wild Type	3	0.00087%	Wild Type	N/A	Wild Type
LVS2A	280	30	5	0.01277%	Wild Type	3	0.00766%	Wild Type	N/A	Wild Type
LVS2B	200	30	5	0.00912%	Strong positive	3	0.00547%	Strong positive	ITD	Strongly positive for ITD
LVS3	73	30	5	0.00078%	Weak positive	3	0.00047%	Equivocal	WT	Equivocal
LVS4	142	30	5	0.00277%	Wild Type	3	0.00166%	Wild Type	N/A	Wild Type
LVS5	200	30	5	0.00324%	Equivocal	3	0.00195%	Wild Type	N/A	False positive
LVS6	333	30	5	0.00357%	Equivocal	3	0.00214%	Weak positive	ITD	Weakly positive for ITD

B

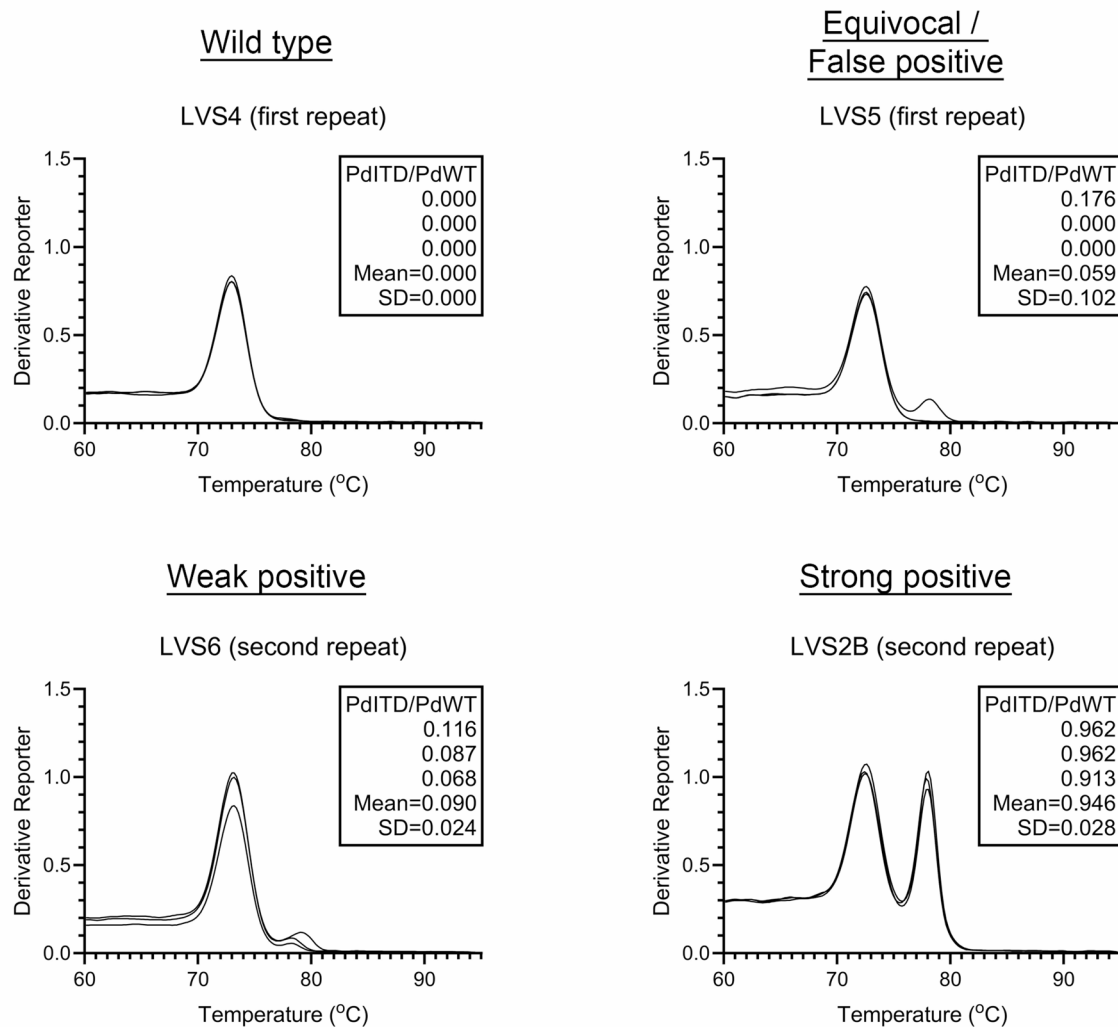


Fig. 6 Detection of *KIT* exon 11 ITD in cell-free DNA from plasma. **A** Summary of results of minA assays on cfDNA isolated from plasma samples. The percentage of the total circulating plasma volume that each sample represented could be calculated knowing the average weight for male and female dogs of each breed [52] and assuming a circulating whole blood volume of 90mls/kg and assuming a hematocrit of 50% (see Additional File 15 for the calculations). The amount of eluate recovered after cfDNA extraction and the amount of this that went into each reaction could then be used to determine what percentage of the total circulating plasma volume was assessed in each assay. **B** Examples of melt curves from assays producing only Wild Type curves (LVS4 first repeat), equivocal (potentially false positive) results (LVS5 first repeat), weak positive ITD detection (LVS6 second repeat) and strong positive ITD detection (LVS2B second repeat). Melt curves for all samples are shown in Additional Files 16 and 17

blood required and turnaround time [70]. Furthermore, some cancers (e.g. CNS or urinary tract) are less likely to release ctDNA into plasma and alternative approaches may be needed to sample them [70].

To our knowledge, use of the minA assay for *KIT* exon 11 ITDs in cf/ctDNA of dogs with cMCT is the first report in a veterinary context of a tumour-agnostic approach to cf/ctDNA analysis using a validated mutation for which there is a specific licenced therapy (masitinib). We tested cf/ctDNA from eight individuals (nine samples) with a confirmed cMCT diagnosis. In one sample (LVS2B), there was a clear strong ITD signal in the minA assay. It was notable that this sample was taken one month after a sample (LVS2A) which was negative for the ITD, and that this time period corresponded with a period of treatment (first half of 2023) in which the dog had been switched from masitinib to toceranib due to a shortage of supply of masitinib in the UK (Additional File 21). The clinical picture combined with the results of the minA assay are consistent with tumour heterogeneity. Either the dog had multiple independent MCTs, some of which were *KIT* exon 11 WT and some with the *KIT* exon 11 ITD, or a single origin tumour containing both WT and ITD clones. The initial masitinib therapy was controlling the ITD population, and partially controlling the WT population, but the switch to toceranib allowed the ITD population to escape.

Two samples, LVS3 and LVS6, had low level minA signals on the borderline of detection. LVS6 was confirmed as carrying a duplication by sequencing but LVS3 remains an equivocal result. A fourth sample, LVS5 appears to be a false positive, but it cannot be definitively excluded that this is a genuine detection of a very rare ITD fragment in a low overall input assay (as in the situation in Additional File 20 A). Indeed, based on Ct values, LVS5 had the lowest amount of sample input of any of the cf/ctDNA plasma samples (Additional File 15).

The most surprising result of the analysis of cf/ctDNA isolated from plasma was the lack of detection of the ITD in the sample from case FNA4, having demonstrated by analysis of the lymph node FNA from this case that the dog did have an MCT which was positive for the ITD. It is clear, therefore, that there is not a straightforward correlation between the presence of an ITD in the primary tumour mass and the ability to detect it in liquid biopsy.

In liquid biopsy, the overall amount of template going into each reaction is determined by the concentration of circulating DNA fragments in the plasma from an individual dog, the amount of plasma sampled and the percentage of the overall circulating plasma volume that sample represented. Considering just the second sample from LVS2 (a female Pug, predicted circulating blood volume 730.8 mls) and the sample from FNA4 (a male Labrador Retriever, predicted circulating blood volume 3111.3 mls), 0.00912% and 0.00547% of the predicted total circulating plasma volume was sampled in each reaction from the first and second repeats (respectively) of the LVS2 sample, whereas just 0.00032% of the predicted circulating plasma volume of FNA4 was sampled in each reaction, more than 20 times less. If the concentration per ul of cell-free DNA fragments in plasma was similar for both dogs, then there must have been 20 times less template per reaction in the FNA4 samples compared to the LVS2B samples. At least one recommendation for standardising comparison of liquid biopsy results between animals, and across different time points, is to take a standard amount of blood for analysis based on body weight e.g. 500ul blood for every 10 kilos weight (based on the sample taken from LVS2).

We do not have stage/grading information on the samples used for liquid biopsy, and while the presence of a *KIT* ITD is associated with higher grade tumours, it is not necessarily the case. Therefore, differing tumour grade may be a confounding factor when comparing between animals. Finally, tumour-derived ctDNA may be diluted out to a greater or lesser extent by non-tumour cfDNA from a variety of sources, depending on when the animal

last ate, whether they had a recent trauma, were pregnant or had a co-existing infectious/inflammatory condition, as all these factors are known to increase the levels of cfDNA in the plasma [82–86] and could therefore dilute out a tumour-derived signal. As we have shown, if fewer than 1 in 25 DNA fragments carry the ITD, they are unlikely to be detectable and the lower the overall amount of DNA tested in each assay, the more variable the results will be. Indeed, given that the FNA4 primary tumour was clinically a highly inflamed lesion (GE, personal communication), an inflammatory contribution to plasma cfDNA diluting out tumour-derived cfDNA is another potential confounding factor, in addition to the relatively small proportion of total plasma tested, which may have contributed to a negative test result in this sample. Although we have demonstrated the feasibility of the approach here, prospective longitudinal studies are required with standardised sample collection protocols to better understand the limits of ITD detection in plasma as well as how the detection of the ITD (or otherwise) reflects clinical behaviour.

Our study has limitations. We have no information on sample autolysis time prior to fixation, fixation procedure or fixation time. As the samples were collected as part of routine clinical management of patients from multiple referring practices, it is unlikely they were standardised. Poor DNA quality as a result of variable fixation conditions is likely to be a major contributor to the difficulties experienced with samples AM13, 14 and 17. Retrospective FFPE studies must take such DNA variations into account.

The polymerase enzymes we have used for PCR do not have a proof-reading function. Therefore, it is possible that errors could have occurred during sequencing reactions. However, this is mitigated by the short length of most of the amplicons generated for sequencing, and indeed with one exception the only variations seen in exon 11 sequence were at the two known polymorphisms, and the variants seen were known alleles at those polymorphisms. We can therefore be confident about the sequencing results. The one exception is the sequencing result of the LVS6 ITD amplified by low template input minB qPCR (Additional File 18D). This contains a T>C substitution in the 3' arm of the ITD resulting in a Lysine> Proline missense change. It is unclear if this is genuine or a PCR error.

The low template input minB PCR approach to isolating a pure ITD for sequencing also has the limitation that as the chr13:47940672 T/C polymorphism is located in the reverse minB priming site, then the results of the sequencing of this polymorphism depend on the primers used (RminB or Rmin-ALT), not on the actual sequence of the template. Therefore, the allele present at this polymorphism cannot be determined in amplicons from minB sequencing.

Conclusion

The minA assay is a rapid, quantitative approach to determining *KIT* exon 11 status in canine cutaneous MCT. It offers high sensitivity and specificity with samples including diagnostic FFPE material and fine needle aspirates prepared for cytology. The main determinants of the reproducibility of the assay are the amount of total sample input and the concentration of exon 11 ITD DNA sequences within that sample (which can be as low as 1 in 25). The ability of the assay to detect ITD sequences when diluted out in a background of WT sequences makes the assay suitable for use with sentinel lymph node sampling. The assay can also detect *KIT* exon 11 ITDs in cf/ctDNA isolated from plasma but better standardisation of sample collection and longitudinal studies are required to understand the clinical significance of detecting (or not detecting) an ITD in liquid biopsy from a cMCT patient. Overall, this assay represents a significant advance in the management of dogs with cMCT as well as establishing a new tool to address research questions around tumour heterogeneity and response to therapy.

Abbreviations

cfDNA	Cell-free DNA
ctDNA	Circulating tumour DNA

cf/ctDNA	Cell-free/circulating tumour DNA
CNVs	Copy number variations
FFPE	Formalin fixed paraffin embedded
FNA	Fine needle aspirate
gDNA	Genomic DNA
H&E	Haematoxylin and eosin
ITD	Internal tandem duplication
LOH	Loss of Heterozygosity
MCT	Mast cell tumour
MDCK	Madin-Darby canine kidney cells
qPCR	Quantitative real-time PCR
TBE	Tris base (90mM) Boric acid (90mM) EDTA (2mM)
WT	Wild type

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1186/s12917-026-05950-6>.

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Authors' contributions Authors' contributions (CRediT Taxonomy) Conceptualization – MJS, GT; Data curation – MJS; Formal analysis – MJS, LSH, JOE, GT; Funding acquisition – MJS; Investigation – MJS, LSH, JOE, GT; Methodology – MJS, JOE, GT; Project administration – MJS; Resources – SB, MD, GE; Supervision – MJS; Visualization – MJS; Writing – original draft – MJS. Writing – review & editing – MJS, JOE, LSH, GT, SB, GE, MD.

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Data availability The datasets generated and analysed during the current study are available in the Genbank repository, with accession numbers PZ349546 (AM1), PZ349547 (AM2), PZ349548 (AM3 WT), PZ349549 (AM4), PZ349550 (AM5 WT), PZ349551 (AM6 WT), PZ349552 (AM7), PZ349553 (AM8), PZ349554 (AM9 WT), PZ349555 (AM10), PZ349556 (AM11), PZ349557 (AM12), PZ349558 (AM13), PZ349559 (AM15 WT), PZ349560 (AM16 WT), PZ349561 (AM18), PZ349562 (AM19 WT), PZ349563 (AM6 ITD), PZ349564 (AM9 ITD), PZ349565 (AM15 ITD), PZ349566 (AM16 ITD), PZ349567 (AM3 ITD), PZ349568 (AM5 ITD), PZ349569 (AM19 ITD), PZ349570 (LVS2B ITD), PZ349571 (LVS6 ITD), and PZ349572 (FNA4 ITD). Sequences include details of alleles at UU_Cfam_GSD_1.0/ canFam4 chr13:47940619 and chr13:47940672.

Declarations

Ethics approval and consent to participate This study was approved by the Bristol University/Bristol Veterinary School Animal Welfare and Ethics Review Body (VIN number 22–041). Samples from AML Laboratory (Antwerp, Belgium; arranged through SB) were confirmed by the provider that the material is archival veterinary diagnostic material and that there were no ethical limitations to the use of the material in scientific research or the subsequent publication of this research. Non-archival samples from Bristol University/Langford Vets were obtained with fully informed consent of owners and reflected surplus tissue after completion of clinical tests.

Consent for publication Not applicable.

Competing interests This study is subject to a patent filing in the UK (application reference 2602035.4) and the min assay has been licenced for commercial use to CVS/Axiom. CVS group supplied samples for use in the study but had no control over the direction of the study, the analysis or the decision to publish. Sam Beck is Director of Independent Anatomic Pathology Ltd. Melanie Dobromylskyj is a full-time employee of The Veterinary Pathology Group. Langford Vets is a business separate to Bristol Veterinary School.

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Authors and Affiliations

Liam S. Hill¹ · Jacob O. Evans¹ · Giusy Tornillo¹ · Melanie Dobromylskyj² · Sam Beck³ · Grace L. Edmunds⁴ · Matthew J. Smalley¹

✉ Matthew J. Smalley
SmalleyMJ@cardiff.ac.uk

¹ The European Cancer Stem Cell Research Institute, School of Biosciences, Cardiff University, Maindy Road, Cardiff CF24 4HQ, UK

² The Veterinary Pathology Group, Unit 1b, Babbage Way, Science Park, Clyst Honiton, Exeter EX5 2FN, UK

³ Independent Anatomic Pathology, Ltd, c/o Moore Scarrott, Calyx House, South Road, Taunton TA1 3DU, UK

⁴ Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA