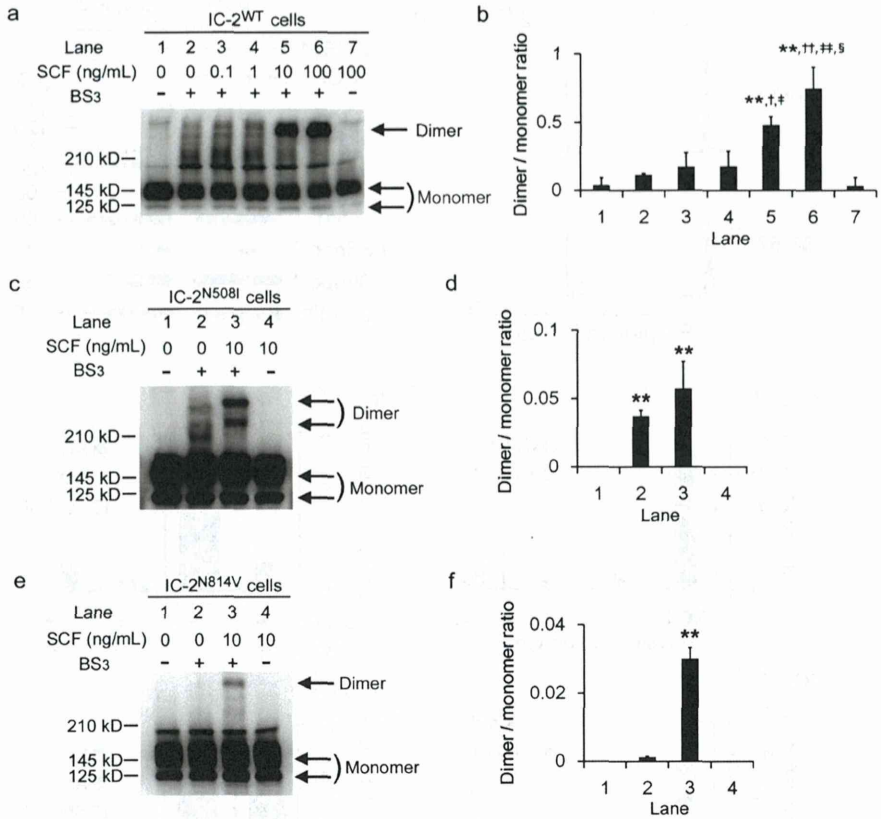

Figure 3 | KIT dimerization in IC-2 sublines. (a) western blot analysis of IC-2^{WT} cells for KIT. Cells were treated with indicated concentrations of SCF, followed by the BS_3 crosslinker (1 mM). (b) the mean dimer/monomer ratios ± SD of KIT observed in 3 independent experiments are indicated. Lane numbers correspond to those shown in Figure 3a. Arrows indicate monomeric or dimeric forms of KIT. **, $p < 0.01$ compared to lane 2; †, ‡‡, $p < 0.05$, 0.01 compared to lane 3; †, ‡‡, $p < 0.05$, 0.01 compared to lane 4; and §, $p < 0.01$ compared to lane 5, respectively. (c, e) western blot analysis of IC-2^{N508I} cells (**c**) and IC-2^{N814V} cells for KIT (**e**). Cells were treated with the indicated concentrations of SCF and/or 1 mM BS_3. Arrows indicate monomeric or dimeric KIT. (d, f) the mean dimer/monomer ratios ± SD of KIT from 3 independent experiments are shown. Lane numbers correspond to those shown in Figure 3c and e, respectively. ** $p < 0.01$ compared to lane 1.

immunohistochemistry (Fig. 4b). The *inhibitory effect of STI571* was also examined in these *in vivo* models. Daily oral administration of 100 mg/kg STI571 attenuated the growth of xenograft IC-2^{N508I} tumors by approximately 50% (Figs 4b and c). All mice were sacrificed at 11 days after STI571 administration, after which tumor tissues were collected. At this point, most tumor tissues from STI571-treated mice were necrotic. In these tumor tissues, KIT phosphorylation and Ki-*67 positivity* were markedly reduced compared to levels observed in vehicle-treated mice (Fig. 4c).

Structural modeling of wild-type and N508I KIT proteins. The data above suggest that dimerization of wild-type KIT led to the activation of the receptor and downstream signaling molecules only in the presence of SCF, while ligand-independent dimerization of N508I KIT resulted in tumorigenesis by causing aberrant signaling activations. To determine the molecular mechanism of N508I KIT dimerization, the structures of both wild-type and N508I KIT were simulated. The dimeric form of wild-type canine KIT was modeled based on the known crystal structure of human KIT[34]. Molecular modeling predicted that Asn508 residues (located in the fifth Ig-like domain) faced each other and formed hydrogen bonds in the dimerized state (Fig. 5a). Circle values, reflecting the stability of modeled structures[35], were calculated to compare differences in stability following dimerization. Under SCF-free conditions, the circle value for amino acid residue Asn508 was 1.39 for the N508I KIT mutant, which was markedly higher than that for the wild-type KIT (0.29; Fig. 5b). An increase in the circle values for the extracellular domain-mutant KIT was also confirmed in human KIT, which has been reported in AML and GIST patients (Table S1) [16–20].

Effects of KIT inhibitors on KIT dimerization. Finally, to clarify the effect of KIT inhibitors on KIT dimerization, IC-2^{N508I} cells were treated with STI571, followed by treatment with a crosslinker BS_3. We observed that the degree of KIT dimerization was markedly increased by STI571 treatment, while KIT phosphorylation was not (Figs 5c, d and e). To exclude the possibility of structure-specific KIT crosslinking by STI571, the effect of another ATP-competitive KIT inhibitor AMN107[36] was examined. The IC_{50} value of AMN107 in IC-2^{N508I} cells was 10.1 ± 0.4 nM. A dose-dependent increase in KIT dimerization was also observed following AMN107 treatment (Figs 5c, d and e), although the total amount of KIT protein expressed was unaffected (Fig. 5f). In addition, we studied the effects of KIT inhibitors on *de novo* KIT synthesis and internalization by RT-PCR and flow cytometry and observed that neither mRNA nor surface KIT expression levels were altered by STI571 and AMN107 treatment (Figs S2a, b and c).

Discussion

In this study, we demonstrated that an Asn508Ile mutation in the extracellular domain of KIT is tumorigenic in mast cells. Including our findings, the schema of KIT mutants with their activation patterns and STI571 sensitivities is presented in Fig. 6. The N508I mutant KIT conferred cytokine-independent growth of IC-2 cells

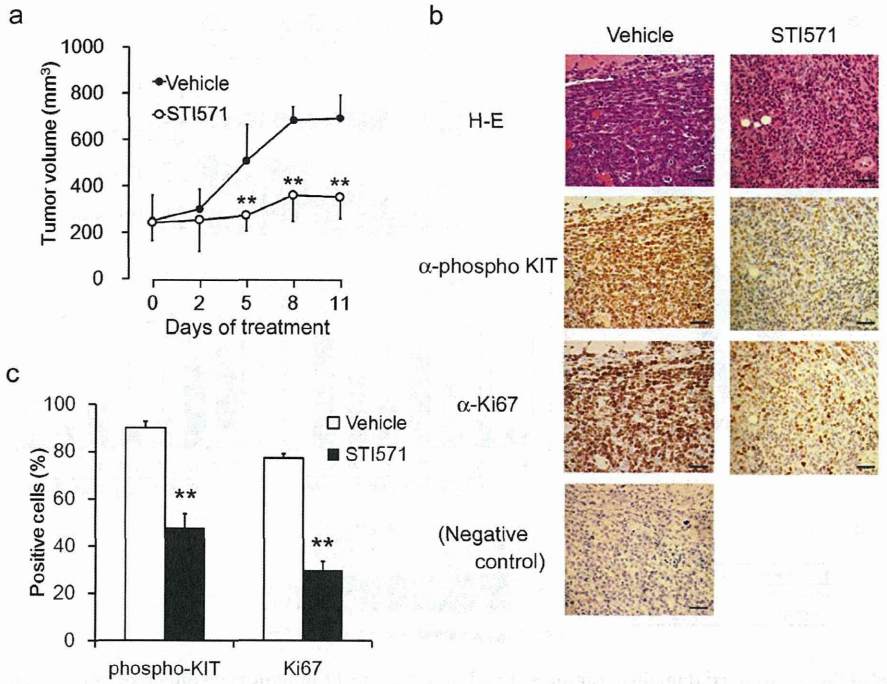

Figure 4 | *In vivo* growth and STI571 sensitivity of IC-2^{N508I} cells. (a) growth curves of IC-2^{N508I} cells *in vivo*. A total number of 5×10^6 cells were injected subcutaneously into the flanks of BALB/c-*nu/nu* mice, and tumor sizes were measured every 2 or 3 days. STI571 (100 mg/kg) was administered orally daily, starting 10 days after tumor cell transplantations (Day 0 in the graph). Each data point represents the mean ± SD for 6 animals in each group. ** $p < 0.01$, compared to vehicle-treated mice. (b) immunohistochemical analysis of IC-2^{N508I} cells. Tissues were collected on Day 11, and phospho-KIT and Ki-67 staining was conducted. Original magnification, × 200. Bar; 100 μm. (c) percentages of phospho-KIT- or Ki-67-positive cells are indicated as means ± SD obtained from 5 randomly selected microscopic fields. ** $p < 0.01$, compared to vehicle-treated.

and induced tumorigenicity both *in vitro* and *in vivo*, as a result of SCF-independent KIT dimerization and autophosphorylation.

Replacement of Asn508 residue with the hydrophobic amino acid isoleucine resulted in the enhancement of circle values, indicating that the hydrophobicity of residue Asn508 stabilizes the KIT dimer. Because spontaneous, SCF-independent dimerization of wild-type KIT (Figs 3a and b) caused neither autophosphorylation nor cytokine-independent growth in IC-2^{WT} cells, N508I mutation probably results in the conformational changes of KIT for activation. It also suggests that dimer formation does not always result in their activation, as reported in erythropoietin receptor and ErbB2/HER2 tyrosine kinases[37–39]. The glycosylated, mature form of KIT dimerizes in response to SCF binding, as observed with other receptor tyrosine kinases[40–42]. The phenomenon was also observed in a KIT variant with a mutation in the tyrosine kinase domain. Multiple bands with differing molecular weights were observed in western blots of IC-2^{N508I} cell lysates treated with BS_3. This observation suggests that the N508I mutation induces aberrant dimer formation including the unglycosylated, immature form, which in turn may trigger abnormal downstream signaling and tumor promotion. The Tyr418 and Asn505 residues in the fifth Ig-like domain play a critical role in the dimerization of human KIT[34]. Because Asn508 in canine KIT corresponds to Asn505 in the human counterpart, a reasonable hypothesis is that the N508I mutation impairs the regulation of KIT dimerization, resulting in the constitutive KIT activation. Our results raise the possibility that mutations around either Tyr418 or Asn505 in human KIT can induce constitutive KIT dimerization and autophosphorylation. In agreement, most KIT mutants examined in this study exhibited higher circle values than wild-type KIT. In addition to the KIT receptor, equivalent mutations have been discovered in other receptor tyrosine kinases[22,23,43–45]. For example, extracellular domain mutations in the fibroblast-growth factor receptor 2 (FGFR2) have been implicated in congenital malformations, such as Crouzon or Apert syndrome[43,44]. Most FGFR2 mutations associated with these diseases are located in its third Ig-like domain, and Robertson *et al.*[45] demonstrated that mutant FGFR2 variants dimerized in the absence of the natural ligand, as observed with the N508I mutant KIT in this study. Collectively, these data indicate that extracellular domain mutations, at least those in the Ig-like domains, can lead to the ligand-independent dimerization of receptors, resulting in the aberrant phosphorylation of various kinds of tyrosine kinase receptors.

Interestingly, treatment with KIT inhibitors increased the amount of activation-null KIT dimerization. Because neither STI571 nor AMN107 affected the *de novo* synthesis or internalization of KIT, inactive dimer formation may have resulted from physical interactions of these agents with KIT proteins. The degree of inactive dimer formation was not dependent on the kinase inhibitory potentials or chemical structures of those agents, but did depend on the concentration used. In addition, the increase in production of KIT dimers after STI571 or AMN107 treatment was comparable despite their distinct IC_{50} values on IC-2^{N508I} cells. The formation of inactive epidermal growth factor receptor (EGFR) dimers was observed when cells were treated with tyrosine kinase inhibitors that react with its active form[46–48], suggesting these inhibitors induced an EGFR conformation similar to that of the activated state, without actually activating the kinase domain. Inactive KIT dimers formed by STI571 or AMN107 treatment may resemble inactive EGFR dimers. Though our results demonstrated the efficacy of tyrosine kinase inhibitors on N508I KIT, agent-dependent inactive dimer formations may modify outcomes in clinical cases by altering the duration of KIT turnover.

To the best of our knowledge, this is the first report demonstrating the tumorigenicity of an extracellular domain KIT mutation causing auto-dimerization in mast cells. These results aid in the understanding of the effects of KIT mutations, not only in mast cells, but also in other types of malignancies harboring mutations in tyrosine kinase-type receptors.

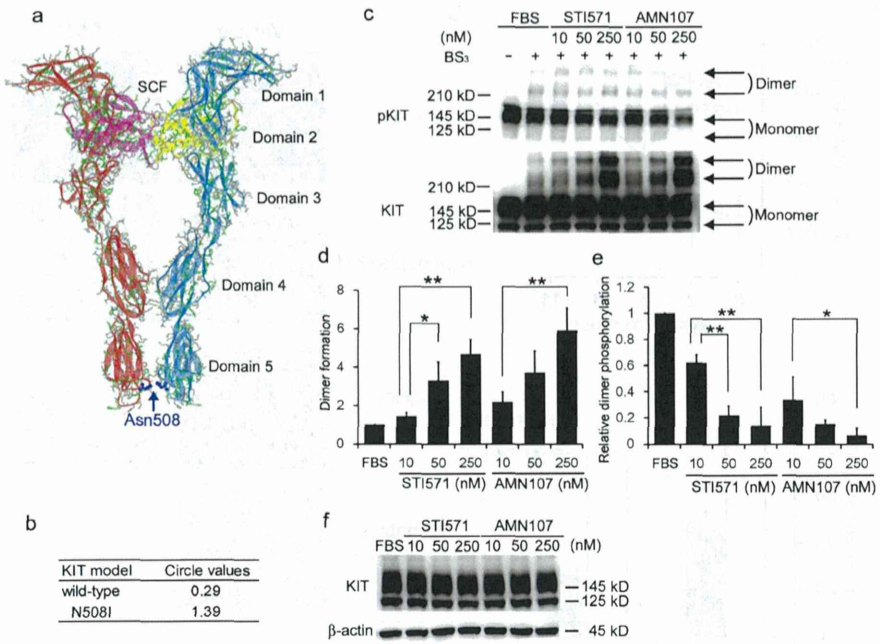

Figure 5 | Structure model of the extracellular domain of canine KIT and the effect of KIT inhibitors on dimerization. (a) modeling of the extracellular KIT domain in the SCF-bound condition. Red and blue ribbons indicate main-chains, green wires indicate the hydrophobic residues, and the blue ball & stick models indicates the residue Asn508. Pink and yellow ribbons indicate stem cell factor. (b) comparison of circle values between wild-type and N508I mutant canine KIT. Circle values for residue 508 in wild-type and N508I canine KIT are indicated. (c) western blot analysis of IC-2^{N508I} cells treated with STI571 or AMN107. Cells were treated with either reagent for 4 h, followed by chemical crosslinking with BS_3. The monomeric and dimeric forms of KIT are indicated with arrows. (d) the relative mean dimer/monomer ratios ± SD from 3 independent experiments are shown. The dimer/monomer ratios of cells cultured in FBS-containing medium were set to 1. *, **, $p < 0.05$, 0.01, compared to treatment with 10 nM STI571 or AMN107, as indicated. (e) relative mean dimer phosphorylation levels ± SD from 3 independent experiments are indicated. The dimer/monomer ratio observed in cells cultured in FBS-containing medium was set to 1. *, **, $p < 0.05$, 0.01 compared to cells treated with 10 nM of the indicated reagents. (f) western blot analysis of IC-2^{N508I} cells treated with STI571 or AMN107. Cells were treated by each agent for 4 h and lysed without BS_3 treatment.

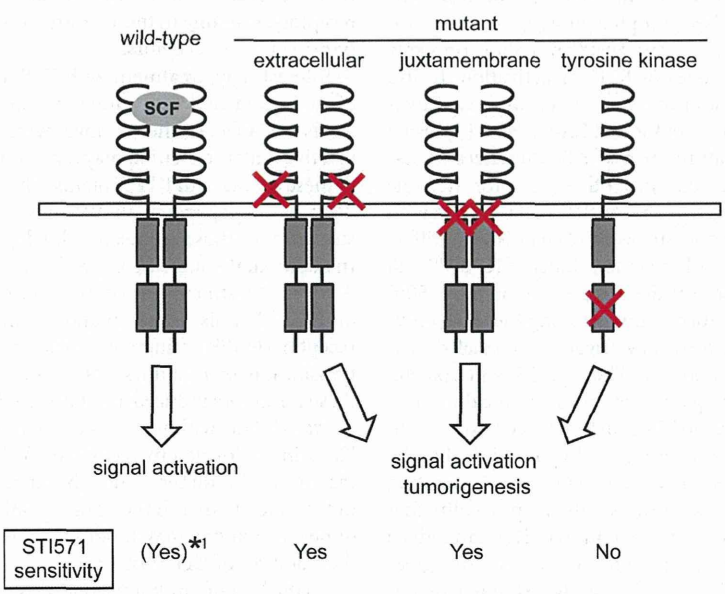

Figure 6 | Schematic representation of mutant KIT phenotypes. This diagrammatic representation describes the correlation of known KIT mutations with activation patterns. While wild-type KIT dimerizes and gets activated only in the presence of SCF, KIT with mutations in either the extracellular or the juxtamembrane domain dimerizes and becomes activated independently of SCF binding. The variant KIT with a mutation in the tyrosine kinase domain does not require SCF stimulation or dimerization for activation. STI571 sensitivities are also indicated. *1, sensitive to STI571, but less sensitive than mutant KIT.

Materials and methods

MCTs and sequence analysis of c-*kit*. All dogs included in this study were previously referred for MCTs to the Animal Medical Center at Tokyo University of Agriculture and Technology. After appropriate surgical removal or fine needle aspiration of MCT specimens, total RNA from each sample was extracted by using Isogen (Nippon Gene, Toyama, Japan) and cDNA was synthesized with PrimeScript (Takara, Otsu, Japan). Polymerase chain reaction (PCR) was performed using a c-*kit*-specific forward primer (5′-GGA ATT CGC CAC CGC GAT GAG AGG CGC TCG CGG CGC CT-3′), a c-*kit*-specific reverse primer (5′-CTC TGC GGC CGC TCA CAC ATC TTC GTG TAC CAG CA-3′), and PrimeSTAR Max DNA Polymerase (Takara), according to the manufacturer's instructions. The c-*kit* gene was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Gaithersburg, MD), forward primers corresponding to bases 243–263, 648–667, 1050–1069, 1484–1504, 1843–1862, 2205–2225, and 2639–2657, and a reverse primer corresponding to bases 386–406 (GenBank accession no. AF044249). Samples were analyzed using the ABI PRISM 3100-*Avant* Genetic Analyzer (Life Technologies). All experiments using clinical samples complied with the standards specified in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Cell culture. The IC-2 mast cell line was a generous gift from Dr. Y. Kitamura (Osaka University, Osaka, Japan). IC-2 cells are phenotypically similar to mast cells, except that they lack KIT expression[14]. IC-2 cells were cultured in alpha-minimum essential medium (α-MEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS), antibiotics, and 10 ng/mL recombinant murine IL-3 (R&D systems, Minneapolis, MN).

Retroviral vector construction. Full-length wild-type c-*kit* cDNA was amplified using template cDNA from an HRMC mast cell line[49], as described above. The PCR product was ligated into the pMXs-IRES-GFP plasmid (Cell Biolabs, San Diego, CA) using conventional methods and designated pMXs-KITWT-IRES-GFP. A plasmid encoding an Asn508Ile mutant (N508I) of c-*kit* was generated by site-directed mutagenesis and designated pMXs-KITN508I-IRES-GFP.

Retroviral transfer. GP2-293 packaging cells (Clontech, Palo Alto, CA) were cotransfected with the pCMV-VSV-G plasmid (Cell Biolabs) and either the pMXs-IRES-GFP, pMXs-KITWT-IRES-GFP, or pMXs-KITN508I-IRES-GFP retroviral plasmid, using FuGENE 6 (Roche, Indianapolis, IN). The supernatant, containing replication-deficient virus particles, was used to infect IC-2 cells. The resulting IC-2 sublines expressing pMXs-IRES-GFP, pMXs-KITWT-IRES-GFP, or pMXs-KITN508I-IRES-GFP were termed IC-2^{vector}, IC-2^{WT}, and IC-2^{N508I} cells, respectively (Table 1). IC-2^{N814V} cells expressing the Asn814Val mutation (Table 1) were generated as previously described[50]. After establishment of these cell lines, all IC-2 sublines were maintained in α-MEM containing 10% FBS, antibiotics, and 10 ng/mL IL-3.

Western blotting. After serum starvation for 12 h, cells were incubated for 4 h with 10 ng/mL SCF and/or 250 nM STI571 (Novartis Pharmaceuticals, Basel, Switzerland), and immunoblot analysis was conducted as described previously[51]. Primary antibodies including anti-phospho-KIT, anti-total/phospho-Akt, anti-total/phospho-S6 ribosomal protein, and β-actin antibodies purchased from Cell Signaling Technologies (Beverly, MA), and an anti-KIT antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Chemical crosslinking assay. IC-2 sublines were washed and resuspended in phosphate-buffered saline (PBS) containing 1 mg/mL bovine serum albumin. Cells were then incubated for 90 min at 4°C in the presence or absence of recombinant canine SCF and washed 3 times in PBS. In some experiments, cells were treated with STI571 or AMN107 (Selleck Chemicals, Houston, TX) at the indicated concentrations. Subsequently, cells were incubated for 30 min at 22°C in PBS containing 1 mM BS_3 (Sigma-Aldrich Japan, Tokyo, Japan). Cross-linking reactions were terminated by washing the cells in ice-cold PBS, followed by incubation with 150 mM glycine-HCl (pH 7.5) for 5 min at 4°C. Western blot analysis was performed using lysates from these cells.

WST assay. After serum deprivation for 12 h, cells were incubated with or without STI571 for 72 h. A WST assay was performed using the WST-8 Kit (Kishida Chemicals, Osaka, Japan) according to the manufacturer's instructions. Fifty percent IC_{50} values were defined as the concentration of inhibitors at which 50% of cellular activation was attenuated compared to the control.

Flow cytometry. To detect the expression of KIT receptors, cells were stained with an anti-KIT-APC antibody (clone 2B8, BioLegend, San Diego, CA). KIT-positive cells were detected with a MACSQuant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). For cell cycle analysis, cells were harvested after a 24-h incubation with or without STI571 and fixed in 70% ice-cold ethanol, followed by treatment with RNase A and propidium iodide. Data were processed using the FlowJo FACS analysis software ver. 9.5.3 (Tree Star, Inc., Ashland, OR).

Growth assessment of IC-2 sublines *in vivo*. All experiments with animals complied with both the standards specified in the guidelines of the University Animal Care and Use Committee of the Tokyo University of Agriculture and Technology and the guidelines for the use of laboratory animals provided by Science Council of Japan, as well as in accordance with Declaration of Helsinki, and were approved by the institutional committee. A total of 5×10^6 IC-2 sublines were injected subcutaneously into the right and left flanks of 6-week-old female BALB/c-*nu/nu* mice (Charles River Japan, Yokohama, Japan). Tumors were measured with a caliper every 2 or 3 days. Tumor volumes (V) were calculated using the formula, $V = ab^2/2$, where a and b are the length and width of tumor masses in mm, respectively. After 11 days of daily oral administration of 100 mg/kg STI571, mice were sacrificed and tumor tissues were used for immunohistochemical analysis.

Immunohistochemistry. Histological analysis was conducted using IC-2^{N508I} tumor cells according to a previously described method[52]. An anti-phospho-KIT antibody (Abcam, Cambridge, UK) and an anti-Ki67 antibody (Abcam) were used as primary antibodies. Images were captured using a Nikon microscope (Nikon, Melville, NY).

Structural modeling of the extracellular domain of KIT. Wild-type and mutant KIT conformations and KIT dimer stability were simulated using PDFAMS software (In-Silico Sciences Inc., Tokyo, Japan) by referencing the human c-*kit* gene, which shares approximately 80% homology with the canine c-*kit* gene[34]. The stability of the dimeric form was calculated by using circle values[35]. When 2 or more structures were compared by circle values, the structure with the highest value is more stable than the others[35].

Statistical analysis. A Mann-Whitney's U test, Dunnett's test, and a Student's t-test were performed for statistical analysis of the data, and p values of < 0.05 were considered to be statistically significant.

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Additional information

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REVIEW

Crosstalk of carcinoembryonic antigen and transforming growth factor-β via their receptors: comparing human and canine cancer

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Abstract There is accumulating evidence that the transforming growth factor beta (TGF-β) and nuclear factor kappa-B (NFκB) pathways are tightly connected and play a key role in malignant transformation in cancer. Immune infiltration by regulatory T- and B-lymphocytes (Tregs, Bregs) has recently gained increased attention for being an important source of TGF-β. There is a plethora of studies examining the pro-tumorigenic functions of carcinoembryonic antigen (CEA), but its receptor CEAR is far less studied. So far, there is a single connecting report that TGF-β also may signal through CEAR. The crosstalk between cancer tissues is further complicated by the expression of CEAR and TGF-β receptors in stromal cells, and implications of TGF-β in epithelial–mesenchymal transition. Furthermore, tumor-infiltrating Tregs and Bregs may directly instruct cancer cells by secreting TGF-β binding to their CEAR. Therefore, both TGF-β and CEA may act synergistically in breast cancer and cause disease progression, and NFκB could be a common crossing point between their signaling. CEAR, TGF-β1–3, TGF-β-R types I–III and NFκB class I and II molecules have an outstanding human–canine sequence identity, and only a canine CEA homolog has not yet been identified. For these reasons, the dog may be a valid translational model patient for investigating the crosstalk of the interconnected CEA and TGF-β networks.

Keywords Carcinoembryonic antigen (CEA) · CEA-receptor (CEAR) · Transforming growth factor beta (TGF-β) · Cancer immunology · Regulatory · Nuclear factor kappa-B (NFκB)

Abbreviations

AAV	Adeno-associated virus
Akt	Protein kinase B
Breg	Regulatory B-lymphocyte
CEA	Carcinoembryonic antigen (CEACAM5)
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CEAR	Carcinoembryonic antigen receptor
CEARL	Carcinoembryonic antigen receptor, long isoform
CEARS	Carcinoembryonic antigen receptor, short isoform
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor (ErbB1)
EMT	Epithelial-to-mesenchymal transition
FGF	Fibroblast growth factor
HER-2	Human epidermal growth factor receptor 2 (ErbB2)
HGF	Hepatocyte growth factor
hnRNP M4	Heterogeneous nuclear ribonucleoprotein M4 (CEAR)
IGFs	Insulin-like growth factors
IKK	Inhibitor of nuclear factor kappa-B kinase
IKKb	Inhibitor of nuclear factor kappa-B kinase subunit beta
IκB	Inhibitor of kappa-B

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IκBα	Inhibitor of kappa-B subunit alpha
MDCK	Madin–Darby canine kidney cell line
MEK	Mitogen-activated protein kinase kinase
NFκB	Nuclear factor kappa-B
PDGF	Platelet-derived growth factor
RelA	v-Rel avian reticuloendotheliosis viral oncogene homolog A
SMAD	SMA and MAD homolog
TAB1	TAK1-binding protein 1
TAK1	Transforming growth factor-activated kinase-1
TGF-β	Transforming growth factor beta
TGF-β-R	Transforming growth factor beta receptor
Treg	Regulatory T-lymphocyte

Introduction

The strategy of comparative oncology is to find homologous molecules, homologous signaling cascades and homologous immune mechanisms to cure cancer in both humans and pets according to the "One Health" principle [1]. Similar to humans, dogs spontaneously develop malignancies with comparable incidence and prevalence and hence represent a natural model for human cancer. For instance, a Swedish study on 80,000 insured female dogs reported that, dependent on higher age and breed, up to 13 % of female dogs had at least one mammary tumor, with an overall-case fatality of 6 % [2]. In humans, females in more highly developed areas have a cumulative risk of 7.1 % of developing mammary cancer by the age of 75, with a mortality rate of 1.7 % [3]. Mammary carcinoma, among others, is thus a burden in both human and veterinary medicines.

The rationale for favouring this tumor entity for comparative studies derives from the fact that it is wise to have access to primary lesions for monitoring tumor progression by caliper measurements. This facilitates the clinical investigations and also takes into consideration that only few centers have access to imaging facilities. Often more than one mamilla are affected in canine cancer patients and may be compared side by side.

It can further be expected that results from comparative oncology studies, investigating naturally occurring cancers due to distinct risk factors in distinct breeds, have a higher translational potential than studies with genetically highly homologous mouse strains [4]. For example, the epidermal growth factor receptor (EGFR) family members EGFR (ErbB1) and human epidermal growth factor receptor 2 (HER-2 (ErbB2)) are molecules of outstanding homology between humans and dogs, and targeting of these molecules results in the same effects on signaling and cancer biology in both species [5, 6].

A more intricate situation was observed for the carcinoembryonic antigen [CEA, also termed carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5)], which represents a classical soluble as well as membrane-expressed tumor marker in human clinical oncology. Serum levels of soluble human CEA correlate with disease progression [7], and its assessment is recommended in monitoring the treatment course of colorectal cancer in combination with other prognostic markers [8, 9]. However, CEA molecules are structurally and evolutionarily diverse between humans and canines [10, 11]. A direct CEA homolog in dogs has not yet been defined and represents "a missing link" (Table 1). In contrast, overexpression of CEA in humans has been known for over 20 years to play an important role in metastasis and cell motility [12] by acting as a ligand for E- and L-selectins

Table 1 Interspecies amino acid sequence comparisons

Molecule	Human	Canine	Sequence identity (%)	Sequence similarity (%)
CEAR	HNRPM_HUMAN	XP_005633012.1	99.3	99.5
CEA (CEACAM5)	CEAM5_HUMAN	n.d. [20]	–	–
TGF-β-RI	TGFR1_HUMAN	F1PS63_CANFA	91.8	92.2
TGF-β-RII	TGFR2_HUMAN	F1PNA9_CANFA	87.4	90.3
TGF-β-RIII	TGBR3_HUMAN	F1PIG0_CANFA	88.6	93.0
TGF-β1	TGFB1_HUMAN	TGFB1-CANFA	94.1	96.7
TGF-β2	TGFB2_HUMAN	F1PKH0_CANFA	99.5	99.8
TGF-β3	TGFB3_HUMAN	F1PR85_CANFA	88.4	89.5
NFκB1	NFκB1_HUMAN	NFκB1_CANFA	91.0	94.2
NFκB2	NFκB2_HUMAN	E2RLL2_CANFA	92.3	94.9
RelA	TF65_HUMAN	F1PCU1_CANFA	91.2	93.5

Sequences were from UniProt (http://www.uniprot.org/uniprot/) and from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein). Sequences were aligned using a Needleman–Wunsch algorithm (http://www.ebi.ac.uk/Tools/psa/) with a BLOSUM 62 matrix; gap penalty and end penalty were defined as 10.0 and 0.5, respectively

[13] and might have a signaling function probably by interacting with the Wnt pathway [14]. Furthermore, vaccination with an adeno-associated virus (AAV)–CEA vector combined with Toll-like receptor-9 or Toll-like receptor-7 agonists in wild-type mice resulted in enhanced Th1-mediated immunity and protection from challenge with MC38 colon tumor cells expressing CEA, whereas the same CEA vaccines in CEA transgenic animals promoted tumor growth due to tolerance phenomena elicited by dendritic and myeloid cells [15]. Some CEA family members such as CEACAM6 may adhere to and inhibit tumor-infiltrating cytotoxic T cells [16]. CEACAM1, CEACAM5 and CEACAM6 may be released from epithelial tumors in microvesicles, whereas tumor endothelia only contain CEACAM1 which has a receptor function for other CEACAMs, influences T cell behavior [17] and regulates the tumor matrix and microvascularization [18]. Hence, CEA may affect the tumor and its stroma at the same time [19].

CEAR binds TGF-β, a cytokine involved in tolerance induction toward malignant tissue

The scientific history of the carcinoembryonic antigen receptor (CEAR) is much more recent. Interestingly, CEAR showed an outstanding sequence identity of 99 % between the human and canine species [20] (Table 1). The great CEA-receptor homology of humans and dogs on the one hand and the lack of a precise canine CEA equivalent on the other hand are discrepancies and indicate that there could be an alternative ligand. The CEAR was originally described in Kupffer cells and identified as the heterogeneous nuclear ribonucleoprotein M4 (hnRNP M4) [21]. Regarding oncology, it was later also found on colon cancer cells [22]. Moreover, its expression was subsequently also detected in mice in the entire gastrointestinal tract including liver and pancreas [23]. CEAR expression has been connected to inflammation in the liver [24]. In Kupffer cells, a full-length hnRNP M4 (CEARL) and a truncated form (CEARS), generated by alternative splicing, were described [14]. The minimal structural element of human CEACAM5 interacting with hnRNP M4/CEAR was reduced to a peptide of eight amino acids [25].

Surprisingly, a recent study has shown that CEA not only signals via its specific receptor, CEAR, but can also bind to the receptor of the important immunomodulatory cytokine transforming growth factor beta (TGF-β, Fig. 1) [26].

TGF-β sources and its function in the tumor

Three high-affinity membrane-bound receptors for TGF-β are known so far: type I, type II and type III. The classical

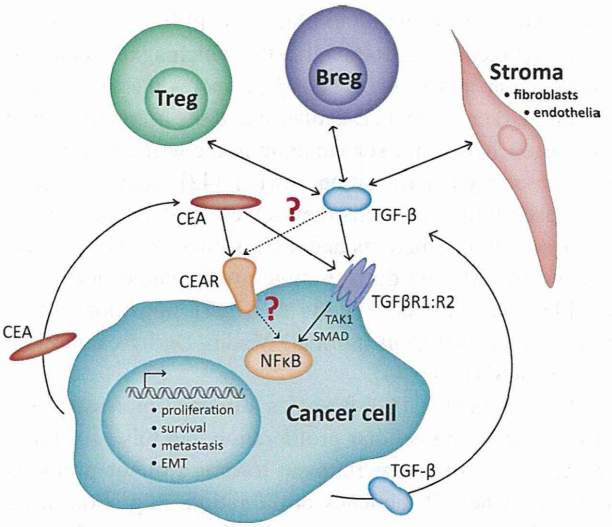

Fig. 1 Interconnected networks of CEA and TGF-β signaling in cancer. The cancer cell is an autocrine source of CEA as well as of TGF-β which bind to their specific receptors, CEAR or TGF-β-RI:RII, respectively; the latter signaling via the NFκB pathway. Recently, it has been recognized that CEA also signals via TGF-β-R and initiates the same biological effects [26]. Additionally, Tregs and Bregs, as well as stroma cells, participate in this network by secreting TGF-β. It remains open whether the reverse is the case, and TGF-β may also interfere with the CEAR pathway, which is much less defined

TGF-β signaling, however, occurs via the heterotetrameric complex of 2 TGF-β-receptor (TGF-β-R) type I and 2 TGF-β-receptor type II transmembrane receptors with serine/threonine kinase activity [27–29]. In the tumor microenvironment, TGF-β is most typically derived from human and canine $Foxp3^+$ regulatory T cells (Tregs). It is well known that Tregs can thereby critically dampen antitumor immunity and tolerize cytotoxic T cells [30–34]. More recently, intratumoral regulatory B cells (Bregs) have gained attention in human oncology [35, 36]. According to Olkhanud et al. [37], tumor-evoked Bregs should phenotypically resemble activated mature B2 cells ($CD19^+$ $CD25^{hi}$ $CD69^{hi}$). Lindner et al. [36] reported that intratumoral Bregs also express granzyme-B (stimulated by IL-21 from Tregs) and a signature of $CD19^+CD38^+CD1d^+IgM^+CD147^+$, as well as including IL-10, CD25 and indoleamine-2,3-dioxygenase. This population seems interesting as a source of TGF-β and for their capacity to suppress intratumoral $CD8^+$ and $CD4^+$ effector T cells. Bregs can even convert naïve $CD4^+CD25^-$ T cells to $Foxp3^+$ Tregs [37]. TGF-β, however, may also be derived from tumor stroma cells [19, 38], where it shapes the microenvironment by interacting with growth factors (epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) [39]), cytokines or

chemokines, crosstalking to fibroblasts [40] and supporting the enrichment of endothelial cells, which again shape the extracellular matrix [41]. TGF-β promotes the loss of epithelial markers such as E-cadherin and the accumulation of the mesenchymal marker vimentin in the process of epithelial–mesenchymal transition (EMT) [42]. Importantly, in this case tumor stem cells themselves show an enrichment of mesenchymal markers and are a source of TGF-β. Most studies on EMT are done in mouse or human cancer models [43], but there are reports that EMT transition can be achieved by TGF-β in (normal) Madin–Darby canine kidney (MDCK) cells [44].

Physiologically, TGF-β acts as a tumor suppressor, negatively regulating cellular proliferation, but this is changed in the cancer microenvironment toward a tumor promoter function, where it mediates proliferation, migration, invasion, EMT and metastasis, associated with high miR-181a expression, and altogether termed the TGF-β-paradox [45]. In this context, it is important to note that canines are much closer to the human species than murine animal models. The appearance of Tregs also negatively correlates with prognosis in dog cancer patients [46].

For instance, naive $CD4^+CD25^-Foxp3^-$ T cells can be converted to $Foxp3^+$ Tregs when adoptively transferred into $Rag^{-/-}$ mice only in the presence of TGF-β-positive tumors [47]. Thus, the intratumoral milieu amplifies the cellular sources for even more immunosuppressive cytokines. It has been recently shown that elevated levels of TGF-β and IL-6 in the tumor microenvironment support Th17 cells and that the resulting inflammation was supporting the clinical development and progression of gastric cancer [48]. Although Li et al. have shown that CEA binds to TGF-β-R [26], it has not yet been investigated whether the reverse is true, and TGF-β (besides acting via its own TGF-β-R) may crosstalk via CEAR, thereby imitating the tumor-progressive properties of CEA. CEA modulates effector–target interaction by binding to lymphocytes [49]. Only CEACAM1 expression was previously described in T cells [50], whereas the expression of CEACAM5 on T cells was excluded. Regarding this, we are not aware of investigations on the expression of CEAR on T- or B-lymphocytes.

TGF-β signaling

In contrast to CEAR, the cellular signaling function of which has to the best of our knowledge not yet been reported, the signaling cascade for the TGF-β-R is well known. The nuclear factor kappa-B (NFκB) is a key master regulator in growth and survival [51, 52]. In normal cells, TGF-β leads to growth inhibition; in short: TGF-β binds to TGF-β-RII, activating TGF-β-RI and then phosphorylating the SMA and MAD homologs SMAD2 and SMAD3, which associate with SMAD4 and together translocate to the nucleus for transcription of genes. All of this is inhibited by SMAD7 [53]. Interestingly, the TGF-β-R-initiated SMAD pathway was shown to target CEACAM5 (and CEACAM6) genes leading to CEA secretion as a mechanism for proliferation in gastric cancer cells [54]. It will be interesting in the future to investigate whether a synergistic crosstalk between the CEA and TGF-β signaling cascades in cancer cells exists.

In human head and neck squamous cell carcinoma cell lines, Freudlsperger et al. [53] could further demonstrate that TGF-β signaling resulted in a sequential phosphorylation of the transforming growth factor-activated kinase-1 (TAK1), inhibitor of nuclear factor kappa-B kinase (IKK), inhibitor of kappa-B subunit alpha (IκBα) and the v-rel avian reticuloendotheliosis viral oncogene homolog A (RelA); however, the crosstalk to CEA was not addressed in this study. Nor did this study address the consecutive activation of TAK1/mitogen-activated protein kinase kinase (MEK)/protein kinase B (AKT)/NFκB and SMAD pathways upon TGF-β stimulation as Gingery et al. [55] did in osteoclasts.

In human cancers, mutations in the TGF-β pathways (e.g., TGF-β-RII or SMAD4) are frequently observed [56]. A recent study has indicated that, although most tested colorectal cancer cells displayed an inactivated TGF-β signaling pathway, they actively secreted TGF-β acting on stromal cells and were thus driving metastasis [57]. In other cancer cell types, TGF-β signaling is intact, but aberrant NFκB activation and NFκB/RelA stimulate proliferation. In this respect, it should be emphasized that NFκB is constitutively activated in a number of hematologic and solid tumors and is one of the major transcription factors associated with cancer progression, inhibition of apoptosis, limitless replicative potential, tissue invasion and metastasis [58].

The TGF-β-R and NFκB pathways are connected via the TAK-1, which (independently, but parallel to SMAD activation) by phosphorylating IKK can directly stimulate the nuclear factor-κB (NFκB) pathway [55]. It is tempting to speculate that CEA may induce similar signals by interacting with TGF-β-R [26]. TAK1 was expressed in head and neck cancers, where nuclear activation of RelA of the NFκB family also took place. TGF-β induced sequential phosphorylation of several targets including TAK1, IKK, IκBα and RelA; additionally, TAK1 again enhanced TGF-β induced NFκB activation [53]. In human neutrophils, a constitutive association of TAK1 and inhibitor of kappa-B (IκB) was recently reported, indicating a close association of these pathways in inflammatory cells [59]. Neil et al. could show that the TAK1-binding protein 1 (TAB 1) forms complexes with IκB kinase b (IKKb) resulting in stimulation of the TAK1:IKKb:RelA pathway. The authors concluded that this

axis, including the NFκB elements, is pivotal in the oncogenic transformation of breast cancer [60]. The fact that NFκB plays a critical role in both intrinsic and acquired resistance against endocrine therapy in human breast cancer cells may additionally complicate the situation [61].

Conclusion

Generally, the dog represents an optimal model organism to study cancer biology in a comparative setting, as many genes represent a great degree of homology to their human counterparts [62]. Even with respect to noncoding RNAs, the significance of similarities between human and dog has recently been acknowledged [63]. Furthermore, the intriguing amino acid homogeneity among human and canine CEAR, TGF-β and TGF-β-R isoforms, NFκB and RelA are given in Table 1, indicating again an advantage of the dog patient in comparative oncology.

We propose that understanding of the crosstalk between CEA and TGF-β signaling toward NFκB as a key cancer regulator, as well as understanding of the Treg and Breg action in tumor tissue, should be extended, possibly with prognostic value. The dog may be a relevant translational model to study these interactions, in line with the comparative oncology strategy [64]. In the future, novel drugs may target the Achilles heel of both obviously interconnected networks.

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