Studies on molecular-targeted therapy in canine mast cell tumor

(イヌの肥満細胞腫における分子標的治療に関する研究)

Yoshinori Takeuchi

竹 内 由 則

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General Introduction

Mast cell tumor (MCT) is one of common skin tumors in dogs and accounts for 7 to 21% of all canine cutaneous tumors (Welle et al., 2008) (Fig. 1). MCTs also arise in extracutaneous sites (visceral MCT) including the conjunctiva, salivary gland, nasopharynx, larynx, oral cavity, gastrointestinal tract, urethra and spine. The clinical behavior of canine MCTs is variable, ranging from mild to severe (Dobson and Scase, 2007). Common prognostic factors of the disease include World Health Organization clinical stage (Owen, 1989), histological grade (Patnaik et al., 1984), the relative frequency of argyrophilic nucleolar organizer regions (AgNORs), the percentage of proliferation cell nuclear antigens (PCNA) and Ki-67 immunoreactivity (Welle et al., 2008). Based on the presence or absence of these prognostic factors, treatment for canine MCTs is selected from surgical resection, irradiation, chemotherapy or combination of these modalities (Welle et al., 2008).

Although the etiology of canine MCTs is still unknown, several studies have indicated that approximately 15 to 40% of canine MCTs had contained *KIT* mutation (Letard et al., 2008; Welle et al., 2008). The *KIT* gene encodes type III receptor protein tyrosine kinase, c-Kit receptor, which is the receptor of stem cell factor (SCF) (Roskoski, 2005b). The c-Kit receptor is also known as CD117 or SCFR. The protein is composed of extracellular ligand binding domains (encoded by exons 1-9 of *KIT*), a single transmembrane segment (exon 10), and an intracellular domain (Fig. 2). The intracellular domain is further divided into a juxtamembrane domain (exons 11-12) and a cytoplasmic tyrosine kinase domain which is split by an insert into ATP-binding (exon 13) and phosphotransferase lobes (exon 17). Binding of SCF to c-Kit receptor leads to

receptor dimerization and autophosphorylation (Roskoski, 2005a). This in turn initiates the intracellular signaling pathways such as Shc/Ras/MAPK, JAK/STAT and PI3K cascades (Gilfillan and Rivera, 2009). The c-Kit receptor signaling is important in erythropoiesis, lymphopoiesis, mast cell development and function, megakaryopoiesis, gametogenesis, and melanogenesis (Roskoski, 2005b).

Recent advances in molecular biology have led to the recognition of the molecular pathology of many tumors. Molecular targeted therapy is a novel and selective anticancer strategy that inhibits the proliferation of tumor cells by interfering with tumor-specific molecules needed for tumorigenesis and cell growth (Ciavarella et al., 2010). Since conventional anticancer agents target DNA or RNA in cells, they injure not only tumor cells but also normal rapidly-dividing cells, and result in serious side effects in cancer patients (Fig. 3A). In contrast, molecular targeted agents cause relatively mild adverse effects since they are designed to inhibit the tumor cell-specific molecules (Fig. 3B). The candidates of therapeutic molecular target include growth factor receptors, signal transducers, molecular chaperones, cell cycle regulators, transcriptional factors and elsewhere (Fig. 3C). In human medicine, several favorable outcomes of molecular targeted therapies, such as imatinib targeting mutant fusion kinase BCR-ABL for chronic myeloid leukemia (CML), rituximab, a chimeric IgG monoclonal antibody against CD20 for B-cell lymphoma, and bortezomib which inhibited the chymotrypsin-like activity of proteasome for multiple myeloma (MM) have been reported (Ciavarella et al., 2010; Mateos and San Miguel, 2007). Recently, the clinical effectiveness of several tyrosine kinase

inhibitors (TKIs) targeting c-Kit receptor, such as imatinib mesylate, masitinib mesylate and toceranib phosphate for canine MCT cases, has also been studied (Hahn et al., 2008; Isotani et al., 2008; London et al., 2009).

Several studies revealed that both *KIT* mRNA and c-Kit receptor protein were expressed in canine normal and malignant mast cells (London et al., 1996; Reguera et al., 2000). In addition, dissimilar cytoplasmic and cell membrane expression of c-Kit receptor has been found between normal and neoplastic mast cells and aberrant cytoplasmic c-Kit receptor expression due to *KIT* mutation has been associated with poor prognosis (Gil da Costa et al., 2007; Preziosi et al., 2004; Reguera et al., 2000; Webster et al., 2004; Webster et al., 2006b; Webster et al., 2007; Webster et al., 2008). Furthermore, several mutations in *KIT,* which have been identified in canine MCT cases, cause a constitutive autophosphorylation and activation of c-Kit receptor without binding of the ligand (Letard et al., 2008; London et al., 1999; Ma et al., 1999). Such aberrant autophosphorylated c-Kit receptors can cause the unregulated downstream signaling, thereby promoting uncontrolled cell proliferation and survival (Roskoski, 2005b). Several studies reported that the TKIs could inhibit c-Kit receptor activation and proliferation of canine MCT cells (Gleixner et al., 2007; Liao et al., 2002). A clinical study reported that canine MCT cases with *KIT* mutation responded to treatment with imatinib mesylate (Isotani et al., 2008). Other studies indicated that canine MCT cases with *KIT* mutations were more likely to respond to treatment with toceranib phosphate or masitinib mesylate than those without mutations (Hahn et al., 2008; London et al., 2009).

Since the overall response rates of canine MCT cases treated with TKIs

were only 42 to 48% (Isotani et al., 2008; London et al., 2009), the tumors could show various sensitivity to TKIs. Most of the initial reports on canine MCT were derived from the studies using C2 cells, a canine MCT cell lines with internal tandem duplication (ITD) of *KIT*. Meanwhile, some dogs with MCT which had no *KIT* mutation showed beneficial response to imatinib mesylate treatment (Isotani et al., 2008). If not only c-Kit receptor but also other tyrosine kinases, which are inhibited by imatinib, can play a role in growth and survival of canine neoplastic mast cells, the tyrosine kinases may become novel therapeutic targets.

The aim of this study was to clarify the molecular basis associated with molecular targeted therapy in canine MCT cell lines and tumor cells obtained from dogs with MCT. In chapter 1, genetic statuses of *KIT* and c-Kit receptor phosphorylation were characterized and SCF/c-Kit receptor interaction was examined in various canine MCT cell lines. In chapter 2, the biological effects of several TKIs and the implication of ABCB1 on these effects were investigated in the canine MCT cell lines with various *KIT* statuses. In chapter 3, the mRNA expression and phosphorylation statuses of several kinases in several canine MCT cell lines were assessed to identify the candidate of therapeutic targets. The inhibitory effect of specific inhibitors was also examined in the cell lines. In chapter 4, genetic statuses of *KIT* and c-Kit receptor phosphorylation were characterized in canine MCT cases.

Chapter 1

Aberrant autophosphorylation of c-Kit receptor in canine mast cell tumor cell lines

Abstract

The *KIT* mutation could cause ligand-independent activation of c-Kit receptor in canine MCT. In this chapter, four canine MCT cell lines (HRMC, VIMC1, CoMS1 and CMMC1) were subjected to analyses of *KIT* mutation, c-Kit receptor phosphorylation, SCF expression, and the effects of SCF stimulation. The SCF/c-Kit receptor autocrine mechanism was also verified in HRMC. HRMC cells expressed wild type c-Kit receptor. Both VIMC1 and CoMS1 cells had the same one amino acid (AA) substitution of c-Kit receptor. CMMC1 cells had the three variants of c-Kit receptor, such as one AA deletion, one AA substitution as well as an ITD, and a nonsense mutation. The c-Kit receptors were phosphorylated in all cell lines. Phosphorylation of c-Kit receptor in HRMC, VIMC1 and CoMS1 was enhanced by SCF whereas no enhancement was observed in CMMC1. There was no effect of SCF stimulation on proliferation of all cell lines. SCF protein was detectable in only HRMC. A TKI, Dasatinib (internal inhibitor) inhibited c-Kit receptor phosphorylation in HRMC whereas anti-canine SCF antibody (external inhibitor) did not. Thus there could be an internal SCF/c-Kit receptor autocrine mechanism within HRMC. The results indicated that c-Kit receptor phosphorylation could be caused by the stimulation with autocrine SCF in HRMC while it could be caused by functional mutations of *KIT* in VIMC1, CoMS1 and CMMC1. Since the four canine MCT cell lines had various aberrations associated with c-Kit receptor phosphorylation, *KIT* mutation and SCF expression, such molecular

biological diversity might reflect the different biological behavior in canine MCT.

Introduction

Recently, several studies revealed that approximately 15 to 40% of canine MCTs contain *KIT* mutation (Letard et al., 2008; Welle et al., 2008). The *KIT* gene encodes type III receptor protein tyrosine kinase, c-Kit receptor, which is the receptor of SCF (Roskoski, 2005b). Several mutations in *KIT,* which have been identified in canine MCT cases, cause a constitutive autophosphorylation and activation of c-Kit receptor without binding of the ligand (Letard et al., 2008; London et al., 1999; Ma et al., 1999). The previous *in vitro* studies of TKIs targeting c-Kit receptor demonstrated inhibition of proliferation of canine MCT cell lines and primary neoplastic mast cells, associated with cell-cycle arrest and apotosis (Gleixner et al., 2007; Liao et al., 2002; Peter et al., 2010). Some clinical studies reported that canine MCT cases with *KIT* mutations were more likely to respond to treatment with TKIs than those without mutations (Hahn et al., 2008; Isotani et al., 2008; London et al., 2009).

SCF is known as c-Kit receptor ligand or mast cell growth factor (de Paulis et al., 1999). SCF is expressed in a wide range of tissues including the brain, endothelium, gametes, heart, kidney, lung, melanocytes, skin, liver, thymus and stromal cells of bone marrow (Roskoski, 2005a). SCF exists as membrane-bound and soluble forms that arise from alternative RNA splicing and proteolytic processing; both isoforms contain initially an extracellular domain, a transmembrane segment and intracellular components (Broudy, 1997; Roskoski, 2005a; Shull et al., 1992). Some studies revealed that SCF was produced and stored in normal and malignant human mast cells, and raised the

possibility that there was an autocrine or a paracrine growth and differentiation loop for mast cells (Akin et al., 2002; de Paulis et al., 1999; Welker et al., 1999). However, there has been no report that revealed the evidence of an autocrine SCF/c-Kit receptor loop in canine MCTs.

The objective of this chapter was to investigate the mechanism of c-Kit receptor activation in canine MCT. To study the pathogenesis of canine MCT, genetic statuses of *KIT* and c-Kit receptor phosphorylation were characterized, and SCF/c-Kit receptor interaction was examined in several canine MCT cell lines.

Materials and Methods

Canine mast cell tumor cell lines

Four canine MCT cell lines (HRMC, VI-MC, CoMS and CM-MC) were used in the present chapter. HRMC and CM-MC were originated from cutaneous MCT (Ohmori et al., 2008; Takahashi et al., 2001). VI-MC and CoMS were derived from visceral MCT (Ishiguro et al., 2001; Takahashi et al., 2001). Each cell line was cloned by limiting dilution method and the cloned VI-MC, CoMS and CM-MC were renamed as VIMC1, CoMS1 and CMMC1, respectively. HRMC cells were cultured in serum-free AIM-V medium (Invitrogen, Carlsbad, CA). VIMC1, CoMS1 and CMMC1 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen) with 10% fetal bovine serum (FBS), 100 unit/ml penicillin and 100 μg/ml streptomycin.

Nucleotide sequence of KIT

Total RNA was extracted from HRMC, VIMC1, CoMS1 and CMMC1 cells with RNeasy mini KIT (QIAGEN, Valencia, CA). Single-strand cDNA was synthesized from total RNA by SuperScript III Reverse Transcriptase (Invitrogen). Oligonucleotide primers to amplify the open reading frame (ORF) of canine *KIT* were described previously (Ohmori et al., 2008). Using this primer pair, *KIT* cDNA was amplified with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen). The PCR products were cloned into a plasmid

vector using a pGEM-T Easy Vector system (Promega, Madison, WI), followed by transformation of *Escherichia coli* competent cells. The PCR products cloned into a plasmid were sequenced by BigDye Terminator v3.1 Cycle Sequencing KIT (Applied Biosystems, Foster City, CA) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The each PCR product obtained from at least 2 PCR reactions was cloned and more than 8 independent clones of the each plasmid were sequenced.

c-Kit receptor phosphorylation

Whole cell lysates were extracted from HRMC, VIMC1, CoMS1 and CMMC1 cells in ice-cold lysis buffer [50 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM EDTA, 2 mM Na_3VO_4, 10 mM NaF and Protease Inhibitor Cocktail (Complete Mini; Roche Diagnostics, Manheim, Germany)]. Protein concentrations were determined with a Lowry protein assay kit (Bio-Rad, Hercules, CA). The equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (GE Healthcare, Buckinghamshire, England). Membranes were blocked in 2% bovine serum albumin/tris-buffered saline with Tween20, and then incubated with primary antibodies against c-Kit receptor (1:1,000, DAKO, Carpinteria, CA), phosphorylated c-Kit receptor (p-Kit) at tyrosine 719 (1:1,000, Cell Signaling Technology, Danvers, MA), and β-actin (1:1,000, Cell Signaling Technology). After incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG

(1:5,000, GE Healthcare), positive immunoreactivity was detected with ECL Plus Western Blotting Detection Reagents (GE Healthcare) and visualized with ChemDoc XRS-J (Bio-Rad).

Effect of SCF stimulation

For SCF stimulation test, HRMC, VIMC1, CoMS1 and CMMC1 cells were cultured in serum-free AIM-V medium for 2 hours at 37 °C before incubation with SCF. Each 10^6 cells /ml were treated with phosphate buffered saline (PBS) or various concentrations of recombinant canine SCF (*r*cSCF) (R&D systems, Mineapolis, MN) for 15 minutes at 37 °C, then collected and lysed in lysis buffer, and the protein concentrations were measured. Following SDS-PAGE of equal amounts of protein, c-Kit receptor and phosphorylated c-Kit receptor were evaluated by Western blotting analysis as described above.

To determine the effect on cell viability of canine MCT cell lines by SCF stimulation, each 10^5 cells /ml were cultured in serum-free AIM-V medium in a 96-well plate with PBS or *r*cSCF at various concentrations for indicated time periods. The cell viability in the wells was measured by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) with WST-8 as a substrate. Absorbance was measured at 450 nm using Model 680 Microplate Reader (Bio-Rad).

Detection and sequence of SCF mRNA

The primers to amplify an ORF of *SCF* were described previously (Shin et al., 2001). Using the primers, *SCF* cDNA was amplified by RT-PCR with

KOD FX (TOYOBO, Osaka, Japan) from single-strand cDNAs of canine MCT cell lines. The PCR products of *SCF* cDNA were subcloned into pCR Blunt Vector (Invitrogen) and sequenced as described above. More than 8 independent clones of the each plasmid were sequenced.

Detection of SCF protein

The equal amounts of protein on HRMC, VIMC1, CoMS1 and CMMC1 cells were separated by SDS-PAGE, and transferred to a PVDF membrane. The blotted membrane was blocked in 5% skimmed milk/tris-buffered saline with Tween20, and then probed with anti-canine SCF antibodies (1:1,000, R&D systems). After incubation with HRP-conjugated anti-goat IgG (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA), immunoreactivity was detected with ECL Plus Western Blotting Detection Reagents and visualized with ChemDoc XRS-J.

To detect SCF protein in the supernatant of cultured HRMC cells, the protein in the HRMC supernatant was concentrated by a centrifugal ultrafiltration method using Vivaspin centrifugal concentrator (GE Healthcare). The same liquid volume of concentrated HRMC supernatant and various concentration of *r*cSCF were subjected to SDS-PAGE and transferred to a PVDF membrane. Then canine SCF protein was detected by Western blotting analysis as described above.

Evaluation of signaling pathways activated by external or internal SCF/c-Kit receptor loops on HRMC

HRMC cells (10^6 /ml) were cultured in serum-free AIM-V medium with various concentrations of anti-canine SCF antibodies (as an external inhibitor) or the tyrosine kinase inhibitor Dasatinib (LC laboratories, Woburn, MA), which could inhibit canine c-Kit receptor phosphorylation (Gleixner et al., 2007) and act internally, for 15 minutes at 37 °C, and then the protein was extracted. Following SDS-PAGE of equal amounts of the protein, c-Kit receptor and phosphorylated c-Kit receptor were evaluated by Western blotting analysis as described above.

Results

Genetic statuses of KIT and c-Kit receptor phosphorylation

Genetic statuses of *KIT* in HRMC, VIMC1, CoMS1 and CMMC1 cells were analyzed and compared with the ORF of normal canine c-*kit* (Genbank accession no. AY313776). The ORF of *KIT* in HRMC cells showed two different nucleotide substitutions [159 (C → T) and 507 (A → G)] although the substitutions led to no amino acid change (Table 1). The results were consistent with the previous report (Ohmori et al., 2008). Both VIMC1 and CoMS1 cells had the same two different nucleotide substitutions [1275 (A → G) and 1523 (A → T)] of *KIT* (Table 1). While the latter led to an amino acid conversion ($Asn^{508}Ile$) in the extracellular domain (exon 9), the former did not. CMMC1 cells had at least three variants of *KIT* (Table 1). The *KIT* variant A of CMMC1 had a 3-bp deletion ($^{763\text{-}765}$Del CAG) which led to one amino acid deletion (^{255}Del Gln) in the extracellular domain (exon 5). The variant B led to an amino acid substitution ($Lys^{415}Glu$) in the extracellular domain (exon 8) and an ITD in the juxtamembrane domain ($ITD^{573\text{-}590}$) (exon 11). The variant C had a nonsense mutation in the extracellular domain. In addition, these three variants had some single nucleotide substitutions [159 (C → T), 507 (A → G), 282 (A → G) and 1275 (A → G)] which led to no amino acid change (Table 1).

To assess the function of these *KIT* mutations to c-Kit receptor activation, c-Kit receptor phosphorylation was evaluated with Western blotting analysis in the cell lines. Two kinds of c-Kit receptor were observed in all cell lines (Fig. 4)

due to differential glycosylation of the protein (approximately 145 kDa for the mature form and approximately 125 kDa for the immature form) (London et al., 1999). Apparent phosphorylation of c-Kit receptor was detected in all the cell lines (Fig. 4) although these cells were cultured with *r*cSCF-free medium. While both mature and immature forms of c-Kit receptor were substantially phosphorylated in CMMC1 cells, the immature form was slightly phosphorylated in other cell lines (Fig. 4).

Effect of SCF stimulation

Next, the effect of *r*cSCF stimulation on c-Kit receptor phosphorylation in canine MCT cell lines was examined. Phosphorylation of c-Kit receptor in HRMC, VIMC1 and CoMS1 cells was enhanced following incubation with *r*cSCF for 15 minutes whereas no enhancement of phosphorylation in CMMC1 cells was observed (Fig. 5). The enhancement was observed in only mature form of c-Kit receptor in HRMC, VIMC1 and CoMS1 cells (Fig. 5).

To evaluate the effect of *r*cSCF stimulation on proliferation of the cell lines, the cells were cultured in serum-free AIM-V medium with various concentrations of *r*cSCF. The viability of the cells was assessed at 24, 48, 72 and 96 hours (HRMC and CMMC1) or 12, 24, 36 and 48 hours (VIMC1 and CoMS1) depending on the doubling times of the cells (Ishiguro et al., 2001; Ohmori et al., 2008; Takahashi et al., 2001). There was no effect of *r*cSCF supplementation on the proliferation of all the cell lines (Fig. 6).

Expression and sequence of SCF mRNA

To evaluate the possibility that c-Kit receptor of the canine MCT cells was activated by autocrine SCF, the expression of *SCF* mRNA was examined with RT-PCR. As shown in Fig. 7A, *SCF* mRNA was amplified in all the cell lines. The 825 bp of *SCF* was a soluble form and the 741 bp of *SCF* was a membrane-bound form (Shull et al., 1992). Both forms were detected in HRMC, VIMC1 and CMMC1 cells whereas there was only a soluble form in CoMS1 cells. Compared with the ORF of normal canine *SCF* (Genbank accession no. NM_001012735), there was no mutation in the *SCF* mRNA of all the cell lines.

Production of SCF protein

The production of SCF in the canine MCT cell lines was evaluated with Western blotting analysis. As shown in Fig. 7B, SCF was detectable in only the HRMC cell lysate and undetectable in those of other cell lines.

On Western blotting analysis conducted in the present study, the detectable limit for canine SCF was 100 ng/ml (Fig. 7C). The concentration of SCF in the culture supernatant of HRMC cells was less than 1 ng/ml since SCF protein was undetectable in a hundredfold concentrated culture supernatant.

Evaluation of signaling pathways activated by external or internal SCF/c-Kit receptor loops on HRMC

According to the manufacture's instruction (R&D systems), 100 ng/ml of anti-canine SCF antibody can neutralize 1 ng/ml of extracellular SCF. A

maximum of 1,000 ng/ml of antibody led to no change of c-Kit receptor phosphorylation levels of HRMC cells (Fig. 8A).

Since the antibody can not diffuse into the cell, it has no inhibitory effect on intracellular SCF activity. By contrast, tyrosine kinase inhibitors, such as Dasatinib, are known to diffuse into the cytoplasm and can inhibit c-Kit receptor phosphorylation. In the present chapter, Dasatinib could reduce c-Kit receptor phosphorylation levels of HRMC cells in a dose dependant manner (Fig. 8B).

Discussion

In the present chapter, apparent c-Kit receptor phosphorylation was found in HRMC, VIMC1, CoMS1 and CMMC1 cells although these cells were cultured with *r*cSCF-free medium. Asn^{508}Ile substitution, which is found in *KIT* of VIMC1 and CoMS1 cells, has been previously reported in 1.6% (3/191) of canine MCT cases and led to constitutive c-Kit receptor activation without SCF binding (Letard et al., 2008). The mutation is located on exon 9 of *KIT* which encodes an extracellular ligand-binding domain of c-Kit receptor. The *KIT* mutations in the extracellular domain have been also found in human acute myeloid leukemia (AML) (Gari et al., 1999), gastrointestinal stromal tumor (GIST) (Miettinen and Lasota, 2006) and pediatric mastocytosis (Bodemer et al., 2010). A previous study suggested that oncogenic *KIT* mutations in this region led to ligand-independent c-Kit receptor dimerization by enhancing the binding affinity (Yuzawa et al., 2007). Therefore, the ligand-independent c-Kit receptor phosphorylation could be caused by the amino-acid substitution (Asn^{508}Ile) in VIMC1 and CoMS1 cells. Interestingly, both VIMC1 and CoMS1 cells had the same mutation in *KIT*. The mutation (Asn^{508}Ile) might frequently occur in this type of MCT since both cells were derived from canine visceral MCT (Ishiguro et al., 2001; Takahashi et al., 2001).

CMMC1 cells might have *KIT* gene amplification and/or chromosomal aneuploidy since the cells had at least three variants of *KIT*. A previous study indicated that chromosomal amplification of *KIT*, c-*myc* and *PAX3* occurred in canine MCT cell line CL1 (Lin et al., 2009). The ITD in the juxtamembrane

domain of *KIT*, which has been identified in variant B of CMMC1, is a common mutation in canine MCT and observed in approximately 9 to 45% of the diseases (Downing et al., 2002; Isotani et al., 2008; Letard et al., 2008; London et al., 1999; Pryer et al., 2003; Webster et al., 2006a; Zemke et al., 2002). The ITD is also found in human juvenile AML (Corbacioglu et al., 2006) and GIST (Miettinen and Lasota, 2006). The ITD may disrupt its inhibitory motif and cause ligand-independent c-Kit receptor phosphorylation since the enzyme activity of wild type c-Kit receptor in the absence of SCF is inhibited by its juxtamembrane domain (Roskoski, 2005b). The mutations in the extracellular domain of *KIT*, such as [^{255}Del Gln] in variant A or Lys^{415}Glu substitution in variant B, could also lead to ligand-independent c-Kit receptor phosphorylation as mentioned above. Multiple mutations might lead to c-Kit receptor activation in CMMC1 cells.

Consistent phosphorylation of c-Kit receptor was noted in HRMC cells although the cells had no *KIT* mutation. By contrast, there was no evidence of c-Kit receptor autophosphorylation in another canine MCT cell line, CL1, which expressed wild type c-Kit receptor (Lin et al., 2009). It was hypothesized that c-Kit receptor could be activated by external or internal autocrine SCF/c-Kit receptor loops mechanism in HRMC cells. In the external autocrine mechanism, SCF secreted from the cells might be presumed to activate its own c-Kit receptor. Neutralisation by anti-canine SCF antibody failed to alter the c-Kit receptor phosphorylation status although the SCF protein was detected in the HRMC cell lysate and was undetectable in the culture supernatant. Thus there could be no external autocrine mechanism in HRMC cells. In the internal

autocrine mechanism, intracrine SCF might be presumed to bind and activate intracellular c-Kit receptor within the cells. This unique autocrine mechanism has been described for VEGF/VEGFR loops of hematopoietic stem cell (Gerber et al., 2002) and AML cells (Santos and Dias, 2004) and angiopoietin-2/Tie-2 loops of endothelial cells (Scharpfenecker et al., 2005). The internal autocrine mechanism can be impervious to extracellular inhibitors such as antibodies for ligands since the activation events can occur within the cells. In HRMC cells, the internal inhibitor Dasatinib inhibited the c-Kit receptor phosphorylation whereas the antibody for SCF had no inhibitory effect. Therefore, there could be the internal autocrine mechanism in HRMC cells. Meanwhile, both soluble and membrane-bound forms of *SCF* mRNA were detected in HRMC cells. It was difficult to identify which form of SCF protein was expressed in HRMC cells since the antibody used in this chapter recognized both forms and the molecular weight of both forms could range from 28 to 40 kDa reflecting both N- and O-linked glycosylation (Broudy, 1997).

SCF protein was undetectable in VIMC1, CoMS1 and CMMC1 cells although mRNA expression of *SCF* was detected. In these cell lines, the amount of produced SCF protein could be too low to detect by Western blotting analysis or there could be no produced SCF protein at all by halting protein synthesis from mRNA.

Stimulation by *r*cSCF had no effect on proliferation of HRMC, VIMC1 and CoMS1 cells although it enhanced c-Kit receptor phosphorylation. Since these cells could grow in serum-free medium (Fig. 6A-C), c-Kit receptor activation might be enough for the growth to plateau before SCF stimulation.

By contrast, *r*cSCF stimulation could induce c-Kit receptor phosphorylation and enhance cell proliferation in CL1 cells which innately had no phosphorylated c-Kit receptor (Lin et al., 2009).

The *KIT* mutations in CMMC1 cells might disrupt the activation mechanism of c-Kit receptor by SCF binding since stimulation by *r*cSCF provided no enhancement of c-Kit receptor phosphorylation as well as proliferation. On the other hand, c-Kit receptor with $Asn^{508}Ile$ substitution made the activation mechanism by *rc*SCF remained in VIMC1 and CoMS1 cells. However, 250 ng/ml of recombinant murine SCF provided no enhancement on phosphorylation of $Asn^{508}Ile$ mutant c-Kit receptor transfected into murine cells (Letard et al., 2008). The discrepancy could be explained by the assumption that the $Asn^{508}Ile$ mutated canine c-Kit receptor activity might change in murine cells, or that the reactivity of the mutant c-Kit receptor to murine SCF might be weaker than that of canine SCF.

The *KIT* mRNA sequence of AY313776 was referred as that of a normal canine *KIT* in this chapter. Two different series of the canine *KIT* mRNA sequence (AY313776 and NM_001003181) is available in Genbank. Although the foremost published one was NM_001003181, the mRNA sequence of canine *KIT* collected from 191 dogs in the previous study (Letard et al., 2008) was consistent with the sequence of AY313776 (Tsai et al., 2003) whereas different from that of NM_001003181 at three nucleotides. Therefore, NM_001003181 could contain some errors or minor polymorphisms of canine *KIT* (Letard et al., 2008). Based on this information, AY313776 was considered to be more suitable as a reference sequence of canine *KIT* mRNA.

The tyrosine residues of c-Kit receptor collected from all cell lines were phosphorylated in the present chapter. Not only *KIT* mutation and canine SCF but also several cytokines in FBS might have potentials to facilitate phosphorylation of c-Kit receptor. In the present chapter, VIMC1, CoMS1 and CMMC1 cells were unable to be cultured continuously in serum-free medium while HRMC cells could grow. Since no phosphorylation of c-Kit receptor was observed in another canine MCT cell line CL1, which expressed the wild type of c-Kit receptor, after FBS starvation for 2 hours (Lin et al., 2009), the influence of FBS on the phosphorylation might dissolve for 2 hours. Therefore, the influence of FBS on the results of SCF stimulation effect on c-Kit receptor phosphorylation and cell viability might be little since all cell lines were incubated in serum-free condition for 2 hours. In addition, c-Kit receptor collected from VIMC1, CoMS1 and CMMC1 cells was still phosphorylated after FBS-free cell culture for 2 hours (Fig 5). Considering those findings, the effect of factors containing in FBS could be practically nought.

In this chapter, the four canine MCT cell lines had various aberrations associated with c-Kit receptor phosphorylation, *KIT* mutation and SCF expression. Such molecular biological diversity may reflect the different biological behavior in canine MCT. Not only mutated *KIT* but also SCF/c-Kit receptor autocrine loops can be potential therapeutic targets by TKI since c-Kit receptor of HMRC cells has consistently phosphorylated. The unique cell lines presented here will be useful for understanding the etiology and developing novel diagnostic and therapeutic procedures in mast cell malignancies.

Chapter 2

Biological effect of tyrosine kinase inhibitors on three canine mast cell tumor cell lines with various *KIT* statuses

Abstract

Clinical importance of TKIs for the treatment of canine MCT has been noticeable. In addition, some TKIs were identified as substrates for ABCB1. In this chapter, the inhibitory effect of four TKIs (axitinib, imatinib, masitinib and vatalanib) for proliferation and phosphorylation of c-Kit receptor as well as the expression and function of ABCB1 were investigated in three cMCT cell lines (HRMC, VIMC1 and CMMC1). The functional ABCB1 seemed to be expressed in only the VIMC1. The IC_{50} values of axitinib, imatinib, masitinib and vatalanib were > 10 μM, > 10 μM, 8.4 μM and > 10 μM in HRMC, 9.0 nM, 42.3 nM, 52.1 nM and 549.4 nM in CMMC1 and 4.4 nM, 157.8 nM, 133.7 nM and 611.3 nM in VIMC1, respectively. VIMC1 required higher concentrations for the inhibition of c-Kit receptor phosphorylation than those in cell growth. The CsA treatment slightly increased the effects of the TKIs on VIMC1. The results indicated that sensitivity to TKIs in a canine MCT cell line harboring wild-type *KIT* was lower than that in canine MCT cell lines with mutated *KIT.* However, the growth of VIMC1 was seemingly reduced by TKIs through the inhibition of the activity of other tyrosine kinases than c-Kit receptor. Moreover, ABCB1 can play a role in resistance to the treatment of TKIs in canine MCT, on which ABCB1 is overexpressed. The results obtained in this chapter will be helpful to understand the different sensitivity to TKIs in canine patients with MCT.

Introduction

TKIs are designed as small organic molecules blocking the ATP-binding site of kinases (Bennasroune et al., 2004). Some studies reported that the some TKIs could inhibit c-Kit receptor activation and proliferation of canine MCT cells with *KIT* mutations (Gleixner et al., 2007; Liao et al., 2002; Peter et al., 2010). The clinical efficacy of several TKIs for canine MCT cases has been studied (Hahn et al., 2008; Isotani et al., 2008; Lin et al., 2009; London et al., 2009). A clinical study reported that 100% of canine MCT cases with *KIT* mutation responded to treatment with imatinib (Isotani et al., 2008). Other studies indicated that canine MCT cases with *KIT* mutations were more likely to respond to treatment with toceranib or masitinib than those without mutations (Hahn et al., 2008; London et al., 2009). Meanwhile, some dogs with MCT which had no *KIT* mutation showed beneficial response to imatinib treatment (Isotani et al., 2008). Previous chapter revealed that the c-Kit receptor was consistently phosphorylated in HRMC cells although the cells had no *KIT* mutation, indicating a SCF/c-Kit receptor autocrine loop. Based on these result, I hypothesized that not only mutated c-Kit receptor but also SCF/c-Kit receptor autocrine loops could be potential therapeutic targets by TKIs.

ABCB1, also known as P-glycoprotein and encoded by *MDR1* gene, is a member of the ATP-binding cassette transporter superfamily and functions as an ATP-dependent efflux transporter (Zhou, 2008). ABCB1 is membrane-bound protein comprising 12 transmembrane domains, two ATP binding/hydrolysis sites and a phosphorylated linker region, and can efflux a

diverse range of structurally and functionally unrelated compounds (Zhou, 2008). In both human and veterinary medicine, overexpression of ABCB1 is considered to be one of the common mechanism of multidrug resistance on tumor cells since its substrates includes some chemotherapeutic agents such as vinca-alkaroids, anthracyclines and antifolates (Schrickx and Fink-Gremmels, 2008; Zhou, 2008). Several studies revealed that both *MDR1* mRNA and ABCB1 protein were expressed in canine MCT cells (Miyoshi et al., 2002; Nakaichi et al., 2007). According to the recent studies (Hegedus et al., 2009; Kitazaki et al., 2005; Mahon et al., 2003), some TKIs were also identified as the substrate for ABCB1. Therefore, there could be the implications of ABCB1 overexpression for drug-resistance of TKIs in canine MCT.

The objective of this chapter was to investigate the biological effects of several TKIs and the implication of ABCB1 on these effects in canine MCT. In the present chapter, the expression and function of ABCB1 were characterized, and inhibitory effects of the TKIs on cell proliferation and phosphorylation of c-Kit receptor were evaluated in 3 canine MCT cell lines with various *KIT* statuses. In addition, the influences of ABCB1 function were examined using inhibition of the drug efflux by another ABCB1 substrate, cyclosporine (CsA).

Materials and Methods

Canine mast cell tumor cell lines

Three canine MCT cell lines (HRMC, CMMC1 and VIMC1) were used in this chapter. A study in the previous chapter in this thesis revealed that the c-Kit receptor of these cell lines was consistently phosphorylated in stem cell factor-free medium. The study revealed that HRMC cells expressed wild type c-Kit receptor. CMMC1 had multiple mutations of *KIT* including ITD within its juxtamembrane domain, and VIMC1 had a point mutation within *KIT* extracellular domain (Table 1).

Tyrosine kinase inhibitors

Four TKIs (axitinib, imatinib, masitinib and vatalanib) were used in this chapter. Axitinib inhibits activity of vascular endothelial growth factor receptors (VEGFRs) 1, 2 and 3, platelet-derived growth factor receptors (PDGFRs) α and β and c-Kit receptor (Hu-Lowe et al., 2008). Imatinib inhibits c-Abl, PDGFRβ and c-Kit receptor (Manley et al., 2002). Masitinib inhibits PDGFRs α and β, Lyn and c-Kit receptor (Dubreuil et al., 2009). Vatalanib inhibits VEGFRs 1 and 2, PDGFRβ, c-Fms and c-Kit receptor (Hess-Stumpp et al., 2005). Axitinib was purchased from LKT Laboratories (Westin St.Paul, MN). Imatinib, masitinib and vatalanib were purchased from LC laboratories (Woburn, MA).

Detection of MDR1 mRNA

Total RNA extracted from HRMC, CMMC1 and VIMC1 cells were used for cDNA synthesis as described previously (Chapter 1). The primers to amplify canine *MDR1* were described previously (Nakaichi et al., 2007). Using the primers, *MDR1* cDNA was amplified by RT-PCR with KOD FX from single-strand cDNAs of canine MCT cell lines.

Detection of ABCB1 protein

Whole cell lysates were extracted from HRMC, CMMC1 and VIMC1 cells as described previously (Chapter 1). Protein concentrations were determined with the Lowry protein assay kit. The equal amounts of protein were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in 5% skimmed milk/tris-buffered saline with Tween20, and then incubated with primary antibodies against ABCB1 (1:100, Merk Calbiochem, Darmstadt, Germany) and β-actin (1:1,000, Cell Signaling Technology). After incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase (1:5,000, GE Healthcare), positive immunoreactivity was detected with ECL Plus Western Blotting Detection Reagents and visualized with ChemDoc XRS Plus (Bio-Rad).

Flowcytometric detection of rhodamine 123 efflux function

HRMC, CMMC1 and VIMC1 cells (10^6 cells) were incubated with 200

ng/ml of Rhodamine 123 (Rh123) (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 medium at 37 °C for 20 minutes. After washing twice in phosphate buffered saline (PBS), the cells were then incubated in Rh123-free medium at 37 °C for 60 minutes, either with or without 2 μM of CsA (Wako Pure Chemical Industries, Osaka, Japan). After the incubation, the cells were washed with ice-cold PBS in 3 times, placed on ice in the dark, and subjected to flowcytometric analysis. The cells that had not been exposed to Rh123 were used as controls. Ten thousands events were counted by a flow cytometer (FACScan; Becton Dickinson, San Jose, CA) equipped with 488 nm argon laser and 530 nm bandpass filter.

Antiproliferation effect of tyrosine kinase inhibitors

To determine the antiproliferative effect of canine MCT cell lines by TKIs, each 2 × 10^5 cells /ml were cultured in appropriate medium in a 96-well plate with or without axitinib, imatinib, masitinib and vatalanib at various concentrations for indicated time periods. VIMC1 cells were also cultured in RPMI-1640 medium containing 5 μM of CsA with various concentrations of the TKIs. The cell viability in the wells was measured by using Cell Counting Kit-8. Absorbance was measured at 450 nm using Model 680 Microplate Reader. In addition, 50% inhibitory concentration (IC_{50}) values of the TKIs were determined after a 48-hour culture of the cell lines.

Inhibitory effect of tyrosine kinase inhibitors on c-Kit receptor

phosphorylation

HRMC, CMMC1 and VIMC1 cells (10^6 /ml) were cultured with various concentrations of axitinib, imatinib, masitinib and vatalanib for 4 hours at 37 °C, and then the proteins were extracted. In addition, VIMC1 cells were also cultured in RPMI-1640 medium containing 5 μM of CsA with the same concentrations of the TKIs, and then the protein was collected. Following SDS-PAGE of equal amounts of the protein, c-Kit receptor and phosphorylated c-Kit receptor were evaluated by Western blotting analysis as described previously (Chapter 1).

Results

Expression of MDR1 mRNA and ABCB1 protein

The expression of *MDR1* mRNA and ABCB1 protein were examined with RT-PCR and Western blotting analysis, respectively, in canine MCT cell lines. As shown in Fig. 9A, the *MDR1* mRNA was amplified in CMMC1 and VIMC1 cells, whereas not in HRMC cells. The *MDR1* mRNA in VIMC1 cells was expressed stronger than in CMMC1 cells.

On Western blotting analysis, three kinds of ABCB1 protein were observed in CMMC1 and VIMC1 cells (Fig. 9B) due to differential glycosylation of the protein (approximately 140 kDa for the mature form and approximately 120 and 100 kDa for the immature forms) (Blott et al., 1999), whereas weakly in HRMC cells.

Rhodamine 123 efflux function

Although the intracellular accumulation of Rh123, which is the dynamic traffic of a fluorescent dye, occurs by passive effusion, the efflux from cells actively depends on ATP-dependent transport through ABCB1 (Tang et al., 2004). To evaluate ABCB1 function as a transporter in canine MCT cell lines, the inhibition of Rh123 efflux by CsA was measured using flowcytometry. In VIMC1 cells, the addition of 2 μM CsA increased Rh123 accumulation compared to that for the cells without CsA treatment (Fig. 10C). On the other hand, the Rh123 accumulation for HRMC and CMMC1 cells hardly changed by

the addition of CsA (Figs. 10A and B). Therefore, the functional ABCB1 seemed to be expressed in only VIMC1 cells.

Antiproliferation effect of tyrosine kinase inhibitors

To assess the antiproliferative effect of TKIs on canine MCT cell lines, the cells were cultured with axitinib, imatinib, masitinib and vatalanib at various concentrations based on the maximum plasma concentration (C_{max}) values of the TKIs (Hahn et al., 2008; Ishizuka et al., 2007; Jost et al., 2006; Pithavala et al., 2010). The viability of the cells was assessed at 24, 48, 72 and 96 hours (HRMC and CMMC1) or 12, 24, 36 and 48 hours (VIMC1) depending on the doubling times of the cells (Ohmori et al., 2008; Takahashi et al., 2001). The proliferation of HRMC cells was consistently inhibited by 10 μM masitinib (Fig. 11C) and slightly by 10 μM imatinib (Fig. 11B), whereas hardly inhibited by axitinib and vatalanib (Figs. 11A and D). That of CMMC1 cells was inhibited by at least 10 nM axitinib (Fig. 11E), 0.1 μM imatinib (Fig. 11F), 0.1 μM masitinib (Fig. 11G) and 1μM vatalanib (Fig. 11H). VIMC1 cells were unable to proliferate in 10 nM axitinib (Fig. 11I), 1 μM imatinib (Fig. 11J), 1 μM masitinib (Fig. 11K) and 1μM vatalanib (Fig. 11L). The IC_{50} values of these TKIs determined after a 48-hour culture of the cell lines are shown in Table 2.

Inhibitory effect of tyrosine kinase inhibitors on c-Kit receptor phosphorylation

The inhibitory effect of TKIs on c-Kit receptor phosphorylation was

evaluated by Western blotting analysis in the cell lines. As shown in Fig. 12A, 10 μM of imatinib and masitinib inhibited slightly the phosphorylation of c-Kit receptor in HRMC cells, whereas axitinib and vatalanib did hardly. The phosphorylation of c-Kit receptor in CMMC1 and VIMC1 cells was inhibited by all four TKIs in a dose dependent manner (Figs. 12B and C). The signal of c-Kit receptor phosphorylation was strongly reduced by all TKIs in CMMC1 cells (Fig. 4B). In contrast, the inhibitory effect in VIMC1 cells was milder than that in CMMC1 cells (Fig. 12C). The TKI treatments did not change the expression level of c-Kit receptor in all cell lines.

Influences of ABCB1 function on biological effects of tyrosine kinase inhibitors in VIMC1 cells

To determine the implications of ABCB1 for biological effects of the TKIs, the changes in the outcome of CsA treatment were evaluated in VIMC1 cells. As shown in Fig. 13, the treatment with 5 μM of CsA increased the sensitivities to the TKIs in VIMC1 cells. The IC_{50} values of axitinib, imatinib, masitinib and vatalanib in VIMC1 cells cultured without CsA were 1.4 times, 1.7 times, 2.3 times and 1.3 times higher than those in the cells cultured with 5 μM of CsA, respectively (Table 2). The CsA treatment also reinforced slightly the inhibitory effects of TKIs on c-Kit receptor phosphorylation in VIMC1 cells (Figs. 4C and D). The CsA treatment did not change the expression level of c-Kit receptor in VIMC1 cells.

Discussion

In the present chapter, both *MDR1* mRNA and ABCB1 protein were detected in CMMC1 and VIMC1 cells. However, CMMC1 cells did not show apparent Rh123 efflux function (Fig. 10B). ABCB1 of CMMC1 cells could lose drug transport function by loss-of-function mutation of *MDR1* or halting its activation process such as binding of ATP, nucleotide or substrate. On the other hand, VIMC1 cells showed apparent Rh123 efflux function (Fig. 2C). The results could reflect the function of ABCB1 of the cells; however, it might not exclude the possibility of Rh123 efflux by other transporters. Rh123 is extruded by another ABC transporter, such as $Arg^{482}Thr$ mutant ABCG2 (Honjo et al., 2001). According to the previous study (Nakaichi et al., 2007) using the parental cells which were not cloned (CMMC and VIMC cells), both mRNA and protein of ABCB1 were not expressed in CMMC cells whereas expressed in VIMC cells. The discrepancy could be explained by the assumption that ABCB1 had expressed in the cloning process of CMMC1 cells.

A maximum of 10 μM of axitinib, imatinib and vatalanib could not inhibit by 50% growth of HRMC cells which express wild type of c-Kit receptor. The IC_{50} value of only masitinib (8.4 μM) for HRMC cells could be calculated. However, the value was also unreasonable clinical concentration since the C_{max} value of masitinib in serum of beagle dogs administrated at an oral dose of 10 mg/kg was 1.3 to 1.5 μM (Hahn et al., 2008). Correspondingly, phosphorylation of c-Kit receptor was slightly inhibited under high concentrations (10 μM) of imatinib and masitinib in HRMC cells (Fig. 12A).

By contrast, reasonable concentration of all four TKIs inhibited the cell proliferation and phosphorylation of c-Kit receptor in CMMC1 cells with ITD of juxtamembrane domain and 3 mutations in the extracellular domain in *KIT*. The results were consistent with previous reports describing that another canine MCT cell line, C2, which had ITD of juxtamembrane domain in *KIT* was sensitive to the several TKIs such as imatinib, midostaurin, nilotinib, dasatinib, toceranib and bafetinib (Gleixner et al., 2007; Liao et al., 2002; Peter et al., 2010). According to some clinical studies, 100% and 60% of canine MCT case harboring ITD of juxtamembrane domain showed beneficial response (complete or partial remission) to imatinib and toceranib, respectively (Isotani et al., 2008; London et al., 2009).

Interestingly, the growth of VIMC1 cells with a point mutation in the extracellular domain of *KIT* was almost completely inhibited by 10 nM of axitinib as well as 1 μM of imatinib, masitinib and vatalanib (Figs. 11I-L) although the concentration of these TKIs required for an inhibition in the phosphorylation of c-Kit receptor was higher level (Fig. 12C). The targets of the TKIs used in this chapter were not only c-Kit receptor but also other known (VEGFRs, PDGFRs, c-Abl, Lyn and c-Fms) and unknown kinases (Dubreuil et al., 2009; Hess-Stumpp et al., 2005; Hu-Lowe et al., 2008; Manley et al., 2002). Therefore, the TKIs could suppress the proliferation of VIMC1 cells by inhibiting the activity of other kinases than c-Kit receptor in the present chapter. A previous study (Rebuzzi et al., 2007) revealed that VEGFRs 1 and 2 were expressed in canine MCT cells. In addition, a clinical study indicated that 31% of the dogs with MCT which had no *KIT* mutation showed beneficial response

to imatinib which could inhibit c-Abl, PDGFRβ and c-Kit receptor (Isotani et al., 2008). According to the present and previous observations, not only c-Kit receptor but also other tyrosine kinases could play a role in growth and survival of canine neoplastic mast cells.

Inhibition sensitivity of the TKIs on phosphorylation in c-Kit receptor with wild type and that with a point mutation in the extracellular domain were lower than that with multiple mutations including ITD of juxtamembrane domain in the present chapter (Fig. 12). The binding affinity to the TKIs of ATP-binding site of c-Kit receptor in CMMC1 cells might be higher than that of wild type c-Kit receptor in HRMC cells since the ITD mutation within the juxtamembrane domain could alter the cytoplasmic structure including the ATP-binding sites. By contrast, the affinity to TKIs of mutated c-Kit receptor in VIMC1 cells might be as low as that of wild type c-Kit receptor since the point mutation in the extracellular domain could not alter the intracellular structure.

CsA mildly increased inhibitory effects of the TKIs on cell proliferation and phosphorylation of c-Kit receptor in VIMC1 cells (Figs. 12C, 12D and 13). The four TKIs could be the substrates for canine ABCB1 since CsA increased the effects of TKIs on VIMC1 cells which expressed functional ABCB1. Therefore, ABCB1 may play a role in resistance to the treatment of TKIs in canine MCT if ABCB1 is overexpressed in neoplastic cells.

In this chapter, sensitivity to TKIs in the canine MCT cell line harboring wild-type *KIT* was lower than that in canine MCT cell lines with mutated *KIT*. However, the growth of the canine MCT cell line with a point mutation in the

extracellular domain of *KIT* was seemingly suppressed by TKIs through the inhibition of the activity of other tyrosine kinases than c-Kit receptor. The results obtained in this chapter will be helpful to understand the different sensitivity to TKIs in canine patients with MCT. In addition, not only imatinib and masitinib but also axitinib and vatalanib will have beneficial applicability in the treatment of canine MCT.

Chapter 3

Screening of therapeutic targets for canine mast cell tumors from a variety of tyrosine kinase molecules

Abstract

Options of systemic treatment for canine MCT have been still limited and most canine cases with MCTs eventually undergo relapses even after achievement of a remission. Thus additional therapies are required to establish for the tumor. To identify the novel candidate therapeutic targets for canine MCT, the mRNA expression and phosphorylation statuses of several receptor or non-receptor kinases as well as the inhibitory effect of 95 specific inhibitors on the growth were assessed in three canine MCT cell lines (HRMC, VIMC1 and CMMC1). Among the 14 targets, the mRNAs of 11, 7 and 7 kinases were amplified in HRMC, VIMC1 and CMMC1, respectively. The mRNAs of *VEGFR3, PDGFRα, SRC, YES, LCK* and *FYN* were detected in all cell lines. The phosphorylation of 12, 8 and 7 kinases was observed by using specific antibody arrays in HRMC, VIMC1 and CMMC1, respectively. DTK, EPHB6, AMPKα1, CREB, STAT5a and STAT5b were phosphorylated in all cell lines. The 10, 9 and 17 inhibitors exhibited the biological activity against the growth of HRMC, VIMC1 and CMMC1, respectively. Only three inhibitors such as SB218078 (for Chk1), PDGF RTK inhibitor IV (for PDGFR) and radicicol (for Hsp90) suppressed the growth of all three cell lines. The present chapter indicated that several kinases could be used as therapeutic targets in the treatment for canine MCT. Further studies and clinical trials are warranted to apply the inhibitors for the treatment of the tumor.

Abbreviations; AMPK: AMP-activated protein kinase, CDK: Cyclin-dependent

AMPK: AMP-activated protein kinase, CDK: Cyclin-dependent kinase, Chk: Checkpoint kinase, CREB: cAMP response element binding protein, CSF1R: Colony stimulating factor 1 receptor, EGFR: Epidermal growth factor receptor, EPHA2: Ephrin type-A receptor 2, EPHB6: Ephrin type-B receptor 6, FLT3: FMS-like tyrosine kinase 3, HDAC: Histone deacetylase, Hsp90: Heat shock protein 90, InsulinR: Insulin receptor, JNK: c-Jun N-terminal kinase, MCT: Mast cell tumor, PKC: Protein kinase C, PKR: Protein kinase RNA-activated, PDGFR: Platelet-derived growth factor receptor, STAT: Signal transducers and activator of transcription, VEGFR: Vascular endothelial growth factor receptor.

Introduction

Recently, clinical efficacy of several TKIs such as toceranib, masitinib and imatinib for canine MCT cases has been studied (Hahn et al., 2008; Isotani et al., 2008; London et al., 2009). However, the overall response rates of the TKIs against canine MCT were only 42 to 48% (Isotani et al., 2008; London et al., 2009). In addition, most canine cases with MCTs eventually undergo relapses even after achievement of a remission with TKIs treatment. Therefore, the efficacy of several novel agents such as HSP90 inhibitor (Lin et al., 2008), HDAC inhibitors (Kisseberth et al., 2008; Lin et al., 2010) and calcitriol which is the ligand for vitamin D receptor (Malone et al., 2010) for canine MCT have recently been examined.

Chapter 1 revealed that the c-KIT receptor of VIMC1 cells, a MCT cell line harboring a point mutation in the extracellular domain of *KIT*, was consistently phosphorylated in ligand-free medium. Chapter 2 subsequently indicated that the concentration of some TKIs required for inhibition of the phosphorylation of c-Kit receptor was higher than that of cell growth in VIMC1 cells. Since targets of the TKIs were not only c-Kit receptor but also other kinases, the growth of VIMC1 cells could be suppressed by TKIs through inhibition of activity of other kinases than c-Kit receptor. A previous study revealed that VEGFR1 and VEGFR2 were expressed in canine MCT cells (Rebuzzi et al., 2007). In addition, a clinical study indicated that 31% of the dogs with MCT harboring wild type *KIT* showed beneficial response to imatinib (Isotani et al., 2008). According to the previous observations, it was

hypothesized that not only c-Kit receptor but also other kinases could play a role in growth and survival of canine neoplastic mast cells.

The objective of the present study was to screen candidate molecular therapeutic targets for canine MCT. The mRNA expression and phosphorylation statuses of several kinases in three canine MCT cell lines were assessed with RT-PCR and specific antibody arrays, respectively. The inhibitory effect of specific inhibitors on the proliferation was also examined in the cell lines.

Materials and Methods

Canine mast cell tumor cell lines

Three canine MCT cell lines (HRMC, VIMC1, and CMMC1) were recruited. HRMC and CMMC1 were originated from cutaneous MCT (Ohmori et al., 2008; Takahashi et al., 2001). VIMC1 was derived from visceral MCT (Takahashi et al., 2001).

Detection of mRNA expression for tyrosine kinases

Total RNA extracted from HRMC, VIMC1 and CMMC1 cells were subjected to cDNA synthesis as described previously (chapter 1). Using the primers listed in Table 3, *VEGFR1, VEGFR2, VEGFR3, CSF1R, PDGFRα, PDGFRβ, EPHA2, FLT3, RET, EGFR, SRC, YES, LCK* and *FYN* were amplified by RT-PCR with KOD-FX. The tyrosine kinases encoded by the genes are inhibited by clinically approved tyrosine kinase inhibitors such as dasatinib, gefitinib, imatinib, sorafenib and sunitinib. The PCR amplification consisted of pre-denaturation (94 °C, 2 min) and 35 cycles of denaturation (98 °C, 30 sec), annealing (60 °C, 30 sec) and extension (68 °C, 1 min), followed by final extension (68 °C, 8 min). The amplified PCR products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide and visualized with ultraviolet illumination.

Analysis of the phosphorylation profiles of receptor tyrosine kinases

The phosphorylation of RTKs in canine MCT cell lines was examined by using Human Phospho-RTK Array Kit (ARY001; R&D systems). The kit was used to identify the phosphorylation profiles of 42 different RTKs. Whole cell lysates were extracted from HRMC, VIMC1 and CMMC1 cells in ice-cold NP-40 lysis buffer consisting of 20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4 and Protease Inhibitor Cocktail. Protein concentrations were determined with Lowry protein assay kit (Bio-Rad). Nitrocellulose membranes spotted in duplicate with the antibodies for RTKs were blocked in Array Buffer 1 supplied in ARY001 (R&D systems) for 1 hr at room temperature, and then incubated with each protein lysate (400 μg total protein) for overnight at 4 °C. After incubation with HRP-conjugated anti-phospho-tyrosine antibody supplied in ARY001 (R&D systems) for 2 hrs at room temperature, positive immunoreactivity was detected with ECL Plus Western Blotting Detection Reagents and visualized with ChemDoc XRS-J (Bio-Rad).

Analysis of the phosphorylation profiles of non-receptor kinases

The protein phosphorylation of non-receptor kinases in the cell lines was examined by using Human Phospho-Kinase Array Kit (ARY003, R&D systems). The kit was used to identify the phosphorylation profiles of 27 different non-receptor kinases. The cells were collected and lysed in Lysis Buffer 6 supplied in ARY003, and the protein concentrations were measured. The membranes spotted in duplicate with the capture antibodies against non-receptor kinases were blocked in Array Buffer 1, and then incubated with

each 400 μg of protein for overnight at 4 °C. After incubation with diluted detection antibody cocktails supplied in ARY003 for 2 hrs at room temperature and streptavidin-HRP supplied in ARY003 for 30 min at room temperature, the immunoreactivity was detected with ECL Plus Western Blotting Detection Reagents and visualized with ChemDoc XRS-J.

Antiproliferation effect of inhibitors

To examine the growth inhibitory effect of canine MCT cell lines by various chemical inhibitors, each 2×10^5 cells /ml were cultured in appropriate medium in a 96-well plate with DMSO or each 1 μM of 95 inhibitors for more than 40 targets supplied in SCADS inhibitor kit III (provided by Screening Committee of Anticancer Drugs, Japan) for indicated time periods at 37 °C. The inhibitors and their targets are represented in Table 5. The cell viability in the wells was measured by using Cell Counting Kit-8 with WST-8 as a substrate. Absorbance was measured at 450 nm using Model 680 Microplate Reader (Bio-Rad).

Results

Detection of mRNA expression for tyrosine kinase

Among the 14 targets, the mRNAs of 11, 7 and 7 kinases were amplified in HRMC, VIMC1 and CMMC1 cells, respectively (Fig. 14). The mRNAs expressed in the cell lines were represented on Table 4. The mRNAs of *VEGFR3, PDGFRα*, *SRC, YES, LCK* and *FYN* were detected in all cell lines.

Phosphorylation profiles of receptor tyrosine kinases and non-receptor kinases

In general, activated RTKs are phosphorylated whereas inactivated RTKs are not. The phosphorylated RTKs captured on membranes were detected by anti-phospho-tyrosine antibodies in ARY001. On the other hand, the specific phosphorylation of activated non-receptor kinases was detected in ARY 003. The results of ARY001 and ARY003 in canine MCT cell lines were shown in Figs. 15 and 16, respectively. The phosphorylated RTKs and non-receptor kinases were listed on Table 4. Among the kinases detected by RT-PCR, the phosphorylation of EGFR and VEGFR2 in HRMC cells and VEGFR2 in VIMC1 cells were observed.

Antiproliferation effect of inhibitors

To evaluate the inhibitory effect of the 95 inhibitors specifically-targeted

to various molecules on proliferation of the cell lines, the cells were cultured with each 1 μM of the inhibitors. The viability of the cells was assessed at 72 (HRMC and CMMC1) or 36 hrs (VIMC1) depending on the doubling times of the cells (Ohmori et al., 2008; Takahashi et al., 2001). As shown in Table 5, 10, 9 and 17 inhibitors suppressed more than 50% growth of HRMC, VIMC1 and CMMC1 cells, respectively.

Discussion

In the present chapter, DTK, EPHB6, AMPKα1, CREB, STAT5a and STAT5b were phosphorylated in all canine MCT cell lines (Figs. 15 and 16). DTK, also known as Tyro-3, is TAM receptor tyrosine kinase and the receptor of both Gas6 and Protein S (Linger et al., 2008). Although DTK is overexpressed in human myeloid leukemia and myeloma cells, its function and downstream signals are poorly understood (Linger et al., 2008). EPHB6 is identified as one of the type B ephrin receptor and plays a role in many biological process such as embryonic development, cell proliferation and differentiation (Castano et al., 2008). AMPKα1 is a subunit of AMPK which is a key enzyme of cellular energy homeostasis (Canto and Auwerx, 2010). The phosphorylation of AMPK leads to the activation of many stimulus-induced transcription factors including CREB (Canto and Auwerx, 2010). The constitutive activation of CREB can play a role in oncogenesis in many human tumors such as acute myeloid leukemia and adult T-cell leukemia (Siu and Jin, 2007). Both STAT5a and STAT5b are also transcription factors and phosphorylated in the course of downstream signaling cascades of many receptors (Buitenhuis et al., 2004). If the kinases play a role in the growth, proliferation and survival of malignant canine mast cells, they could be the therapeutic targets for canine MCT. Unfortunately, the SCADS inhibitor kit III used in this chapter included no inhibitors that targeted those kinases except for AMPK. More than 50% growth of VIMC1 and CMMC1 was suppressed by 1 μM of the compound C, the AMPK inhibitor, whereas that of HRMC cells was not (Table 5). The

AMPK inhibitor could suppress insufficiently the proliferation of HRMC cells since the phosphorylation signal of AMPKα1 in HRMC cells was weaker than that in VIMC1 and CMMC1 cells (Fig. 15).

Among the 95 inhibitors consisted in SCADS inhibitor kit III, only three inhibitors such as SB218078 (for Chk1), PDGF RTK inhibitor IV (for PDGFR) and radicicol (for Hsp90) suppressed more than 50% growth of all three cell lines (Table 5). Chk1 is a serine/threonine kinase and regulates both S and G_2-M checkpoints (Tse et al., 2007). If cells are exposed to the genotoxic stress such as radiation and chemotherapy, Chk1 is phosphorylated, resulting in a swift G_1-S delay following DNA damage. Some phase I trials of the combination therapy with the inhibitors and DNA-damaging agents have already been performed since the Chk1 inhibitor can sensitize the tumor cells to DNA damage (Tse et al., 2007). Since PDGFRs have been demonstrated to play important roles in many human cancers, a large number of PDGFR inhibitors have been developed (Dai, 2010). The phosphorylation of PDGFRα was undetectable in all canine MCT cell lines (Fig. 15) although the *PDGFRα* mRNA was detected in the cells (Fig. 14). The protein of canine PDGFRα might be unable to be captured by the antibody in ARY001 while it was developed as an antibody recognizing human PDGFRα. Nevertheless, further studies are required to determine whether PDGFRα is a potential therapeutic target for canine MCT. Hsp90 is a cellular chaperone and works as a master regulator of the stability and activity of multiple oncoproteins such as c-Kit receptor, EGFR, B-Raf and AKT in many human cancers (Porter et al., 2010). Therefore, the inhibition of Hsp90 retains the potential to shut down multiple oncogenic

signaling pathways simultaneously. A previous study suggested that the Hsp90 inhibitor, STA-9090, down-regulated expression and phosphorylation of both mutated and wild type c-Kit receptors and suppressed the growth of both normal and malignant canine mast cells (Lin et al., 2008). According to the observations, Hsp90 could be a potent therapeutic target for canine MCT.

Although the phosphorylation of VEGFR2, EGFR, InsulinR, p38α and JNK was observed in HRMC cells, 1 μM of the inhibitors targeting the kinases hardly suppress the growth (Table 5). The discrepancy could be explained by the assumption that the kinases might be less important for the growth and survival of the cells. In contrast, one of the inhibitors of AKT, which was phosphorylated in HRMC cells, exhibited biological activity against the cell lines (Table 5). AKT is a serine/threonine protein kinase that plays a key role in multiple cellular signaling such as cell proliferation, apoptosis, transcription and cell migration (Carnero et al., 2008). Since AKT activation is a secondary signaling event of the phosphorylation of c-Kit receptor (Gilfillan and Rivera, 2009) and c-Kit receptor of HRMC cells has been consistently phosphorylated (chapter 1), the AKT of the cells can be phosphorylated. In addition, 1 μM of the inhibitors of CDK, PKC or Aurora kinase also suppressed more than 50% growth of HRMC cells (Table 5). CDK is a crucial regulator of cell cycle progression and its overactivation has been observed in many human cancer cells (Shapiro, 2006). PKC is a serine/threonine protein kinase that regulates various cellular functions including adhesion, secretion, proliferation and apoptosis (Konopatskaya and Poole, 2010). Aurora kinase is also a serine/threonine kinase which functions as a key regulator of the mitosis

process and has been overexpressed in a number of human cancers (Lok et al., 2010). HRMC cells which expressed wild type c-Kit receptor showed low sensitivity to some clinically approved TKIs (chapter 2) and a considerable number of canine MCT patients without *KIT* mutation hardly responded to the TKI treatments (Hahn et al., 2008; Isotani et al., 2008; London et al., 2009). According to the present findings, the inhibitor targeting Chk1, PDGFR, Hsp90, AKT, CDK, PKC or Aurora kinase might be effective in canine MCT cases without *KIT* mutation.

The inhibitors of AMPK, FGFR, PDGFR, InsulinR, c-Kit receptor, PKR, Raf, Hsp90 and VEGFR suppressed more than 50% growth of VIMC1 cells in this chapter (Table 5). Chapter 2 suggested that the growth of VIMC1 cells could be suppressed by TKIs through the inhibition of the activity of not only c-Kit receptor but also other kinases. The activation of the kinases might be important in the growth and survival of VIMC1 cells. Since SU4984 (for FGFR, PDGFR and InsulinR), SU11652 (for PDGFR, VEGFR, FGFR and c-Kit) and VEGFR RTK inhibitor II (for VEGFR and c-Kit) were multiple kinase inhibitors, it was difficult to identify the actual target of the inhibitors for VIMC1 cells. The 2 specific VEGFR inhibitors (VEGF receptor 2 kinase inhibitor I and SU1498) hardly suppressed the growth of VIMC1 cells (Table 5) although both mRNA expression and phosphorylation of VEGFR2 were observed in the cells (Figs. 14 and 15). Therefore, further studies on the genetic mutation as well as the protein activity are warranted to confirm this observation.

CMMC1 cells were relatively sensitive to a number of inhibitors consisted in SCADS inhibitor kit III (Table 5). The cells harboring ITD

mutation in the juxtamembrane domain of *KIT* also showed high sensitivity to some TKIs in chapter 2. It could be explained by the assumption that the TKIs could suppress the proliferation of CMMC1 cells by inhibiting the activity of not only c-Kit receptor but also the other kinases.

SRC family kinases including SRC, YES, LCK and FYN are non-receptor tyrosine kinases and initiate intracellular signaling in response to ligation of antigen-receptors in lymphocytes, mast cells and macrophages (Bradshaw, 2010). Although mRNAs of the four kinases were expressed in all cell lines (Fig. 13), no phosphorylation of those was observed. In addition, the inhibitors of SRC family kinases hardly suppressed the proliferation of all three cell lines except for the LCK inhibitor in CMMC1 cells (Table 5). Considering the findings, SRC family kinases were unlikely to be therapeutic targets for canine MCT.

The important limitation of this chapter was whether the antibodies in ARY001 and ARY003 could recognize canine proteins. Further studies are crucial to confirm the phosphorylation profiles of the kinases in the cell lines.

In the present chapter, several kinases retaining the potential to become therapeutic targets against canine MCT were screened. Inhibitors of the candidate kinases should be selected by specificity for tumor cells since the kinases also play important roles in various cellular functions of many normal cells. In addition, the three canine MCT cell lines showed various sensitivities to the inhibitors in SCADS inhibitor kit III. Such diversity might reflect the different biological behavior in canine MCT. The present findings would pave the way for an additional medical treatment for canine MCTs.

Chapter 4

Genetic statuses of *KIT* and phosphorylation statuses of c-Kit receptor in canine mast cell tumor patients

Abstract

Some recent studies have revealed that 15 to 40% of canine MCTs contained *KIT* mutation. However, there are few studies that investigate the full-length of *KIT* ORF in canine MCTs. In this chapter, the nucleotide sequence of *KIT* ORF and phosphorylation of c-Kit receptor were examined in the 36 canine MCT samples obtained from 33 dogs. In 3 dogs, tissue samples were collected twice when the tumors relapsed. The ORF (1 – 2928) of *KIT* in 35 canine MCTs and a partial sequence (772 - 2928) of 1 tumor were determined. *KIT* sequences of the 3 relapse tumors were identical to those of the respective original tumors. Genetic alterations of *KIT* were found in 25 (75.8%) of the 33 dogs. The *KIT* alterations included the mutations, such as 23-bp deletion of exon 2 (n = 1), complete deletion of exons 6 and 7 (n = 1), 3-bp deletion (n = 1), point mutation (n = 1) and ITD mutation (n = 5) of exon 11. Two genetic alterations, which were observed in normal canine *KIT* variations, such as 3-bp deletion of exon 5 (n = 16) and 12-bp insertion at the junction of exons 9 and 10 (n = 5), were also identified. The phosphorylation of c-Kit receptor was observed in 12 tumors with and without *KIT* mutation. In addition, the Rh123 efflux function was assessed in the tumor cells obtained from an imatinib resistant canine MCT case with ITD mutation of *KIT*. As a result, there was no function of ABC transporters in the tumor cells. In this chapter, the various known and unknown mutations of *KIT* were found in canine MCTs. The c-Kit receptor activation could play a role in the proliferation and survival of the canine MCTs without *KIT* mutation. The results obtained in this chapter will

be helpful in clarifying the implication between the *KIT* mutation and the prognosis and tumorigenesis of canine MCT.

Introduction

Traditionally, based on the World Health Organization clinical stage (Owen, 1989) and histological grade (Patnaik et al., 1984), treatment for canine MCTs is selected from surgical resection, irradiation, chemotherapy or combination of those (Welle et al., 2008). In addition to the conventional therapy, the clinical effectiveness of several TKIs, such as toceranib, masitinib and imatinib for canine MCT cases has recently been studied (Hahn et al., 2008; Isotani et al., 2008; London, 2009; London et al., 2009). Some clinical studies reported that canine MCT cases with *KIT* mutations were more likely to respond to treatment with TKIs than those without mutations (Hahn et al., 2008; Isotani et al., 2008; London et al., 2009). Therefore, detection of *KIT* mutations in canine MCTs can predict the efficacy of treatment with TKI.

However, most of the studies investigated only the partial sequences of *KIT* mRNA, such as parts of extracellular domain (exons 8 and 9) (Letard et al., 2008), transmembrane domain (exon 10) (Letard et al., 2008), juxtamembrane domain (exons 11 and 12) (Downing et al., 2002; Letard et al., 2008; London et al., 1999; Ma et al., 1999; Pryer et al., 2003; Webster et al., 2006b; Webster et al., 2007; Webster et al., 2008; Zemke et al., 2002) and kinase domain (exons 16 to 20) (Letard et al., 2008; Webster et al., 2006a) in clinical canine MCT cases. The mutational profiles in other exons of *KIT* of canine MCT cases are still unclear.

In the present chapter, to clarify the implication between *KIT* mutation and c-Kit receptor activation in canine MCT, genetic statuses of *KIT* and phosphorylation statuses of c-Kit receptor were examined in some canine MCT

cases. In addition, I described a canine MCT case with ITD mutation. The MCT acquired resistance to imatinib and relapsed during the observation period although the patient had achieved a remission with the treatment. Nucleotide sequence of *KIT* was assessed before and after evolution of resistance to imatinib, and Rh123 efflux function was analyzed after relapse.

Materials and Methods

Canine mast cell tumor cases

Tumor samples of canine MCTs from the dogs referred to the Veterinary Medical Center of the University of Tokyo, Nihon University Animal Medical Center, the Veterinary Medical Teaching Hospital of Nippon Veterinary and Life Science University and Japan Small Animal Cancer Center were used in the present study. The samples obtained by surgical resection, punch biopsy or tru-cut biopsy were divided into 3 parts: each one was routinely fixed in 10% phosphate-buffered formalin for pathological diagnosis, the other was submerged immediately in RNAlater for sequencing analysis of *KIT*, the remaining was frozen and stored at –80 °C for western blotting analysis.

Histological evaluations

The formalin fixed tumor samples were routinely paraffin-embedded, and processed. The histologic diagnoses were made on hematoxylin-eosin stain. The MCTs were classified according to the grading system defined by Patnaik et al. (Patnaik et al., 1984) as grade I (well-differentiated), grade II (moderately-differentiated) and grade III (poorly-differentiated).

Nucleotide sequence of KIT

Total RNA extracted from the tumor samples with RNeasy Fibrous

Tissue Midi Kit (QIAGEN) was used for cDNA synthesis as described previously (Chapter 1). The primers to amplify an ORF of *KIT* were described previously (Ohmori et al., 2008). Using the primers, *KIT* cDNA was amplified by RT-PCR with KOD FX from single-strand cDNAs of canine MCT cell lines. The PCR products of *KIT* cDNA were subcloned into pCR Blunt Vector and sequenced as described in chapter 1. More than 3 independent clones of the each plasmid were sequenced.

c-Kit receptor phosphorylation

For the analysis of c-Kit receptor phosphorylation, frozen tumors were homogenized and then lysed with HNTG lysis buffer (50 mM HEPES, 150 mM NaCl, 10% grycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF and Protease Inhibitor Cocktail). The protein was immunoprecipitated overnight 4 °C with an agarose-conjugated anti-c-Kit receptor antibody (Santa Cruz). Immune complexes were washed with HNTG lysis buffer. The protein was eluted by boiling in Laemmli Sample Buffer (Bio-Rad), separated by SDS-PAGE, and transferred to a PVDF membrane. Membranes were probed with an anti p-Kit antibody (1:1,000, Cell Signaling Technology) and then stripped with Restore Western Blot Stripping Buffer (Thermo scientific, Rockford, IL). To detect total c-Kit receptor levels, membranes were reprobed with anti c-Kit receptor antibody (1:1,000, DAKO).

Flowcytometric detection of rhodamine 123 efflux function

Fresh tumor tissues were minced with surgical scalpels in PBS. The tumor cells were then collected by filtration through 40 μm cell strainers (BD Falcon, Bedford, MA). The viability of mast cells, determined by trypan blue staining exclusion, was > 90%, and the preparations contained > 80% mast cells assessed by cytospin analysis with Wright-Giemsa stain. The Rh123 efflux function of tumor cells was determined by flowcytometric analysis as described previously (chapter 2).

Results

Population of cases

The 36 canine MCT samples obtained from 33 patients were used in this chapter (Table 6). In three dogs (C9, 14, 31), the samples were collected twice when the tumors relapsed after the achieving remission by surgical resection or chemotherapy. The median age of the 33 dogs was 10 years (y) and 8 months (m), ranging from 4y11m to 14y5m of age. The dogs represented 15 breeds including mixed breed dogs (10), Labrador retrievers (4), American cocker spaniels (3), Golden retrievers (2), Shetland sheepdogs (2), Shiba dogs (2), Miniature schnauzers (2) and 8 additional breeds that were represented by single dog. The sex distribution of the cases consisted of 10 intact and 11 spayed female dogs, as well as 7 intact and 4 castrated male, and the sex of one dog was unavailable. According to the Patnaik histologic grading system for canine MCTs, 36 tumors were divided into 3 groups; grade I (n = 3), grade II (n = 25) and grade III (n = 8). Histologic grades of three relapsed tumors [C9-2 (II), 14-2 (II) and 31-2 (III)] were the same as those of their original tumors (C8, 13 and 31).

Genetic statuses of KIT

The ORF (1 – 2928) of *KIT* in 35 tumors obtained from 32 canine MCT patients was determined and compared with the ORF of normal canine *KIT*

(Genbank accession no. AY313776). *KIT* sequences of the three relapse tumors (C9-2, 14-2 and 31-2) were identical to those of the respective original tumors (C9, 14 and 31). In one sample (C7), a partial sequence (772 - 2928) was amplified instead of the ORF of *KIT*. Genetic alterations of *KIT* were found in 25 (75.8%) of the 33 dogs (Table 7 and Fig. 17). Among them, the most frequent (n = 16) alteration was a 3-bp deletion ($^{763\text{-}765}$Del CAG) which led to one AA deletion (^{255}Del Gln) in exon 5. Five dogs had 12-bp insertion ($^{1537\text{-}1538}$Ins 12) which led to 4 AA insertion ($^{512\text{-}513}$Ins 4) at the junction of exons 9 and 10. Within exon 11, various mutations which led ITD mutation of 10 to 19 AA (n = 5), a single AA deletion (^{558}Del Val) (n = 1) or a single AA conversion (Leu^{575}Pro) (n = 1) were observed. A complete deletion of exons 6 and 7 ($^{935\text{-}1240}$Del 306) were detected in one dog. Some dogs had more than two mutations simultaneously. Two dogs had at least two variants of *KIT*: one dog (C13) had 3-bp deletion ($^{763\text{-}765}$Del CAG) in exon 5 and 23-bp deletion ($^{321\text{-}343}$Del 23) in exon 2 which led downstream nonsense mutation, and the other (C29) had 12-bp insertion ($^{1537\text{-}1538}$Ins 12) at the junction of exons 9 and 10 and ITD (ITD$^{1709\text{-}1738}$) in exon 11. In addition, some single nucleotide substitutions which led to no amino acid change were observed (Table 7).

Phosphorylation profile of c-Kit receptor

The phosphorylation of c-Kit receptor was evaluated with immunoprecipitation and Western blotting analysis in 34 tumors obtained from 31 dogs. The protein of c-Kit receptor was detected in 13 samples among them.

Of the 13 tumor samples, c-Kit receptor of 12 samples was phosphorylated whereas that of 1 case (C3) did not (Fig. 18). The 12 tumor samples with c-Kit receptor phosphorylation had various *KIT* statuses, such as 3-bp deletion ($^{763\text{-}765}$Del CAG) in exon 5 (n = 4), both ($^{763\text{-}765}$Del CAG) and 12-bp insertion ($^{1537\text{-}1538}$Ins 12) at the junction of exons 9 and 10 (n = 1), ITD (n = 3), a single AA deletion (^{558}Del Val) (n = 1), or a single AA conversion (Leu^{575}Pro) (n = 1) in exon 11 and without mutation (n = 2).

Case report (case C31)

A dog affected by MCT (C31) was 6-year old intact male Miniature schnauzer referred to the Veterinary Medical Center of the University of Tokyo with multiple nodules in the inguinocrural skin. Cytological examination by fine-needle aspirations (FNA) of the nodule disclosed a number of mast cells. Histopathological examination of one of the inguinocrural nodules obtained by surgical resection confirmed the diagnosis of grade III MCT. Sequencing analysis of *KIT* in the tumor tissue revealed that ITD mutation (ITD$^{572\text{-}590}$) in the juxtamembrane domain. The dog was treated with imatinib mesylate (Gleevec; Novartis, East Hanover, NJ, 100 mg/head, PO, q24h), then the remaining inguinocrural nodules were immediately disappeared. However, after 6 months from start of imatinib administration, inguinal and popliteal lymphoadenopathy was observed. FNA cytology of the both lymph nodes revealed a number of atypical mast cells observed initially. Histological examination of popliteal lymph node revealed that the normal structures were

almost completely replaced by a diffuse proliferation of mast cells and confirmed the relapse of grade III MCT. Sequencing analysis of *KIT* in the lymph node disclosed the completely same sequence of the original tumor without additional mutation. To investigate the implication of ABC transporters on the resistance to imatinib, Rh123 efflux function of the tumor cells was assessed. As a result, there was no function of ABC transporters in the tumor cells since the Rh123 accumulation hardly changed by the addition of CsA (Fig. 19).

Discussion

In this chapter, various types of known and unknown mutations of *KIT* were found in canine MCTs. The exon 11 of *KIT* encodes the juxtamembrane domain of c-Kit receptor and is the most well-researched region in canine MCT. Some previous studies found not only ITD mutation but also point mutation, in-frame mutation and deletion of the exon in canine MCT cell lines and clinical cases (Letard et al., 2008; London et al., 1999; Ma et al., 1999). The ITD mutation in the exon 11 of *KIT* is known to be a common mutation and observed in approximately 9 to 45% of canine MCTs (Downing et al., 2002; Isotani et al., 2008; Letard et al., 2008; London et al., 1999; Pryer et al., 2003; Webster et al., 2006a; Zemke et al., 2002). The ITD mutation is known to cause the activation of c-Kit receptor without binding of the ligand (Letard et al., 2008; London et al., 1999). The point mutation [1724 (T → C)] and 3-bp deletion ($^{1672\text{-}1674}$Del TTG) in exon 11, that observed in canine MCT cases in this chapter, were also identified in a canine MCT cell line, BR cells, and a previous canine MCT patient, respectively (Ma et al., 1999). As well as ITD mutation, these two mutations led to ligand-independent c-Kit receptor activation (Ma et al., 1999). In the present chapter, strong phosphorylation of c-Kit receptor was observed in the canine MCT cases with ITD, point mutation [1724 (T → C)] and 3-bp deletion ($^{1672\text{-}1674}$Del TTG) in the exon 11 of *KIT* (Fig. 18). The results were consistent with the previous findings (Letard et al., 2008; London et al., 1999; Ma et al., 1999).

The most frequent genetic alteration of *KIT* in this chapter was the 3-bp deletion ($^{763\text{-}765}$Del CAG) in exon 5 (n = 16, 48.5%). However, the alteration was also observed in a sequence of *KIT* obtained from normal canine cerebellum (Genbank accession no. AF099030) (London et al., 1999). Also, the 12-bp insertion ($^{1537\text{-}1538}$Ins 12) at the junction of exons 9 and 10, which identified in 5 cases in this study, was observed in the AF099030 and AY296484 which was a sequence obtained from the renal cortex of Dalmatian (Tsai et al., 2003). Therefore, these two alterations were probably not genetic mutation of canine *KIT*. However, the phosphorylation of c-Kit receptor was detected in the tumor samples not only with the 3-bp deletion ($^{763\text{-}765}$Del CAG) and the 12-bp insertion ($^{1537\text{-}1538}$Ins 12) but also without *KIT* mutation (Fig. 18). As described in chapter 1, there could be SCF/c-Kit receptor autocrine loop in canine MCT cells. In addition, SCF is produced by bone marrow endothelial cells, fibroblasts and eosinophils (Broudy, 1997; Hartman et al., 2001). Based on these previous and present findings, the c-Kit receptor phosphorylation might be caused by the stimulation with autocrine or paracrine SCF in the canine MCT cases without mutation.

In addition to the *KIT* mutations described in previous reports (Letard et al., 2008; London et al., 1999; Ma et al., 1999), various *KI*T mutations were identified in the primary tumor specimens obtained from dog patients with MCT. A canine MCT case (C13) had the 23-bp deletion ($^{321\text{-}343}$Del 23) in exon 2 which led downstream nonsense mutation. Since the case also possessed the *KIT* sequence with only the 3-bp deletion ($^{763\text{-}765}$Del CAG), there could be no deficiency of c-Kit receptor. Case C7 had the wide range deletion ($^{935\text{-}1240}$Del

306) which caused the complete deletion of exons 6 and 7. The tumor could have splicing abnormality since the 5'-end sequence (1 - 771) of *KIT* was not amplified in the case. However, there was no denying that the *KIT* sequence of C7 was one of splicing variant of canine normal *KIT*. Unfortunately, the c-Kit receptor phosphorylation was not examined in the case since the frozen sample was not available.

A canine MCT case (C31) with ITD mutation in exon 11 initially showed beneficial response to the treatment with imatinib. The findings were consistent with the previous clinical study which reported that 100% of canine MCT cases with *KIT* ITD mutation responded to imatinib treatment (Isotani et al., 2008). The tumor eventually relapsed during the treatment, indicating the development of resistance to imatinib. In human CML, most important mechanism of resistance to imatinib therapy is the additional point mutation in the kinase domain of BCR-ABL (Quintas-Cardama et al., 2009). In addition, a previous study indicated that the overexpression of ABCB1 could lead to the resistance to imatinib in human CML by upregulating the efflux of imatinib (Galimberti et al., 2005). As described in chapter 2, imatinib could be a substrate of canine ABCB1. However, there were no evidences of the additional mutation of *KIT* and the upregulation of ABCB1 function in the relapse tumor (C31-2). Considering these results, there might be other mechanisms to develop to imatinib tolerance, such resistance to apoptosis or increasing of imatinib metabolism (Bergman, 2003; Quintas-Cardama et al., 2009) in the case.

In this chapter, the protein of c-Kit receptor was detected in only 13

tumors although the 34 samples were assessed by immunoprecipitation and Western blotting analysis. Among the 13 cases, the phosphorylation of c-Kit receptor was observed in the 12 tumors. Some immunohistochemical studies indicated that most canine MCT expressed the protein of c-Kit receptor (Morini et al., 2004; Webster et al., 2004; Webster et al., 2007). However, the expression level of c-Kit receptor could be low in canine MCT in which the c-Kit receptor was not activated. Also, the protein of the tumors could be degraded by freezing and thawing methods or during transportation.

The present sequencing analysis found some genetic alterations of *KIT* in the regions other than exons 8, 9, 11, and 17 in which the mutations had already been identified in canine MCT. Nevertheless, the further researches by comparing the sequences of large number of normal dogs are warranted to confirm the genetic alteration as the true genetic mutation. In this chapter, the c-Kit receptor phosphorylation was detected in the tumor samples with and without *KIT* mutation. Therefore, c-Kit receptor activation could play a role in the proliferation and survival of the canine MCT without *KIT* mutation. The results will be helpful in clarifying the implication between the *KIT* mutation and the prognosis and tumorigenesis of canine MCT.

Conclusion

In chapter 1 of the present thesis, genetic statuses of *KIT* and c-Kit receptor phosphorylation were characterized and the SCF/c-Kit receptor interaction was examined in four canine MCT cell lines to investigate mechanisms of c-Kit receptor activation in various canine MCTs. HRMC expressed wild type c-Kit receptor whereas VIMC1, CoMS1 and CMMC1 harbored some *KIT* mutations. The c-Kit receptors were phosphorylated in all cell lines. SCF stimulation enhanced the phosphorylation of c-Kit receptor in HRMC, VIMC1 and CoMS1. There was no effect of SCF on proliferation of all cell lines. SCF protein was detectable in only HRMC. Since an internal inhibitor suppressed c-Kit receptor phosphorylation in HRMC whereas an external inhibitor did not, there could be an internal SCF/c-Kit receptor autocrine mechanism within HRMC. The results indicated that c-Kit receptor phosphorylation could be caused by the stimulation with autocrine SCF in HRMC while it could be caused by functional mutations of *KIT* in other three cell lines.

In chapter 2, the inhibitory effect of four TKIs for proliferation and phosphorylation of c-Kit receptor as well as the expression and function of ABCB1 were investigated in three canine MCT cell lines (HRMC, VIMC1 and CMMC1). The IC_{50} values of the TKIs in HRMC were clearly higher than those in CMMC1 and VIMC1. In HRMC and CMMC1, both the growth and phosphorylation of c-Kit receptor were suppressed proportionally by the TKIs. VIMC1 required higher concentrations for the inhibition of c-Kit receptor phosphorylation than those in cell growth. The treatment with CsA increased the effects of the TKIs on VIMC1, since ABCB1 was expressed in the cells. The

results indicated that canine MCT cell lines harboring wild-type *KIT* had lower sensitivity to TKIs. The growth of VIMC1 was seemingly suppressed by TKIs through the inhibition of other tyrosine kinases. ABCB1 can play a role in resistance to the treatment of TKIs in canine MCT.

In chapter 3, the mRNA expression and phosphorylation statuses of several kinases in three canine MCT cell lines (HRMC, VIMC1 and CMMC1) were assessed to identify the candidate of therapeutic targets. The inhibitory effect of 95 specific inhibitors was also examined in the cell lines. As results, the mRNAs of 11, 7 and 7 kinases and the phosphorylation of 12, 8 and 7 kinases were observed in HRMC, VIMC1 and CMMC1, respectively. The 10, 9 and 17 inhibitors exhibited the biological activity against the growth of HRMC, VIMC1 and CMMC1, respectively. The chapter indicated that several kinases could be used as therapeutic targets in the treatment for canine MCT.

In chapter 4, genetic statuses of *KIT* and phosphorylation statuses of c-Kit receptor were determined in 36 canine MCTs obtained from 33 dogs. Genetic alterations of *KIT* were found in 25 (75.8%) of the 33 dogs. The *KIT* alterations included the mutations such as 23-bp deletion of exon 2, complete deletion of exons 6 and 7, 3-bp deletion, point mutation and ITD mutation of exon 11. Two genetic alterations observed in normal canine *KIT* variations such as 3-bp deletion of exon 5 and 12-bp insertion at the junction of exons 9 and 10 were also identified. The phosphorylation of c-Kit receptor was observed in 12 tumors with and without *KIT* mutation. The results of the chapter indicated that the c-Kit receptor activation could play a role in the proliferation and survival of the canine MCTs without *KIT* mutation.

Recent clinical studies reported that canine MCT cases with ITD mutations in exon 11 of *KIT* were more likely to respond to treatment with TKIs than those without mutations (Isotani et al., 2008; London et al., 2009). Therefore, detection of *KIT* ITD mutations in canine MCTs is known to predict the efficacy of treatment with TKIs. However, another study identified the mutations in not only exon 11 but also exons 8, 9 and 17 of *KIT* in canine MCTs (Letard et al., 2008). Several mutations were also identified in exons 2, 6 and 7 of *KIT* in canine MCT in chapter 4 of this study. Furthermore, the c-Kit receptor was phosphorylated in canine MCT cells without *KIT* mutation (chapters 1 and 4). In addition, 30% of canine MCT cases without ITD mutation responded to treatment with TKI (Isotani et al., 2008; London et al., 2009). Based on these findings, only the investigation of ITD mutation in exon 11 is not enough to predict the efficacy of TKI treatment in canine MCTs.

In chapter 2, the wild type c-Kit receptor showed low sensitivity to TKIs even though it was phosphorylated. The inhibitor of Hsp90, cellular chaperone responsible for c-Kit receptor, suppressed the proliferation of canine MCT cells with and without *KIT* mutation by down-regulating the expression of c-Kit receptor (Lin et al., 2008). Chapter 3 in this study also revealed that radicicol, an Hsp90 inhibitor, suppressed the growth of all three canine MCT cell lines. If the inhibitors showed high selectivity for tumor cells as compared to normal cells, Hsp90 inhibitors would become a potent remedy for canine MCTs.

The present studies in this thesis demonstrated that canine MCT cells harbored various *KIT* sequence, c-Kit receptor phosphorylated statuses, and

sensitivity to TKIs and other inhibitors. Such biological diversity may reflect the different biological behavior and treatment response in canine MCTs. These findings would provide the fundamental observations for the molecular targeted therapy for the management of canine MCTs.

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Table 1. Genetic statuses of *KIT* in canine mast cell tumor cell lines

Cell lines		*KIT* mutation*	Amino acid change	Mutated exon	Mutated domain
HRMC		159 (C → T)	-	-	-
		507 (A → G)	-	-	-
VIMC1		1275 (A → G)	-	-	-
		1523 (A → T)	$Asn^{508}Ile$	Exon 9	Extracellular
CoMS1		1275 (A → G)	-	-	-
		1523 (A → T)	$Asn^{508}Ile$	Exon 9	Extracellular
CMMC1					
	Variant A	159 (C → T)	-	-	-
		507 (A → G)	-	-	-
		$^{763\text{-}765}$Del CAG	^{255}Del Gln	Exon 5	Extracellular
	Variant B	282 (A → G)	-	-	-
		1243 (A → G)	$Lys^{415}Glu$	Exon 8	Extracellular
		1275 (A → G)	-	-	-
		$ITD^{1718\text{-}1771}$	$ITD^{573\text{-}590}$	Exon 11	Juxtamembrane
	Variant C	1275 (A → G)	-	-	-
		$^{1521\text{-}1522}$Ins T	Nonsense mutation	Exon 9	Extracellular

* Compared with the sequence of normal canine *KIT* (Genbank accession no. AY313776)

Table 2. IC_{50} values for the tyrosine kinase inhibitors (TKIs) in the canine MCT cell lines.

Cell lines	IC_{50} of TKI			
	Axitinib	Imatinib	Masitinib	Vatalanib
HRMC	> 10 μM	> 10 μM	8.4 μM	> 10 μM
CMMC1	9.0 nM	42.3 nM	52.1 nM	549.4 nM
VIMC1	4.4 nM	157.8 nM	133.7 nM	611.3 nM
VIMC1 + CsA	3.1 nM	91.3 nM	58.7 nM	472.7 nM

Table 3. Nucleotide sequences of RT-PCR primers.

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)
VEGFR1	Forward	ATCTGCCTGTGGAAGGAATG	550
	Reverse	TCTTGTGTTCAAGGCAGTGG	
VEGFR2	Forward	CACGGAGTTGAGCTGTCTGTTGGA	466
	Reverse	CTCACTTCCATAATCGTCAGG	
VEGFR3	Forward	GAAGAATAAGACGGTGAGCAAGCT	301
	Reverse	CACGTTTTTGCAGTCGAGCA	
CSF1R	Forward	AGTTCACCGAGACCCAGGGCTACCAAT	471
	Reverse	CACCTTGACTTGGAGATCGAC	
PDGFR α	Forward	CTACATCGGCGTCACCTACAAAA	303
	Reverse	GAAGCTGTCCTCTACCAGGTCTGA	
PDGFR β	Forward	CCTTGGTGGTTCTCACAATCATCT	301
	Reverse	GCTGTGGATTTCAGCATCTTGACT	
EPHA2	Forward	CCTTGTCAACAGCAACCTGGTC	315
	Reverse	ATAAGCTGGTAGATGGCGGAGG	
FLT3	Forward	AAAGAGAGGCTCTCATGTCTGAACTC	301
	Reverse	CCTGAGATGTGATCCAAGTCCTG	
RET	Forward	TGACTCCCTGCTTTATGACGATG	305
	Reverse	CCCTTGTGAGTCCATTACCTTTCA	
EGFR	Forward	AGGTCTTGAAGGCTGTGCAAAAC	305
	Reverse	GGATCCAGAGTCCCTTGTACACTG	
SRC	Forward	AAGTCAGATGTGTGGTCCTTTGG	250
	Reverse	GGCTCGGTGGATGTGAAGTAGT	
YES	Forward	CAATCAAATGGACAGCTCCTGAG	310
	Reverse	GGTACTGTGGCTCTGTAGCAGTGA	
LCK	Forward	GAAGAGCGGAATTATATCCACCG	301
	Reverse	CCTCGTTCCAGGTTCTGAATCA	
FYN	Forward	GTGTGAACTCTTCATCTCATACCGG	303
	Reverse	CAAAGGACAAAAGCTGTCGCTC	

Table 4. The mRNAs detected with RT-PCR and phosphorylated kinases assessed by ARY001 and ARY003 in canine MCT cell lines. The RTKs and non-receptor kinases are represented in each upper and under row, respectively.

Cell lines	Expressed mRNAs	Phosphorylated kinases
HRMC	*VEGFR1, VEGFR2, VEGFR3, PDGFR α, FLT3, RET, EGFR*	VEGFR2, EGFR, DTK, InsulinR, EPHB6
	SRC, YES, LCK, FYN	p38α, JNK, AMPKα1, AKT, CREB, STAT5a, STAT5b
VIMC1	*VEGFR2, VEGFR3, PDGFR α*	VEGFR2, DTK, EPHB6
	SRC, YES, LCK, FYN	AMPKα1, AMPKα2, CREB, STAT5a, STAT5b
CMMC1	*VEGFR2, VEGFR3, PDGFR α*	DTK, EPHB6
	SRC, YES, LCK, FYN	p38α, AMPKα1, CREB, STAT5a, STAT5b

Table 5. The viability of the canine MCT cell lines cultured with 1μM of the inhibitors supplied in SCADS inhibitor kit III.
The viability of less than 50% are indicated by boldface. Data represent the mean of triplicate culture.

Targets	Inhibitor	Viability (% of control)			Targets	Inhibitor	Viability (% of control)		
		HRMC	VIMC1	CMMC1			HRMC	VIMC1	CMMC1
AK	ABT-702	101.8	89.0	84.0	GSK	Indirubin-3'-monoxime	105.1	91.1	85.5
AKT	Akt Inhibitor IV	**31.9**	96.3	83.9	HER2	AG825	84.7	86.8	94.6
AKT	Akt Inhibitor VIII	87.9	86.4	81.9	IGF-IR	AG1024	90.1	93.0	88.1
AKT	Akt Inhibitor XI	96.9	94.3	80.3	IGF-IR	AGL 2263	79.5	97.1	89.3
AMPK	Compound C	95.7	**10.7**	**10.9**	IKK	BMS-345541	80.7	88.9	85.7
ATM	ATM/ATR kinase inhibitor	98.0	93.8	96.0	IKK	IKK-2 inhibitor VI	90.9	75.5	54.2
ATM	ATM kinase inhibitor	98.9	93.6	99.8	IRAK	IRAK-1/4 inhibitor	95.3	88.7	78.1
Aurora/CDK	Aurora kinase/cdk inhibitor	74.8	91.1	78.9	JAK	JAK Inhibitor I	83.5	63.2	**41.4**
Aurora	Aurora kinase inhibitor II	92.6	88.9	69.9	JAK	JAK3 Inhibitor VI	65.2	62.3	**38.9**
Aurora	Aurora kinase inhibitor III	**45.3**	93.9	63.4	JNK	SP600125	96.2	87.3	86.2
Bcr-abl	AG957	97.1	93.4	83.1	JNK	JNK inhibitor VIII	97.3	85.9	84.5
BTK	LFM-A13	91.4	88.0	84.8	Lck	Damnacanthal	92.0	92.0	81.8
BTK	Terreic acid	96.0	91.7	84.3	Lck	PP2	88.4	64.7	**45.3**
CAMKII	KN-93	96.6	91.3	87.7	MAPK	ERK inhibitor II	88.0	91.8	93.5
CAMKII	KN-62	91.9	87.2	99.1	MEK	PD98059	98.6	83.2	83.5
CAMKII	Lavendustin C	97.9	94.4	92.5	MEK	U-0126	92.9	93.8	102.2
CDK	Kenpaullone	106.4	95.4	87.2	MEK	MEK inhibitor I	103.6	93.3	94.1
CDK	Purvalanol A	90.9	92.5	83.6	Met	SU11274	77.5	79.0	84.5
CDK	Olomoucine	92.9	94.8	89.8	MLCK	ML-7	60.5	94.1	86.7
CDK/GSK3b	Alsterpaullone, 2-cyanoethy	**32.4**	66.8	**33.8**	p38	SB202190	86.2	93.7	89.4
CDK	Cdk1/2 inhibitor III	**11.0**	70.3	**38.1**	p38	SB239063	75.4	91.0	83.3
CDK	Cdk2/9 inhibitor	**19.4**	57.1	**45.8**	PDGFR	AG1296	91.7	74.7	60.6
CDK	NU6102	95.4	90.6	89.1	Multi-target*	SU11652	75.5	**7.3**	**10.1**
CDK	Cdk4 inhibitor	91.6	77.7	81.7	PDGFR	PDGF RTK inhibitor V	95.0	76.2	75.5
CDK	NSC625987	100.0	89.5	88.6	PDGFR	PDGF RTK inhibitor IV	**10.5**	**7.1**	**10.0**
Chk	SB218078	**40.0**	**31.4**	**21.9**	PI3K	LY-294002	88.1	89.2	87.2
Chk	Isogranulatimide	103.0	80.4	76.4	PI3K/MLCK	Wortmannin	87.5	82.3	53.1
Chk	Chk2 inhibitor	82.2	90.7	85.1	PKA	H-89	85.8	85.6	81.1
Chk	Chk2 inhibitor II	83.6	89.5	87.3	PKA	4-cyano-3-methylisoquinoline	86.1	90.1	89.6
CK	Ellagic acid (Dihydrate)	95.8	90.7	84.6	PKC	Bisindolymaleimide I, HCl	**49.8**	82.5	75.5
CK	TBB	101.0	89.4	95.8	PKC	Go7874	**48.4**	57.6	51.6
CK	DMAT	107.9	96.3	97.1	PKG	Rp-8-CPT-cGMPS	94.8	91.4	98.1
CK	D4476	83.6	93.4	89.4	PKG	KT5823	105.0	93.8	108.6
Clk	TG003	89.1	92.0	83.9	PKR	PKR inhibitor	66.5	**28.5**	**20.5**
DGK	Diacylglycerol kinase inhibitor	91.2	94.1	83.2	Raf	RAF1 kinase inhibitor I	96.6	92.8	88.8
DNA-PK	IC60211	80.8	88.4	87.1	Raf	ZM 336372	91.6	**48.4**	**13.5**
eEF2	TX-1918	85.9	90.4	58.8	ROCK	H-1152	64.0	78.4	80.9
EGFR	BPIQ-Ⅱ	98.3	93.5	93.8	ROCK	Y-27632	85.9	85.9	93.7
EGFR	AG1478	96.6	92.0	98.2	Hsp90	Radicicol	**36.8**	**8.3**	**11.7**
EGFR	AG490	95.7	99.7	103.9	Src	PP1 analog	97.5	88.7	96.4
FGFR/PDGFR/ InsulinR	SU4984	91.5	**11.9**	**18.7**	Syk	Syk inhibitor	95.8	82.0	98.1
					TGF-bRI	SB431542	91.2	93.9	95.0
FGFR	SU5402	90.7	93.1	79.0	TGF-bRI	TGF-b RI kinase inhibitor II	84.7	93.4	106.5
Flt-3	Flt-3 Inhibitor	99.9	71.7	38.6	Tpl2	Tpl2 kinase inhibitor	92.2	93.3	83.3
Fms	cFMS RTK Inhibitor	83.6	88.5	84.0	TrKA	TrkA inhibitor	88.2	78.1	67.5
Fyn	SU6656	93.6	80.6	75.0	VEGFR/c-KIT	VEGFR RTK inhibitor II	92.7	**10.3**	**11.2**
GSK	GSK-3 inhibitor IX	105.6	50.1	**44.1**	VEGFR	VEGF recptor 2 kinase	97.4	86.8	100.1
GSK	1-Azakenpaullone	114.2	99.1	96.1	VEGFR	SU1498	97.0	92.2	107.8

* The targets of SU11652 are PDGFR, VEGFR, FGFR and c-Kit receptor

Table 6. Profiles of the 36 canine MCT samples obtained from 33 patients.
In three dogs (C9, 14, 31), the tumor samples were collected twice.

No.	Breed	Age	Sex	Grade
C1	Miniature pinscher	10y7m	Female	I
C2	Golden retriever	7y8m	Spayed female	I
C3	Mixed breed	12y5m	Spayed female	I
C4	Labrador retriever	10y	Castrated male	II
C5	Mixed breed	11y	NA	II
C6	Golden retriever	8y9m	Spayed female	II
C7	Labrador retriever	8y2m	Female	II
C8	Mixed breed	6y5m	Castrated male	II
C9	American cocker spaniel	9y2m	Male	II
C9-2	-	10y3m	Male	II
C10	Labrador retriever	10y9m	Spayed female	II
C11	Miniature dachshund	10y10m	Male	II
C12	American cocker spaniel	13y	Female	II
C13	Pug	10y8m	Spayed female	II
C14	Shiba inu	12y10m	Female	II
C14-2	-	13y1m	Female	II
C15	Mixed breed	10y6m	Male	II
C16	Shetland sheepdog	10y11m	Female	II
C17	Papillon	9y9m	Female	II
C18	Miniature schnauzer	4y11m	Female	II
C19	French bulldog	11y5m	Male	II
C20	Shetland sheepdog	14y5m	Female	II
C21	Mixed breed	13y5m	Spayed female	II
C22	Mixed breed	12y8m	Castrated male	II
C23	Mixed breed	10y4m	Castrated male	II
C24	Shiba inu	10y9m	Male	II
C25	Mixed breed	14y4m	Spayed female	II
C26	Mixed breed	13y1m	Female	II
C27	Bernese mountain dog	6y7m	Female	III
C28	Labrador retriever	8y11m	Spayed female	III
C29	Shih tzu	14y2m	Spayed female	III
C30	American cocker spaniel	9y	Male	III
C31	Miniature schnauzer	6y4m	Male	III
C31-2	-	6y10m	Male	III
C32	Pembroke welsh corgi	5y7m	Spayed female	III
C33	Mixed breed	11y9m	Spayed female	III

Abbreviations, y: years, m: months, NA: not available.

Table 7. Summary of mutaions in the *KIT* fouund in canine MCT cases.

KIT mutation*	Amino acid change	No. Dogs (% of Total)
Exon 2		
$^{321\text{-}343}$Del 23	Nonsense mutation	1 (3.0%)
Exon 5		
$^{763\text{-}765}$Del CAG	^{255}Del Gln	16 (48.5%)
Exon 6-7		
$^{935\text{-}1240}$Del 306**	$^{312\text{-}413}$Del 102, Thr^{414}Ala	1 (3.0%)
Exon 9-10		
$^{1537\text{-}1538}$Ins 12	$^{512\text{-}513}$Ins 4	5 (15.2%)
Exon 11		
$^{1672\text{-}1674}$Del TTG	^{558}Del Val	1 (3.0%)
1724 (T → C)	Leu^{575}Pro	1 (3.0%)
ITD$^{1714\text{-}1758}$	ITD$^{572\text{-}586}$	1 (3.0%)
ITD$^{1726\text{-}1770}$	ITD$^{576\text{-}590}$	1 (3.0%)
ITD$^{1718\text{-}1765}$	ITD$^{573\text{-}588}$	1 (3.0%)
ITD$^{1709\text{-}1738}$	ITD$^{570\text{-}579}$	1 (3.0%)
ITD$^{1714\text{-}1770}$	ITD$^{572\text{-}590}$	1 (3.0%)
Total ITD		5 (15.2%)

* Compared with the sequence of normal canine *KIT* (Genbank accession no. AY313776)

** Complete deletion of exons 6 and 7

Table 8. Single nucleotide substitutions of *KIT* which led to no amino acid change observed in canine MCT cases.

KIT substitution*	Amino acid change	No. Dogs (% of Total)
21(C → A)	-	1 (3.0%)
159 (C → T)	-	7 (21.2%)
414 (C → T)	-	4 (12.1%)
507 (A → G)	-	8 (24.2%)
1275 (A → G)	-	20 (60.6%)
1731 (C → T)	-	7 (21.2%)
1809 (T → C)	-	1 (3.0%)
1971 (A → G)	-	1 (3.0%)
2355 (G → A)	-	1 (3.0%)

* Compared with the sequence of normal canine *KIT* (Genbank accession no. AY313776).

Figure legends

Fig. 1. The gross, cytological and histopathological images of canine MCT. Multiple nodules of MCT over the face (A) and the breast skin (B). (C) A cytological finding of well-differentiated canine MCT cells. The tumor cells contain a round nucleus with dense chromatin pattern and an abundance of fine cytoplasmic granules. (D) Histopathological finding of well-differentiated canine MCT. Note that tumor cells are loosely arranged and separated by collagen bundle. They show mild anisocytosis. (E) A cytological finding of poorly-differentiated canine MCT. The tumor cells possess round to oval nucleus with a fine chromatin pattern and moderately basophilic cytoplasm containing a few granules. The cells show marked anisocytosis and occasional bi-nucleation. (F) A histopathological finding of poorly-differentiated canine MCT. The tumor cell shows marked anisocytosis, marked anisokaryosis and bi-nucleation.

Fig. 2. The organization of normal canine c-Kit receptor (A) and the correlation between each domain of c-Kit receptor and exons of *KIT* mRNA (B). TM: Transmembrane domain, JM; Juxtamembrane domain, TK1: Proximal kinase domain, KI: Kinase insert domain, TK2: Distal kinase domain.

Fig. 3. The conceptual schemes of molecular targeted therapy for cancer. Conventional anticancer agents injure both tumor and normal cells (A). In contrast, molecular targeted agents show the selectivity for tumor cells as

compared to normal cells (B). (C) The candidate target molecules in tumor cells.

Fig. 4. The result of Western blotting analyses of c-Kit receptor, phosphorylated c-Kit receptor (p-Kit) and β-actin in the cell lysate of canine MCT cell lines. The c-Kit receptor and phosphorylated c-Kit receptor are detected in all the cell lines. Phosphorylation of c-Kit receptor in CMMC1 is higher than those of other cell lines.

Fig. 5. The effect of SCF stimulation on c-Kit receptor phosphorylation in HRMC (A), VIMC1 (B), CoMS1 (C) and CMMC1 cells (D). In HRMC, VIMC1 and CoMS1 cells, the addition of *r*cSCF enhances phosphorylation of a mature form of c-Kit receptor. In contrast, no enhancement of phosphorylation of c-Kit receptor is observed in CMMC1 cells. Phosphorylated c-Kit receptor is abbreviated as p-Kit.

Fig. 6. The effect of SCF stimulation on proliferation in HRMC (A), VIMC1 (B), CoMS1 (C) and CMMC1 cells (D). A maximum of 500 ng/ml *r*cSCF supplementation have no effect on proliferation of all cell lines. HRMC, VIMC1 and CoMS1 cells can grow in serum-free AIM-V medium while CMMC1 cells can hardly grow. The data represents mean $\pm$ S. D. of triplicate independent experiments.

Fig. 7. The results of SCF expression analyses in canine MCT cell lines. (A) RT-PCR

for *SCF* reveals the presence of *SCF* mRNA in all cell lines. Both soluble and membrane-bound forms are detected in HRMC, VIMC1 and CMMC1 cells while only a soluble form is detected in CoMS1 cells. (B) SCF protein is detected in the cell lysate of only HRMC cells by Western blotting analysis. The native form of SCF has been cleaved proteolytically and shrinks as the mature conformation. The molecular weight of SCF protein in HRMC cells is larger than that of *r*cSCF. While *rc*SCF takes the mature form, SCF of HRMC cells can be in the native form. (C) SCF protein is undetectable in both unconcentrated and a hundredfold concentrated culture supernatants of HRMC cells by Western blotting analysis. The anti-canine SCF antibody can detect more than 100 ng/ml of SCF.

Fig. 8. The results of inhibitory effect of anti-SCF antibody and Dasatinib on c-Kit receptor phosphorylation in HMRC cells. (A) A maximum of 1,000 ng/ml of anti-canine SCF antibody (an external SCF inhibitor) showed no inhibitory effect on c-Kit receptor phosphorylation. (B) By contrast, a tyrosine kinase inhibitor, Dasatinib (an internal inhibitor) can inhibit c-Kit receptor phosphorylation in a dose dependant manner.

Fig. 9. The results of *MDR1* mRNA and ABCB1 protein expression analyses in canine MCT cell lines. (A) RT-PCR for *MDR1* reveals the presence of *MDR1* mRNA in both CMMC1 and VIMC1 cells, whereas not in HRMC cells. The expression of the mRNA in VIMC1 cells is higher than that in CMMC1 cells. (B) The mature and immature forms of ABCB1 protein are consistently detected

in both CMMC1 and VIMC1 cells by western blotting analysis, whereas slightly in HRMC cells.

Fig. 10. The histogram data of Rh123 accumulation by flowcytometry in HRMC (A), CMMC1 (B) and VIMC1 (C). The curves with black and white area represent fluorescent intensity of the cells incubated with Rh123 and with Rh123 following CsA (Rh123+CsA), respectively. The curves with gray area represent untreated controls. The mean fluorescent intensity (MFI) of both HRMC and CMMC1 cells cultured with Rh123 are similar to those of the cells cultured with Rh123 followed by CsA. In contrast, the MFI of VIMC1 cells cultured with Rh123 is lower than that of the cells cultured with Rh123 followed by CsA. The transverse axes represent fluorescence intensity (FL1) and the vertical axes represent number of the cells.

Fig. 11. The effect of the TKIs on cell proliferation in canine MCT cell lines. Cell proliferation of HRMC (A-D), CMMC1 (E-H) and VIMC1 (I-L) cells, under the effect of various concentration of the TKIs (axitinib, imatinib, masitinib and vatalanib), were assessed by WST-8 assay. The results are expressed as the mean $\pm$ S. D. The transverse axes represent culture hours and the vertical axes represent OD_{450} of triplicate cultures, which is directly proportional to the number of viable cells.

Fig. 12. The results of inhibitory effect of the TKIs on c-Kit receptor phosphorylation in canine MCT cell lines. The c-Kit receptor and p-Kit were detected by

western blotting analysis in HRMC (A), CMMC1 (B) and VIMC1 (C) cells after incubation for 4 hours in control medium or various concentration of TKIs (axitinib, imatinib, masitinib and vatalanib). The same western blotting analysis was also carried out in VIMC1 cells after incubation for 4 hours with both 5 μM CsA and various concentration of TKIs (D).

Fig. 13. The dose-response curve of cytotoxic effect of axitinib (A), imatinib (B), masitinib (C) and vatalanib (D) in VIMC1 cells cultured with (dotted lines) or without (solid lines) 5 μM of CsA. The viable cells, determined by WST-8 assay, relative to those measured in DMSO-treated control samples were plotted against the log of TKIs concentrations, and exponential fit was applied. Data represent the mean ± S. D. of triplicate cultures. The transverse axes represent concentration of TKIs and the vertical axes represent the relative cell viability.

Fig. 14. The results of RT-PCR of 14 tyrosine kinases in HMRC (A), VIMC1 (B) and CMMC1 cells (C). The amplicon sizes of the mRNA are represented in Table 1. The positions of molecular size marker (200 and 600 bp) are represented on the right side.

Fig. 15. The results of phosphorylation profiles of 42 different RTKs in HMRC (A), VIMC1 (B) and CMMC1 cells (C) examined with ARY001. The positive signals (phosphorylation) are indicated in the black squares. a: EGFR, b: InsulinR, c: DTK, d: VEGFR2, e: EPHB6. The white squares on the four corners of each membrane represent positive controls (cont).

Fig. 16. The results of phosphorylation profiles of several non-receptor kinases in HMRC (A), VIMC1 (B) and CMMC1 cells (C) examined with ARY003. The positive signals (phosphorylation) are indicated in the black squares. a: p38α, b: JNK, c: AMPKα1, d: AKT, e: CREB, f: AMPKα2, g: STAT5b, h: STAT5a/b, i: STAT5b. The gray squares on the upper-left and bottom-left corners of each membrane represent positive controls (cont).

Fig. 17. Location and frequency of *KIT* mutations identified in canine MCTs. The number of dogs with each mutation is shown in parentheses. TM: Transmembrane domain, JM; Juxtamembrane domain, TK1: Proximal kinase domain, KI: Kinase insert domain, TK2: Distal kinase domain.

Fig. 18. The result of Immunoprecipitation/Western blotting analyses of c-Kit receptor, and p-Kit in the canine MCT cases. Among the 13 tumors in which the protein of c-Kit receptor was detected, phosphorylated c-Kit receptor are observed in 12 tumors, except for one case (C3) harboring 3-bp deletion ($^{763\text{-}765}$Del CAG) of *KIT*.

Fig. 19. The histogram data of Rh123 accumulation by flowcytometry in the tumor cells obtained from canine MCT case C31-2. The curves with black and white area represent fluorescent intensity of the cells incubated with Rh123 and with Rh123 following CsA (Rh123+CsA), respectively. The curves with gray area represent untreated controls. The MFI of the cells cultured with Rh123 is

similar to that of the cells cultured with Rh123 followed by CsA. The transverse axes represent fluorescence intensity (FL1) and the vertical axes represent number of the cells.

Fig. 1

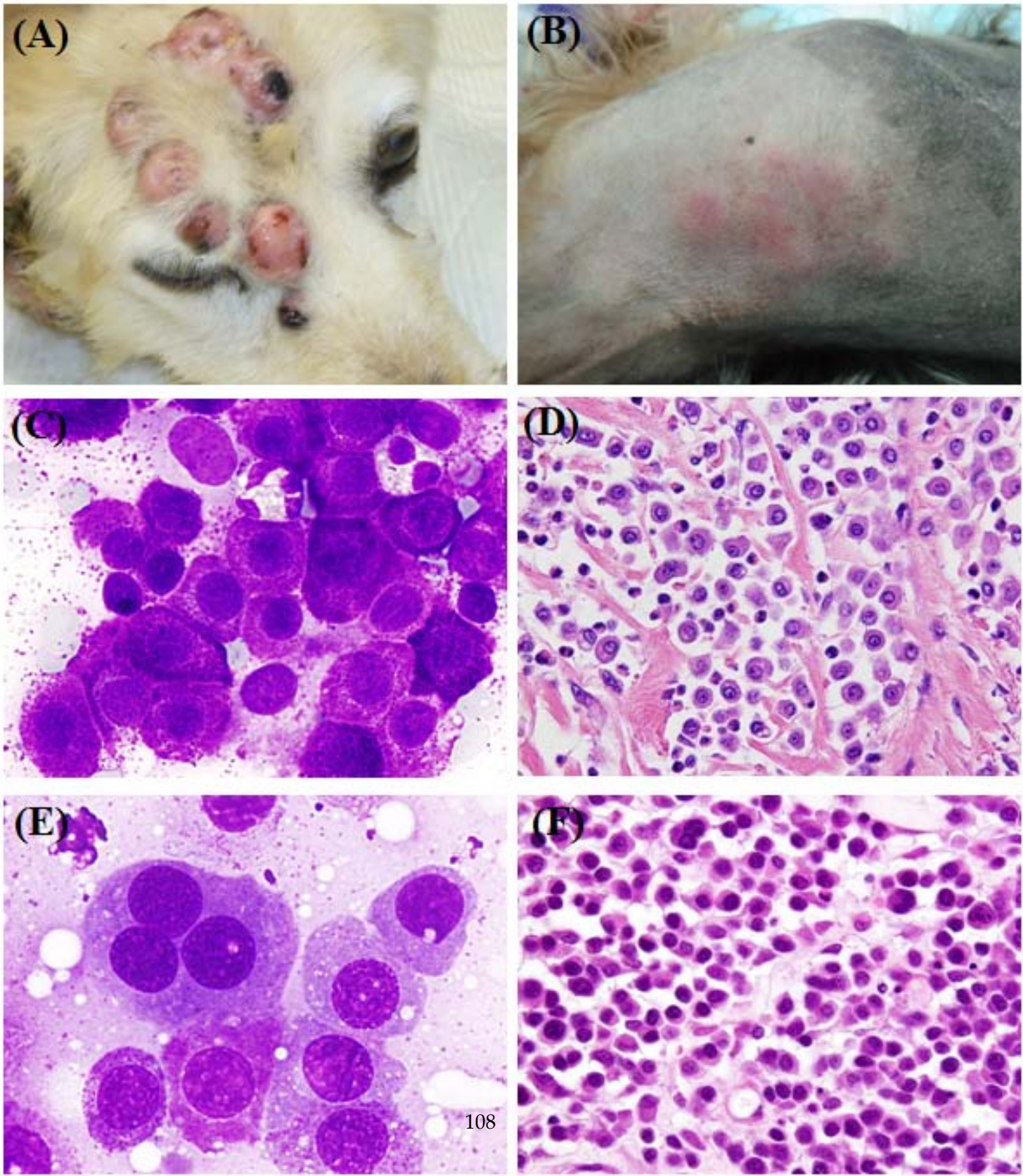
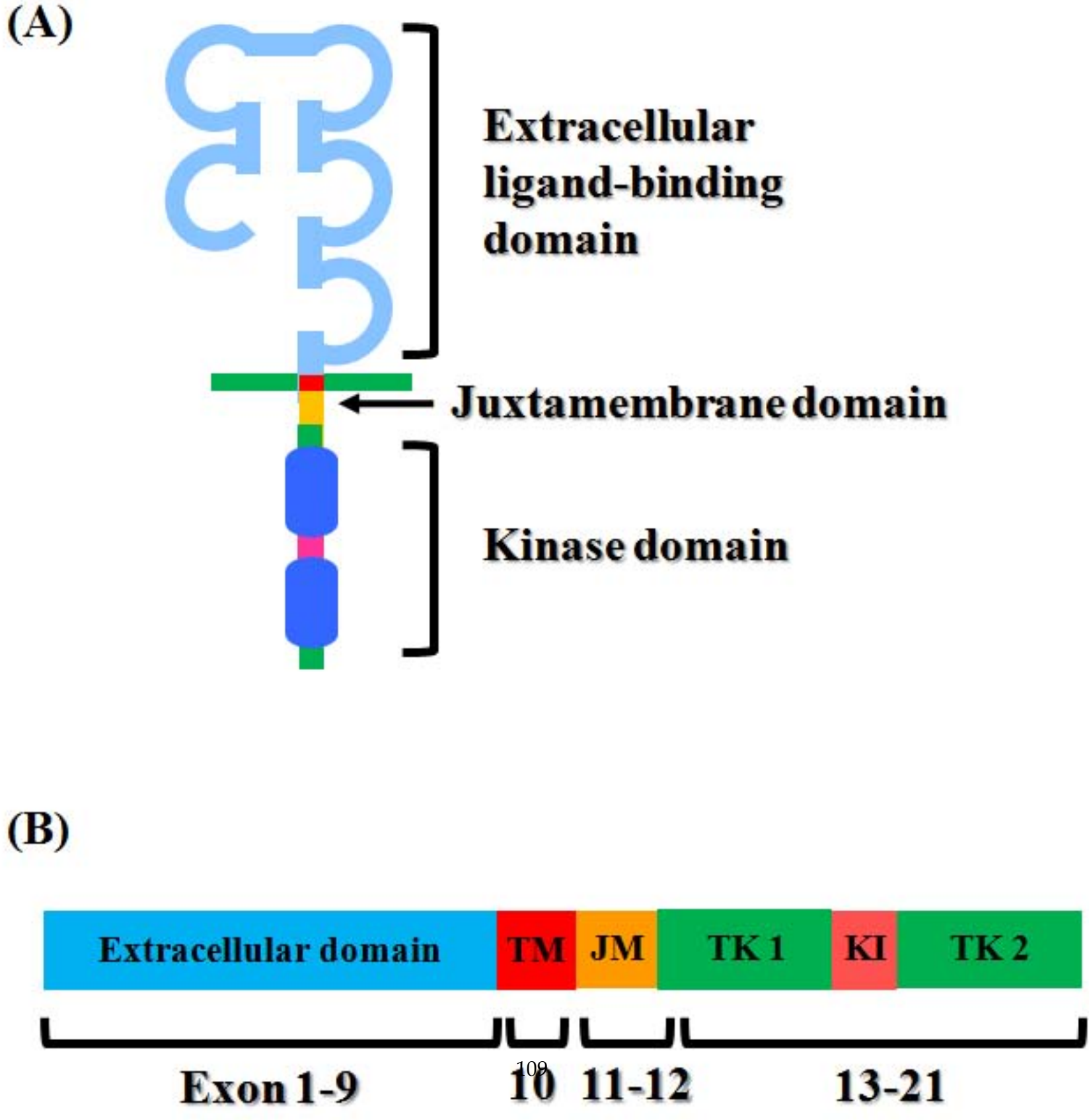


Fig. 2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Juxtamembrane domain
Kinase domain
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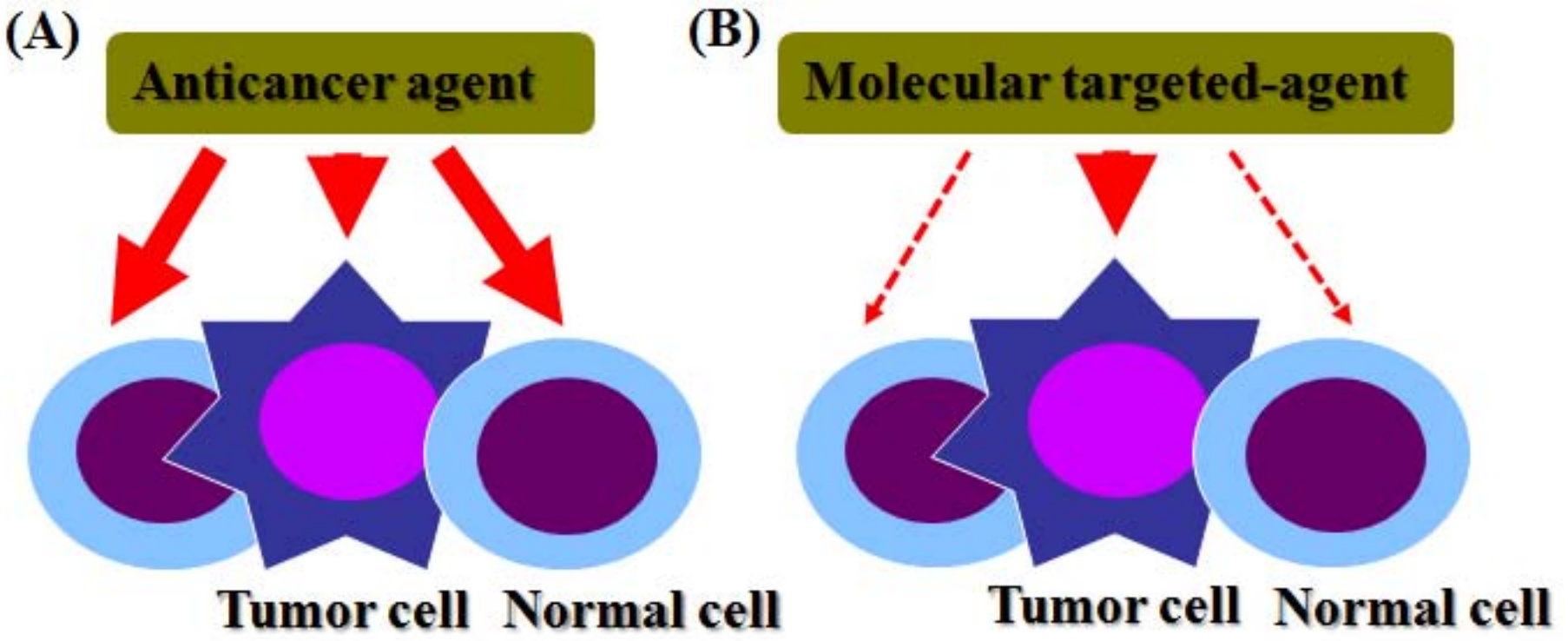
KI
TK 2
Exon 1-9
10
11-12
13-21

Fig. 3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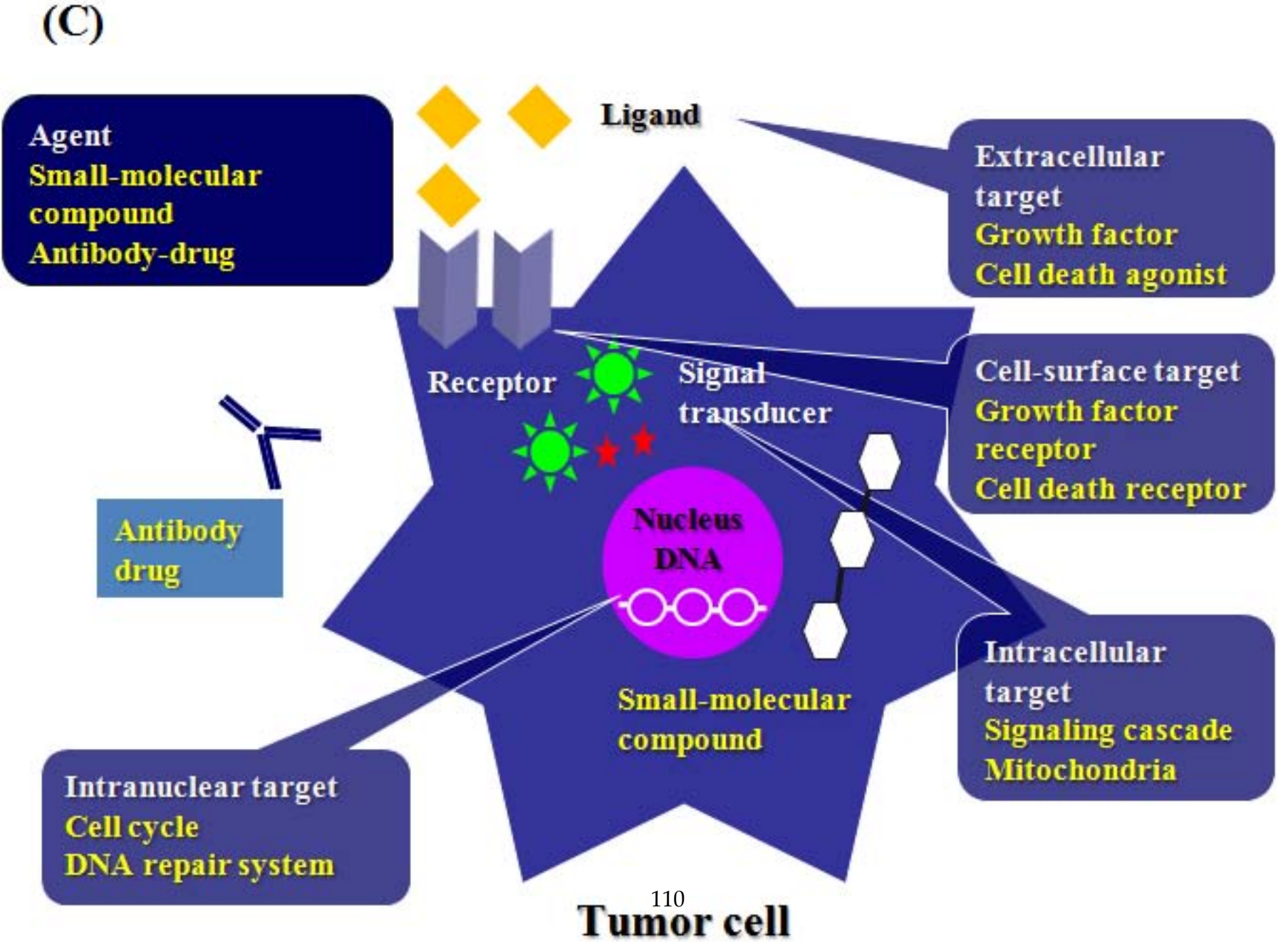
Tumor cell
Normal cell
(B)
Molecular targeted-agent
Tumor cell
Normal cell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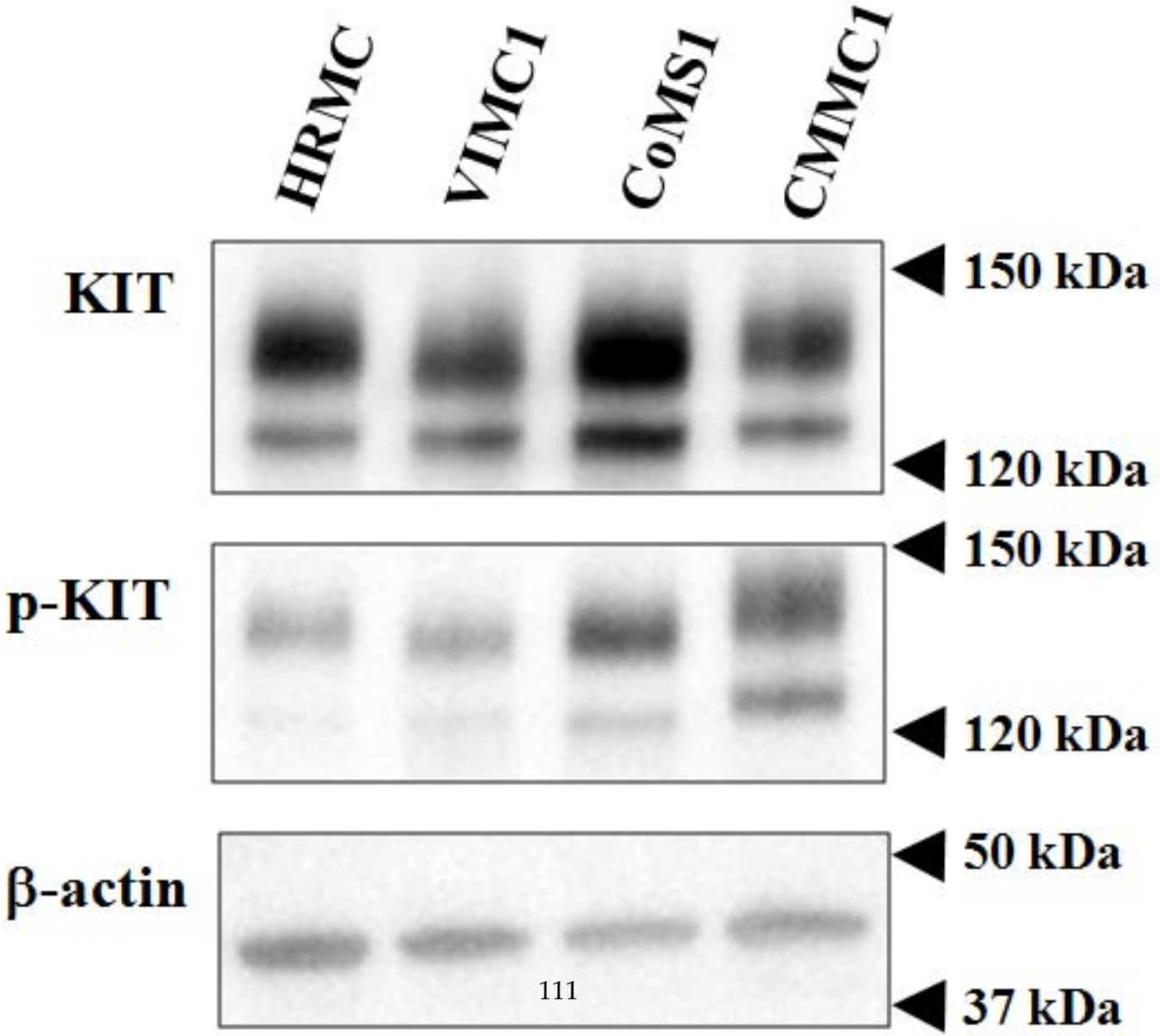
Small-molecular compound
Antibody-drug
Ligand
Extracellular target
Growth factor
Cell death agonist
Receptor
Signal transducer
Cell-surface target
Growth factor receptor
Cell death receptor
Antibody drug
Nucleus
DNA
Small-molecular compound
Intracellular target
Signaling cascade
Mitochondria
Intranuclear target
Cell cycle
DNA repair system
Tumor cell

Fig. 4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CMMC1
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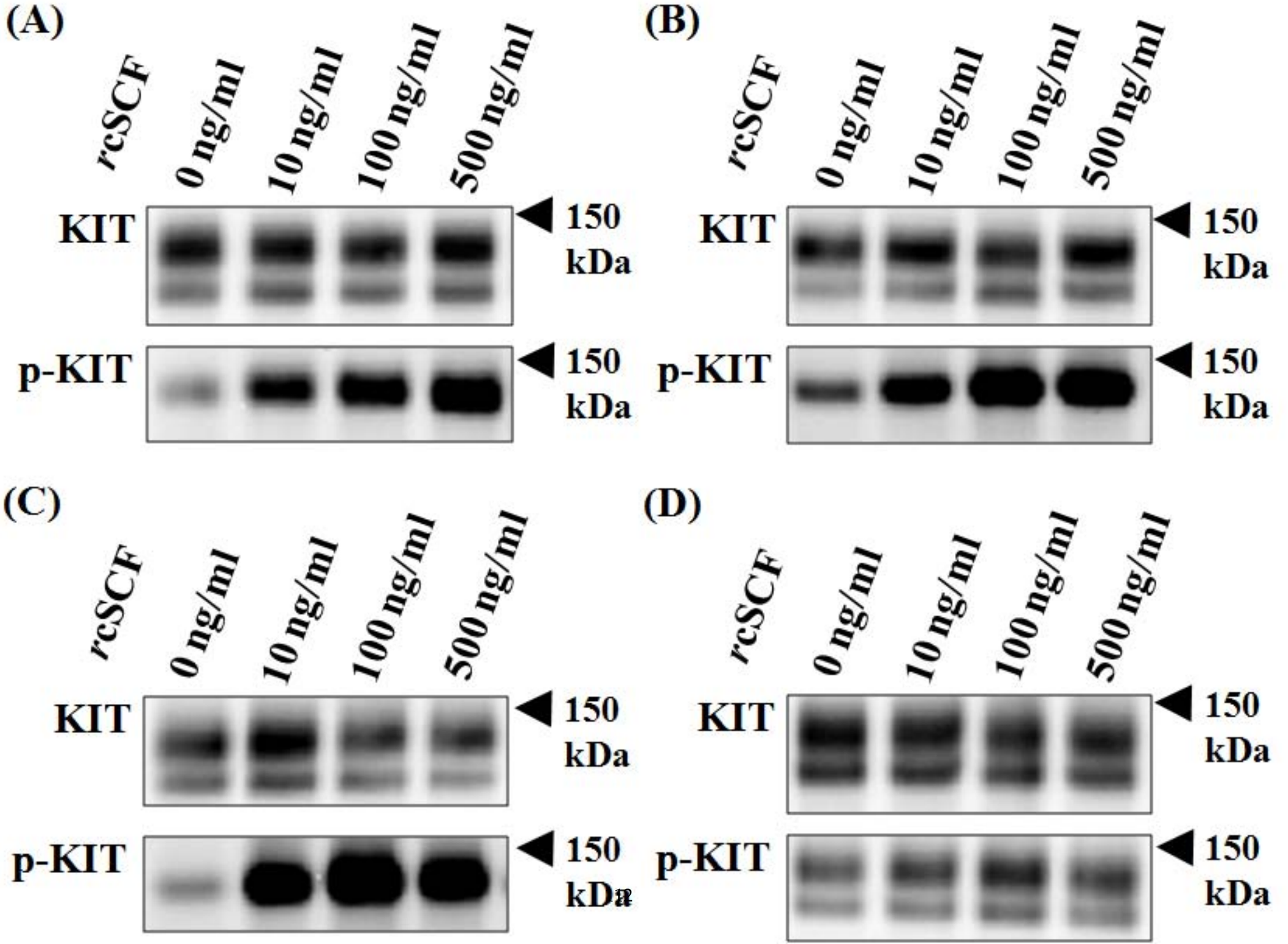
150 kDa
120 kDa
β-actin
50 kDa
37 kDa

Fig. 5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150 kDa
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150 kDa
p-KIT
150 kDa
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500 ng/ml
KIT
150 kDa
p-KIT
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KIT
150 kDa
p-KIT
150 kDa

Fig. 6

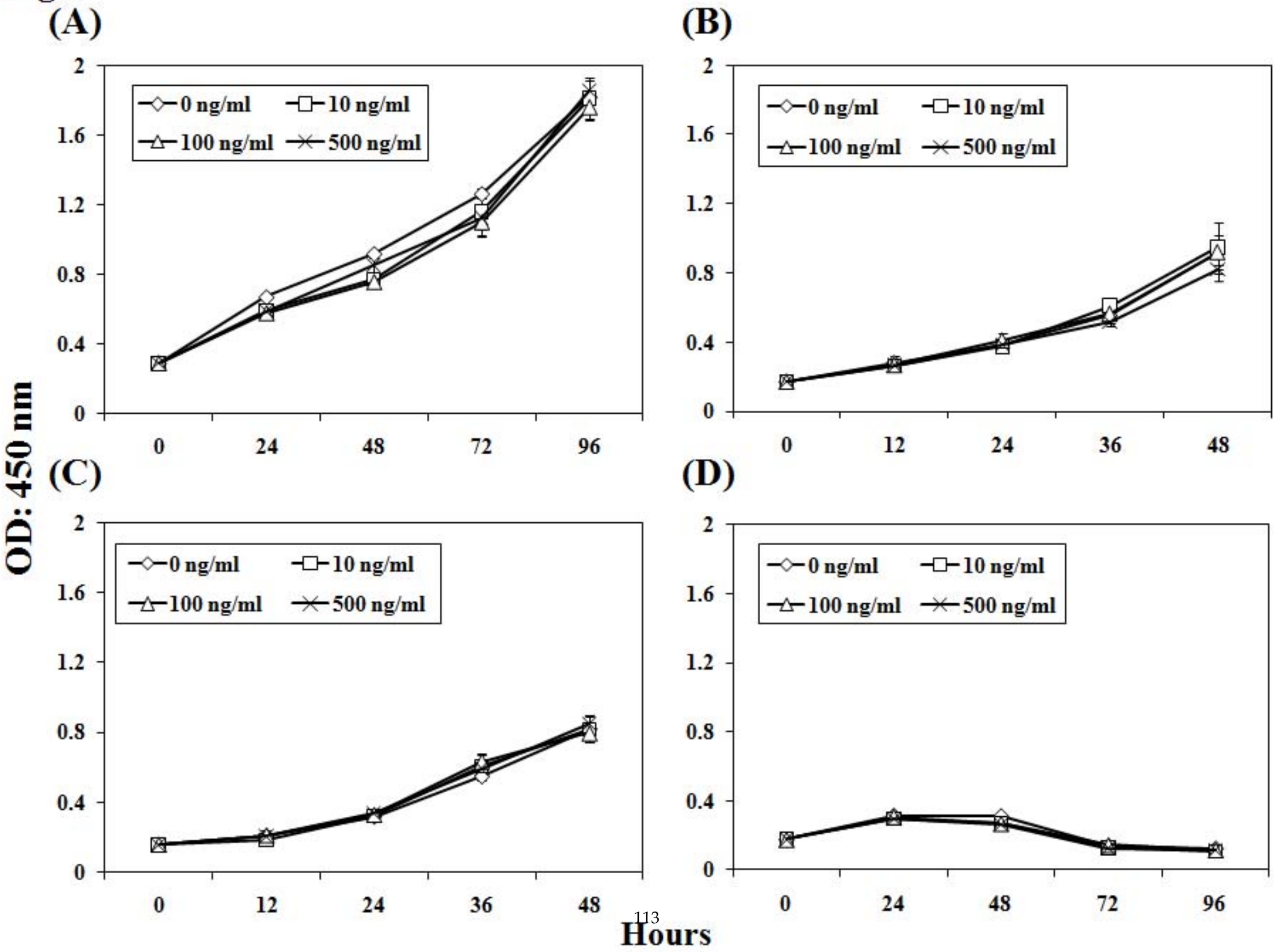
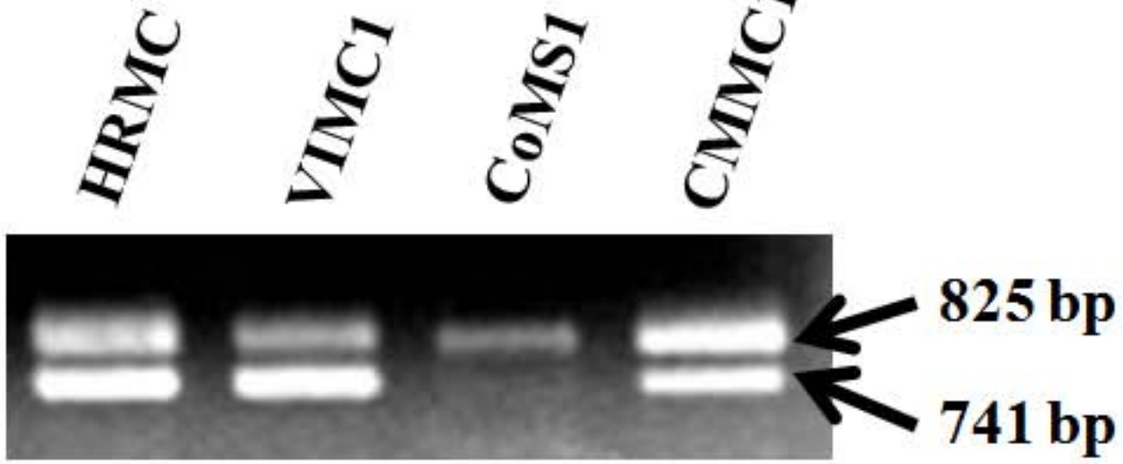

(A)
(B)
(C)
(D)
0 ng/ml
10 ng/ml
100 ng/ml
500 ng/ml
OD: 450 nm
Hours
0
0.4
0.8
1.2
1.6
2
0
12
24
36
48
72
96

Fig. 7

(A)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CMMC1
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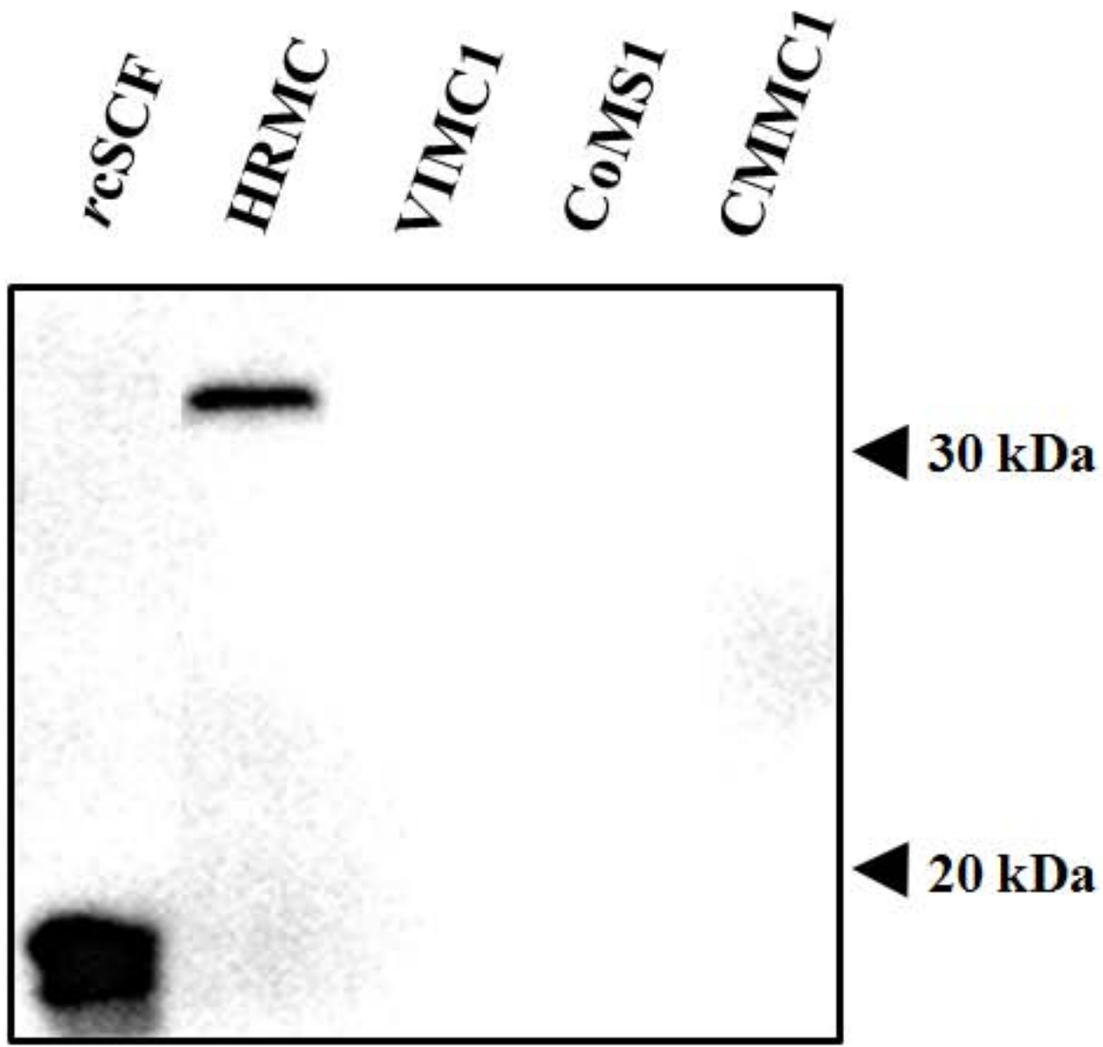
741 bp

(B)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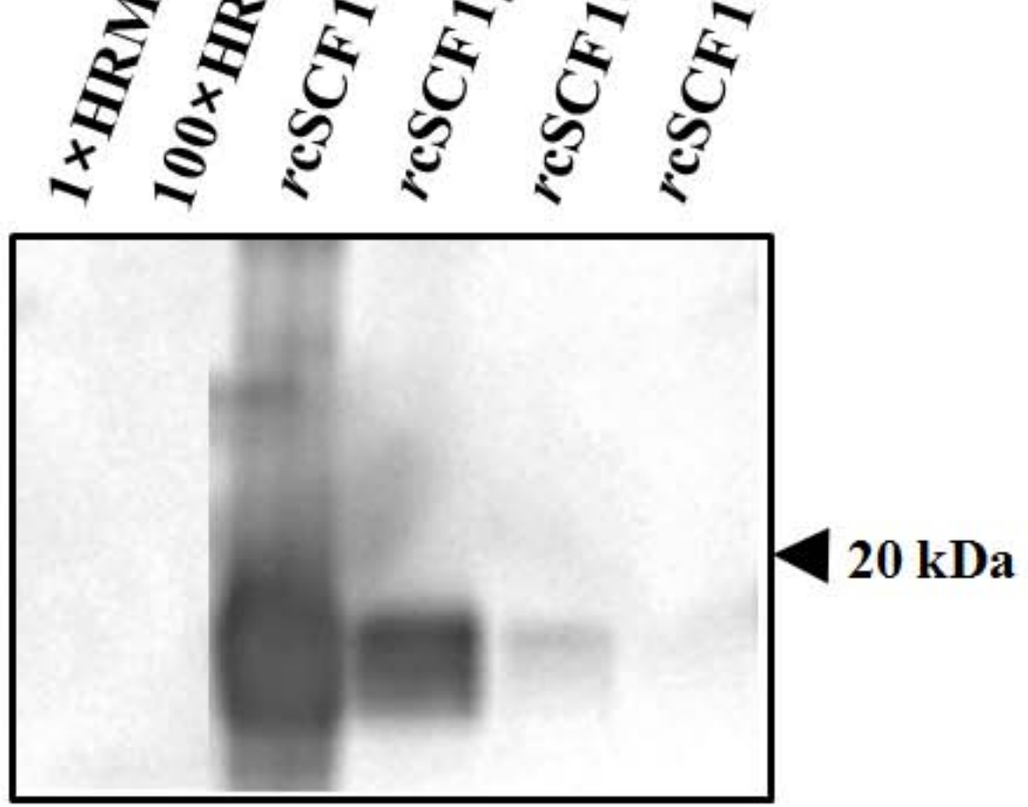
CMMC1
30 kDa
20 kDa

(C)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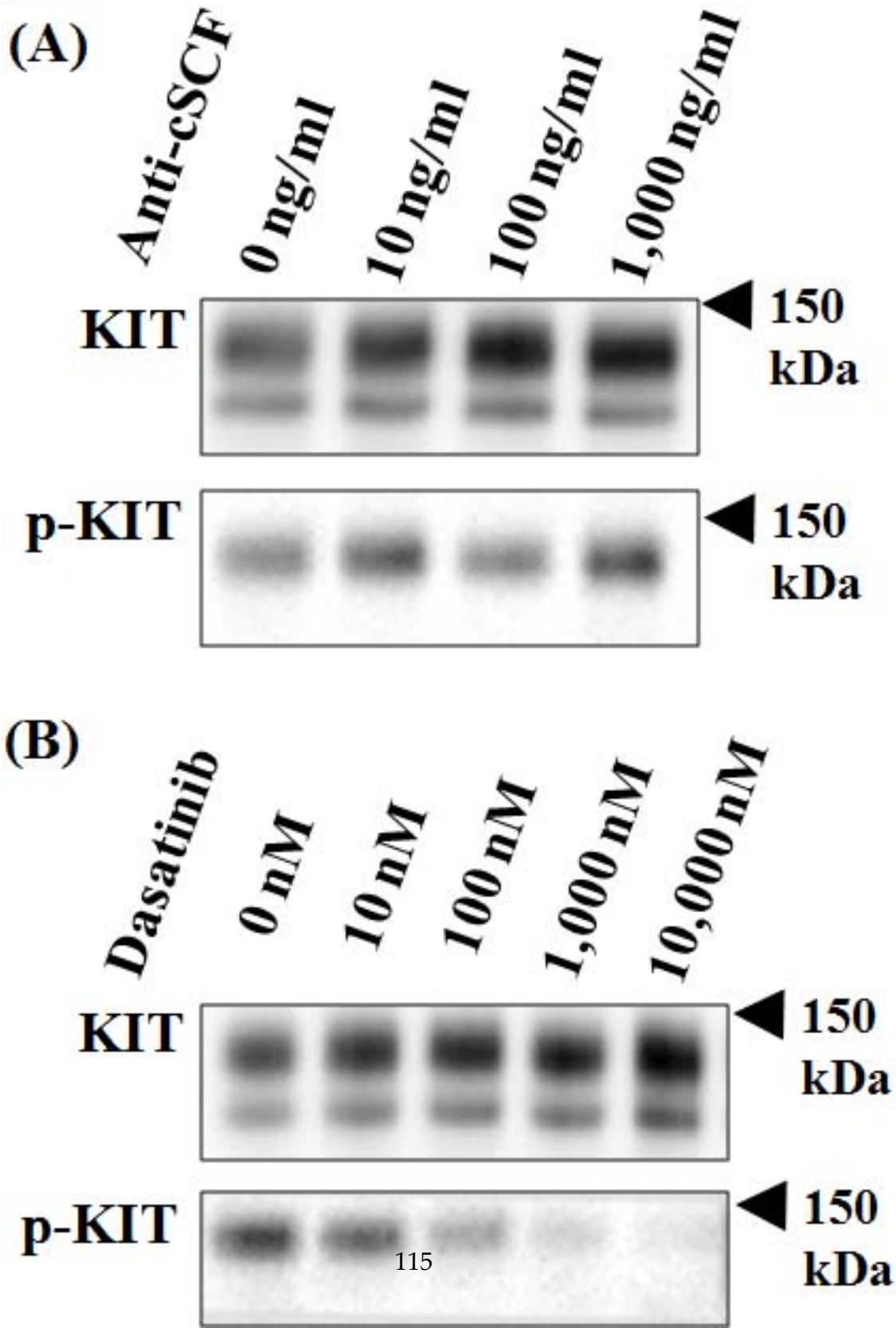
rcSCF1μg/ml
rcSCF100 ng/ml
rcSCF10 ng/ml
20 kDa

Fig. 8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Anti-cSCF
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10 ng/ml
100 ng/ml
1,000 ng/ml
KIT
150 kDa
p-KIT
150 kDa
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Dasatinib
0 nM
10 nM
100 nM
1,000 nM
10,000 nM
KIT
150 kDa
p-KIT
150 kDa

Fig. 9

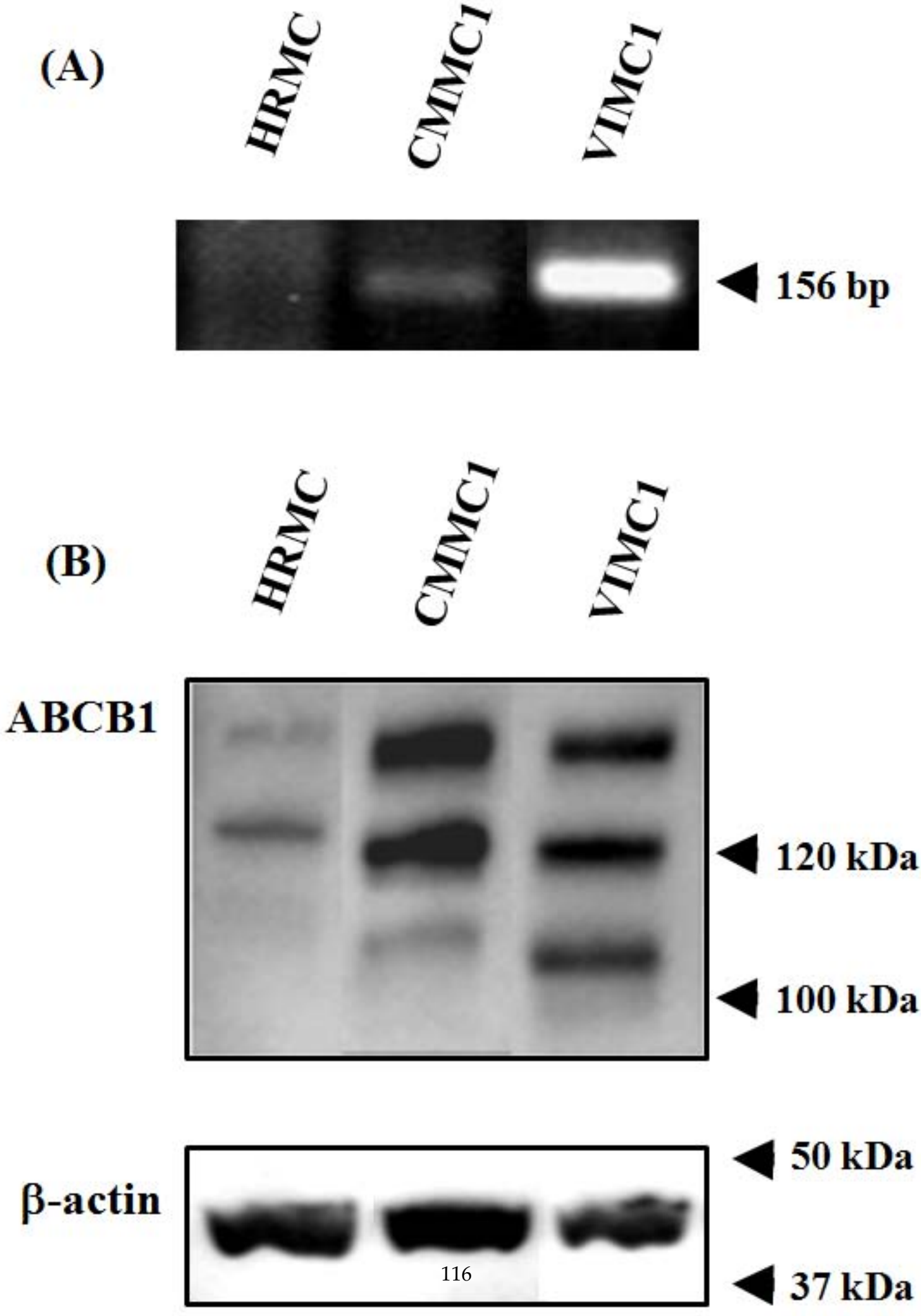

(A)
HRMC
CMMC1
VIMC1
156 bp
(B)
HRMC
CMMC1
VIMC1
ABCB1
120 kDa
100 kDa
β-actin
50 kDa
37 kDa

Fig. 10

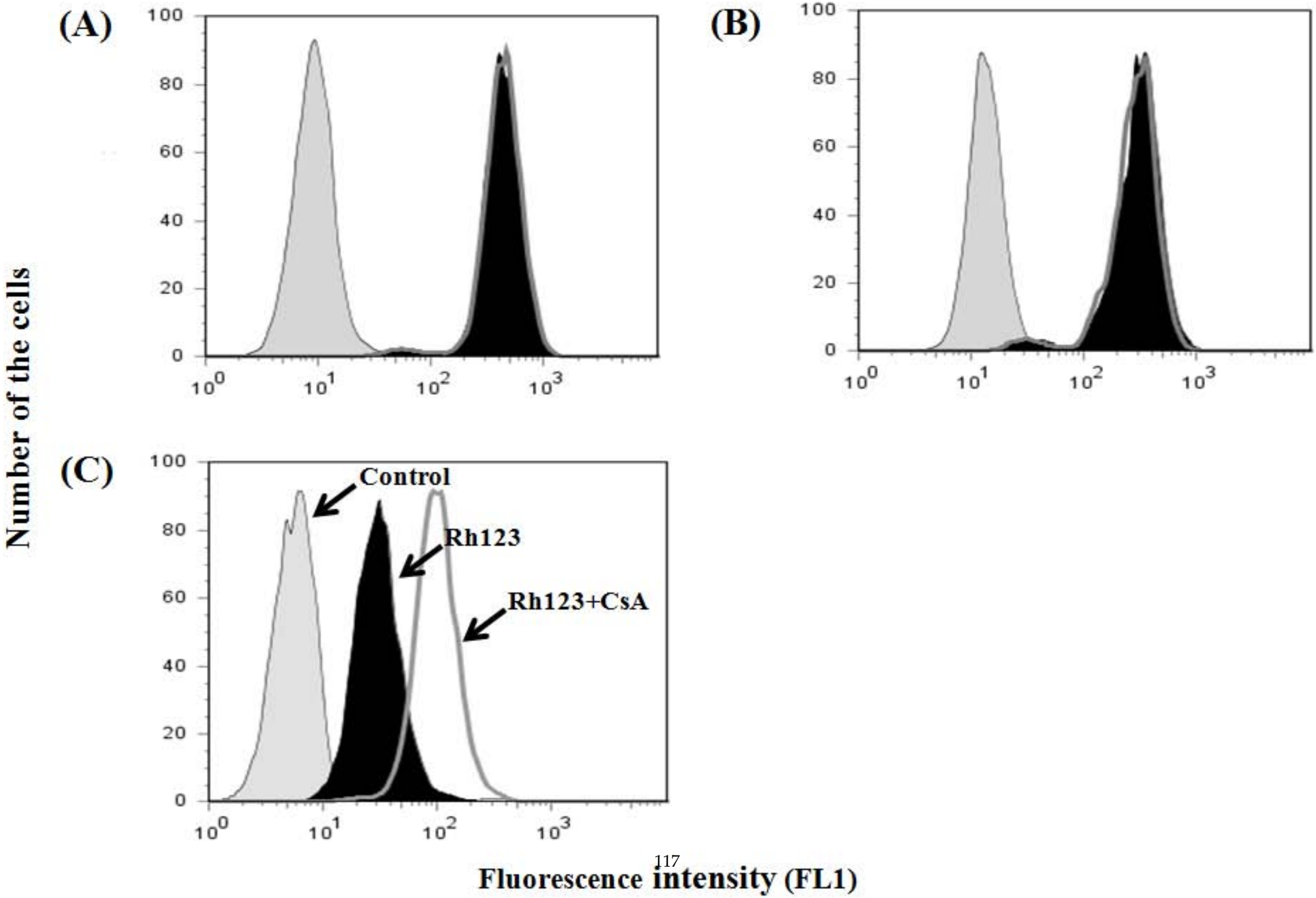

(A)
(B)
(C)
Control
Rh123
Rh123+CsA
Number of the cells
Fluorescence intensity (FL1)
0
20
40
60
80
100
10^0
10^1
10^2
10^3

Fig. 11

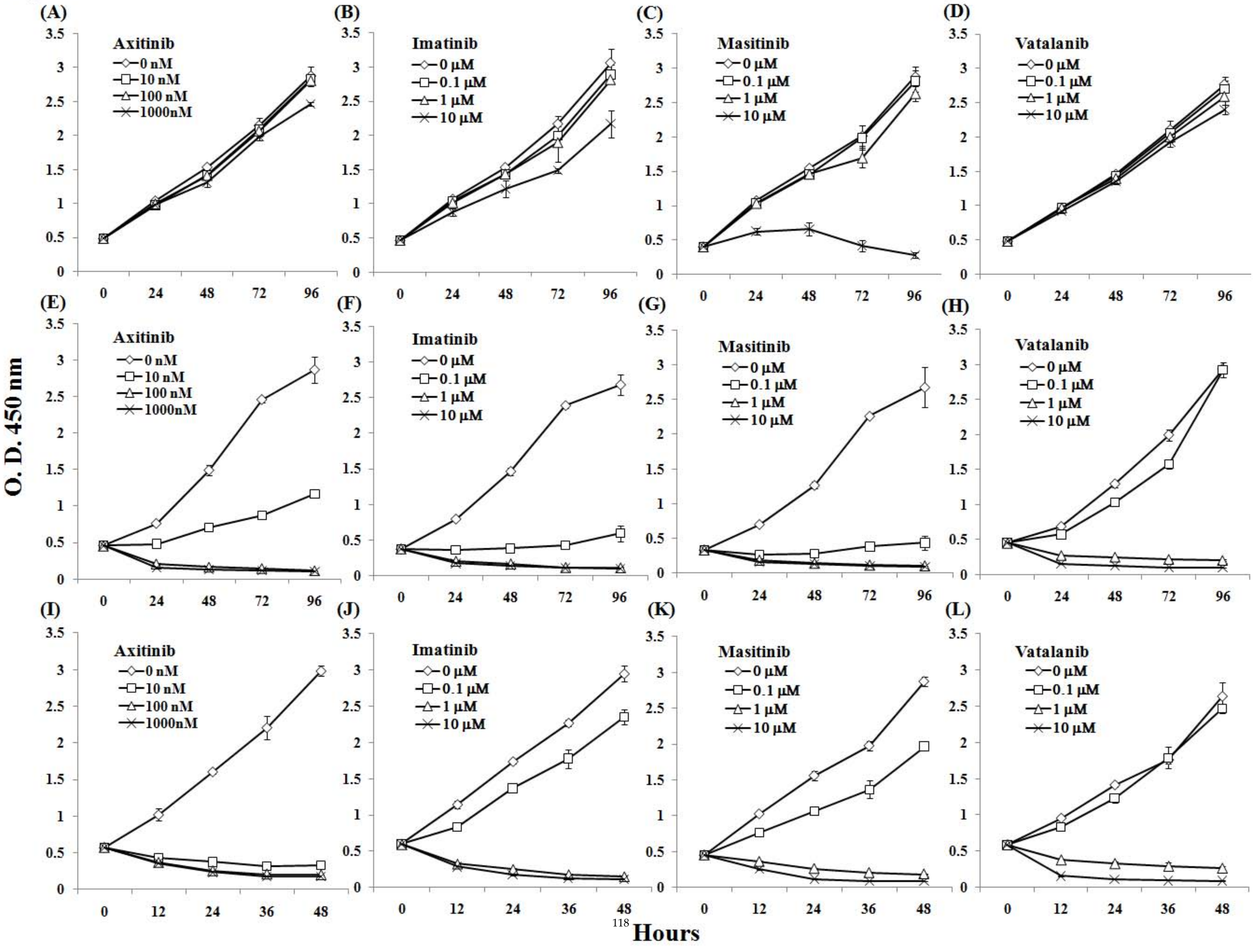

(A)
Axitinib
0 nM
10 nM
100 nM
1000nM
(B)
Imatinib
0 μM
0.1 μM
1 μM
10 μM
(C)
Masitinib
0 μM
0.1 μM
1 μM
10 μM
(D)
Vatalanib
0 μM
0.1 μM
1 μM
10 μM
(E)
Axitinib
0 nM
10 nM
100 nM
1000nM
(F)
Imatinib
0 μM
0.1 μM
1 μM
10 μM
(G)
Masitinib
0 μM
0.1 μM
1 μM
10 μM
(H)
Vatalanib
0 μM
0.1 μM
1 μM
10 μM
(I)
Axitinib
0 nM
10 nM
100 nM
1000nM
(J)
Imatinib
0 μM
0.1 μM
1 μM
10 μM
(K)
Masitinib
0 μM
0.1 μM
1 μM
10 μM
(L)
Vatalanib
0 μM
0.1 μM
1 μM
10 μM
O. D. 450 nm
Hours

Fig. 12

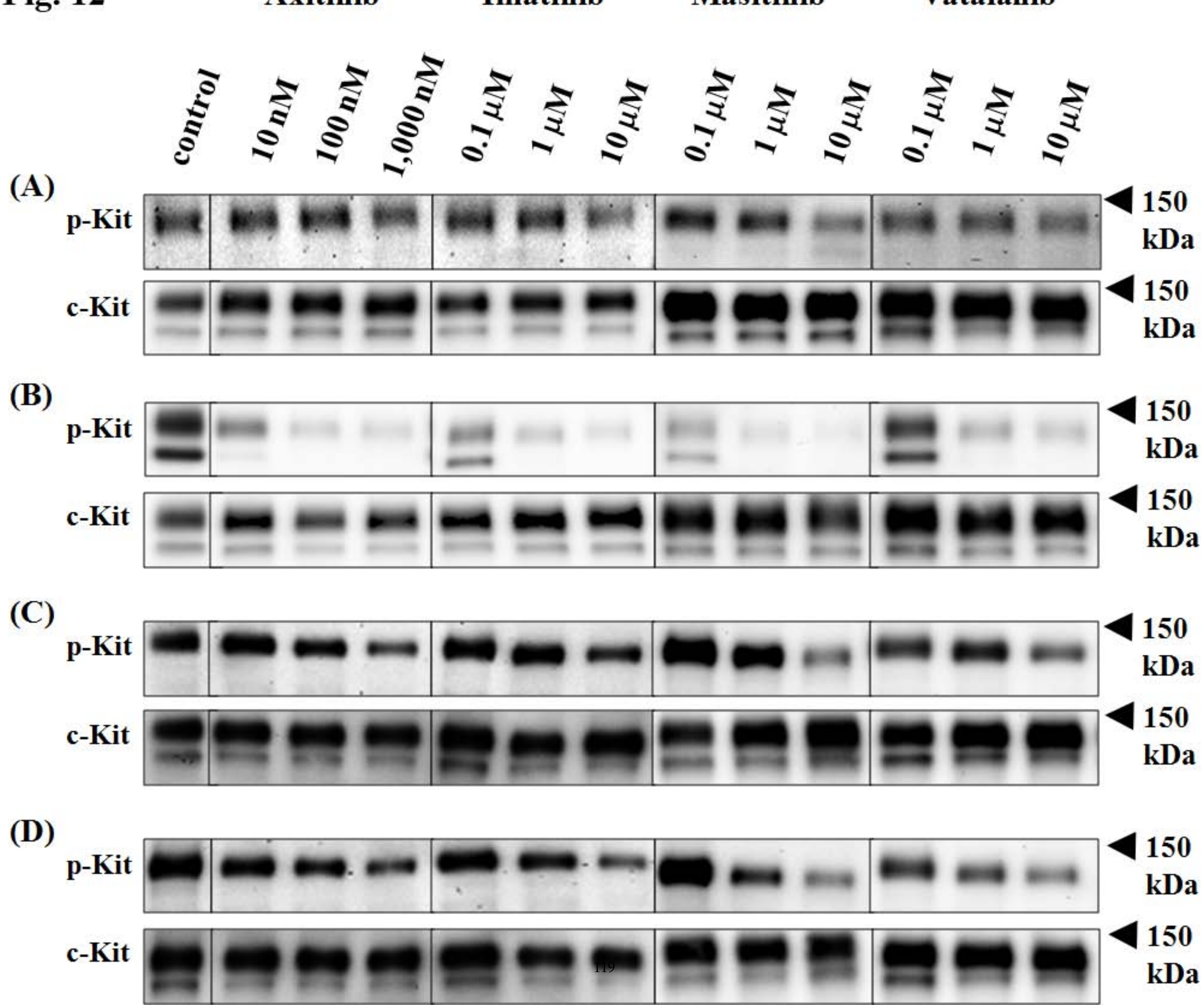

Axitinib
Imatinib
Masitinib
Vatalanib
control
10 nM
100 nM
1,000 nM
0.1 μM
1 μM
10 μM
0.1 μM
1 μM
10 μM
0.1 μM
1 μM
10 μM
(A)
p-Kit
c-Kit
150 kDa
150 kDa
(B)
p-Kit
c-Kit
150 kDa
150 kDa
(C)
p-Kit
c-Kit
150 kDa
150 kDa
(D)
p-Kit
c-Kit
150 kDa
150 kDa

Fig. 13

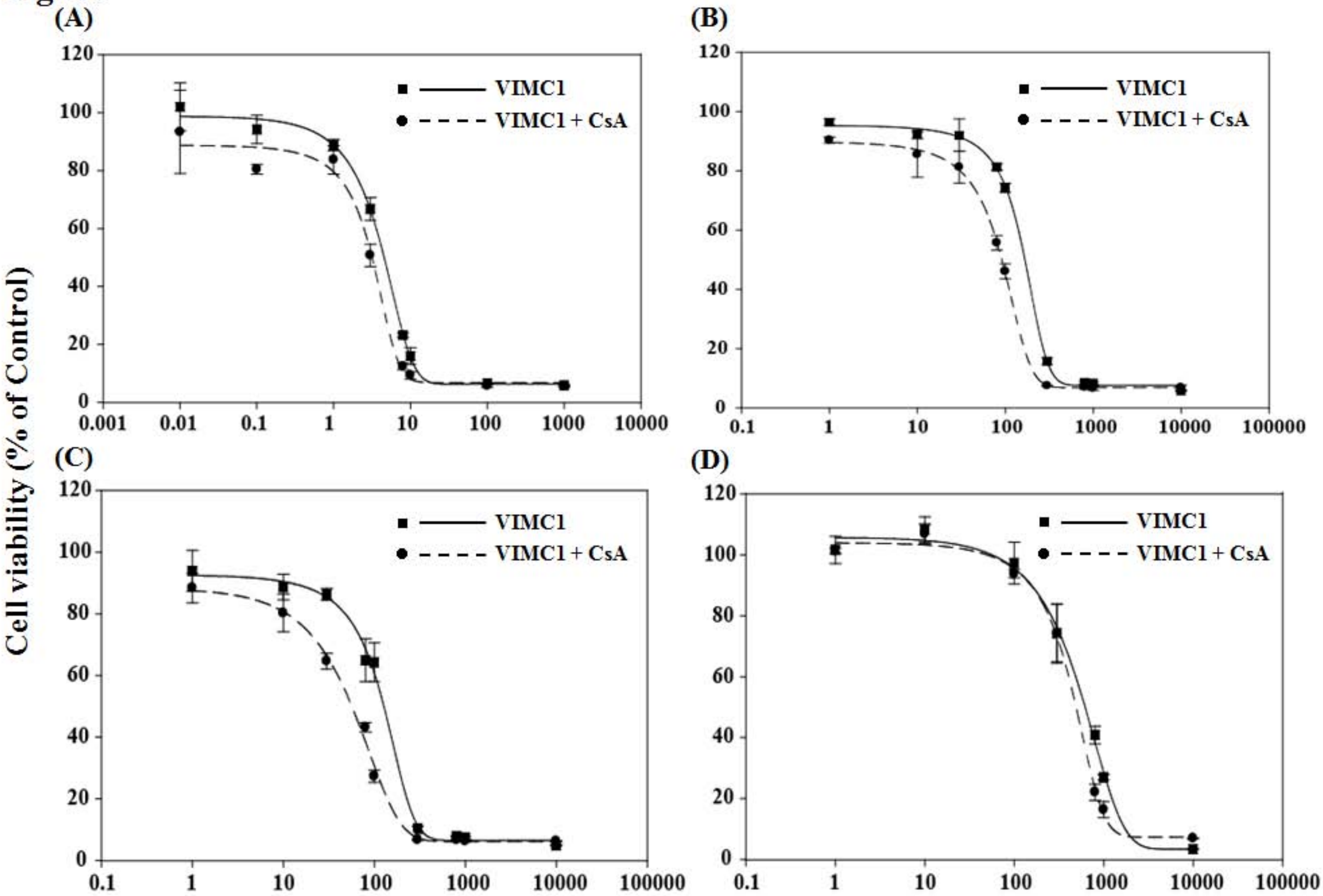

(A)
(B)
(C)
(D)
VIMC1
VIMC1 + CsA
Cell viability (% of Control)
Concentration of TKIs (nM, log)
0
20
40
60
80
100
120
0.001
0.01
0.1
1
10
100
1000
10000
100000

Fig. 14

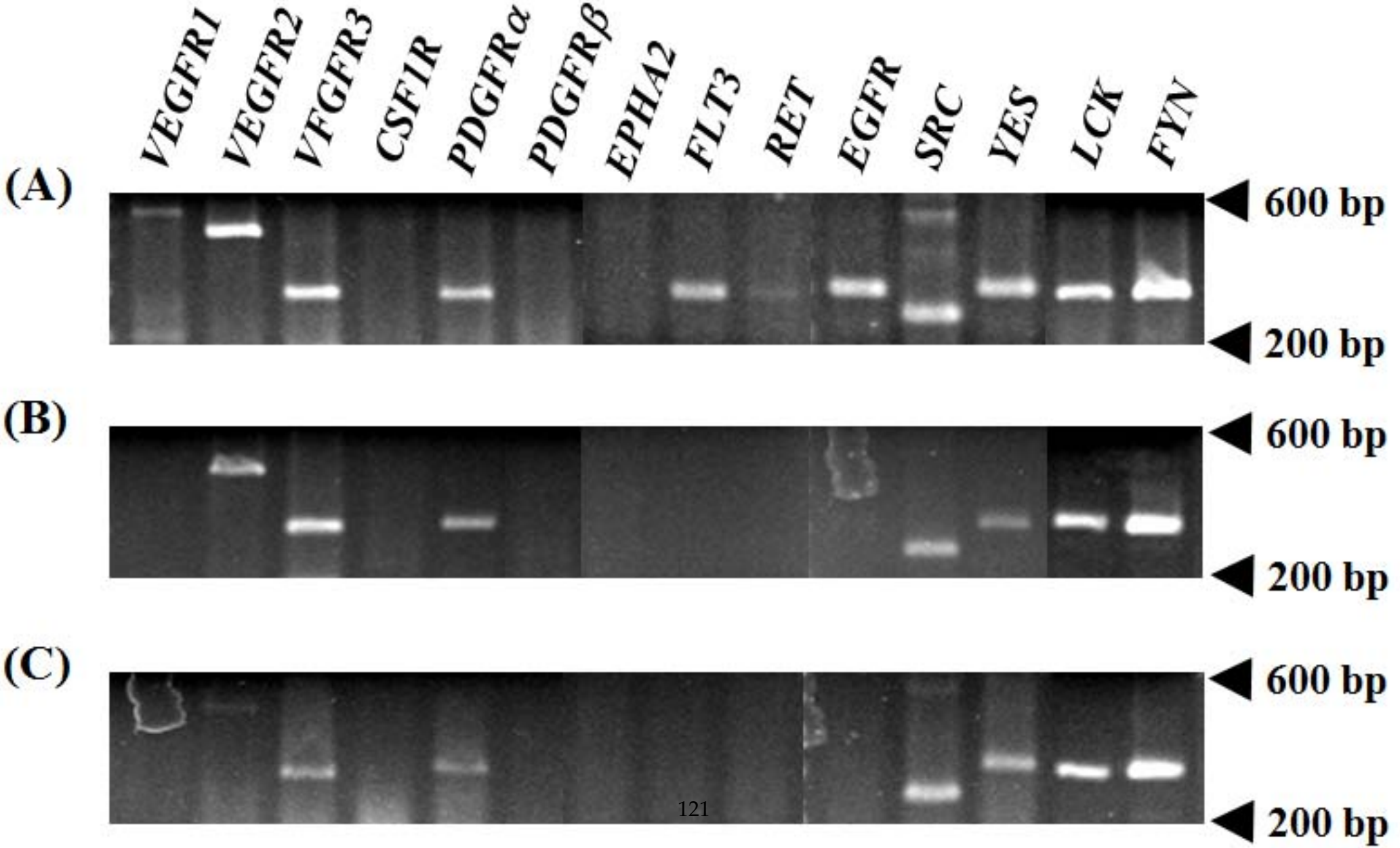
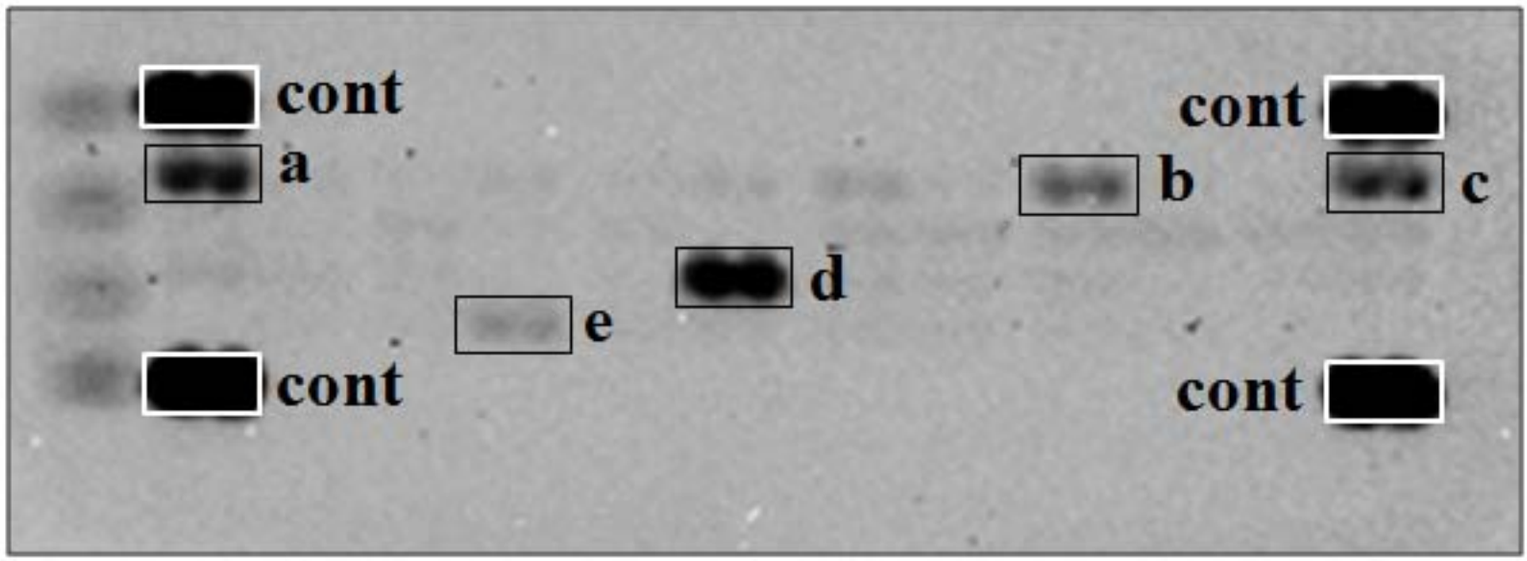
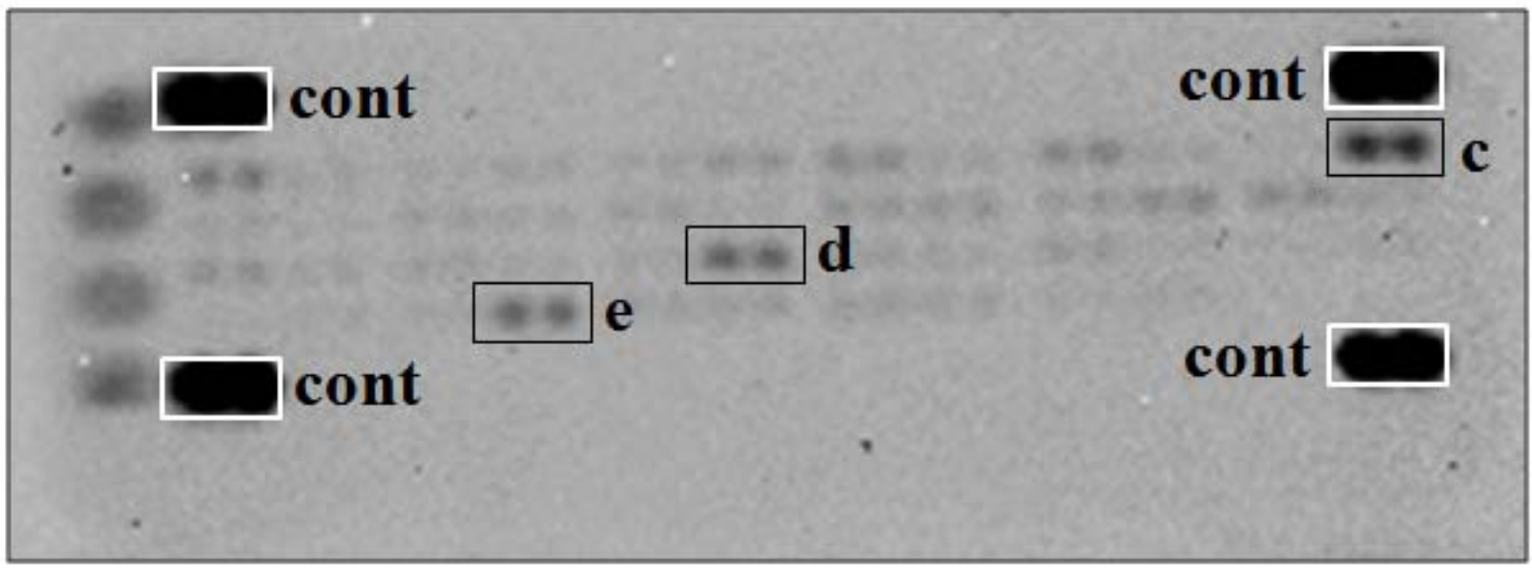

VEGFR1
VEGFR2
VFGFR3
CSF1R
PDGFRα
PDGFRβ
EPHA2
FLT3
RET
EGFR
SRC
YES
LCK
FYN
(A)
600 bp
200 bp
(B)
600 bp
200 bp
(C)
600 bp
200 bp

Fig. 15

(A)


cont
a
cont
e
d
cont
b
cont
c
cont

(B)


cont
cont
e
d
cont
cont
c

(C)

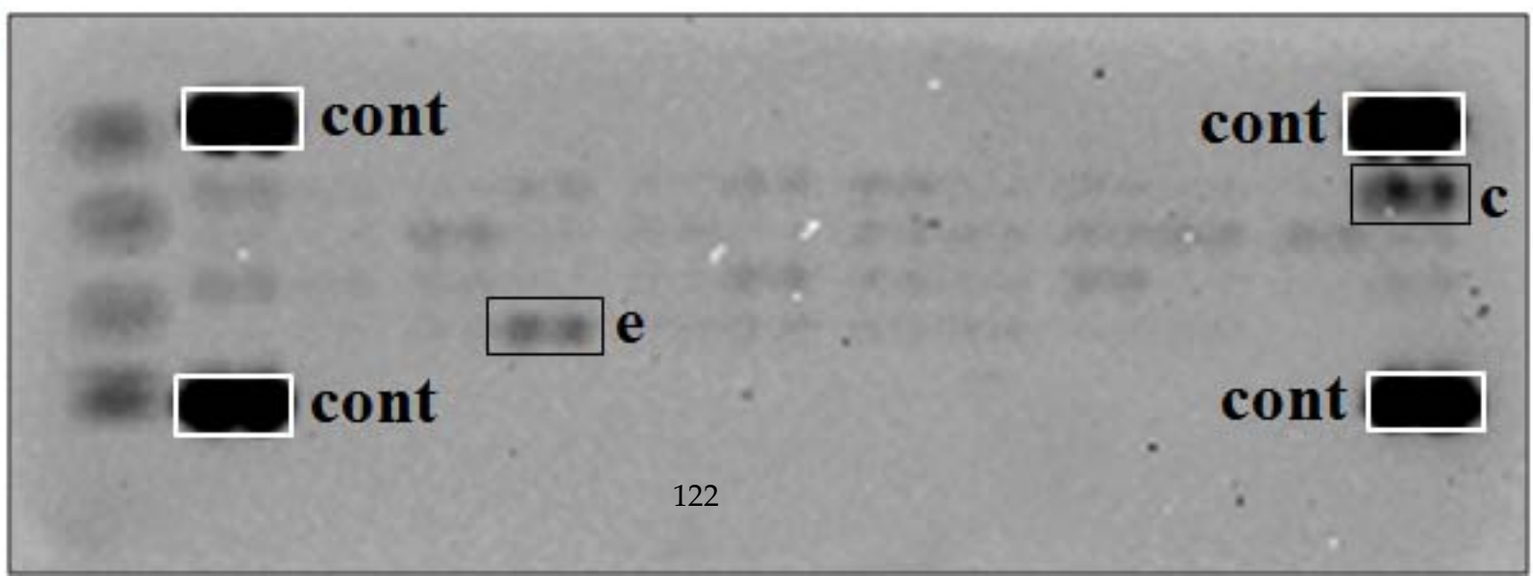
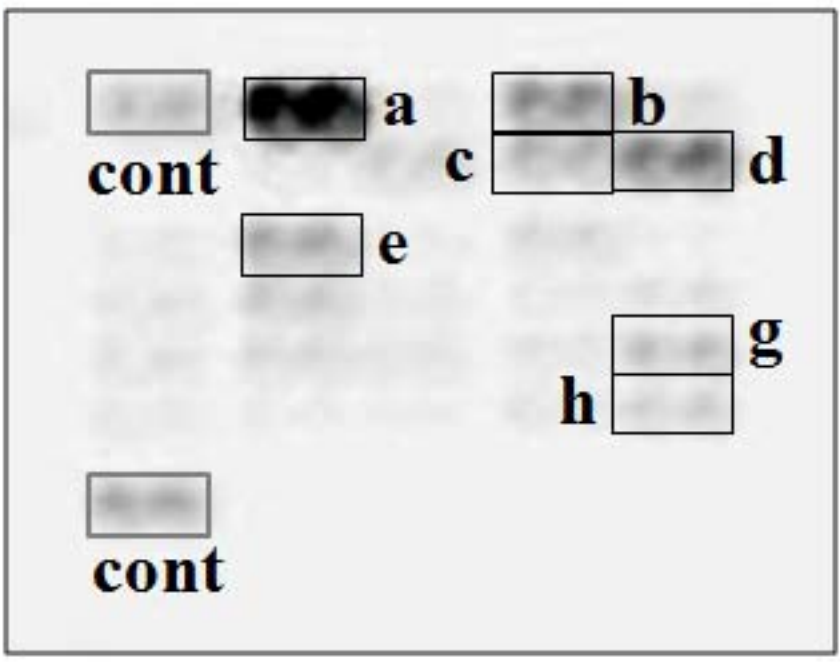
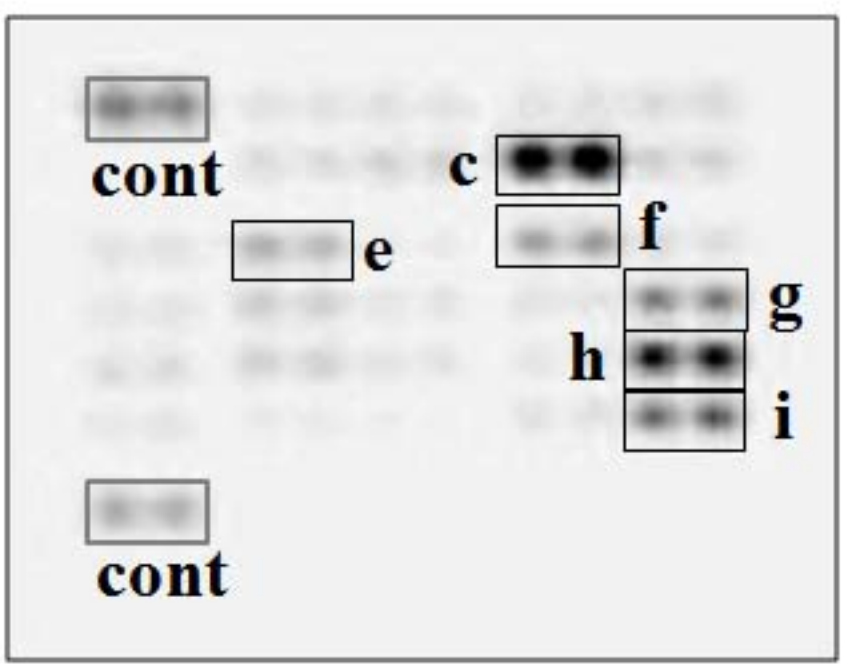

cont
cont
e
cont
cont
c

Fig. 16

(A)



(B)



(C)

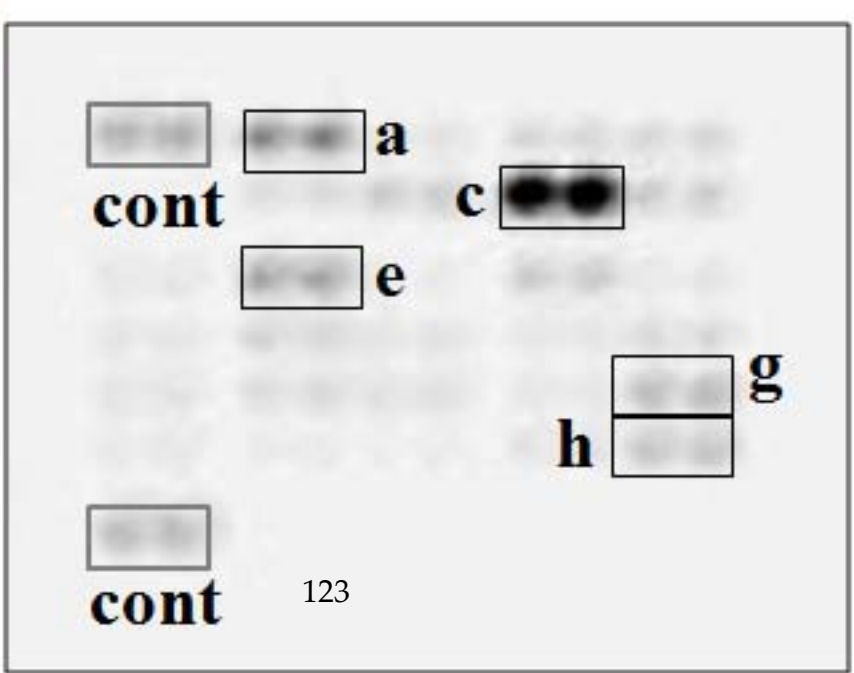


Fig. 17

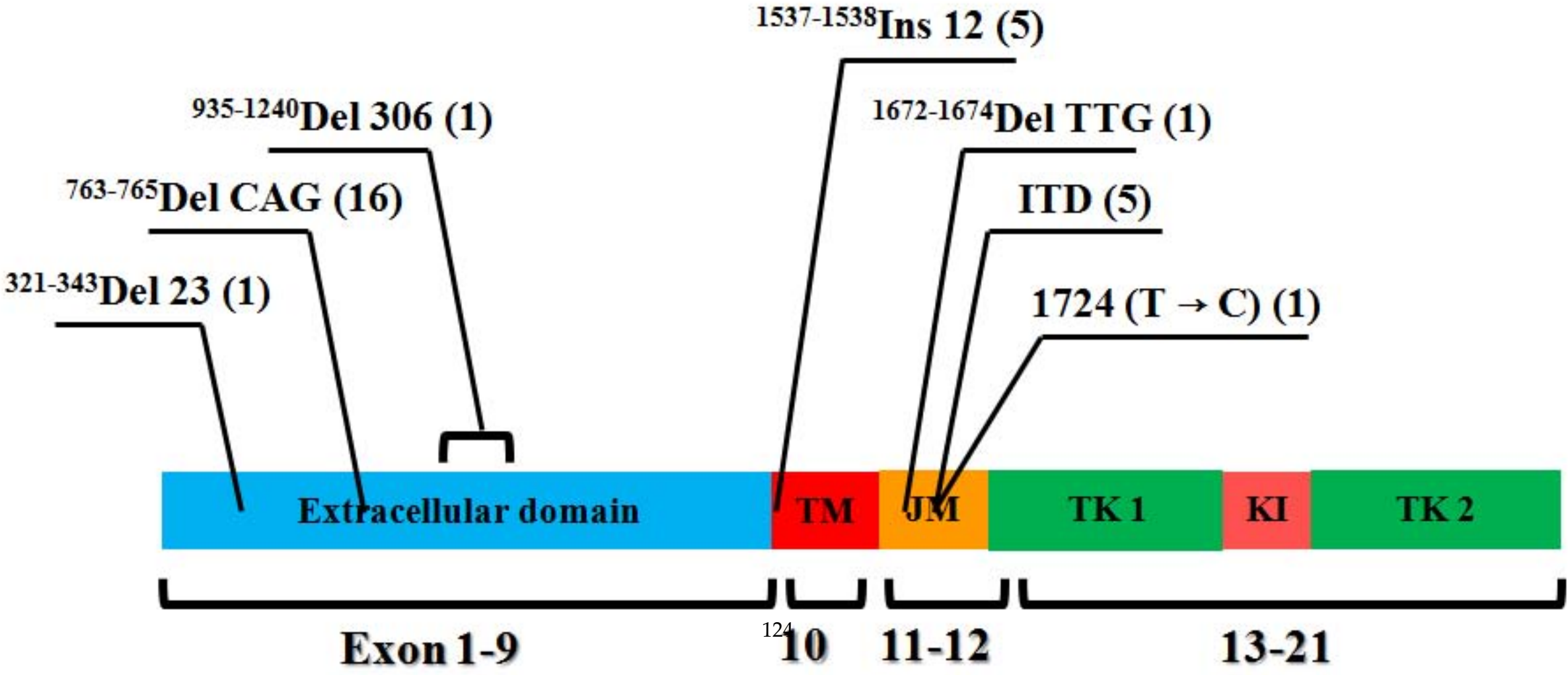

1537-1538Ins 12 (5)
935-1240Del 306 (1)
1672-1674Del TTG (1)
763-765Del CAG (16)
ITD (5)
321-343Del 23 (1)
1724 (T → C) (1)
Extracellular domain
TM
JM
TK 1
KI
TK 2
Exon 1-9
10
11-12
13-21

Fig. 18

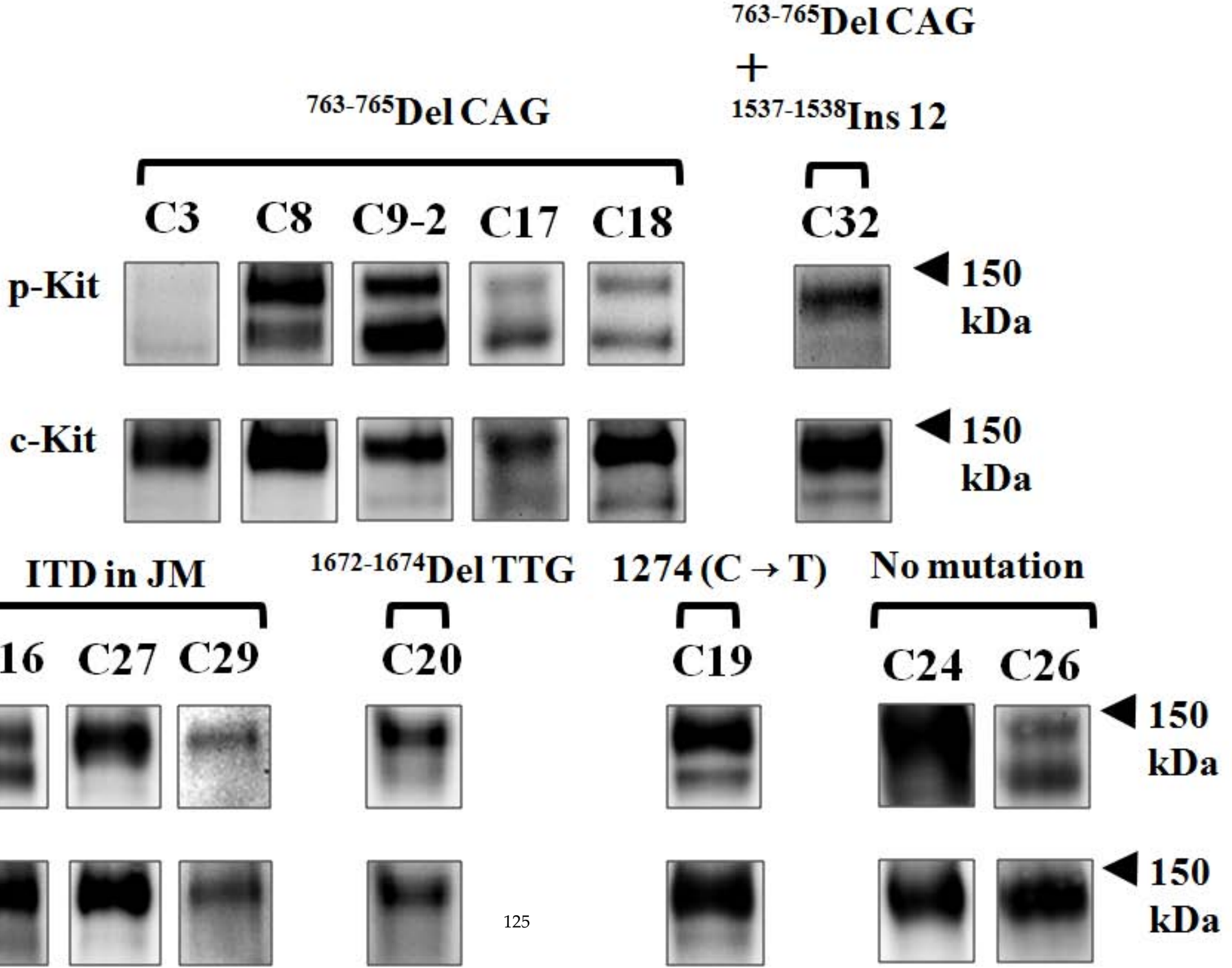
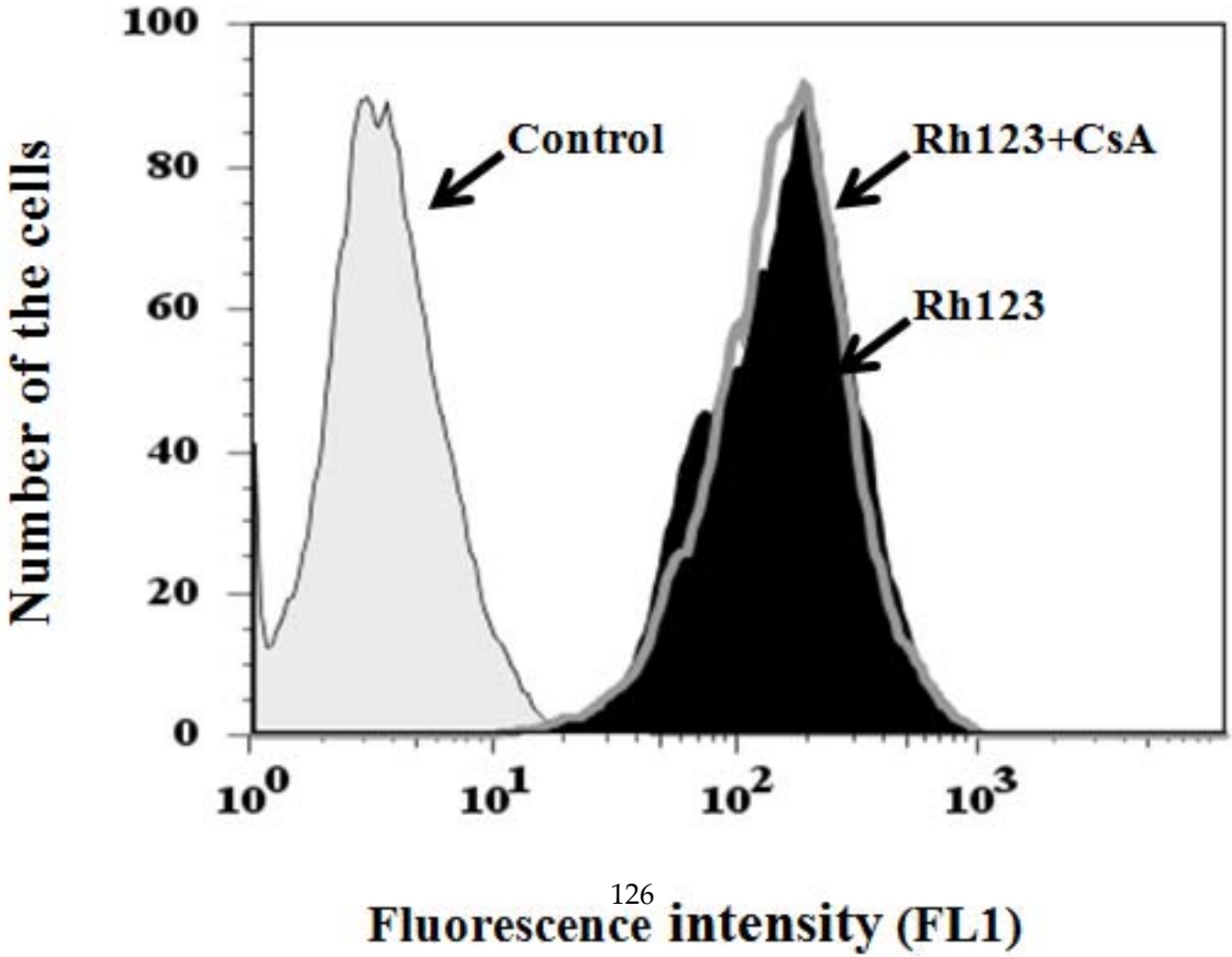

763-765Del CAG
763-765Del CAG
+
1537-1538Ins 12
C3
C8
C9-2
C17
C18
C32
p-Kit
c-Kit
150 kDa
150 kDa
ITD in JM
1672-1674Del TTG
1274 (C → T)
No mutation
C16
C27
C29
C20
C19
C24
C26
p-Kit
c-Kit
150 kDa
150 kDa

Fig. 19


100
80
60
40
20
0
Number of the cells
Control
Rh123+CsA
Rh123
10^0
10^1
10^2
10^3
Fluorescence intensity (FL1)