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Expression of Hepatocyte Growth Factor and the proto-oncogenic receptor c-Met in canine osteosarcoma.

H. Fieten, B. Spee, J. IJzer, M.J. Kik, L.C. Penning* and J. Kirpensteijn*

* equal contribution.

Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, The Netherlands (HF, BS, LCP, JK);

Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands (JIJ, MK);

Department of Morphology and Molecular Pathology, University Hospitals Leuven, Belgium (BS)

Corresponding author:

Hille Fieten, Yalelaan 108, 3508 TD, Utrecht

Telephone: 0031-302534647

FAX: 0031-302539393

E-mail: H.Fieten@uu.nl

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Abstract:

Hepatocyte Growth Factor and the proto-oncogenic receptor c-Met are implicated in growth, invasion and metastasis in human cancer. Little information is available on the expression and role of both gene products in canine osteosarcoma. We hypothesize that the expression of c-Met is associated with malignant histological characteristics, a short survival time, and reduced disease free interval in canine osteosarcoma. Quantitative real-time PCR was used to analyze the mRNA expression of both Hepatocyte Growth Factor and c-Met in 59 canine osteosarcoma samples. The relation between Hepatocyte Growth Factor and c-Met expression, patient outcome and histological characteristics of the tumor were studied. Western blot analysis was performed to investigate the presence of active Hepatocyte Growth Factor protein. The expression pattern of c-Met in 16 slides of canine osteosarcoma was identified by immunohistochemistry.

Co-expression of Hepatocyte Growth Factor and c-Met mRNA in all canine osteosarcoma samples suggested autocrine or paracrine receptor activation. A significant, moderately positive correlation was found between c-Met and HGF mRNA expression. c-Met mRNA expression was not associated with survival time or disease free interval. Expression of c-Met was significantly associated with metastasis via the lymphogenic route. Immunolabeling with c-Met revealed a cytoplasmic staining pattern in all osteosarcoma cell types.

In this study, c-Met mRNA expression in canine osteosarcoma was found to be of no influence on survival time and disease free interval. Further studies are necessary to confirm the involvement of the c-Met pathway in the lymphogenic route of metastasis.

Osteosarcoma, the most common primary bone tumor in dogs, accounts for 85% of all skeletal malignancies.[6] It primarily affects large- and giant-breed dogs of middle to older age, and is a locally aggressive tumor, with the appendicular skeleton most frequently affected. Metastatic disease is very common and occurs mainly to the lungs via the hematogenous route.[6] In most cases, dogs will die or euthanasia is indicated because of malaise due to metastatic disease.

Canine osteosarcoma resembles osteosarcoma in humans. Histologically, canine and human osteosarcomas are alike, i.e. both are of high histological grade. The tumor metastasizes rapidly to the lungs and survival is improved with adjuvant chemotherapy in both species. Other similarities include the relative size of patients (with larger individuals being affected more often), and presentation at metaphyseal locations of major weight-bearing bones.[27]

Canine osteosarcoma do differ in some respects from the human counterpart: dogs are affected at a later stage in life, and the disease has a higher prevalence and a more rapid progression in the dog.[27]

The etiology of osteosarcoma in both species is largely unknown. The ligand Hepatocyte Growth Factor (HGF) and the proto-oncogenic receptor c-Met have been implicated in the genesis and malignant progression of several human malignancies, including osteosarcoma.[2] A causative role for HGF/c-Met signaling in the development and/or progression of canine osteosarcoma is still under debate.

HGF, first identified as a potent mitogen of primary cultured hepatocytes,[18] is produced by several mesenchymal cells and secreted as the biologically inactive precursor: pro-HGF. Pro-HGF (92 kDa) is proteolytically activated by HGF-activator. The activated product, a disulfide-linked heterodimer, consists of a 69-kDa α-subunit and a 34 kDa β-subunit. The latter is involved in c-Met binding and subsequent transmembrane signaling.[10] Research on human and canine

osteosarcoma cell lines has shown a possible role for HGF in enhanced proliferation, invasion and scattering of tumor cells after stimulation of c-Met.[3,5,8] More recently, Patanè et al found that overexpression of c-Met, obtained by lentiviral vector-mediated gene transfer, resulted in the conversion of human osteoblasts into osteosarcoma cells.[20]

Co-expression of HGF en c-Met has been detected in a variety of human tumors including breast, lung, pancreatic and thyroid cancers, glioma and myeloma.[13] Autocrine stimulation of the c-Met receptor by HGF may lead to a more malignant phenotype: autocrine stimulation of c-Met by HGF implicates an advantage for tumor cells in colony forming in anchorage independent conditions.[16] In addition, the non-tumorigenic mouse cell line C127 (expressing very low levels of HGF and c-Met proteins), was engineered to overexpress both HGF and c-Met proteins. This cell-line became phenotypically transformed, highly tumorigenic and metastatic *in vivo*. Increased levels of either HGF or c-Met alone did not result in a tumorigenic switch.[12]

A body of publications discuss expression of HGF and c-Met in human osteosarcoma samples [1,8,9,17,19,21,22,26], but only one study reports on canine osteosarcoma, noting c-Met expression in 5 of 7 clinical samples.[7] HGF expression, and histological malignancy characteristics and patient outcome in relation to c-Met expression, however, were not addressed in that study.

In the study reported here, mRNA expression levels of both HGF and c-Met were measured in 59 canine osteosarcoma samples with quantitative real time PCR (Q-PCR) and the data were related to histological malignancy characteristics and patient outcome. The amount of proteolytically activated HGF in tumor tissue was determined with Western blot.

The cell-specific expression pattern of c-Met in histological slides of canine osteosarcoma was identified by immunohistochemistry.

Materials and methods

Dogs

A total of 59 osteosarcoma samples were obtained from dogs that were referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, The Netherlands, between 1993 and 2004. The age of the dogs at presentation ranged from 1.0 to 13.0 years (median: 7.4 years). The most common breeds from which osteosarcoma were obtained were Rottweiler (*n* = 17), Cross breed (*n* = 6), Labrador retriever (*n* = 5), Great Dane (*n* = 4) and Dobermann (*n* = 4). The remaining 23 dogs comprised several breeds. Weight of the dogs ranged from 16.0 to 87.0 kg (median: 41.0 kg). The majority of the dogs suffered from appendicular osteosarcoma (*n* = 51), seven dogs had axial osteosarcoma and one dog was diagnosed with an extra-skeletal osteosarcoma. Clinical data were collected from the medical records. Follow-up information was obtained from the medical records or via direct communication with owners or referring veterinarians. None of the dogs received chemotherapy prior to harvesting of tumor material.

Sample handling

Osteosarcomas were harvested under sterile conditions during surgery or necropsy within 30 minutes after euthanasia of the dog. Samples were immediately snap-frozen in liquid nitrogen and stored at -70°C. An adjacent sample was fixed in paraformaldehyde (4%) for at least 24 hours, decalcified in EDTA (10%) and embedded in paraffin. Sections (4 μm) were stained with haematoxylin and eosin for confirmation of the diagnosis and to determine the histological classification.

Prior to mRNA and protein extraction, samples were pulverized, under cooling in liquid nitrogen, using a 5 mm ball bearing in a Braun Mikro-dismembrator U

(Braun Biotech International, Melsungen, Germany) at 2,000 rounds per minute for 45 sec in RNA-se free plastic containers.

Histological classification

Osteosarcoma were classified by one certified veterinary pathologist (MK), using the osteosarcoma grading system described previously.[14] Osteosarcoma were sorted into osteoblastic and mixed type histological subtypes. Three grades with increasing histological malignancy characteristics were distinguished; these characteristics included: nuclear pleomorphism, amount of matrix, percentage of tumor cells, number of mitotic cells, extent of necrosis, number of multi-nucleated giant cells, number of whirls and the extent of tumor cell invasion in vessels or lymph nodes. The association between c-Met mRNA expression and these histological characteristics was studied.

RNA isolation and reverse-transcription polymerase chain reaction.

Total cellular RNA was isolated from each tumor powder specimen using RNeasy Mini Kit® (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. An additional pretreatment with >600 mU/ml Proteinase K® (Qiagen, Venlo, The Netherlands) was used to improve the RNA isolation procedure. The RNA samples were treated with Dnase-I (Qiagen, Venlo, The Netherlands) to exclude contamination with traces of genomic DNA. The amount of isolated RNA was quantified with a Nanodrop-1000® spectrophotometer (Thermo scientific, Wilmington, DE). In total 3 µg of RNA was incubated with oligo(dT) primers at 42°C for 45 min in a 60 µl reaction volume, using the Reverse Transcription System® (Promega Benelux, Leiden, The Netherlands), according to the manufacturer's instructions.

Quantitative PCR

Quantitative real-time PCR (Q-PCR) was performed on HGF and c-Met and reference genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT). Real-time PCR was based on the high affinity double-stranded DNA-binding dye SYBR green I (SYBR® green I, BMA, Rockland, ME) and was performed in triplicate in a spectrofluorometric thermal cycler (iCycler®, BioRad, Veenendaal, The Netherlands). For each PCR reaction, 2 µl (of the 2 × diluted stock) of cDNA was used in a reaction volume of 50 µl containing 1× manufacturer's buffer, 2 mM $MgCl_2$, 0.5 × SYBR® green I, 200 µM dNTP's, 20 pmol of both primers, 1.25 units of AmpliTaq Gold (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands), on 96-well iCycler iQ plates (BioRad). Primer pairs, depicted in Table 1, were designed using PrimerSelect software (DNASTAR Inc., Madison, WI). All PCR protocols included a 5-minute polymerase activation step and continued with for 40 cycles (denaturation) at 95°C for 20 sec, annealing for 30 sec, and elongation at 72°C for 30 sec with a final extension for 5 min at 72°C. Annealing temperatures were optimized at various levels ranging from 56°C till 58°C (Table 1). Melt curves (iCycler, BioRad), agarose gel electrophoresis, and standard sequencing procedures were used to examine each sample for purity and specificity (ABI PRISM 3100 Genetic Analyser, Applied Biosystems). Standard curves constructed by plotting the relative starting amount *versus* threshold cycles were generated using serial 4-fold dilutions of pooled cDNA fractions from 44 samples. The amplification efficiency, $E\ (\%) = (10^{(1/-s)}-1)\cdot 100$ (s = slope), of each standard curve was determined and appeared to be > 95 %, and < 105 %, over a wide dynamic range. For each experimental sample the amount of the gene of interest, and of the endogenous references glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyl transferase (HPRT) were determined from the appropriate standard curve in autonomous

experiments. If relative amounts of GAPDH and HPRT were constant for a sample, data were considered valid and the average amount was included in the study (data not shown). Results were normalized according to the average amount of the endogenous references.

Western blot analysis for HGF protein

Extracted protein from 6 osteosarcoma samples was used in the Western blot assay. Two samples with the highest HGF mRNA expression were selected as well as two samples with the lowest HGF mRNA expression. In addition two samples with medium expression were analyzed. Normal canine liver tissue was taken as a positive control. Extracted protein from a canine bile duct epithelial cell-line, which has no endogenous HGF production, was taken as a negative control. The cell-line was cultured in house as described before.[23]

For the extraction of soluble protein, 50 mg of frozen, pulverized tumor tissue (and frozen liver tissue as positive control) was used. The extraction of protein, measurement of protein concentration, electrophoresis, Western blotting procedure, immunodetection and exposure were carried out according to our standard laboratory protocols as previously described.[24] The primary HGF antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was diluted 1:1,000 with TBS-T containing 4% bovine serum albumine (BSA). Beta-Actin (Neomarkers, Lab Vision Corporation, Fremont, CA) was used as a loading control in a dilution of 1:2,000 in TBS-T containing 4% BSA. After washing with TBS-T, the membranes were incubated with their respective secondary antibody; chicken anti goat IgG conjugated with horseradish peroxidase (HRP) (dilution 1:20,000) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) or anti-mouse IgG HRP-conjugated (dilution 1:20,000) (R&D systems, Minneapolis, MN) for 1 hour at room temperature.

Immunohistochemistry for c-Met

Sixteen large intact histological osteosarcoma samples were selected for immunohistochemical staining for c-Met. Excluded were samples of core biopsies or samples with mainly necrotic areas. Seven were from an appendicular location and 2 were from axial osteosarcoma. Seven samples were of the osteoblastic subtype and 9 samples were of the mixed subtype. Two of these samples were classified low malignant (grade 1), 4 showed a medium malignant grade (grade 2) and 10 samples were of a high malignant grade (grade 3). Freshly-cut paraffin sections (4 μm) were mounted on 3-aminopropyltriethoxysilan (APES) coated slides, dried for 48 hours at 40°C and stored at 4°C until use. Deparaffinized sections were treated with 0.3% H_2O_2 in methanol for 20 minutes at room temperature to block endogenous peroxidase activity. After rehydration, antigen retrieval was performed by boiling the sections in 0.01M citrate pH = 6.0 in a microwave oven for 10 minutes. The sections were quickly cooled down by rinsing in de-mineralized water, followed by washing in Tris buffered saline (TBS). Background staining was blocked by incubating sections with normal rabbit serum 1:50 diluted for 15 minutes at room temperature. The sections were incubated with primary antibody (human c-Met, clone 8F11, cat. no. MONX10170 Monosan, Uden, The Netherlands), diluted 1:1,000 in TBS/BSA 1%, overnight at room temperature. After washing in TBS, slides were incubated with rabbit-anti-mouse biotinylated secondary antibody (1:200 in TBS/BSA 1%) (Dako Cytomation, Glostrup, Denmark) at room temperature, followed by washing in TBS and 30 minutes incubation with Horseradish Peroxidase/Streptavidin complex (Dako Cytomation, Glostrup, Denmark). Colour was developed in 3-3'-diaminobenzidine (0.5 mg/ml) and 0.02 % H_2O_2 in Tris buffer (pH 7.6). Sections were counterstained in Mayer's hematoxylin. Positive control tissue consisted of human prostate (n = 1), canine prostate (n = 1), and canine liver (n = 1), all without EDTA-treatment,

as well as human osteosarcoma (n = 3) and normal canine humerus (n = 2), decalcified in EDTA as described above. In negative controls, the first antibody was omitted.

Statistical analysis

Statistical analyses were carried out with SPSS 15.0 statistical package (SPSS Benelux BV, Gorinchem, The Netherlands).
A multivariate stepwise linear regression was performed to evaluate the association of c-Met mRNA expression with histological characteristics. Predictors included were: histologic subtype, nuclear pleomorphism, amount of matrix, percentage of tumor cells, number of mitotic cells, extent of necrosis, number of multi nucleated giant cells and number of whirls. The level of significance for inclusion was set at $p < 0.05$ and the level of significance for exclusion was set at $p > 0.10$. Association of c-Met mRNA expression and tumor invasion in lymph nodes and vessels was analyzed in a separate analysis using ANOVA, because values for these characteristics were missing (in 3 samples no vessels were present and in 37 cases no draining lymph nodes were available for analysis). c-Met mRNA expression in relation to tumor grade was analysed separately using ANOVA, because grade is a derivate of the histologic characteristics (save the histological subtype).

Log values for HGF and c-Met mRNA expression, corrected for the values of the reference genes were distributed normally and the relationship between expression levels of HGF and c-Met mRNA were evaluated using the Pearson correlation coefficient.

A univariate Cox regression model was used to compare c-Met mRNA expression with survival data. Survival time and disease free interval were studied. Survival time in days was defined as the time between presentation in the

clinic and death of the dog. Disease free interval in days was defined as the time between the day of surgery and recurrence or the appearance of metastases. Dogs that had died from non-osteosarcoma related causes were counted as censored cases. None of the dogs was alive at the time of analysis.

Results

Detection of c-Met and HGF on mRNA level with Q-PCR

Samples of 59 canine osteosarcoma were analyzed with Q-PCR for c-Met and HGF mRNA expression levels.

In all 59 samples, both c-Met and HGF mRNA were detected. The specimens showed a great variation in expression levels of both c-Met and HGF (Figure 1 and 2). Expression levels were related to expression of the reference genes. Expression of c-Met and HGF exceeded mean expression in respectively 29% and 47% of cases. Expression of both c-Met and HGF exceeded mean expression in 24% of cases. A significant, moderately positive correlation was found between c-Met and HGF mRNA expression (Figure 3).

Western blot analysis for HGF protein

Proteolytically-activated HGF-α protein was detected in the liver sample and in all six osteosarcoma samples (Figure 4). The canine bile duct epithelial cell-line remained negative for HGF-α, verifying the specificity of the antibody. The osteosarcoma samples showed no large differences in protein expression for HGF, although there was a wide variation in the mRNA expression levels as measured with Q-PCR. Therefore, HGF mRNA expression levels were not used for outcome analysis and association with histological characteristics.

Association between c-Met mRNA expression level and histological characteristics in canine osteosarcoma

The majority of osteosarcoma was of high histological grade: 47 samples were classified as high malignant, 10 samples as medium malignant and 2 samples as low malignant. Twenty six were of the osteoblastic subtype, the remaining 33 samples were mixed-type tumors. A negative association between the number of mitosis and c-Met mRNA expression level ($p = 0.036$) was revealed by multivariate stepwise linear regression analysis for histological characteristics. As shown through ANOVA, c-Met mRNA expression was not associated with grade and metastasis in blood vessels. Draining lymph nodes of 23 dogs were evaluated. Of these lymph nodes, 3 contained metastatic osteosarcoma cells. A significant positive association of c-Met mRNA expression and metastasis in lymph nodes was discovered with ANOVA $F(1,22) = 7.042$, $p = 0.015$.

Effects of c-Met mRNA expression on survival and disease free interval

Dogs that were euthanized on the day of diagnosis, or were treated conservatively, were excluded from the analysis ($n = 21$). Of the remaining 38 dogs, 32 dogs suffered from appendicular osteosarcoma, 5 had an axial osteosarcoma, and one presented with an extraskeletal osteosarcoma. The majority of the osteosarcoma were graded as highly malignant ($n = 29$), followed by medium malignant ($n = 8$) and low malignant ($n = 1$).
From this group of patients, 8 dogs underwent surgery without additional chemotherapy. Two dogs underwent limb amputation for appendicular osteosarcoma, in 2 dogs a mandibulectomy was performed, 2 dogs underwent ulnectomy, and in 2 dogs an osteosarcoma of the rib was resected.

The remaining 30 dogs received chemotherapy in addition to surgical resection of the primary tumor.

Twenty-one dogs of the chemotherapy group underwent total limb amputation for appendicular osteosarcoma. Four dogs underwent scapulectomy and in one dog the arcus zygomaticus was removed. In one dog, an extraskeletal osteosarcoma in the pectoral region was removed. In three dogs, limb sparing surgery for osteosarcoma of the distal ulna (n = 1) and distal radius (n = 2) was performed. Fifteen of these dogs received lobaplatin at a dose of 35 mg/m^2 i.v., once every three weeks, for a maximum of 4 doses as described previously [15], 5 dogs received carboplatin 300 mg/m^2 every 3 weeks and 8 dogs received alternating carboplatin 300 mg/m^2 and doxorubicin 30 mg/m^2 every three weeks. One dog received carboplatin and after metastatic disease was discovered, lobaplatin was given. One dog received doxorubicin monotherapy (30 mg/m^2 every three weeks).

For statistical analysis of survival time, 38 patient records could be used, and for the analysis of disease free interval, 35 records. Three dogs were excluded from the disease free interval analysis: one was excluded because the primary tumor in the vertebrae could not be completely resected, another was excluded because lung metastasis were present at time of diagnosis, and a third developed lung metastasis, but the dog suffered from a histiocytic sarcoma at the same time, so the origin of the metastases was inconclusive. Four cases were censored in both analyses: one dog was euthanized due to a thoracic fibrosarcoma, one dog died after surgery for a herniated disc in private practice, one dog developed an anaplastic carcinoma at the amputation site, which had been metastasized to the lungs, and the last dog was euthanized due to an adenocarcinoma in the nasal sinus.

In this analysis no effect of c-Met mRNA expression levels on survival time ($p = 0.795$) or disease free interval ($p = 0.939$) was detected.

Immunohistochemistry for c-Met

In normal canine bone samples, osteoblasts, osteoclasts and the vast majority of all bone marrow cells showed strong positively staining cytoplasm for c-Met antibody. Some background reactivity was present in arterial smooth muscle cells, while endothelial cells, osteocytes and striated muscle cells remained negative. Negative controls of bone tissues (n = 2) remained negative. Both human and canine prostate positive controls showed a strong cytoplasmic staining pattern in epithelial cells. The human osteosarcoma samples exhibited a strong cytoplasmic staining pattern in tumor cells. All tested canine osteosarcoma stained positive for c-Met antibody, with minor inter- and intra-tumor variability for the specific cell types. Background staining corresponded to the decalcified controls. Generally, neoplastic osteoblasts, fibroblasts, chondroblasts, and multi-nucleated giant cells exhibited strong cytoplasmatic positivity (Figure 5).

Discussion

In human carcinogenesis, the HGF and c-Met signaling systems have been confirmed to play an important role in stimulating cell division and motility, resulting in the promotion of progression and metastasis in various epithelial and mesenchymal malignancies.[2] Co-expression of HGF and c-Met is reported in human osteosarcoma, and indicates an autocrine or paracrine mediated signal transduction pathway.[8] The co-expression of HGF and c-Met in canine cancers had not previously been investigated in a large cohort. In the current study, we found expression of HGF in c-Met in all canine osteosarcoma samples. The difference between previously reported data on c-Met expression in canine osteosarcoma[7] and the current report is most likely caused by the higher sensitivity of the Q-PCR detection method, compared to Northern blotting.

It is suggested that autocrine or paracrine stimulation, or over-expression of c-Met is responsible for activation of the receptor.[8] In the current study, HGF and c-Met mRNA expression were detectable in all osteosarcoma samples. This means HGF is produced locally, suggesting an autocrine activation loop in our samples. Thus, the receptor is probably being activated through autocrine or paracrine stimulation.

For both HGF and c-Met, mRNA expression levels showed a wide variation. In the majority of cases, the expression of HGF and c-Met decreased compared to the reference genes. Variation in expression levels of HGF and c-Met might be caused by the great intra- and inter-tumor variability in morphologic appearance in canine osteosarcoma.

Since HGF is post-translationally modified, mRNA expression levels do not necessarily indicate the amount of active protein. Furthermore, the HGF protein can be produced by various tissues throughout the body.[28] Together, these two facts indicate that HGF mRNA levels in the tumor do not necessarily reflect the amount of active protein present locally. Therefore, Western blot

analysis was added to measure the availability of active HGF protein. HGF protein levels were more or less comparable between the samples with highest and lowest mRNA expression levels (Figure 4). This may indicate that besides locally produced HGF, HGF supplied from surrounding tissues is present in the osteosarcoma samples. In conclusion, based on our findings, both an autocrine and paracrine mode of activation might play a role in the activation of the c-Met receptor in canine osteosarcoma.

This study showed no significant correlation between patient outcome and c-Met mRNA expression. A limitation in this study is the wide variability of ascertained cases and different treatment strategies. However, there was no significant difference in survival time between different treatment strategies applied. The findings reported here confirm results from a previous study that investigated correlation of c-Met expression and outcome in dogs.[7] Although, an 8-fold larger number of cases was analyzed and a more sensitive technique was used (QPCR in our study vs Northern blotting in the previous study), the lack of correlation of c-Met expression and patient outcome is concluded in both the Ferracini study[7] and in the current study. Similar conclusions were reported in human osteosarcoma.[17,19] Based on our findings, c-Met expression in tumor samples can not be used as a predictor for prognosis in canine osteosarcoma. A crucial causative role for the c-Met pathway in progression and hematogenous metastasis of canine osteosarcoma is not confirmed based on our results.

A positive relationship was found between lymph node metastasis and c-Met mRNA expression. Canine osteosarcoma metastasize mainly through the hematogenous route and lymph node metastases are rare.[6] The 3 dogs in our study with lymph node metastasis all had high grade osteosarcoma of the appendicular skeleton and were treated with amputation and chemotherapy: one dog died due to lung

metastasis, the other two dogs (with both increased c-Met and HGF expression) did not develop lung metastasis. This correlation between c-Met expression and the lymphogenic metastatic pathway has not been described previously in either human or canine osteosarcoma, however c-Met and stromal HGF were found to significantly correlate with regional lymph node metastasis in human head and neck squamous cell carcinoma, salivary gland carcinoma and mammary carcinoma .[4,11,25] Determining the significance of the role of c-Met in lymphogenic metastasis in canine osteosarcoma requires studies of a larger number of dogs with lymph node metastases.

A negative association was detected between number of mitoses, counted histologically, and c-Met expression. This was an unexpected finding, as the c-Met pathway has been previously reported to be associated with a stimulation in cell division.[2] To confirm the importance of this finding and quantify expression levels of HGF and c-Met in a cell-specific way, laser micro-dissection of tumor cells and subsequent RNA isolation and Q-PCR are recommended to exclude noise due to expression of HGF and c-Met in reactive bone and stromal component present in whole tumor samples.

In the immunohistochemical study with c-Met antibody, a cytoplasmatic staining pattern was detected in the majority of tumor cells. The histology is similar to the staining pattern in the human osteosarcoma samples, which were used as a positive control. Several immunohistochemical studies for c-Met are performed on human osteosarcoma. A diffuse, cytoplasmatic staining pattern with or without a linear membranous staining pattern is described in human osteosarcoma, which is concordant with what we described in canine osteosarcoma. [1,9,17,19,22,26] The proportion of c-Met positive tumors in human studies varies between studies. Some studies report c-Met positivity in the majority of tumors [1,22,26], whereas other studies detect c-Met positivity only

in several samples [9,17,19]. c-Met positivity in osteoblastic cells, multi nucleated giant cells and fibroblastic tumor cells between canine and human osteosarcoma is comparable, however, one study describes a negative staining in fibroblastic tumor cells. In this study however, the overall positivity of c-Met in tumor samples as well as in cellular subtypes, except for the chondroblastic subtype, was low.[17] Differences in c-Met detection with immunohistochemistry could be due to differences in c-Met expression. In the cited human studies here, no expression profiling had been performed to study c-Met expression on the mRNA level. Important factors that will account for differences in number of positive tumors are, differences in tissue preparation, fixation (frozen versus formalin sections), formalin fixation time, decalcification (time and method) and used antibodies. For future immunohistochemical studies we propose a standardized protocol for decalcification and formalin fixation for osteosarcoma samples to obtain comparable staining patterns.

This study is the first to describe mRNA expression levels of HGF and c-Met in a large number of canine osteosarcoma samples. This co-expression of HGF and c-Met indicates an autocrine activation loop. In addition, Western blot analysis of active HGF protein indicates that a paracrine loop of activation might occur as well. Immunohistochemistry detected c-Met protein of all different cell-types within the osteosarcoma cells. No major effect of c-Met expression on patient outcome is detected, therefore c-Met expression in tumor tissue is not recommended as a prognostic indicator. c-Met seems involved in the lymphogenic route of metastasis, but a larger number of samples is necessary to prove this effect. We conclude that the c-Met pathway is present in canine osteosarcoma. The effects described here are very similar to effects previously described in human osteosarcoma, but the specific effects of the c-Met pathway in canine osteosarcoma on the biological level require further

investigation. For further studies we would propose tissue collection and array analysis in a matched fashion. To elucidate c-Met related and other pathways in canine osteosarcoma we would propose micro-array analysis for a hypothesis free approach.

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Request reprints from Hille Fieten, Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 108, 3508 TD, Utrecht, The Netherlands. E-mail: H.Fieten@uu.nl

Figure legends

Figure 1: Relative gene-expression c-Met. Data depicted as individual relative gene-expression after normalization against endogenous reference genes.

Figure 2: Relative gene-expression HGF. Data depicted as individual relative gene-expression after normalization against endogenous reference genes.

Figure 3: Correlation between HGF and c-Met mRNA expression
Pearson's correlation coefficient: 0.323 (p=0.013). Samples are depicted in order of increasing c-Met expression (no 1 – 59)

Figure 4: Western blot analysis for HGF alpha

In brackets: relative HGF mRNA expression levels of canine osteosarcoma

1: Liver

2: Bile duct epithelium

3: OS (0.1)

4: OS (0.34)

5: OS (3.33)

6: OS (9.71)

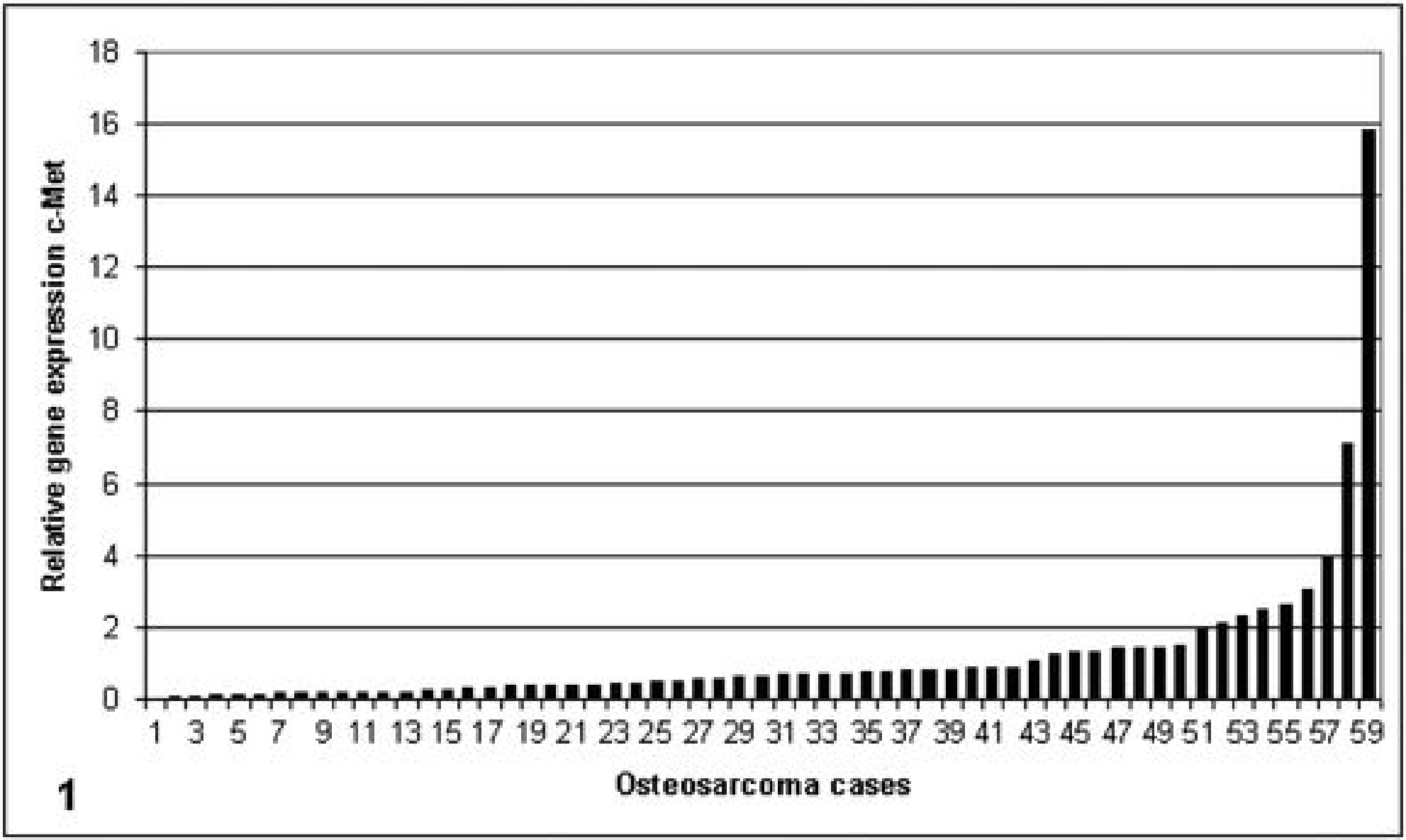
7: OS (20.67)

8: OS (24.27)

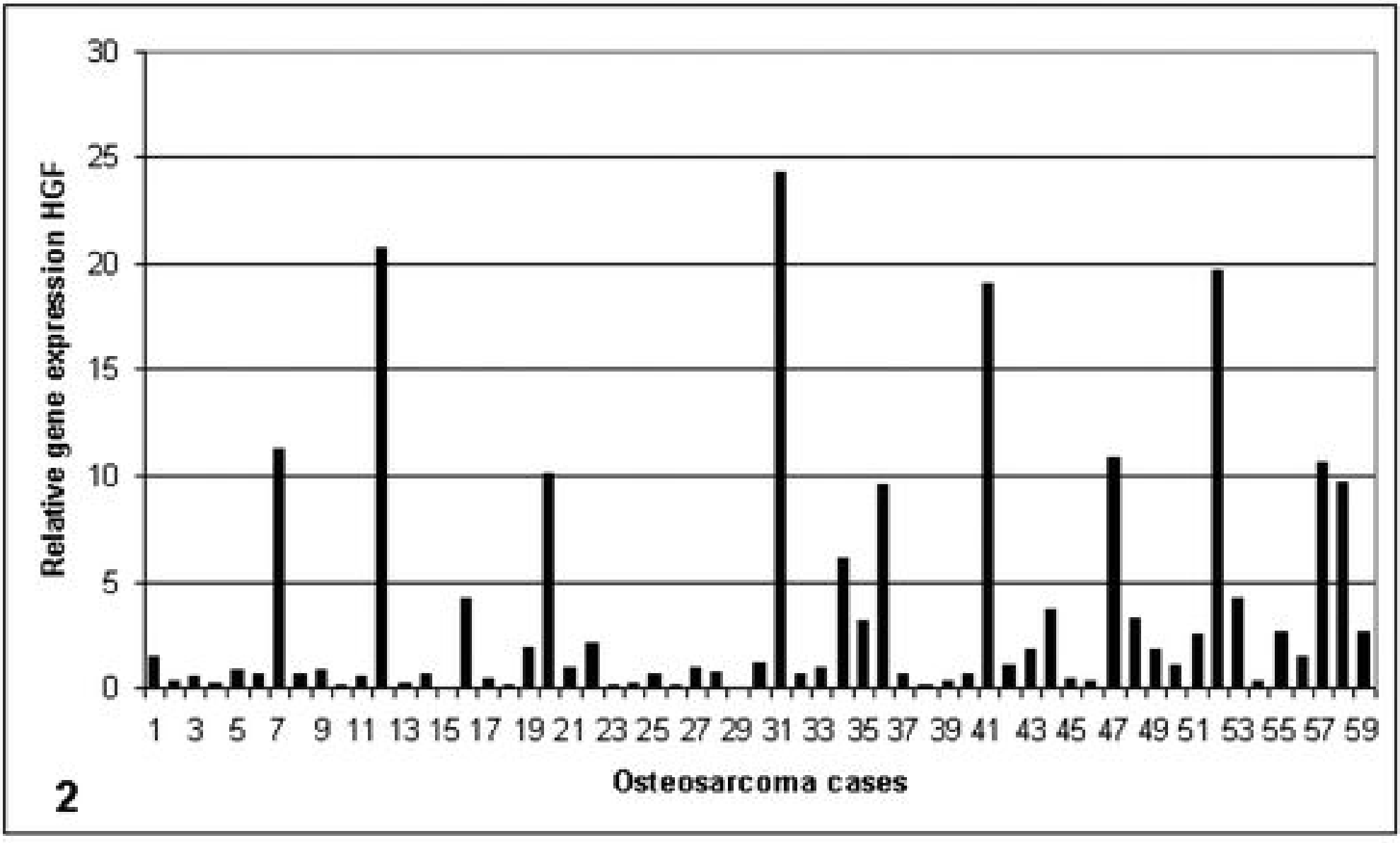
Figure 5: Scapula: mixed type osteosarcoma grade III, dog. Strong cytoplasmic staining pattern (brown) is present in multi-nucleated giant cells (asterisk), neoplastic osteoblasts (horizontal arrow), and neoplastic fibroblasts (vertical arrow). c-Met immunolabeling, Mayer's hematoxylin counterstain. Bar = 50 μm.

Table 1: Nucleotide sequences of dog-specific primers for real-time quantitative PCR

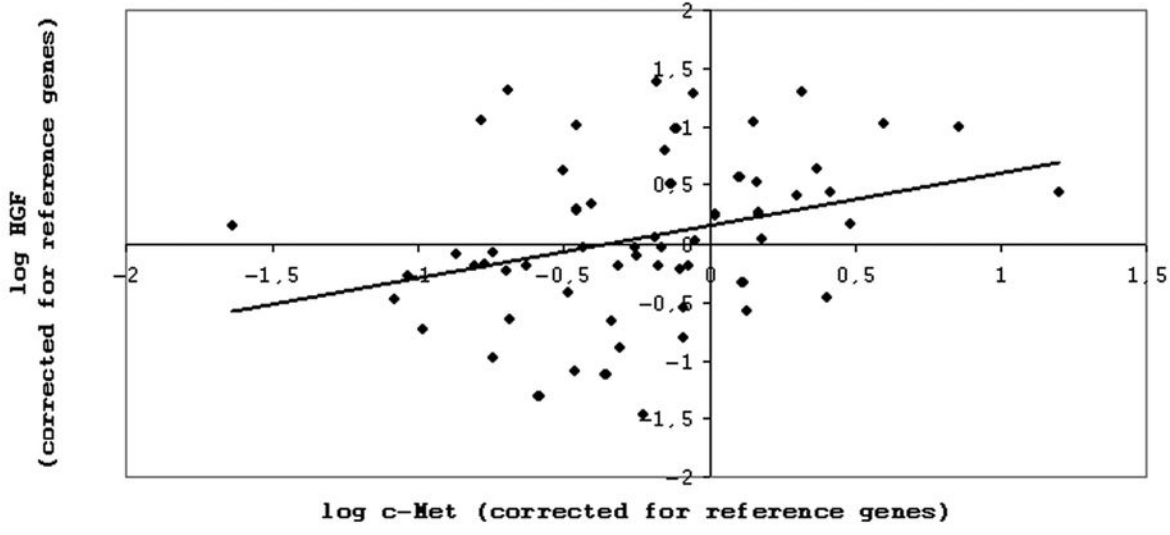
Gene	Primer	Sequence (5′ → 3′)	Tm (˚C)	Product size (bp)
GAPDH	Forward	TGT CCC CAC CCC CAA TGT ATC	58	100
	Reversed	CTC CGA TGC CTG CTT CAC TAC CTT		
HPRT	Forward	AGC TTG CTG GTG AAA AGG AC	56	100
	Reversed	TTA TAG TCA AGG GCA TAT CC		
HGF	Forward	AAA GGA GAT GAG AAA CGC AAA CAG	58	92
	Reversed	GGC CTA GCA AGC TTC AGT AAT ACC		
c-Met	Forward	TGT GCT GTG AAA TCC CTG AAT AGA ATC	58	112
	Reversed	CCA AGA GTG AGA GTA CGT TTG GAT GAC		


Relative gene expression c-Met
18
16
14
12
10
8
6
4
2
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Osteosarcoma cases

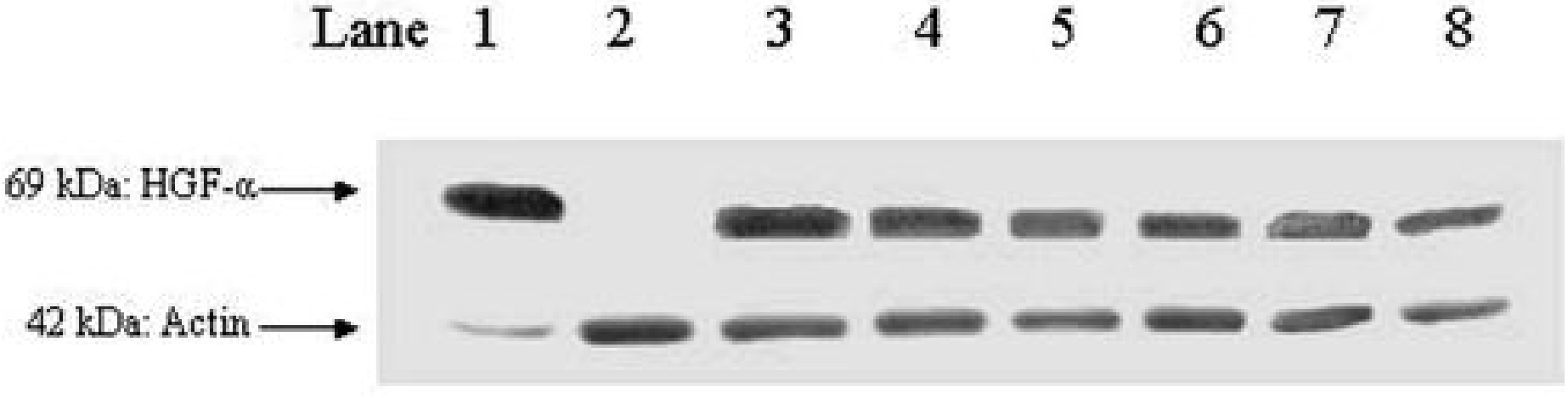
1


Relative gene expression HGF
30
25
20
15
10
5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Osteosarcoma cases

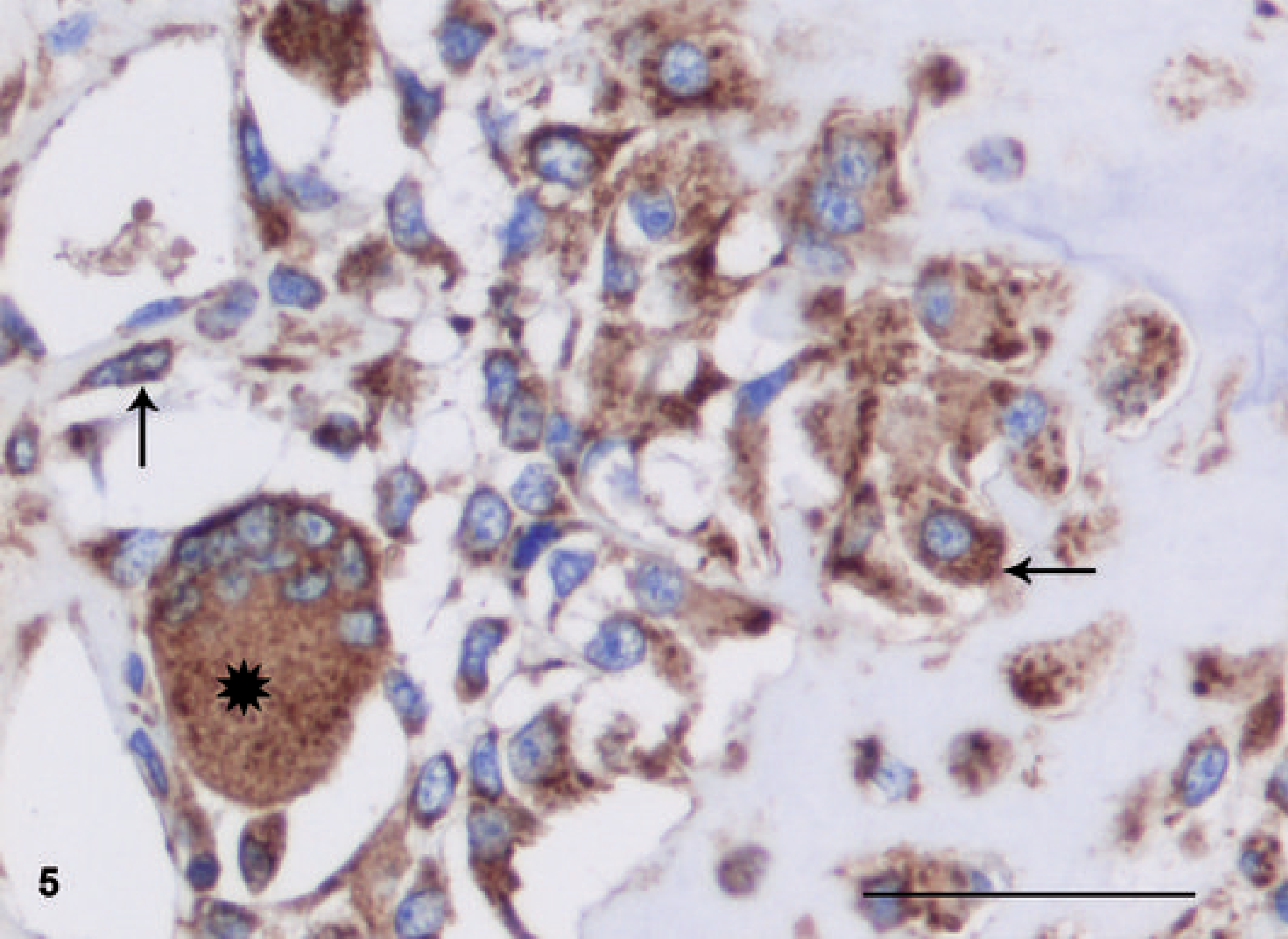
2


log HGF
(corrected for reference genes)
2
1,5
1
0,5
0
-0,5
-1
-1,5
-2
-2
-1,5
-1
-0,5
0
0,5
1
1,5
log c-Met (corrected for reference genes)

3



4



5