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***met* oncogene activation qualifies spontaneous canine osteosarcoma as a suitable pre-clinical model of human osteosarcoma[†]**

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[†]The authors declare that there are no conflicts of interest.

Keywords: osteosarcoma; *MET* oncogene; receptor tyrosine kinase; immunohistochemistry

ABSTRACT

The Met receptor tyrosine kinase (RTK) is aberrantly expressed in human osteosarcoma and is an attractive molecular target for cancer therapy. We studied spontaneous canine osteosarcoma (OSA) as a potential pre-clinical model for evaluation of Met-targeted therapies. The canine *MET* oncogene exhibits 90% homology compared with human *MET*, indicating that cross-species functional studies are a viable strategy. Expression and activation of the canine Met receptor were studied utilizing immunohistochemical techniques in 39 samples of canine osteosarcoma, including 35 primary tumours and four metastases. Although the Met RTK is barely detectable in primary culture of canine osteoblasts, high expression of Met protein was observed in 80% of canine osteosarcoma samples acquired from various breeds. Met protein overexpression was also concordant with its activation as indicated by phosphorylation of critical tyrosine residues. In addition, Met was expressed and constitutively activated in canine osteosarcoma cell lines. OSA cells expressing high levels of Met demonstrated activation of downstream transducers, elevated spontaneous motility, and invasiveness which were impaired by both a small molecule inhibitor of Met catalytic activity (PHA-665752) and *met*-specific, stable RNA interference obtained by means of lentiviral vector. Similar to observations in human OSA, these data suggest that Met is commonly overexpressed and activated in canine OSA and that inhibition of Met impairs the invasive and motogenic properties of canine OSA cells. These data implicate Met as a potentially important factor for canine OSA progression and indicate that it represents a viable model to study Met-targeted therapies. Copyright © 2009 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons, Ltd.

INTRODUCTION

Many of the significant advances in cancer management in recent years have centred on the development of molecularly targeted therapies, including monoclonal antibodies and tyrosine kinase inhibitors. The receptor tyrosine kinase encoded by the human *MET* proto-oncogene 1 activates a unique physiological programme leading to morphogenesis, known as 'invasive growth' 2. When Met is deregulated in cancer, the invasive growth programme contributes to cell transformation and tumour progression 3. Therefore inhibition of Met receptor function is a new and challenging approach to hamper both the tumourigenic and the metastatic process 4–7.

A growing body of data has highlighted the association between overexpression of the *MET* oncogene and the onset and progression of human osteosarcoma (OSA) 8–12. In 70–85% of human OSAs, we and others found the *MET* oncogene to be aberrantly expressed and its

expression was correlated with *in vivo* proliferation, metastatic capacity, and poor prognosis 8–10, 13. We also demonstrated that overexpression of the *MET* oncogene causes and sustains the transformation of primary human osteoblasts 14.

Human OSA is more frequent in children and adolescents 15. The age of onset is socially important and notably limits the possibility of testing new therapies. Aggressive treatment modalities such as wide tumour resection and adjuvant chemotherapy have improved the cure rate of patients with localized OSA, but nearly one-third of all patients experience recurrent or progressive disease. Patients with metastatic disease at presentation have a poor prognosis 16 and the need for new systemic therapies is apparent.

The similarities between human and canine OSA have already suggested that the latter could be a suitable model to study novel therapeutic approaches for the former 11, 17. Spontaneous tumours in dogs and cats—humans' favoured companions—are suitable models of human cancer (see the website <http://ccr.ncifcrf.gov/resources/cop/default.asp> and refs 18–20). The comparable incidence of some cancers, large body size, and shorter overall life span are advantageous factors. Furthermore, they share the same environment as humans. Above all, companion animals and their tumours are genetically heterogeneous, as are randomly selected groups of cancer patients. The *met* gene was found to be expressed in canine OSA cell lines 11 and a *met* polymorphism was found in Rottweiler dogs, which are predisposed to OSA 21. This suggested that canine OSA might be a pre-clinical model of human OSA.

MATERIALS AND METHODS

Tumour samples and cell lines

Thirty-five primary canine OSA specimens (28 osteoblastic, six chondroblastic, and one fibroblastic) and four metastatic lymph nodes were harvested from patients treated at the University Teaching Hospital of Grugliasco (Turin). The D17 and D22 canine OSA cell lines and the MDCK (Madin–Darby canine kidney) normal cells were obtained from the American Type Culture Collection (ATCC). The canine DK (dog kidney normal cells) and TH (embryonic thymocytes) cells were kindly provided by the Istituto Zooprofilattico at Brescia (Italy). The CO (canine osteosarcoma) cell lines were propagated as previously described 22. Primary cultures of canine osteoblasts were harvested, propagated, and characterized as previously described 14.

Antibodies and growth factors

The canine Met receptor was labelled with either a rabbit polyclonal antibody (sc-162; Santa Cruz Biotechnology) or the 3D4 monoclonal antibody (Zymed Laboratories). The latter was also used for immunoprecipitation. Both antibodies have already been proved to label both the human and the mouse Met protein. Anti-phospho-Met 1234/1235 rabbit polyclonal antibody was from Cell Signalling Technology. Anti-phospho-tyrosine mouse monoclonal antibodies were from Upstate (clone 4G10). Anti-human phospho AKT and phospho ERK1/2 mouse monoclonal antibodies were from Cell Signalling and Sigma, respectively. The purified rabbit β -tubulin H235 antibody was from Santa Cruz Biotechnology. Human recombinant HGF was from Immunotools.

Immunohistochemical analysis

Sections of formalin-fixed, paraffin-embedded tissues were either incubated at room temperature for 1 h with the indicated anti-Met antibody or incubated with anti-phospho-Met antibody overnight at 4 °C after antigen retrieval 23. Visualization of the immunostaining was performed using an EnVision kit, which carried the secondary antibody (Dako). The score was evaluated by light

microscopy by two independent observers, using a Zeiss apparatus equipped with an MRc5 Axiocam. Morphometric analysis was performed using the Image-Pro Plus software (Media Cybernetics) on 15 000 pixel areas (40x). The specificity of the immunostaining reaction was assessed by either omitting the primary antibody or pre-incubating the primary antibody with the blocking peptide, ie the peptide used for immunization, at a 12-fold excess for 2 h at room temperature. Antibody specificity was further confirmed by the labelling of the canine Met receptor in cell lines with western blot analysis (see below).

Total RNA extraction and quantitative PCR

Total RNA was prepared as previously described 14. To determine the amount of specific RNA products of the canine *met*, *hgf*, and β -*actin* genes, cDNA was subjected to quantitative PCR, using Syber green Master MIX (Applied Biosystem) and the MyiQ Single-Color Real-Time PCR Detection System (Biorad). Each experiment was repeated three times independently, to ensure reproducibility of the results. Primer sequences are available from the authors.

Mutational analysis

Total cDNA encoding for canine *met* transcript was amplified, purified with the Ampure kit, and automatically sequenced. Genomic DNA was extracted and sequenced as previously described 24. Primer sequences are available from the authors.

Immunoprecipitation and western blot analysis

Western blot analysis was carried out as previously described 25. Proteins were visualized with horseradish peroxidase-conjugated secondary antibody using Super Signal West Pico Chemiluminescent Substrate (Pierce). Band relative intensity was measured using the Chemi Doc XRS (Biorad) and the Quantity One v. 4.6 program.

Expression of canine *met*-specific shRNA by means of cell transduction with lentiviral vectors

Lentiviral vector generation, production, and cell transduction were carried out as previously described 14. The pLKO1 lentiviral vector with a hairpin sequence of 21 base-pair sense and antisense stem and a 6 base-pair loop was purchased from Open Biosystem. Control shRNAs were obtained by sub-cloning a scrambled sequence.

Cell proliferation and cell viability assays

On day 0, 1000 cells were resuspended in 50 μ l of complete growth medium and seeded in either six-well or 96-well plastic culture plates. For the proliferation assay, cells were exposed to 250 nM drug for 3 days and counted every 24 h. For the viability assay, on day 1, 50 μ l of drug or vehicle (DMSO) serially diluted in complete medium was added to cells. On day 5, cell viability was assessed by ATP content using a luminescence assay (ATPlite 1 step kit, Perkin Elmer). All measurements were recorded using a DTX 880-Multimode plate reader (Beckman-Coulter).

Motility and Matrigel[®] invasion assay

Motility and invasion assays were performed in Transwell[®] chambers (Costar) as previously described 14. Cells that migrated to the lower side were fixed with 11% glutaraldehyde in PBS and stained with 0.1% crystal violet in 20% methanol. The filters were photographed and cells in five microscopic images were counted. To test the effects of the Met kinase inhibition, cells were pre-incubated with 500 nM PHA-665 752 at room temperature for 30 min and then added to each chamber.

RESULTS

Expression and activation of the *met* oncogene in canine OSA specimens

We evaluated Met protein expression utilizing immunohistochemical techniques (Figure 1) in a total of 35 primary canine OSA specimens and four metastatic lymph node lesions, collected immediately after excision (Figure 1). All samples were obtained from treatment-naïve animals and adjuvant chemotherapy was administered only 3–5 days after surgery. All the samples obtained were classified at histology as high-grade OSAs, and features commonly included extreme cellular pleomorphism, a variable number of mitoses, small to moderate amounts of matrix, a high percentage of tumour cells, and minimal to moderate amounts of necrosis. In some cases, evidence of vascular invasion or infiltration was also found.

Out of the 39 OSA samples, 80% scored positive for expression of the Met receptor. Immunoreactivity was localized to both the cell membrane and the cytoplasm of neoplastic cells. This was expected, as in Met-overexpressing cells the protein has been demonstrated to accumulate in the cytoplasm 26. Morphometric analysis indicated that the number of positive cells ranged from 2% to 40% (average 12%). The highest level of expression was found in specimens derived from metastatic lesions (see Figures 1g–1i). The activation status of Met was evaluated by immunohistochemistry in samples overexpressing the receptor, utilizing an anti-phospho-Met antibody (Figure 2). In seven out of 11 samples (nine from osteoblastic and two chondroblastic OSA), the activated state of the Met receptor was detectable.

Expression and activation of the *met* oncogene in canine OSA cell lines

The Met receptor was characterized by immunoblot in the D22 and D17 canine OSA cell lines and compared with the Madin–Darby canine kidney cells and TH canine embryonic thymocytes (Figures 3a and 3b). In all cell lines, the M_r of the Met receptor corresponded to those reported previously in MDCK and canine prostatic cells 27 and was almost identical to that of the human receptor. This was expected, as the canine *met* oncogene has 90% homology with the human *MET* gene and the precursor is likely to be processed similarly to the human one.

Figure 3. Expression of the *met* gene in canine cells. Osteosarcoma cell lines (listed in Table 1) which have been propagated from a number of primary osteosarcomas and from a metastasis were studied. *met* gene expression was determined by western blot analysis (a) and measured using quantitative real-time RT-PCR (b). In a, Met antibody raised against a cytoplasmic domain of the mouse receptor recognizes the p170 Met precursor and p145 Met β chain separated in PAGE gel under reducing conditions (upper panel). As a control, the Met receptor was visualized in two primary cultures of canine osteoblasts (COB1 and COB2) and in three normal canine cell lines (MDCK and DK kidney cells, and TH embryonic thymocytes), which are known to express the Met receptor. The same gel was re-probed with a β -tubulin antibody to check for equal loading. In the lower panel, band relative intensities are compared with that of the COB1 cells. In b, the amount of *met* transcript in the OSA cell lines was measured; the expression levels were all compared with that of the mRNA of the COB2 primary cultured cells, which is the primary culture that expressed the highest level of Met protein. Each point shows the mean expression variations (\pm SD) measured using mRNAs prepared from three experiments carried out independently. The mean fold change in expression of the target gene in each cell line versus COB2 cells was calculated using the formula $2^{-\Delta\Delta C_T} = - \{ (C_{T, Target} - C_{T, \beta-actin})_{cellX} - (C_{T, Target} - C_{T, \beta-actin})_{COB2cells} \}$. In c, phosphorylation of the canine Met receptor was assayed using an anti-phosphotyrosine (anti-P-Tyr) antibody in the western blot analysis of the protein immunoprecipitated with the same antibody

used in panel a from D17 cell extracts before and after Met activation with HGF. The same gel was re-probed with the same anti-Met antibody to check for Met equal amount. The phosphorylation of Met downstream signal transducers AKT and ERK1/2 was assayed on total D17 cell extracts before and after Met activation with HGF. MDCK cells were examined as a positive control. The same gels were re-probed with a β -tubulin antibody to check for equal loading. In d, D17 (upper left) and D22 (upper right) cells were formalin-fixed and paraffin-embedded, and stained with the same Met antibody used in Figure 1. In the lower left panel, the antibody was displaced by an excess concentration of the peptide used to raise it. In the lower right panel, D17 cells were stained with the anti-phospho-Met 1234–1235 antibody used to stain tissues shown in Figure 2

In OSA cell lines, we detected an amount of Met protein similar to that found in the MDCK and TH cells. To assess whether this level of expression was typical of OSA cells or detectable also in the normal counterpart, we examined primary cultures of osteoblasts. In canine osteoblasts, the Met receptor was barely detectable (Figures 3a and 3b).

In order to assess if Met overexpression is a common feature of canine OSA cells, as it is in human OSA cell lines 8, 13, we measured the expression of the canine *met* gene using quantitative RT-PCR. We compared *met* mRNA levels in the D22 and D17 OSA cell lines with other OSA cell lines propagated from OSAs occurring in different dog breeds (characteristics of the cell lines are reported in Table 1) and with the primary cultures of canine osteoblasts. As shown in Figure 3b, the canine *met* gene expression increased in all the OSA cell lines, with three out of six cell lines exhibiting a strikingly higher level. As Met ligand HGF is secreted by cells of mesenchymal origin, we investigated if the cell lines produce and secrete HGF utilizing end-point (ie conventional) RT-PCR. HGF expression was found only in the CO3 and CO7 cells (Table 1).

To evaluate the activation of the Met receptor in canine OSA cells, we measured the level of phosphorylation of the receptor and downstream signal transducers, such as AKT and ERK1/2 (Figure 3c). Met was demonstrated to be constitutively activated (ie phosphorylated) in Met-overexpressing D17 cells in the absence of HGF and was further activated following the addition of exogenous HGF. Also the phosphorylation of ERK1/2 and AKT was increased by HGF (Figure 3c). Immunohistochemical analysis also indicated that Met was overexpressed and activated in D17 cells (Figure 3d) but not in canine osteoblasts (not shown).

To assess if *met* was mutated in OSA cell lines, the full-length *met* cDNA was amplified and sequenced, and compared with the wild-type sequence (NM_001 002 963). Table 1 shows that one new and one previously described 21 polymorphism were identified.

Motility and invasiveness of canine OSA cells triggered by the Met receptor

The Met receptor mediates a complex genetic programme known as 'invasive growth', which results from the integration of apparently distinct biological responses to its ligand, HGF, also known as scatter factors 2. These responses include cell proliferation, survival, motility, and invasion of the extracellular matrix. In human OSA cell lines, we have already demonstrated that migration 12 and tumourigenicity 14 rely on Met receptor expression and kinase activity.

We evaluated the ability of the overexpressed canine Met receptor to trigger the above biological responses in canine OSA cells, by either down-modulating the receptor expression (Figure 4a) using stable RNA interference 28 or inhibiting the Met receptor kinase activity (Figure 4b) with PHA-665 752 29. Both approaches allowed us to study the OSA bulk populations and not selected clones.

PHA-665 752 inhibited not only the auto-phosphorylation of the Met receptor, but also the downstream phosphorylation of AKT and ERK1/2 (Figure 4b). However, PHA-665 752 did not affect canine OSA cell proliferation (data not shown) or survival (Figure 4c). In contrast, spontaneous motility of both the D17 and the D22 cell line was decreased by both RNA interference and PHA-665 752 (Figure 4d).

We also measured the ability of OSA cells to invade a reconstituted basement membrane made of laminin, collagen type IV, and glycosaminoglycans (Matrigel®). This Matrigel® invasion assay is universally thought to be a reliable test to evaluate cancer cell propensity to metastasize. This assay elegantly reflects the actual ability of cancer cells to invade and metastasize *in vivo* in response to Met receptor activation 30. Both the D17 and the D22 cell line demonstrated the ability to invade the basement membrane in the absence of chemo-attractive factors. Invasion of each of these cell lines was reduced by both Met kinase inhibition and the *met*-specific interfering RNA (Figure 4e).

DISCUSSION

This study shows that canine OSA is a suitable model to test Met-targeted approaches for the treatment of human and canine OSA. We have demonstrated that the Met receptor is detectable in a high percentage of canine OSAs by means of immunohistochemistry, and is highly expressed in most OSA samples and cell lines. These findings are similar to those reported for human OSA 8–10, 13. Immunohistochemistry was also successfully applied to identify the tumours where the receptor is constitutively activated. In addition, we have shown that the Met receptor drives motility and invasiveness of OSA cells, which are indeed impaired by Met-inhibiting molecules.

We studied Met protein and gene expression in a series of canine OSA samples and in a number of cell lines. Immunohistochemical analysis of randomly selected samples showed Met positivity in 80% of cases. Met expression was increased in tumour versus normal cells. In the latter, a very low, but detectable, level of *met* expression was found only in immunoblot analysis. This is likely reflective of the presence of either immature osteo-progenitors or haematopoietic cells possibly contaminating the cultures, as commonly occurs during osteoblast preparation 14. These data are comparable to those obtained when studying human OSAs, of which 70–85% scored positive for the Met receptor in different studies 9, 10, 13.

We found a high level of *met* expression in all metastases analysed and in the D17 cell line, which was derived from an OSA metastasis to the lung. Although anecdotic, these observations are in agreement with the role of Met activation in driving the metastatic propensity of tumour cells 2, 3.

Collective data indicate that overexpression may be the mechanism of Met activation in both human and canine OSA. Indeed, we have demonstrated that the overexpression of wild-type *MET* is alone able to transform human osteoblasts 14. In canine OSA cells, *met* overexpression is associated with receptor constitutive phosphorylation, which was not due to an autocrine circuit as the same cells did not produce HGF.

Met is one of the most studied possible targets for therapies with tyrosine kinase inhibitors and antibodies 31 for its role not only in sustaining the cancer phenotype, but also in triggering a cell's propensity to metastasize 3, 5, 7. We and others showed a role for *MET* oncogene expression and the onset and progression of human osteosarcoma 8–12. Here we have shown that the Met receptor is required for cell motility and the invasion of canine OSA cells, and might be impaired by a paradigmatic small molecule inhibitor. Another small molecule Met inhibitor similarly exhibits biological activity against dog OSA cell lines 32. Altogether, the data show that Met is a relevant

target for therapeutic intervention in human OSA and that therapies can be first validated in canine OSA.

Based on data presented at meetings and unpublished information available on the Internet, numerous Met-inhibiting compounds are entering the clinic for initial studies in human oncology. Interim data show that when patients are recruited in a trial on the basis of tumour histology and site, mostly stabilization of the disease and a limited number of partial responses are obtained 33. This is not surprising, as experience with EGFR tyrosine kinase inhibitors has demonstrated that the inhibitors are efficacious in a small subset of tumours: those that exhibit genetic alterations of the EGFR itself 34. In the case of Met, biochemical and biological characterization of many inhibitors has been carried out in cell lines and has shown that some cancer cells display 'addiction' to the *MET* oncogene 35, while in many others, Met is an adjuvant (also called an 'expedient'; see ref 5) for survival, motility, and invasiveness 14, 36.

Thus, patient selection is the challenge to evaluate therapies targeting the Met receptor and this can be realized by identifying potentially responsive cases. In OSA, as in most human cancer, evaluation of response to Met inhibition requires that the molecular link between response and Met expression and activation be ascertained. We have shown here that the Met receptor is overexpressed and activated in canine OSA, and that activation can be detected with a practical approach such as immunohistochemistry in OSA samples. Phosphorylation of Met serves as a surrogate for direct assay of enzymatic activity, which is not presently possible for analyses *in situ*. In addition, there is a well-established correlation between the enzymatic activity and autophosphorylation of Met, which can be reliably assayed by means of phospho-Met-specific antibodies (see, for example, refs 23, 37 and 38).

In conclusion, the data presented here show that canine OSA appears to be an ideal pre-clinical model to test Met-targeted therapies, not only because it recapitulates all the features of human OSA, but in particular because it might allow the rational design of pre-clinical trials with Met kinase inhibitors.

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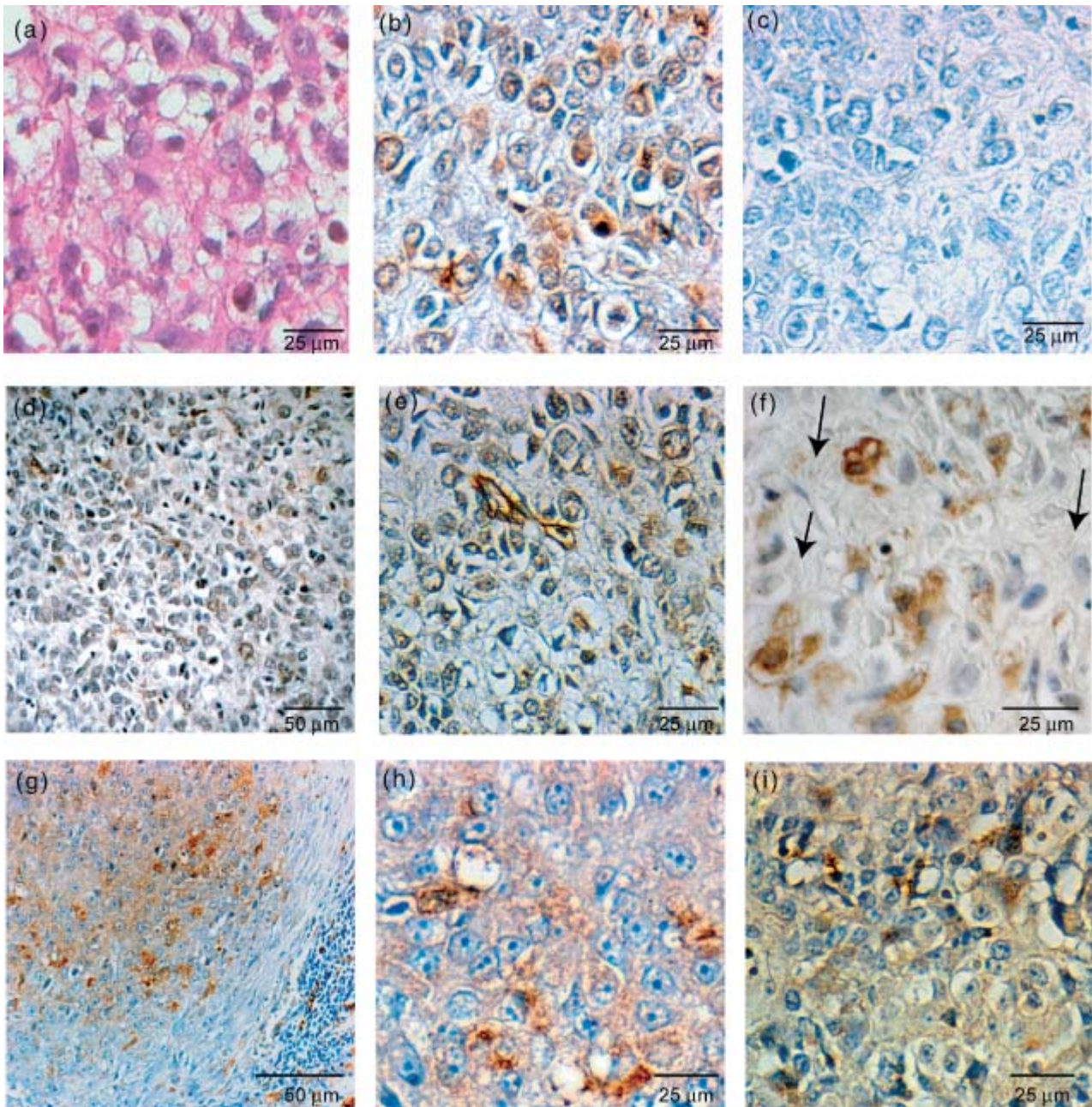


Figure 1. Expression of the Met receptor in canine OSA samples. OSA samples were stained using Met antibody and counter-stained with haematoxylin. We performed a control with the secondary antibody, which did not stain cells (not shown). In a–c, sections of the same primary osteosarcoma sample are stained with haematoxylin and eosin (a), Met antibody (b), and the latter antibody pre-incubated with an excess concentration of the peptide used to raise it (c). Other samples of primary osteosarcoma are shown in d–f. Arrows : bone matrix. In g–i, samples of OSA lymph node metastases are shown.

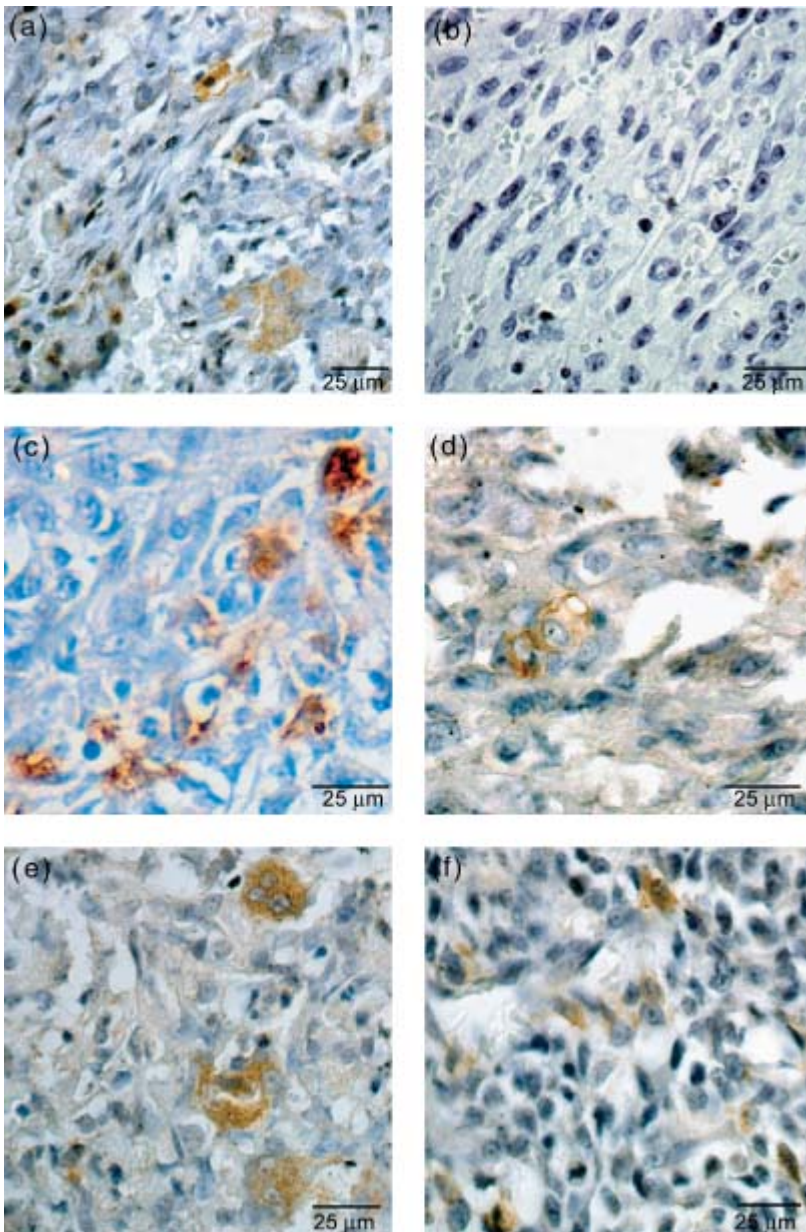


Figure 2. Activation of the Met receptor in canine OSA samples. Five different samples of Met-overexpressing primary canine OSAs were stained with an anti-phospho-Met 1234–1235 antibody (a, c–f). In b, the sample shown in a was incubated with the secondary antibody alone. The correlation between the enzymatic activity and autophosphorylation of Met on tyrosines at residues 1234–1235 (numbered according to GenBank accession number X54559), which are located in the activation loop of the human Met, is well known [39](#)

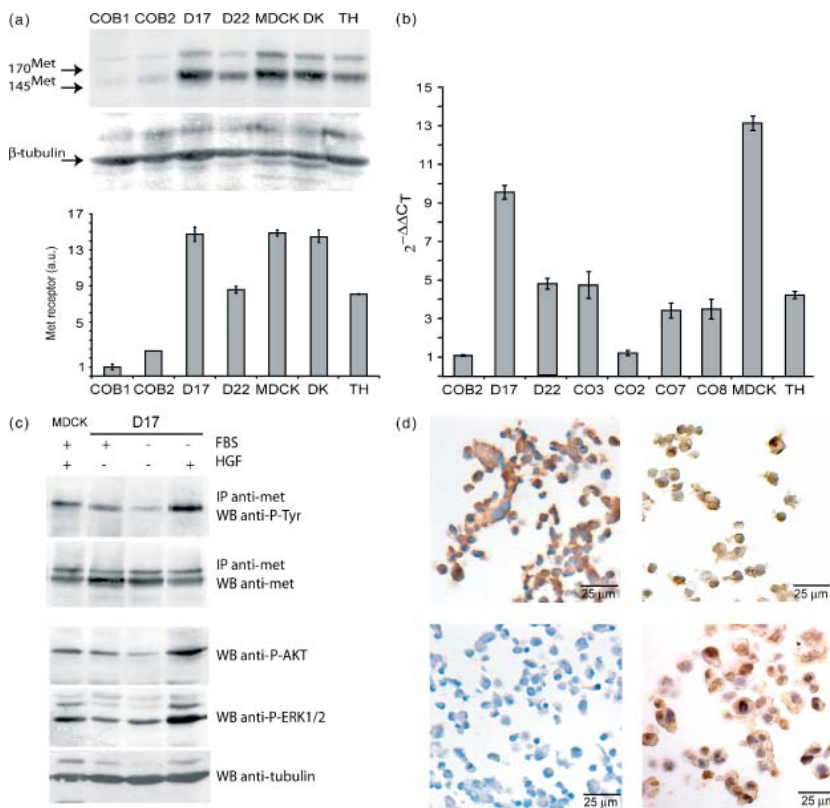


Figure 3. Expression of the *met* gene in canine cells. Osteosarcoma cell lines (listed in Table 1) which have been propagated from a number of primary osteosarcomas and from a metastasis were studied. *met* gene expression was determined by western blot analysis (a) and measured using quantitative real-time RT-PCR (b). In a, Met antibody raised against a cytoplasmic domain of the mouse receptor recognizes the p170 Met precursor and p145 Met β chain separated in PAGE gel under reducing conditions (upper panel). As a control, the Met receptor was visualized in two primary cultures of canine osteoblasts (COB1 and COB2) and in three normal canine cell lines (MDCK and DK kidney cells, and TH embryonic thymocytes), which are known to express the Met receptor. The same gel was re-probed with a β -tubulin antibody to check for equal loading. In the lower panel, band relative intensities are compared with that of the COB1 cells. In b, the amount of *met* transcript in the OSA cell lines was measured; the expression levels were all compared with that of the mRNA of the COB2 primary cultured cells, which is the primary culture that expressed the highest level of Met protein. Each point shows the mean expression variations (\pm SD) measured using mRNAs prepared from three experiments carried out independently. The mean fold change in expression of the target gene in each cell line versus COB2 cells was calculated using the formula $2^{-\Delta\Delta C_T} = -\{(C_{T, Target} - C_{T, \beta-actin})_{cellX} - (C_{T, Target} - C_{T, \beta-actin})_{COB2cells}\}$. In c, phosphorylation of the canine Met receptor was assayed using an anti-phosphotyrosine (anti-P-Tyr) antibody in the western blot analysis of the protein immunoprecipitated with the same antibody used in panel a from D17 cell extracts before and after Met activation with HGF. The same gel was re-probed with the same anti-Met antibody to check for Met equal amount. The phosphorylation of Met downstream signal transducers AKT and ERK1/2 was assayed on total D17 cell extracts before and after Met activation with HGF. MDCK cells were examined as a positive control. The same gels were re-probed with a β -tubulin antibody to check for equal loading. In d, D17 (upper left) and D22 (upper right) cells were formalin-fixed and paraffin-embedded, and stained with the same Met antibody used in Figure 1. In the lower left panel, the antibody was displaced by an excess concentration of the peptide used to raise it. In the lower right panel, D17 cells were stained with the anti-phospho-Met 1234–1235 antibody used to stain tissues shown in Figure 2

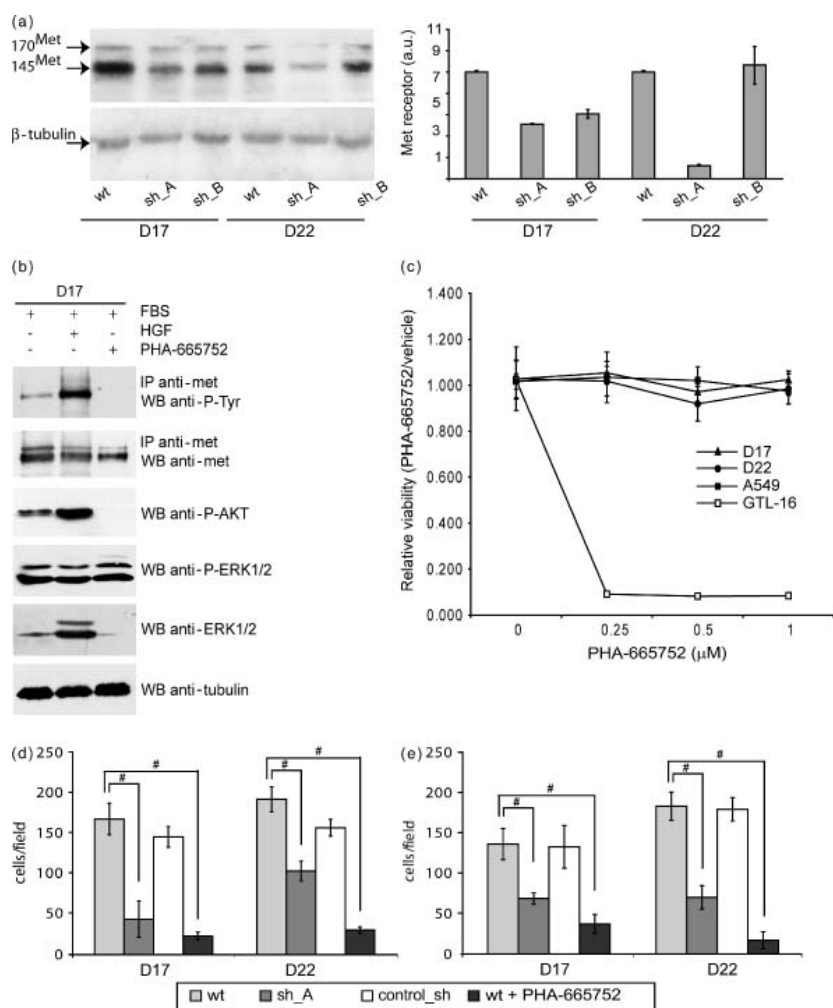


Figure 4. Met receptor-dependent biological activities of the canine D17 and D22 cell lines. Met expression was inhibited using either the Met kinase inhibitor PHA-665752 or *met*-specific shRNA sequences, indicated as *met*_sh, stably expressed in the bulk unselected cell line populations using LV vectors. Met receptor expression (a, left upper panel) was determined by western blot analysis as in Figure 3. The same gel was re-probed with a β -tubulin antibody to check for equal loading (a, left lower panel). In the right panel of a, band relative intensities in each cell line are compared with that of the untransduced cells. We tested two different canine *met*-specific shRNAs (A and B). The panels show that sh_A was effective in down-modulating Met receptor expression in both cell lines. Panel b shows that Met inhibition by PHA-665752 resulted in inhibition of the phosphorylation of both Met receptor and receptor signal transducers studied as in Figure 3c. To assess the biological outcome of the decrease of Met expression, we evaluated cell survival (c), cell motility (d), and invasiveness (e). Panel c shows the percentage of cells that remained viable after treatment with increasing concentration of PHA-665752 versus cells treated with the vehicle alone (DMSO). D17, D22, and the control A549 cells remained viable, while GTL-16 cells were affected, as already described 40. Motility and invasiveness were evaluated in Transwell chambers after either the addition of PHA-665752 or the stable expression of sh_A. In d, cells that migrated through the porous filter were counted after 16 h incubation. In e, cells that invaded the artificial basement membrane (Matrigel[®]) were counted after 24 h incubation. In d and e, the Y-axis shows the average migrated cell number (\pm SE) of triplicate wells in a representative experiment. #Significance of different motility or invasiveness of cells either transduced with *met* shRNA or treated with the inhibitor versus untransduced cells, using Student's *t*-test ($^{\#}p < 0.001$); the control_sh did not change cell motility and invasion significantly.

Table 1. Characteristics of the canine OSA cell lines studied

Cell line	Breed	Age (years)	Bone	Site	<i>Met</i> ² sequence	HGF
D17	Poodle	11	Unknown	Lung mts [†]	D544N	—
D22	Collie	?	Unknown	Primary	wt	—
CO2	Rottweiler	2	Distal femur	Primary	G966S	—
CO3	Golden retriever	5	Distal radius	Primary	wt	+
CO7	Rottweiler	2	Proximal humerus	Primary	G966S	+
CO8	Alaskan malamute	7	Proximal humerus	Primary	wt	—

* The full *met* cDNA was sequenced: wt (wild type) or mutated at the indicated codon.

† mts = metastasis.

In CO2 cells, a guanine-to-adenine base homozygous substitution, and in CO7 cells the same but heterozygous substitution (ie associated with the presence of the wild-type *met* allele) at position 2896, results in the change of glycine 966 with serine in the juxtamembrane Met receptor domain. Both OSA cell lines are from a Rottweiler dog. This change has already been described in the germline of 70% of Rottweiler dogs [21](#). In the D17 cells, there is a novel homozygous substitution (G → A) at position 1630, resulting in the change to aspartate of asparagine 544, located in the extracellular domain of the canine Met receptor. This change is also present in 20% of 39 genomic DNA samples (78 dog chromosomes) from various breeds. This strongly suggests that the amino acid change found in D17 cells is also a polymorphism.