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# Establishment and characterisation of a novel canine mast cell tumour cell line (C18)

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## Abstract

**Background** Mast cell tumour (MCT) is a life-threatening neoplasm commonly found in dogs worldwide. The outcome of treatment for dogs with cutaneous MCT is currently poor, mainly because of the tumour's aggressiveness and the heterogeneity in tumour behaviour. This study established a novel canine MCT cell line and compared with three reference canine MCT cell lines (CMMC, VIMC and CoMS) in terms of their characteristics and tumour sensitivity to immune cell-mediated cytotoxicity.

**Results** Of 18 MCT samples, only one cell line derived from high grade cutaneous MCT was established and referred to as C18 cell line. The C18 cell line could be maintained for over 100 passages while they still exhibited *c-kit*, *tryptase*, *FcεRIα* and *FcεRIβ* expression. The C18 had the longest doubling time and smallest tumour spheroid size when compared to the other three reference cell lines. The C18 also had *c-kit* internal tandem duplication (ITD) in exon 11 and nine single nucleotide polymorphisms (SNPs) in five genes, namely *c-kit*, *HYAL4*, *SEL1L*, *SPAM1* and *TRAF3*. For a comparison of tumour sensitivity to immune cell-mediated cytotoxicity, the percentages of early and total apoptotic cells were significantly increased in all four cell lines. However, the percentages of viable cells were significantly decreased only in C18.

**Conclusion** In conclusion, a novel canine cutaneous MCT cell line was successfully established, in terms of its characteristics, growth behavior and interaction with PBMCs. The C18 cell line holds a potential promise for advancing studies and developing new therapeutic strategies.

**Keywords** Canine, Cell line, Gene mutation, Mast cell tumour, Tumour cytotoxicity

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## Background

Mast cell tumour (MCT) commonly occurs in dogs, accounting for 16–21% of all cutaneous tumours [1–3]. Although the aetiology of MCT is caused by a number of risk factors including dog breed predisposition, gene mutation and environmental factors, the exact mechanism is still largely unknown. The prognosis of canine MCT exhibits significant variability and is contingent upon factors such as tumour location, grade, and disease stage. High grade MCT, particularly those situated in mucosal sites within the oral cavity and gastrointestinal tract, are frequently associated with a less favorable prognosis due to their aggressive nature and heightened propensity for metastasis [3, 4]. Standard therapeutic approaches for canine MCT typically encompass surgical excision, with additional modalities such as radiotherapy or chemotherapy being considered for advanced cases or instances where surgical intervention alone proves inadequate. Recent scientific investigations are placing emphasis on innovative therapeutic strategies, including immunotherapy and targeted chemotherapy. The development of treatment plans is tailored to the individual characteristics of the MCT, considering factors such as tumour grade, stage, and the overall health status of the affected canine. Due to the canine MCT complexity and the high risk of cancer-related deaths, it is challenging to develop an effective model for studying pathogenesis, tumour behaviour and personalised treatment strategies. Several canine MCT cell lines have been established, characterised and tested for mast cell functions [5–10]. However, our knowledge regarding each cell line remains limited, and the interactions between MCT and immune cells are yet to be fully understood. Aside from the more severe systemic symptoms and worse prognosis associated with mucosal MCT [3, 4], the distinction between cutaneous and mucosal tumour origins remains an area that needs more in-depth research. Cell line establishment and in vitro cell-to-cell interaction models will therefore help to understand the cellular mechanisms and to accelerate the discovery of a novel therapy for canine MCT.

Aberrant gene sequence and expression involve pathogenesis, MCT risk and the individual's treatment response. The incidence of the mutation of transmembrane tyrosine kinase receptor (KIT)-encoded gene (*c-kit*) is 8–45% of canine MCT. This mutation is also associated with the pathogenesis and prognosis of the disease [11–16]. The most common type of *c-kit* mutation in canine cutaneous MCT is internal tandem duplication (ITDs) located in exons 8 and 11, which is a negative prognostic factor in canine cutaneous MCT [15–23]. Recently, SNP genotyping has been used for studying genetic variation at a single base position among individuals. Understanding the effects of SNPs on tumorigenesis is critical for tumour diagnosis and treatment. Some SNPs have been reported in canine MCT, such as suppressor of lin-12-like protein 1 (*SEL1L*), sperm adhesion molecule 1 (*SPAM1*) and hyaluronoglucosaminidase 4 (*HYAL4*) [24].

Tumour heterogeneity and the complexity of cellular interplays between the tumour and immune system lead to a large variety of treatment responses among patients. To overcome the limitations, the use of in vitro co-culture models between the tumour and immune cells is a preferred method in several human cancer studies for the personalised investigation of tumour cytotoxicity and anti-tumour immunotherapies [25–31].

The purposes of the present study were to establish a novel in-house canine MCT cell line and to determine its characteristics including cell morphology, growth property, gene and cell marker expression, *c-kit* mutation and SNPs of cancer-related genes. Tumour sensitivity to immune cell-mediated cytotoxicity by co-culturing of the established canine MCT cell line with peripheral blood mononuclear cells (PBMCs) was tested and compared with those of other well-described reference canine MCT cell lines.

## Results

### Establishment of the novel in-house canine MCT cell line

Eighteen canine MCT cases originating from primary tumours were included in this study. Additional File 1 provides the summary description of patient signalment, MCT grading diagnosis, culture conditions, passage number and culture duration. The successful establishment of the cell line originated from a 13-year-old intact female Shih Tzu presenting with a high grade MCT in the right fourth mammary gland. Clinical manifestations included signs of depression, dehydration, and abdominal discomfort. Hematological and blood chemistry analyses revealed anemia, leukocytosis, hypocalcemia, hyperphosphatemia, elevated alkaline phosphatase (ALP), high blood urea nitrogen (BUN), and hypoproteinemia. Unfortunately, the canine subject succumbed to poor physical conditions and died six days post-surgery. The histological examination of this mass elucidated that the infiltrative subcutaneous MCT mass was characterized as a poorly circumscribed, unencapsulated, and densely cellular neoplasm. The round MCT cells were arranged in sheets and rows supported by low numbers of collagenous stroma. These cells were round with distinct cell borders and contained abundant fine basophilic intracytoplasmic granules. Nuclei were round to oval, with finely to coarsely stippled chromatin and with a prominent nucleolus. Anisocytosis and anisokaryosis were moderate (Fig. 1A). The tumour cells were positively stained with toluidine blue (Fig. 1B). Based on the histopathological characteristics of this mass, a high grade mast cell tumour was diagnosed [32].

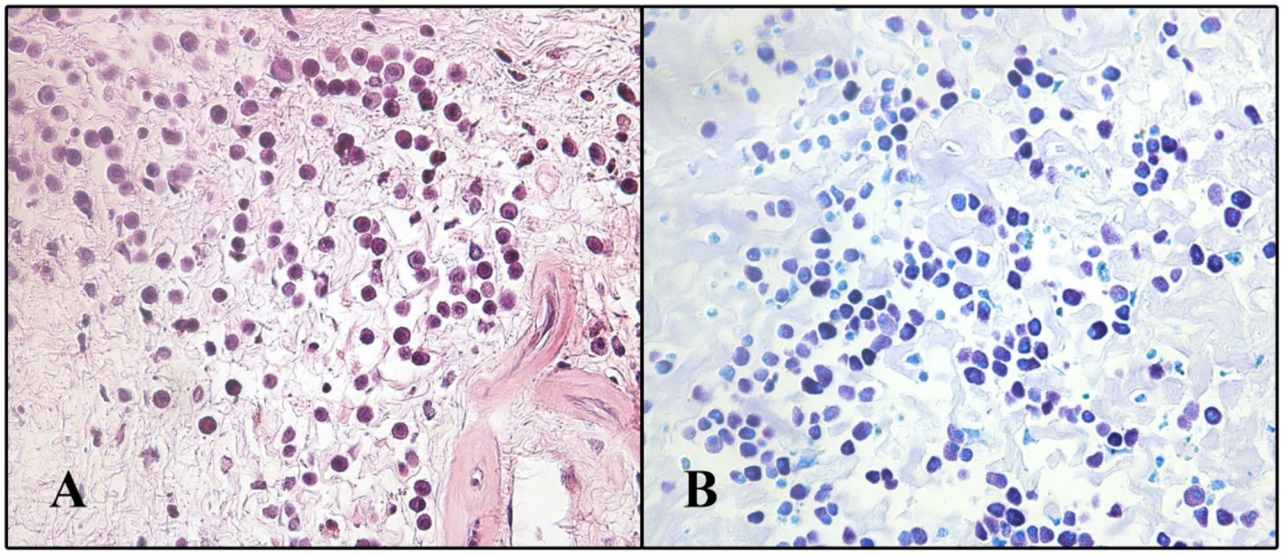


**Fig. 1** Microscopic appearances of canine mast cell tumour. Light microscopic appearances of haematoxylin & eosin staining (**A**) and toluidine blue staining (**B**)

None of the FNA-derived specimens could be cultured for more than two passages. Tissue explant significantly improved the success rate of culture since 6 out of 10 specimens could be cultured with more than three passages. Medium 3 was the only condition that could culture more than three passages in five out of eight specimens (62.5%), resulting in a mast cell tumour cell line (success rate = 12.5%). We collected 18 samples of canine cutaneous MCT to establish the cell line, successfully establishing only one cell line from a high grade MCT dog using the tissue explant method and culture with medium 3. During the first 4 months of culture, small clusters of MCT cells were loosely attached to tumour-associated fibroblasts (Fig. 2A, B). After 4 months of continuous culture, the cells could spontaneously grow and proliferate as suspension cells, independently of the fibroblasts (Fig. 2C). The cells grew mainly as single round cells with variable cell sizes ranging from 13 to 30 µm. Occasionally, multinucleated cells were seen (Fig. 2D).

### Cell morphology and culture characteristics

The established MCT cell line, named C18, was round to oval, with minimal variation in cell size, which ranged from 15 to 20 µm. The average cell sizes of C18 (Fig. 2D), CMMC (Fig. 2F), VIMC (Fig. 2H) and CoMS (Fig. 2J) were 19.34 ± 5.52, 15.64 ± 1.39, 14.11 ± 1.71 and 13.94 ± 1.02 µm, respectively. The C18 cell line showed a significantly larger cell size compared to the other three reference cell lines ($P < 0.001$, Fig. 3A). As shown in Fig. 3B, VIMC had the largest cell aggregate spheroid among the four cell lines, with an average of 443.70 ± 138.49 µm (Fig. 2G), followed by CoMS (421.26 ± 201.48 µm, Fig. 2I), CMMC (319.73 ± 145.78 µm, Fig. 2E) and C18 (108.14 ± 33.52 µm, Fig. 2C). The cell growth of C18 was stable and could be maintained for over 140 passages (more than 1 year of culture).

### In vitro growth characteristics of the cell lines

The doubling time of the C18, CMMC, VIMC and CoMS cell lines was compared in vitro. As shown in Table 1, C18 had the longest doubling time, followed by VIMC, CMMC and CoMS, respectively. All cell lines had an initial lag phase of 48–72 h and a log phase of 4–6 days, followed by stationary and decline phases (Fig. 4).

### Established cell line characteristics

To characterise the mast cell property, the gene expression profile was examined in the four canine mast cell tumour cell lines by reverse-transcription polymerase chain reaction (RT-PCR) (Fig. 5). All cell lines expressed *c-kit*, *tryptase*, *FcεRIα* and *FcεRIβ* genes, whereas *chymase* expression was observed in all cell lines except for C18. Immunofluorescent staining demonstrated the expression of c-kit and tryptase in all cell lines as bright green and red in colour, respectively (Fig. 6). As shown in Fig. 7, flow cytometry indicated the percentage of cells that were positive for c-kit and tryptase in each cell line as follows: C18 with 99.24% and 16.09%, CMMC with 79.86% and 10.60%, VIMC with 99.67% and 72.36%, CoMS with 99.73% and 76.73%.

### Detection of *c-kit* mutation

In our study, no MCT cell line showed mutations of *c-kit* exon 8 (Fig. 8A). Mutation at *c-kit* exon 11 was detected in the C18 cell line as an aberrant band of the PCR

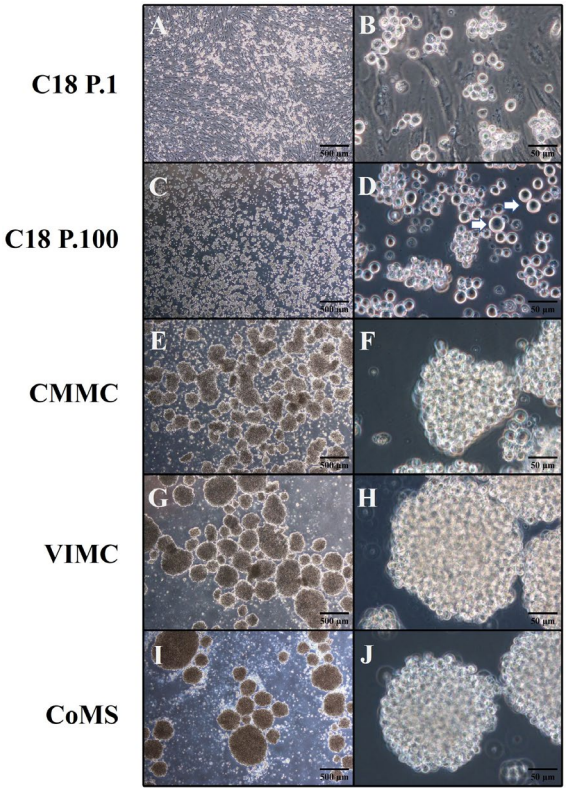


**Fig. 2** Morphology and culture characteristics of the canine MCT cell lines. The C18 cell line in the first month of culturing required co-culture with tumour-associated fibroblasts (**A** and 40× magnification). Aggregated MCT cells attached to fibroblasts (**B** and 400× magnification). C18 cell line after culture for 4 months, which grew as suspended round cells (**C** and 40× magnification). Higher-magnification view of the C18 cells growing as single to small cluster round cells with variable size; white arrows mark the multinucleated cell (**D** and 400× magnification). Lower- and higher-magnification views of the multiple spheroid sizes in CMMC (**E** and **F**), VIMC (**G** and **H**) and CoMS (**I** and **J**)

products, above the wild type one, suggesting duplications (Fig. 8B). Sequence analysis demonstrated *c-kit* ITD of 54 nucleotides, which translated into an insertion of alanine, followed by a copy of 17 amino acids (Fig. 9).

### Detection of SNPs in canine MCT cell lines

In this study, 20 SNPs from six genes were identified in canine MCT cell lines (Fig. 10; Table 2). Among these, *SPAM1* (rs851582160T > TC) was a common SNP found in all cell lines, whereas *SEL1L* (rs24507594A > G and 8:53818371G > A) and *TRAF3* (rs851689319A > T) were found only in cutaneous and mucosal MCT cell lines, respectively. Three cell lines (C18, CMMC and CoMS) had 14:11791385 A > G and 14:11794735 C > T variants of the *HYAL4* as well as 8:53778185T > C variant of *SEL1L*. The rs22299980A > AG variant of *c-kit* was found only in C18, whereas other cell lines were rs22299980A > G. Other variants in *HYAL4*, *TRAF3*, *SEL1L* and *POT1* were carried by at least one of the MCT cell lines.

### Effects of PBMCs on apoptosis and viability of canine MCT cell lines

As shown in Fig. 11, when MCT cell lines were co-cultured with healthy donor PBMCs, there was a significantly increased early and total apoptosis when compared to the control group in all cell lines. Among the four cell lines, C18 had the highest early and total apoptotic cell percentages of 56.71% and 61.27% at a T:E ratio 1:25, 57.39% and 63.20% at 1:50 and 49.03% and 55.85% at 1:100, respectively. Furthermore, only in the C18 cell line, the average number of viable MCT cells in the co-culture condition was significantly lower than that in the control group. Although the difference was not statistically significant, the average number of late apoptotic MCT cells tended to increase while that of viable cells tended to decrease relative to the control group in all cell lines (see Additional File 2).

## Discussion

In this study, we have successfully established and characterised a novel in-house canine cutaneous MCT cell line, named C18, from a high grade MCT case. Only tissue explant culture was successful in achieving long-term cell survival, potentially due to the fragility of MCT cells and their propensity for damage during aspiration. However, cells from explant tissue spontaneously migrated from the tumour mass, thereby minimising cell injury. Our findings suggest that cancer-associated fibroblasts (CAFs) within the tissue explants support cell growth and differentiation, aligning with their known pro-tumorigenic roles in cancer progression [33, 34]. This finding is in agreement with a previous report that CAFs could promote the long-term survival of neoplastic mast cells [35]. We hypothesised that the CAFs may secrete stem cell factor (SCF), crucial for mast cell growth [36, 37]; while mast cells stimulate fibroblast proliferation, migration and collagen synthesis [38, 39].

Our study found most that MCT cells in all cell lines expressed c-kit, but the number of tryptase-positive cells varied, with mucosal MCT cell lines (VIMC and CoMS) showing higher numbers than cutaneous ones (CMMC and C18). This suggests that tumour origin and poor differentiation of cutaneous MCT might influence mast cell granule composition [40–42]. All four canine MCT cell lines formed multicellular spheroids in suspension with distinct layers of necrotic core, quiescent cells and a proliferating outer layer. Factors influencing spheroid size include cell nature, growth parameters, proliferating layer thickness and culture conditions [43–45]. The smallest spheroid size in the C18 MCT cell line correlates with the longest doubling time and fewer outermost proliferating cells [46].

The C18 MCT cell line showed ITD mutation in *c-kit* exon 11, associated with canine MCT development and

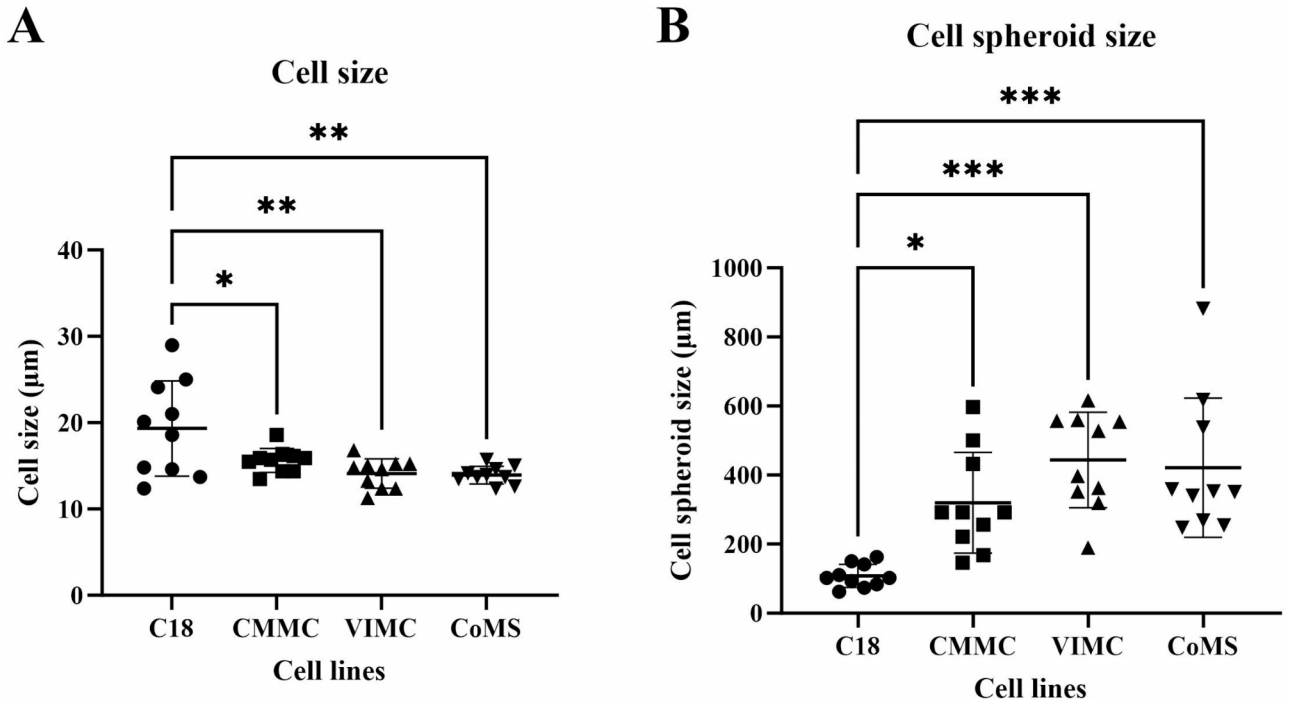


**Fig. 3** Distribution of the cell size (**A**) and spheroid size (**B**) in four canine mast cell tumour cell lines (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

**Table 1** Doubling times of exponentially growing cells in canine mast cell tumour cell lines

| No. | Cell line | Doubling time (h) |
|---|---|---|
| 1 | C18 P.10 | 50.30 ± 1.09 |
| 2 | C18 P.100 | 48.85 ± 0.86 |
| 3 | CMMC | 42.09 ± 4.98 |
| 4 | VIMC | 42.90 ± 1.40 |
| 5 | CoMS | 32.30 ± 1.01 |

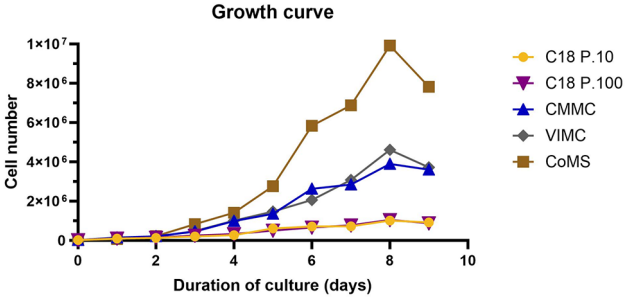


**Fig. 4** Growth curve analysis of canine mast cell tumour cell lines. The cell numbers of each cell line were determined for 5 consecutive days. Each time point represents the mean of triplicate cell counts via the MTT assay

malignancy [15, 16, 22]. We identified 20 SNPs from six genes potentially involved in canine MCT pathogenesis. These genes include hyaluronidase-coding genes *HYAL4* and *SPAM1*, related to cell proliferation and invasion [47, 48], *SEL1L*, linked to reduced tumour aggressiveness [49], *TRAF3*, a tumour suppressor found mutated in canine B cell lymphoma [50, 51], *c-kit*, involved in MCT pathogenesis [8, 12, 13, 16], and *POT1*, associated with genome instability [51, 52]. Despite reporting SNP mutations in canine MCT cell lines, our study was limited by a small sample size, and further studies are needed to better understand the role of functional SNPs in canine MCT development.

In our investigation, peripheral blood mononuclear cells (PBMCs) were utilized to develop a co-culture model involving canine MCT and PBMCs in an initial pilot study. The PBMCs were employed with the objective of specifically targeting and eliminating tumour cells. Among the cell lines, C18 MCT was most susceptible to immune-mediated cytotoxicity. Cutaneous-origin MCT demonstrated increased apoptosis compared to the mucosal counterparts, consistent with their poorer prognosis [3, 4]. Co-culturing significantly enhanced early and total apoptosis across all cell lines, supporting reports of PBMC-induced apoptosis in cancer cells [53, 54]. The PBMCs can initiate both intrinsic and extrinsic apoptosis by releasing perforin and granzyme B and engaging death domains such as FasL/FasR and TNF-α/TNFR1 [55–57]. However, lower apoptosis rates in CMMC, VIMC and CoMS may reflect defects in apoptosis pathways, leading to immune-mediated apoptosis resistance [58, 59], potentially due to increased anti-apoptotic BCL-2 proteins, overexpression of programmed death ligand 1 (PD-L1) leading to T cell exhaustion or mutations in tumour suppressor genes [60–62]. Future research endeavors are planned to delve deeper into the study of CD4 + or

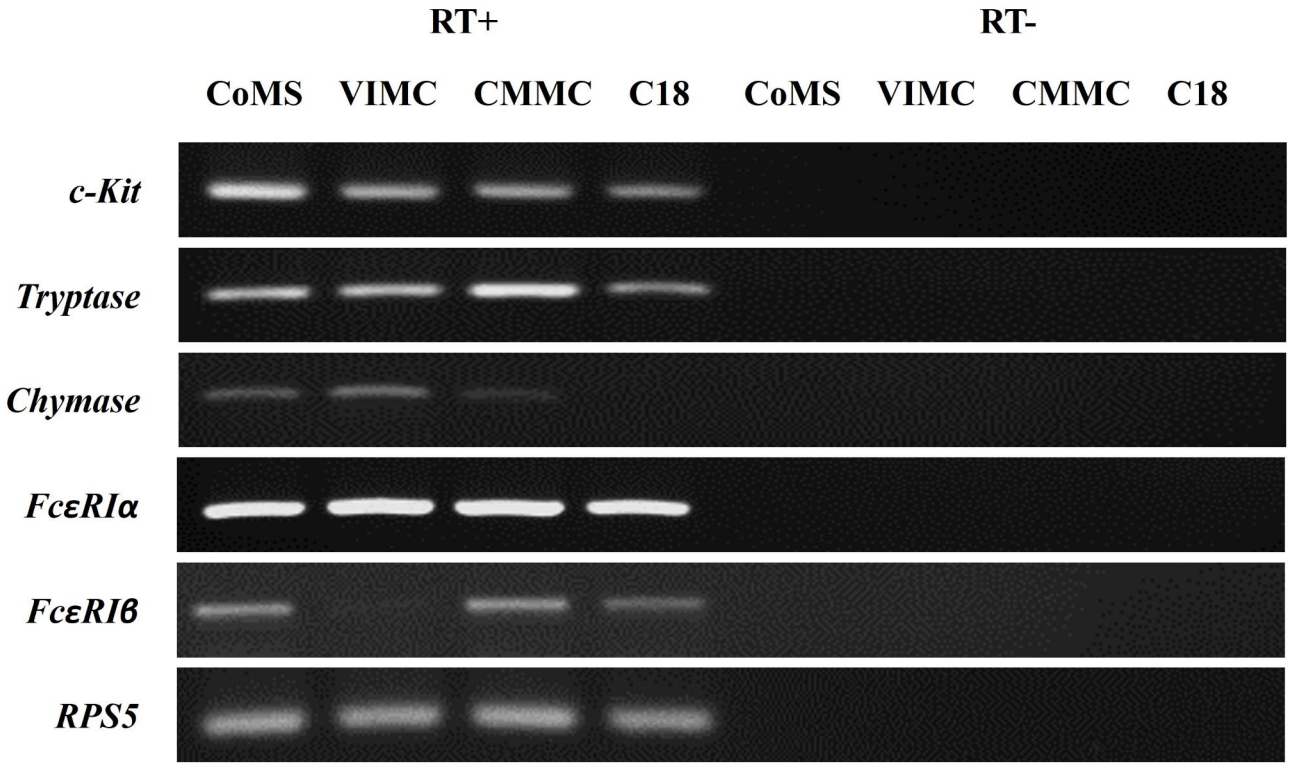


**Fig. 5** Detection of *c-kit*, *tryptase*, *chymase*, *FcεRIα* and *FcεRIβ* gene expression in canine mast cell tumour cell lines by RT-PCR analysis. RT- was used as a negative control

CD8 + cytotoxic T cells, with a particular focus on investigating their interactions with canine MCT.

## Conclusion

In conclusion, we established a novel in-house canine cutaneous MCT cell line, in terms of its characteristics, growth behavior and interaction with PBMCs. We posit that the C18 cell line holds significant potential as a valuable resource for investigating and advancing novel therapeutic strategies targeted against canine MCT. Nevertheless, customization at an individual level is imperative due to the inherent tumour heterogeneity and patient-specific variations in immune capacities.

## Methods

### Experimental design

The canine mast cell tumours samples were collected from the excisional biopsy mass of each patient and the cells were randomly isolated either by fine-needle aspiration or tissue explant. The isolated cells were further cultured under three different culture conditions referred to as medium formula 1, 2 and 3 (see details in 'Establishment of the canine mast cell tumour cell line' section). The resulting cell line was characterised and compared with three canine MCT reference cell lines from the University of Tokyo, Japan: CMMC-cutaneous MCT [5], VIMC-mucosal MCT [5], and CoMS-mucosal MCT [6]. The sensitivity of canine MCT to immune cell-mediated cytotoxicity was tested by co-culturing MCT cells with PBMCs from healthy donor dogs.

### Animal ethics and biosafety

The clinical and experimental protocols were approved by the Chulalongkorn University Guidelines of Animal Care and Use (protocol no.2131049). The biosafety procedure for cell approaches was approved and performed under the permission of the Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (protocol no. 2031029). The CMMC (cutaneous MCT), VIMC and CoMS (mucosal MCT) used in this study were kindly provided by the Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, University of Tokyo.

### Initial clinical specimen for cell collection

All cases were diagnosed and graded by two veterinary pathologists at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand, using the Kiupel grading system for canine cutaneous MCT [32], and the details of patients, diagnosis and conditions for cell culture were shown in Additional File 1. After surgical removal of the mass, the tissue samples were collected in 10% buffered formalin

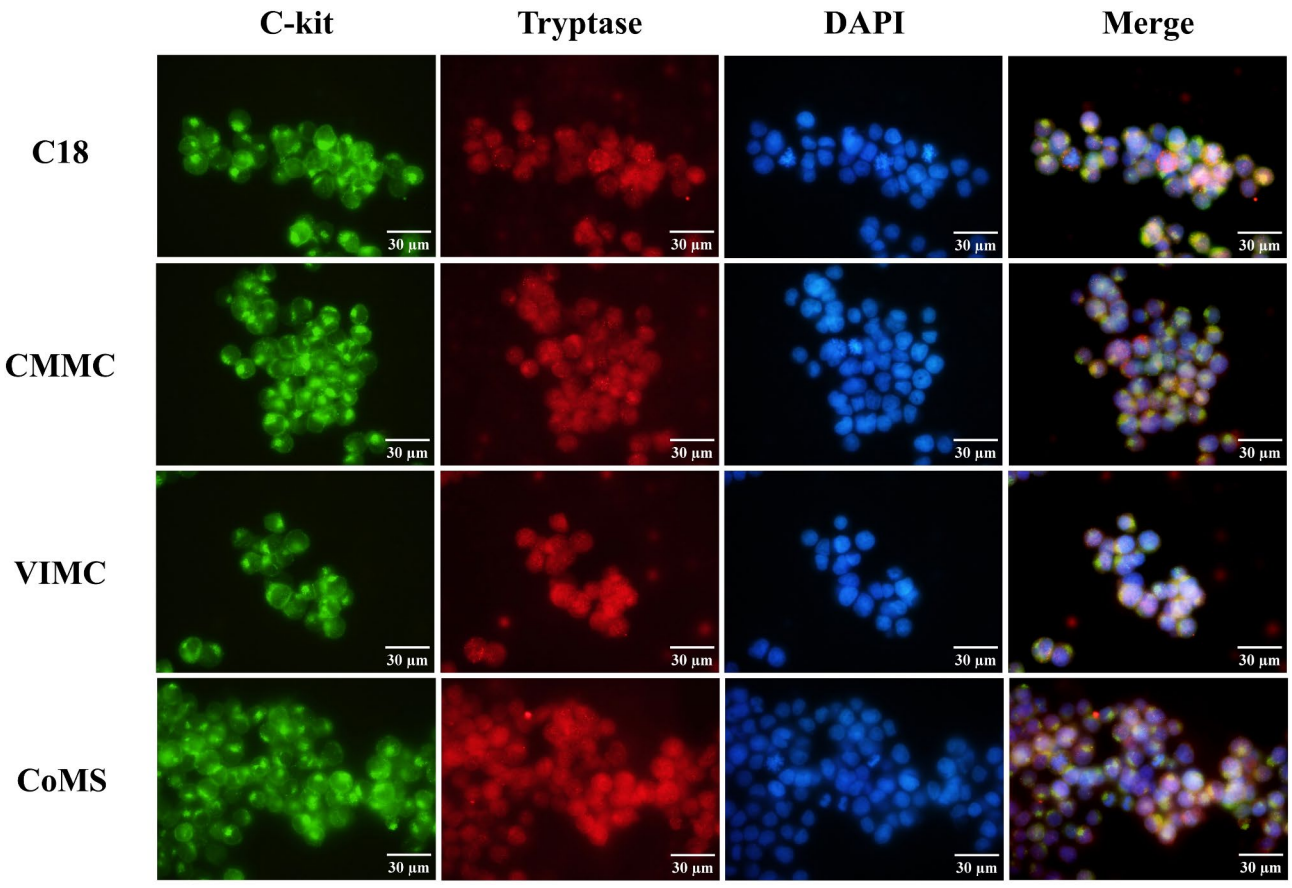


**Fig. 6** Immunofluorescence staining of c-kit (CD117) and tryptase expression in canine mast cell tumour cell lines. In green, cells stained with c-kit; in red, tryptase; in blue, nuclei stained with DAPI

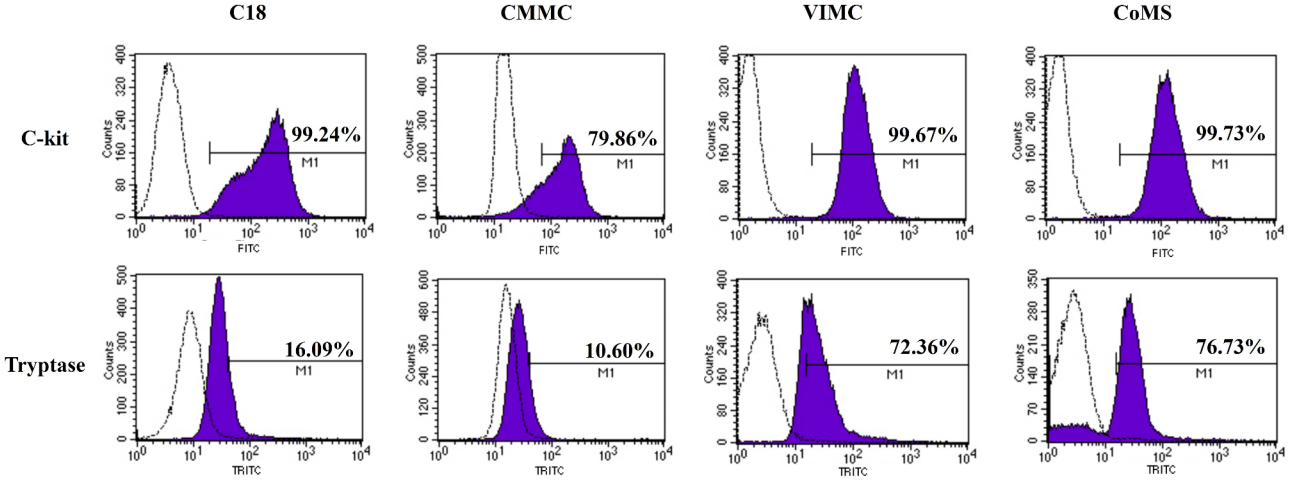


**Fig. 7** Flow cytometry analysis of c-kit and tryptase expression in canine mast cell tumour cell lines

for routine histopathology and in sterile phosphate-buffered saline (PBS) for cell culture.

### Establishment of the canine mast cell tumour cell line

To determine the effects of cell isolation methods on cell line establishment, the tumour cells were randomly collected either by fine-needle aspiration (FNA) or by the explant tissue culture technique. For the FNA method, the sample was repeatedly aspirated using a 21-gauge needle. The cells were then filtered through a 40-µm cell strainer and centrifuged at 1,000 rpm, 4 °C, for 5 min. Excessive red blood cells were removed by Hybri-Max

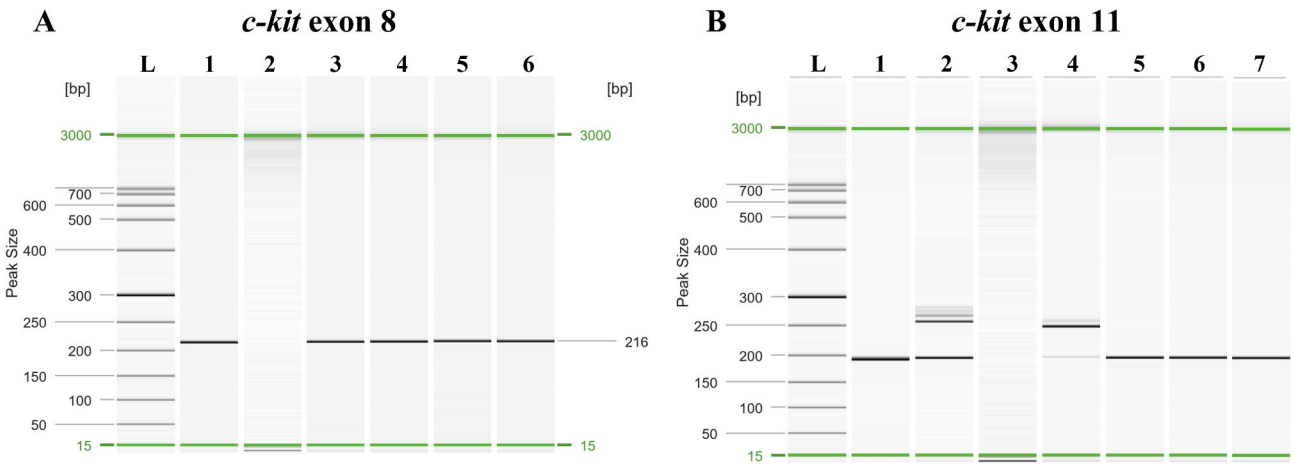


**Fig. 8** Gel image displaying PCR products of exons 8 and 11 of *c-kit* in canine MCT cell lines. In the image, *L* DNA ladder, *lane 1 c-kit* exon 8 wild type, *lane 2 c-kit* exon 8 negative control, *lane 3* C18, *lane 4* CMMC, *lane 5* VIMC, *lane 6* CoMS (**A**). *L* DNA ladder, *lane 1 c-kit* exon 11 wild type, *lane 2 c-kit* exon 11 mutated (positive control), *lane 3 c-kit* exon 11 negative control, *lane 4* C18, *lane 5* CMMC, *lane 6* VIMC, *lane 7* CoMS (**B**)

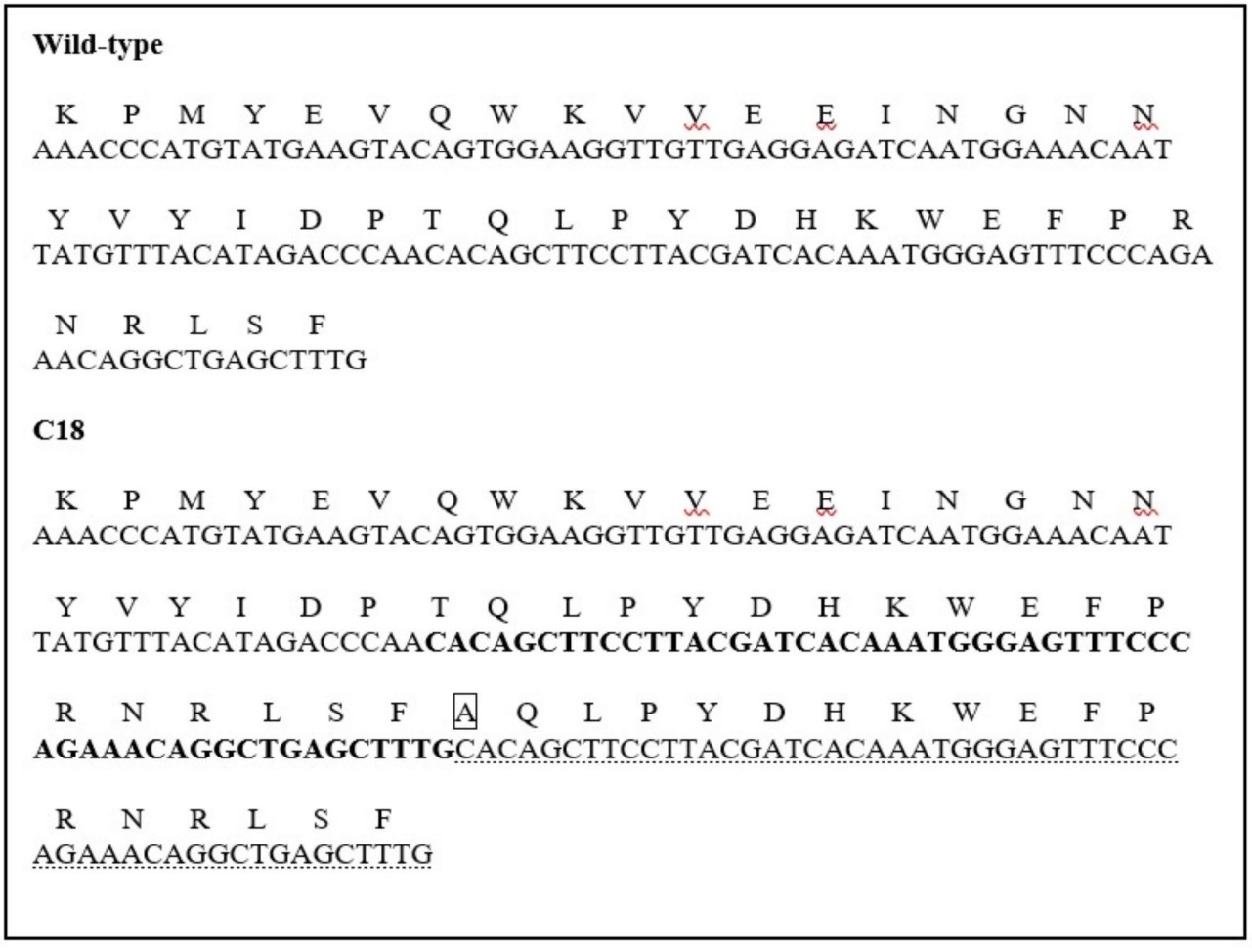


**Fig. 9** Sequence analysis revealed the ITD of 54 nucleotide mutations found in exon 11 of the *c-kit* in C18 cell line. The region of DNA that was duplicated and the actual ITD are indicated by bold and dot lettering, respectively. Amino acid insertion is denoted by boxed letters

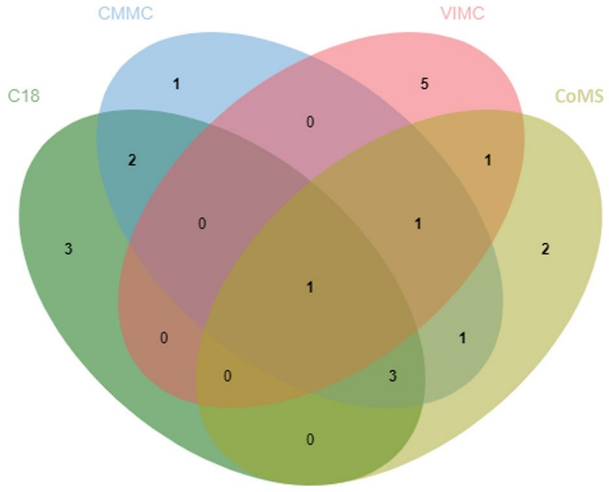


**Fig. 10** Venn diagram illustrating both unique and shared single nucleotide polymorphisms found in each of the canine mast cell tumour cell lines

lysis buffer (Sigma-Aldrich, USA). For the tissue explant technique, the tumour mass was cut to a size of approximately $3 \times 3 \times 3$ $mm^3$ and then placed in a 35-mm culture dish (#353001, Falcon®, USA) with 0.5 mL of culture medium. Three different media were used in this study. Medium (Formula 1) consisted of high-glucose Dulbecco's modified Eagle's medium (DMEM, 12800, Gibco), 10% (v/v) heat-inactivated foetal bovine serum (FBS, 10270, Gibco), 1 mM L-glutamine (GlutaMAX™, 35050-061, Gibco) and antibiotic-antimycotic agents (Anti-Anti, 15240-062, Gibco). Dog serum was used in Medium 2. Medium 3 was similar to Medium 1, except for the addition of 1 mM non-essential amino acid (M7145, Sigma-Aldrich, USA), 1 mM MEM amino acids (M5550, Sigma-Aldrich, USA) and 1 mM nucleosides (EmbryoMax®, Sigma-Aldrich, USA). The cells were cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$. The medium was changed every 3–4 days.

#### Growth characteristics and propagation ability

The growth characteristics and propagation ability of tumour cells during passages 10 and 100 were tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (M6494, Invitrogen, USA). The MTT was assayed in triplicate for the 'in-house' cell line and the other three canine MCT reference cell lines CMMC, VIMC and CoMS. Absorbance was measured by a microplate reader (TECAN, HydroFlex™ Platform, Austria) against a reagent blank at a test wavelength of 570 nm and a reference wavelength of 650 nm. Data are shown as mean ± SD of the average of the cell number on day 5 of each cell line. Population doubling time (PDT) was calculated from the exponential curve (log phase) of each cell line, using the following equation:

$$\mathrm{Td}\cdot = \frac{(\ln 2 - \ln A) - (\ln 1 - \ln A)}{B}$$

where Td = doubling time, $ln1$ = 0, B = number from the exponential curve equation in the form y = $Ae^{Bx}$.

#### Characterisation of the established canine MCT cell line

The characteristics of the established cell line, including gene markers, cell surface markers, *c-kit* exon 8 and 11 mutations as well as SNPs, were compared with those of the three canine MCT reference cell lines from the University of Tokyo, Japan: CMMC (cutaneous MCT), VIMC (mucosal MCT), and CoMS (mucosal MCT).

#### Determination of the canine MCT gene markers

The specific gene expressions of canine MCT were investigated by RT-PCR. Total RNA was extracted and purified from four canine MCT cell lines using the RNeasy mini kit (Qiagen, Hilden, Germany); subsequently, the concentration was determined using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA). The DNase I (RQ1 RNase-Free DNase, Promega WI, USA) was applied to eliminate contaminated genomic DNA. Complementary DNA (cDNA) was synthesised from total RNA using the First-strand cDNA synthesis kit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. A negative control of each sample was duplicated in the same process via reverse transcription, albeit without the SuperScript III reagent. For RT-PCR assays, the specific primers for the canine MCT in this study are listed in Table 3. The *RPS5* gene was used as an internal reference (housekeeping) gene. To amplify the canine MCT gene markers, 200 ng of cDNA template was used in a 25-µL RT-PCR mixture. The mixture contained 10 µm of each primer and 2X GoTaq® Green Master Mix (M7122, Promega, Madison, WI, USA) consisting of 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$. The RT-PCR reaction was performed in a T100™ Thermal Cycler (Bio-Rad, CA, USA) under the following conditions: 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The PCR products were analysed using a 2% agarose gel with 5 µL of Redsafe™ (Intron biotechnology, Korea) and detected using a gel documentation system (GENEFLASH, Syngene, CB, UK).

#### Cell surface marker analyses

The surface markers of the established canine MCT were evaluated by determining the expression of c-kit (CD117) and mast cell tryptase using the immunofluorescence assay (IFA) and flow cytometry. Cells were fixed with 4% (w/v) paraformaldehyde and permeabilised with 0.1% Triton X-100 for 20 min. Afterward, cells were incubated with rabbit anti-human c-kit antibody (1:200; PA5-16770;

**Table 2** Detection of single nucleotide polymorphisms in four canine mast cell tumour cell lines

| Genes | Position | Variants | Allele | Type of MCT | | Amino acid change | Type of mutation |
|---|---|---|---|---|---|---|---|
| | | | | Cutaneous | Mucosal | | |
| *C-kit* | Chr13:47,175,092 | rs22299980 A > G | G | CMMC | VIMC, CoMS | p.Thr425 = | nonsense |
| | | | AG | C18 | - | p.Asn426GlufsTer25 | frameshift |
| | | | A | *wildtype* | | | |
| *HYAL4* | Chr14:11,778,977 | rs8499846 G > A | A | C18 | - | upstream gene variant | upstream gene variant |
| | | | GA | CMMC | CoMS | | |
| | | | G | *wildtype* | | | |
| | Chr14:11,791,385 | 14:11791385 A > G | G | - | VIMC | intergenic variant | intergenic variant |
| | | | GA | C18, CMMC | CoMS | | |
| | | | A | *wildtype* | | | |
| | Chr14:11,794,735 | 14:11794735 C > T | T | - | VIMC | intergenic variant | intergenic variant |
| | | | CT | C18, CMMC | CoMS | | |
| | | | C | *wildtype* | | | |
| *POT1* | Chr14:11,033,763 | c.927delT | Del | - | VIMC | p.Phe309LeufsTer3 | frameshift |
| | | | T | *wildtype* | | | |
| *SEL1L* | Chr8:53,778,185 | 8:53778185 T > C | C | - | VIMC | intron variant | intron variant |
| | | | CT | C18, CMMC | CoMS | | |
| | | | T | *wildtype* | | | |
| | Chr8:53,785,948 | 8:53785948 A > G | GA | CMMC | - | intron variant | intron variant |
| | | | A | *wildtype* | | | |
| | Chr8:53,796,442 | rs24507594 A > G | G | C18, CMMC | - | intron variant | intron variant |
| | | | A | *wildtype* | | | |
| | Chr8:53,818,371 | 8:53818371 G > A | A | C18, CMMC | - | intron variant | intron variant |
| | | | G | *wildtype* | | | |
| | Chr8:52,763,337 | rs24560262 C > A | CA | - | CoMS | intergenic variant | intergenic variant |
| | | | C | *wildtype* | | | |
| *SPAM1* | Chr14:11,704,952 | rs851582160 T > C | TC | C18, CMMC | VIMC, CoMS | p.Lys482ArgfsTer27 | frameshift |
| | | | T | *wildtype* | | | |
| *TRAF3* | Chr8:70,782,945 | rs851689319 A > T | T | - | VIMC, CoMS | p.Lys284Ter | nonsense |
| | | | AT | C18 | - | p.Lys284IlefsTer14 | frameshift |
| | | | A | *wildtype* | | | |
| | Chr8:70,782,999 | c.906delT | Del | - | VIMC | p.Ile302MetfsTer21 | frameshift |
| | | | T | *wildtype* | | | |
| | Chr8:70,783,003 | c.908dupA | A | - | CoMS | p.Arg304GlufsTer9 | frameshift |
| | | | Del | *wildtype* | | | |

ThermoFisher Scientific) and mouse anti-human mast cell tryptase antibody (1:200; clone AA1, M7052; Dako) at 4°C for overnight. After washing with PBS, cells were stained with goat-anti rabbit conjugated with FITC (11-4839-81; Thermo Scientific) for c-kit and goat-anti mouse conjugated with TRITC (T5393; Sigma-Aldrich, USA) for tryptase detection and incubated at 37°C for 1 h. Subsequently, cells were washed and resuspended in fluorescence-activated cell sorter (FACS) buffer and then analysed on a BD FACSCalibur flow cytometer (BD, USA) with the BD CellQuest™ Pro software. The results are presented as percentages of c-kit- and tryptase-positive cells. For IFA, the cell nucleus was counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (D1306, ThermoFisher Scientific) and mounted with antifade mounting medium (VECTASHIELD®, Vector Laboratories, CA, USA) prior to evaluation under a fluorescence microscope (BX51TRF, Olympus).

#### Detection of *c-kit* mutation in exons 8 and 11

Genomic DNA derived from each MCT cell line was evaluated for mutations in exons 8 and 11 of the *c-kit* gene. Specific primers were used to amplify the detection of these mutations, following previous studies [12, 63]. The PCR mixture contained 50 ng of DNA, 2 mM of $MgCl_2$, 10X PCR buffer, 2.5 units of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) and 0.2 µM of each primer, with a total volume of 25 µL. The sample was subsequently amplified using a T100™ Thermal Cycler (Bio-Rad, CA, USA) under the following conditions: 95 °C for 15 min, 40 cycles of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The alterations in *c-kit* exons 8

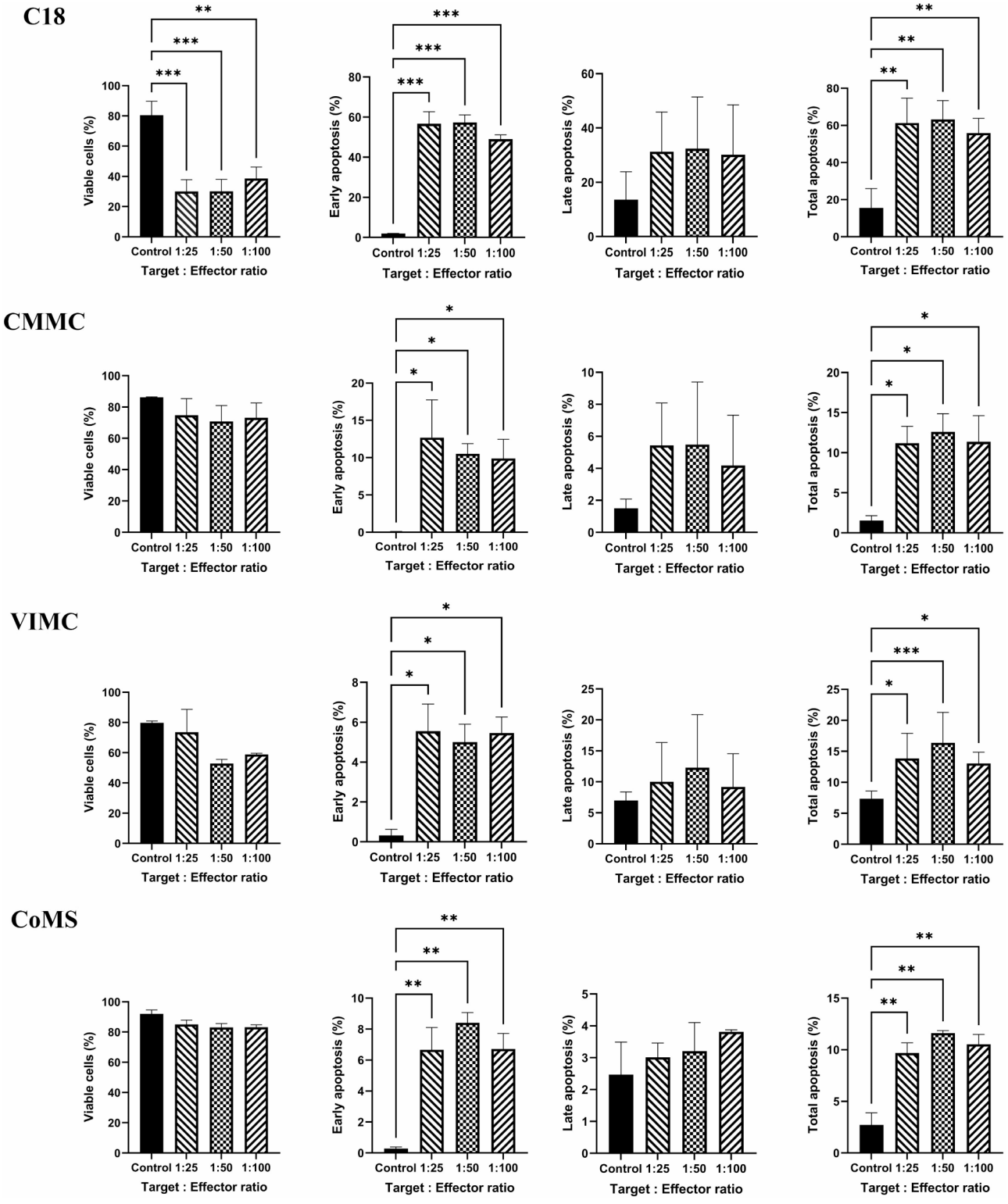


**Fig. 11** Cytotoxicity of healthy donor PBMCs against canine MCT cell lines. Each row represents the percentages of viable, early apoptotic, late apoptotic and total apoptotic MCT cells of each cell line (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

**Table 3** Canine mast cell tumour-specific primer sequences

| Primer name | Primer (5′ to 3′) | Product size (Bps) | Accession No. or reference |
|---|---|---|---|
| *C-kit* | F: CACCGACCCAGGATTTGTCA | 230 | NM_001003181.1 |
| | R: GGACCAGCGTATCATTGCCT | | |
| *Tryptase* | F: ACTACACACCCGAGAATGGG | 243 | NM_001097555.2 |
| | R: GGTGGTATTGCACGTCACAC | | |
| *Chymase* | F: AGTTTGGAAGGAGTGGGAGTG | 282 | U89607.1 |
| | R: ACCTAGTGTGGAGAGGTCAGG | | |
| *FcεRIα* | F: GTCATCAGATACCTT-GAAACCTACA | 294 | NM_001110766.2 |
| | R: ACGTTGGCAGAGGCTTGAA | | |
| *FcεRIβ* | F: TTCTGGGGGTAACACAAAT | 402 | (7) |
| | R: TGCAAAACCCCAGAATAGT | | |
| *RPS5* | F: TCACTGGTGAGAACCCCCT | 141 | XM_533568.4 |
| | R: CCTGATTCACACGGCGTAG | | |

and 11 were analysed and visualised on the QIAxcel Capillary Electrophoresis System (Qiagen, Hilden, Germany). The MCT cell line with *c-kit* mutation was submitted for Sanger sequencing (Bionics Co., Seoul, South Korea) to determine the genetic characterisation.

### Identification of gene polymorphisms

To determine single nucleotide polymorphisms (SNPs) in canine MCT cell lines, we performed MassARRAY SNP genotyping for 41 SNPs in 17 genes (*C-kit*, *FLT3*, *HYALP1*, *HYAL4*, *LMNB1*, *MVB12A*, *MET*, *MYC*, *POT1*, *PTEN*, *SATB1*, *SEL1L*, *SPAM1*, *TP53*, *TRAF3*, *ZC3H7A* and *ZNHIT6*) previously reported as cancer-associated genes in canine lymphoma [64]. The procedures for MassARRAY SNP genotyping were performed according to a previous study [64].

### Co-culture responses of PBMCs to canine mast cell tumour

#### *Isolation of canine PBMCs*

Heparinised peripheral blood samples (15 mL) were collected from three healthy dogs. The blood samples were transferred to 15-mL conical tubes and centrifuged at 3,000 rpm for 10 min at 25 °C. Roswell Park Memorial Institute (RPMI)-1640 medium (31800-022, Gibco) was used to dilute the blood samples before gently layering them onto 4 mL of Histopaque®-1077 (H8889, Sigma, USA). Subsequently, the samples were centrifuged at 2,200 rpm for 20 min at 25 °C. Using a sterile Pasteur pipette, the PBMCs were harvested at the interface layer, washed twice with RPMI-1640 medium and centrifuged at 2,000 rpm for 10 min at 25 °C. The supernatant was removed and resuspended with RPMI-1640 medium supplemented with 10% heat-inactivated FBS (complete RPMI-1640 medium).

#### *Co-culture of canine MCT cell lines with PBMCs*

Prior to the in vitro co-culture assay, the isolated PBMCs were stimulated by co-culture with mitomycin-treated MCT cell lines in a complete RPMI-1640 medium supplemented with 100 U/mL of recombinant human interleukin-2 (rhIL-2) for 14 days. Following treatment with mitomycin C, the MCT cells exhibited a gradual impairment or cessation of growth, ultimately leading to cellular demise within a span of a few days [65, 66]. After 14 days, the remaining cells were activated PBMCs. At the end of the co-culturing period, fresh canine MCT cell lines were seeded at a density of $1 \times 10^5$ cells/well in a 12-well plate for 30 min. After that, the PBMCs were added to each well at target: effector (T: E) ratios of 1:25, 1:50 and 1:100; the control group consisted of MCT cells without PBMCs. After 4 h of co-culture, cells were collected, and the numbers of live and apoptotic tumour cells were analysed when the tumour cells were cultured alone or in the presence of stimulated PBMCs by flow cytometry, using the FITC Annexin V Apoptosis Detection Kit with propidium iodide (640914, BioLegend, San Diego, CA, USA). Briefly, cells were pelleted, washed twice with cold BioLegend's cell staining buffer and resuspended in 100 µL of Annexin V binding buffer. Each sample was stained with 5 µL of FITC Annexin V and 10 µL of propidium iodide and incubated for 15 min at 25 °C in the dark. Subsequently, Annexin V binding buffer was added to each sample to adjust the final volume to 500 µL. The samples were immediately analysed using the BD FACSCalibur flow cytometer (BD, USA) with the BD CellQuest™ Pro software.

### Statistical analysis

The results of cell and spheroid size comparisons as well as doubling time are shown as the means ± standard deviations (SDs) from three independent experiments. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed to determine the statistical differences among the groups in cell and spheroid size comparisons and the co-culture experiment. All statistical analyses were performed using the GraphPad Prism software version 9.4.1; $P < 0.05$ was considered statistically significant.

**Abbreviations**

| | |
|---|---|
| MCT | Mast Cell Tumour |
| ITD | Internal Tandem Duplication |
| SNPs | Single Nucleotide Polymorphisms |
| RT-PCR | Reverse-Transcription Polymerase Chain Reaction |
| PBMCs | Peripheral Blood Mononuclear Cells |
| CAFs | Cancer-Associated Fibroblasts |
| SCF | Stem Cell Factor |
| FNA | Fine-Needle Aspiration |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04603-4.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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**Author contributions**

TT and TK conceptualised and supervised the study. SuBh performed the experiments. SuBh and SuBu analysed flow cytometry data. SuBh, SS, PaPo, AR and PrPh analysed the data on c-kit mutation and gene polymorphisms. SuBh and PT collected the samples. TN and AS provided canine MCT cell lines. SuBh drafted the manuscript. TT and TK reviewed and approved the final manuscript.

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**Data availability**

The datasets generated and/or analysed during the current study are available in the GenBank database of the National Center for Biotechnology Information repository, persistent web link is https://www.ncbi.nlm.nih.gov/, the GenBank accession numbers of ITD found in exon 11 of the c-kit is XM_025435629.3.

## Declarations

**Ethics approval and consent to participate**

The clinical and experimental protocols were approved by the Chulalongkorn University of Animal Care and Use guidelines (protocol no.2131049). The biosafety procedure for cell approaches was approved and performed with the permission of the Biosafety Committee of the Faculty of Veterinary Science, Chulalongkorn University (protocol no. 2031029). The owners of the patients described in this article, who underwent surgical treatment for canine cutaneous MCT, were informed and required to sign treatment consent forms prior to receiving treatment.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

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