J. Comp. Path. 2007, Vol. 136, 65–73

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Clinicopathological Relevance of Tumour Grading in Canine Osteosarcoma

P. Loukopoulos[*,†] **and W. F. Robinson**[*]

[*] *School of Veterinary Science, The University of Queensland, Brisbane, Queensland 4072, Australia, and* [†] *Pathology Laboratory, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki, 54124 Greece*

Summary

Tumour grading assesses biological aggressiveness and is of prognostic significance in many malignancies. The clinicopathological features of 140 primary canine osteosarcomas and their metastases were analysed, and the interrelations between them and an established grading system and its constituent parameters (mitotic index, necrosis, pleomorphism) were examined. Of these tumours, 35% were grade III (high-grade), 37% grade II and 28% grade I. Primary tumours that had metastasized were of significantly higher grade than non-metastatic osteosarcomas. Osteosarcomas belonging to the osteoblastic minimally productive subtype, but not chondroblastic or telangiectatic subtypes, differed from fibroblastic osteosarcomas in being associated with a significantly higher number of high-grade cases. Dogs younger than 4 years of age had osteosarcomas with higher grade, score and mitotic index than did older animals. Appendicular differed from axial tumours in having a higher mitotic index; distal differed from proximal tumours in being of higher grade; cranial tumours differed from tumours in most other sites in being of lower grade and lower mitotic index. Rib osteosarcomas showed a particularly high degree of necrosis. The mitotic index varied widely between tumour locations. Pleomorphism did not have prognostic merit when examined separately, as most osteosarcomas were highly pleomorphic.

Keywords: bone; dog; osteosarcoma; tumour; tumour grading

Introduction

Osteosarcoma is the most frequently diagnosed and reported canine bone tumour (Ling *et al.*, 1974; Jongeward, 1985). It is considered one of the most malignant and aggressive tumours, with a predilection for large breeds (Tjalma, 1966). Osteosarcomas of the appendicular skeleton often metastasize to the lung before the primary tumour is diagnosed (Palmer, 1993).

Osteosarcoma in the dog bears a striking resemblance, both clinically and histopathologically, to osteosarcoma in man (Misdorp, 1980) and is therefore of value as a model of the human disease.

The prognosis of canine osteosarcoma may depend on a number of factors, including age (Spodnick *et al.*, 1992), body weight (Tjalma, 1966; Hammer *et al.*, 1995; Ru *et al.*, 1998), breed (Cohen *et al.*, 1974; Ru *et al.*, 1998), tumour location (Misdorp and Hart, 1979; Hammer *et al.*, 1995; Withrow, 1998), serum alkaline phosphatase concentration (Ehrhart *et al.*, 1998; Garzotto *et al.*, 2000), existence of metastases at presentation (Dernell *et al.*, 1998), completeness of surgical excision (Hammer *et al.*, 1995), tumour diameter and volume (Misdorp and Hart, 1979; Forrest *et al.*, 1992), tumour subtype (Misdorp and Hart, 1979; Hammer *et al.*, 1995), tumour micro-vessel density (Coomber *et al.*, 1998) and tumour necrosis after chemotherapy (Powers *et al.*, 1991). The most serious problem in osteosarcoma is lung metastases, and it is still not possible to predict the metastatic potential of osteosarcoma in individual cases.

Tumour grade, which assesses biological aggressiveness, is being used increasingly as an aid to prognosis and therapy (Mukaratirwa, 2005). Thus, in human beings it has been shown to be of prognostic significance in osteosarcoma (Grundmann *et al.*, 1995),

Correspondence to: P. Loukopoulos, Pathology Laboratory, Faculty of Veterinary Medicine, Aristotle University, Thessaloniki 54124, Greece (e-mail: plouk@vet.auth.gr).

0021-9975/$ - see front matter
doi:10.1016/j.jcpa.2006.11.005

synovial sarcoma (Hasegawa *et al.*, 2001), endometrial stromal sarcoma (Nordal *et al.*, 1996), advanced ovarian cancer (Nyvang *et al.*, 2000), renal cell carcinoma (Onodera *et al.*, 2000), pancreatic ductal carcinoma (Luttges *et al.*, 2000), periampullary adenocarcinoma (Sellner *et al.*, 1999), gastric lymphoma (Ferreri *et al.*, 2001), follicular lymphoma (Martin *et al.*, 1995), neuroblastoma (Layfield *et al.*, 1995), anaplastic glioma (Dropcho and Soong, 1996), and axillary node-positive (Simpson *et al.*, 2000) and other breast carcinomas (Burke and Henson, 1997) but not node-negative invasive breast carcinoma (Younes and Laucirica, 1997).

In the veterinary literature, the prognostic value of tumour grading has been demonstrated in feline mammary carcinomas (based on the degree of myoepithelial differentiation [Castagnaro *et al.*, 1998]), soft tissue fibrosarcomas (Davidson *et al.*, 1997), canine mammary carcinomas (Karayannopoulou *et al.*, 2005), canine sarcomas (e.g., haemangiosarcoma [Ogilvie *et al.*, 1996a, b]), splenic sarcoma (Spangler *et al.*, 1994), multilobular osteochondrosarcoma (Dernell *et al.*, 1998), and synovial sarcoma (Vail *et al.*, 1994); it was not considered sufficiently consistent, however, for evaluating cutaneous mast cell tumours (Northrup *et al.*, 2005). Recently, a tumour grading system incorporating the degree of nuclear pleomorphism, number of mitoses, matrix production, tumour cell density and degree of tumour necrosis was shown to be related to survival time in canine osteosarcoma (Kirpensteijn *et al.*, 2002). Straw *et al.* (1996), however, in a study in which mitotic index, degree of nuclear pleomorphism and degree of tumour necrosis were used to grade canine mandibular osteosarcoma, failed to demonstrate prognostic value, but the number of cases examined was small and the study was limited to tumours in only one site. Similarly, a study of 45 osteosarcomas of flat or irregular bones revealed that a modified World Health Organization staging system (Owen, 1980) had no prognostic significance, but not all cases were available for grading (Hammer *et al.*, 1995).

The aim of the present study was to evaluate the clinical and pathological relevance to canine osteosarcoma of the grading system previously proposed by Straw *et al.* (1996), and to describe the histopathological parameters associated with it.

Materials and Methods

Tumour Samples

The study was based on archived material from canine osteosarcomas. This material included 138 confirmed cases of canine osteosarcoma collected during the period January 1974–January 1999 by the Department of Veterinary Pathology, University of Queensland, Australia, and two confirmed cases from the Veterinary Pathology Laboratory, Aristotle University of Thessaloniki, Greece. In five of the Queensland cases, only the metastases were available for examination. The tissues had been fixed in 10% neutral buffered formalin, processed by routine methods and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin (HE).

Tumour Grading

Mitoses were counted in 10 high-power fields, randomly selected from both central and peripheral areas of the tumour. The mitotic index, the degree of nuclear pleomorphism and the degree of tumour necrosis of osteosarcomas were assessed to give a tumour score (1–10), which was then converted to a grade (I, II, or III), as described previously (Straw *et al.*, 1996) and as shown in Tables 1 and 2.

Histopathological Subtypes

Osteosarcomas were classified into subtypes, as described by Slayter *et al.* (1994), on the basis of: the quality and quantity of the extracellular matrix (ECM) produced (osteoblastic productive, osteoblastic minimally productive, chondroblastic and fibroblastic subtypes); the degree of differentiation (poorly differentiated

Table 1
Tumour scoring system, modified from Straw *et al.* (1996)

Parameter	*Description*	*Value*
Nuclear pleomorphism	None	0
	Mild	1
	Moderate	2
	Marked	3
Mitotic index (number of mitoses per 10 fields at × 400)	1 to 10	1
	11 to 20	2
	21 to 30	3
	> 30	4
Degree of necrosis (%)	None	0
	< 15	1
	15 to 50	2
	> 50	3

Table 2
Tumour grading system (combined scores from nuclear pleomorphism, mitotic index and degree of necrosis) modified from Straw *et al.* (1996)

Histological score	*Histological grade*
1 to 5	I
6 to 7	II
8 to 10	III

subtype); the abundant presence of giant cells (giant cell subtype); and the dominant presence of blood-filled cystic lesions (telangiectatic subtype). Osteoblastic tumours were designated as either productive or minimally productive, when the overall quantity of all matrices produced by the tumour cells was greater or less than 10%, respectively. The term "osteoblastic minimally productive" was used instead of the less accurate term "osteoblastic non-productive" used by Slayter *et al.* (1994).

Osteosarcomas were classified as simple if osteoid or bone was the only type of ECM produced by the malignant cells, or as compound if three were more than one type, provided that no type accounted for more than 75% of the total amount of ECM produced.

Statistical Analysis

To assess the statistical significance of inter-group differences in quantitative data, the Kruskal–Wallis One Way Analysis of Variance (ANOVA) on Ranks or the Mann-Whitney Rank Sum Test was performed. Data grouped in contingency tables were analysed statistically with the χ^2 test or Fisher Exact test for comparison of proportions. Statistical significance was set at $P \leqslant 0.05$. The collected data were analysed with the SigmaStat® Statistical Software (version 2.0; SPSS Inc., Chicago, Illinois, USA).

Results

General Data

The mean age of the cases examined was 8 ± 3.3 years (range 11 months to 15.9 years). There were 66 female (28 entire, 38 neutered) and 60 male (39 entire, 21 neutered) affected dogs; the gender was not recorded in 12 cases. Forty breeds, mostly large or giant, were represented in the osteosarcoma cases. The primary tumours arose in endosteal sites in the appendicular skeleton (98 cases; 70%) or axial skeleton (38 cases; 27.1%), or extraskeletally (four cases; 2.8%). There was no evidence of tumours having arisen on the surface of bones.

All dogs examined *post mortem* had been humanely destroyed because of the diagnosis of osteosarcoma, except for one dog destroyed because of severe emphysema and four that died as a result of disease-related complications. Survival data were not available in respect of a large enough number of animals to permit statistically reliable analysis of the effects of various clinicopathological parameters on survival time.

Metastases were present in 27 of 62 (43.5%) appendicular, 8/24 (33.3%) axial and 2/4 extraskeletal cases in which a complete necropsy was performed (total 37/90; 41.1%).

Cellular features characterizing the tumours varied greatly between cases, between different histological sections of the same tumour, or between samples from the same tumour collected *post mortem* or from an earlier biopsy. Most osteosarcomas, however, were highly cellular, the tumour cell population being characterized by a moderate to high degree of apoptosis, high nucleus to cytoplasm ratio, multiple or prominent nucleoli, vesiculated nuclei, hyperchromatic chromatin, and numerous and bizarre mitoses. Neoplastic cells were mainly large, plump or spindle-shaped, but pyriform, round, polyhedral or epithelial-like cells were also observed. Giant cells within the tumour ranged from occasional to abundant. The giant cell types observed included: giant multinuclear; giant mononuclear, the nuclei sometimes but not always resembling those of mononuclear tumour cells; and osteoclast-like.

Tumour Grade, Mitotic Index, and Degree of Necrosis and Pleomorphism, and their Relation to Other Clinicopathological Parameters

Most osteosarcomas, both primary and secondary, were classified as high grade because of marked pleomorphism, a high mitotic index (MI) and a moderate to high degree of necrosis (Fig. 1). The frequency of occurrence (and percentage) of the tumour grades (TGs) and the values of the various scores (tumour [TS], mitotic (MI), degree of pleomorphism, and degree of necrosis [NS]) in the primary and metastatic tumours are given in Table 3.

The metastatic status, age of the animal, tumour location, proximal or distal site, histopathological subtype, and compound or simple status were factors associated with one or more TG parameters, while the animal's gender and breed were not.

Primary tumours that developed metastases differed in respect of TG from primary tumours that did not ($P = 0.050$) (2.3 ± 0.7 and 1.9 ± 0.8, respectively). They also tended to exhibit a higher degree of necrosis and MI and to consist of a higher proportion of TGIII tumours (13/27 vs 10/40; $P = 0.09$).

Dogs aged $\leqslant 4$ years ($n = 14$) had a significantly higher TG (2.4 ± 0.7) than did dogs aged > 4 years ($n = 97$; TG $= 2.0 \pm 0.8$) ($P = 0.023$). Dogs aged < 5 years had a significantly higher TS (7.3 ± 1.7) (Table 1) and MI (3.2 ± 0.9) than did dogs aged > 5 years (6.5 ± 1.7 and 2.5 ± 1.0, respectively) ($P = 0.023$ and 0.026, respectively). Similarly, cases with a MI of 4 appeared at a significantly younger age ($P = 0.019$) than did cases with a MI of 3 or less (6.9 ± 3.4 and 8.5 ± 3.2, respectively).

Appendicular osteosarcomas had a significantly higher MI ($P = 0.007$) than did axial osteosarcomas (2.3 ± 0.7 and 1.9 ± 0.8, respectively). TG (Table 2) was

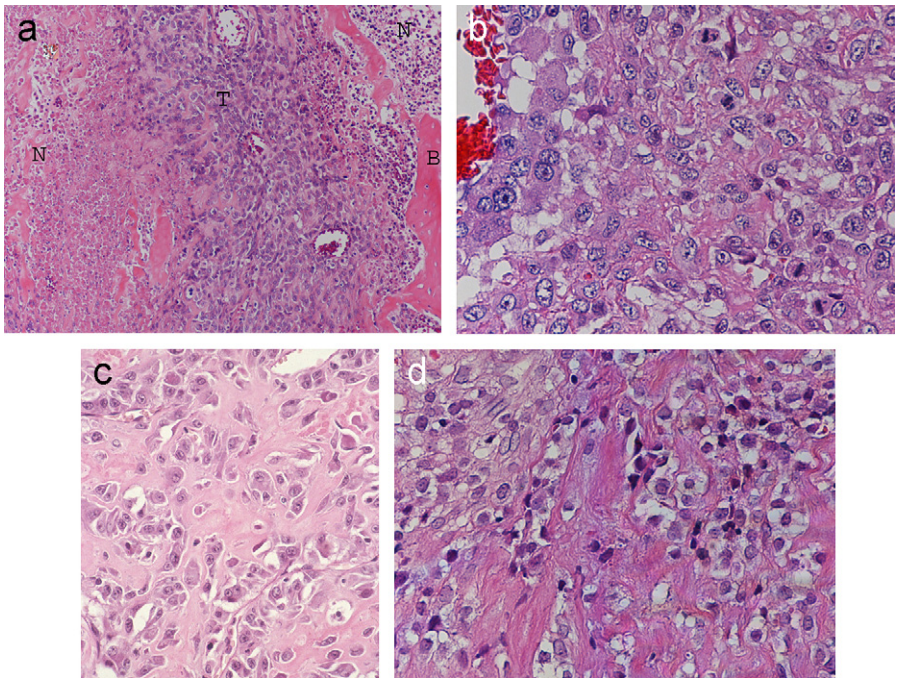

Fig. 1. a–d. Histological features of the various tumour grades in canine osteosarcoma. (a) Grade III. Note large necrotic areas (N), viable tumour cells (T) and viable pre-existing normal bone fragment (b). HE. ×100. (B) Grade III, lung metastasis. Note marked nuclear pleomorphism and the presence of abundant mitotic figures. The tumour also exhibited large areas of necrosis (not shown). The cytological features characterizing the tumour cell population include vesiculated nuclei, large cellular size and prominent and multiple nucleoli. HE. ×400. (c) Grade II. Note moderate nuclear pleomorphism. The tumour exhibited areas of necrosis covering 20% of the area examined and had a mitotic score of 2 (mitoses are not shown here), classifying it as a grade II tumour. HE. ×400. (d) Grade I. Note mild variation in nuclear size and shape, and absence of mitoses or necrotic areas. HE. ×400.

Table 3
Frequency of occurrence of the tumour grade and mitotic score, degree of necrosis and pleomorphism values in the primary and metastatic sites of osteosarcomas examined

		Number (and %) of cases with a Grade/Score of				
Parameter	*Tumour*	1	2	3	4	5
Tumour grade	Primary	…	33 (28.2%)	43 (36.7%)	41 (35.0%)	…
	Metastasis	…	7 (28.0%)	6 (24.0%)	12 (48.0%)	…
Mitotic score	Primary	…	18 (15.0%)	38 (31.6%)	24 (20.0%)	40 (33.3%)
	Metastasis	…	8 (29.6%)	4 (14.8%)	6 (22.2%)	9 (33.3%)
Necrotic score	Primary	23 (20.0%)	35 (30.4%)	32 (27.8%)	25 (21.7%)	…
	Metastasis	2 (8.3%)	7 (29.1%)	8 (33.3%)	7 (29.1%)	…
Pleomorphism	Primary	…	9 (7.2%)	54 (43.2%)	62 (49.6%)	…
	Metastasis	…	2 (7.6%)	14 (53.8%)	10 (38.4%)	…

significantly higher in distal (2.3 ± 0.7) than in proximal (1.9 ± 0.8) osteosarcomas ($P = 0.042$), while there were no differences in any TG parameter when forelimb and hindlimb tumours were compared. The mean TG, TS, MI, NS and pleomorphism values for the various locations of osteosarcoma are given in Table 4.

Appendicular tumours did not differ significantly in TG from each other, but many differed from cranial tumours in being of higher grade. Both TG and TS were significantly higher ($P < 0.05$) in osteosarcomas (1) in antebrachial, radial and rib sites than in cranial sites, and (2) in forelimb, antebrachial and radial sites and in tumours distal to the antebrachiocarpal or tarsocrural joint (DATJ) than in non-gnathic cranial tumours. Moreover, the TG of appendicular tumours was significantly ($P < 0.05$) higher than that of non-gnathic cranial tumours, but not higher ($P = 0.053$) than that of all cranial tumours; the TG was higher in DATJ tumours than in cranial tumours ($P = 0.045$). The TS was significantly ($P < 0.05$) higher (1) in tibial than in cranial tumours, (2) in rib versus non-gnathic cranial tumours and maxillary tumours, and (3) in

Table 4
The mean tumour grade (TG), tumour score (TS), mitotic score (MI), necrotic score (NS) and degree of pleomorphism values for various locations of osteosarcoma

	TG		*TS*		*MI*		*NS*		*Pleomorphism*	
Tumour location	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Appendicular	2.15	0.8	6.88	1.8	2.89	1	1.53	1.1	2.4	0.6
Forelimb	2.17	0.8	6.93	1.7	2.98	1.1	1.52	1.1	2.44	0.6
Scapula	1.67	0.6	5.67	1.5	2	1.7	1.33	1.5	2.33	0.6
Humerus	2.04	0.8	6.64	1.7	2.91	1.1	1.48	1.1	2.29	0.6
Antebrachium	2.43	0.8	7.53	1.4	3.24	0.9	1.57	1.2	2.55	0.6
Radius	2.42	0.8	7.46	1.3	3.29	0.9	1.5	1.2	2.47	0.6
Hindlimb	2.14	0.8	6.82	1.9	2.81	1	1.53	1	2.42	0.6
Femur	2	0.8	6.45	2.1	2.58	1	1.77	1.1	2.26	0.6
Tibia	2.27	0.8	7.46	1.8	3.08	1	1.46	0.9	2.5	0.7
Metatarsal	2.67	0.6	7.5	0.7	3	0	1	1	2.67	0.6
DATJ	2.6	0.6	7.75	1	3	0	1.4	1.1	2.8	0.5
Axial	1.94	0.8	6.46	1.8	2.28	1.1	1.64	1	2.42	0.7
Cranium	1.74	0.7	5.95	1.9	2.21	1.1	1.47	1	2.21	0.8
Non-gnathic cranial	1.5	0.8	5.5	1.8	1.88	1	1.38	1.1	2.13	0.6
Gnathic	1.91	0.7	6.27	2	2.46	1.1	1.55	1	2.27	0.9
Maxilla	1.33	0.6	5.33	1.5	2.33	1.5	0.67	0.6	2.33	1.2
Mandible	2	0.7	6	2.4	1.8	0.5	2	1.2	2.2	0.8
Rib	2.5	0.8	7.54	1.5	2.63	1.2	2.38	0.7	2.75	0.5
Spine	2	0.7	6.2	1.1	2	1.4	1.6	1.1	2.6	0.6
Pelvis	2	1	6.33	1.5	2.67	1.2	1	1	2.67	0.6
Extraskeletal	2.25	0.5	7	0.8	3	0	1.5	0.7	3	0

DATJ, distal to the antebrachiocarpal or tarsocrural joint.SD, standard deviation.

antebrachial, radial and DATJ tumours than in maxillary tumours.

MI was the TG parameter that varied the most between the different tumour locations. The pairs of tumour sites that exhibited statistically significant differences in MI are shown in Table 5.

Rib osteosarcomas were characterized by necrosis, which was significantly greater ($P \leqslant 0.05$) than that of appendicular, hindlimb, cranial, pelvic, metatarsal, or humeral osteosarcomas.

In contrast, tumour location and all other parameters, with the exception of compound or simple status, showed no significant differences in respect of pleomorphism. The mean values for pleomorphism were generally high in all tumour locations, the smallest being 2.1 ± 0.6. Pleomorphism tended to be greater in osteosarcomas of the ribs than in other sites, particularly femoral ($P = 0.074$) and cranial ($P = 0.084$) sites.

Histopathological Subtypes and their Relationship to Tumour Grade Parameters

Osteosarcomas were classified into eight histopathological subtypes, mainly based on criteria described previously (Slayter *et al.*, 1994). The subtype most frequently observed in the primary osteosarcomas and their metastases was the osteoblastic productive one, representing the conventional form of osteosarcoma, in which the dominant extracellular matrix produced by the tumour cells is osteoid or bone.

The TG, TS, MS, NS and pleomorphism values for the various histopathological subtypes of osteosarcoma are given in Table 6. Chondroblastic and osteoblastic minimally productive osteosarcomas had significantly higher TS ($P < 0.05$) but not TG values than did fibroblastic osteosarcomas, the aggressive morphology of which tended to be less than that shown by other subtypes. Osteoblastic minimally productive ($P = 0.025$) —but not chondroblastic ($P = 0.071$) or telangiectatic ($P = 0.089$)—osteosarcomas tended to show a significantly higher number of high grade (III or II) tumours than did fibroblastic osteosarcomas. ($P < 0.89$ to < 0.025). Chondroblastic osteosarcomas tended to be more pleomorphic than the other subtypes; there were, however, no statistically significant differences between the different subtypes in respect of individual tumour grade parameters.

Discussion

This study demonstrated the clinical and pathological relevance to canine osteosarcoma of the grading system proposed by Straw *et al.* (1996). The significance of the classification of osteosarcoma into histopathological subtypes was also established.

The clinicopathological features of spontaneous osteosarcoma cases that occurred in the Australian canine population were analysed, and the interrelations between them and a tumour grading system and its constituting parameters were examined. The pathological features of osteosarcoma have been described extensively (Pool, 1990; Palmer, 1993), but quantitative approaches to the study of the interrelations between clinicopathological parameters have rarely been used previously.

Table 5
Tumour location and mitotic score: pairs of tumour sites exhibiting statistically significant differences in respect of the mitotic score (MI)

*Site 1**	*Site 2*	*P-value*
Appendicular	Axial	0.007
Forelimb	Axial	0.009
Hindlimb	Axial	0.034
Humeral	Axial	0.047
Antebrachial	Axial	0.007
Radial	Axial	0.009
Tibial	Axial	0.04
Appendicular	Cranial	0.017
Forelimb	Cranial	0.017
Hindlimb	Cranial	0.045
Antebrachial	Cranial	0.009
Radial	Cranial	0.011
Tibial	Cranial	0.044
Appendicular	Non-gnathic cranial	0.015
Forelimb	Non-gnathic cranial	0.016
Hindlimb	Non-gnathic cranial	0.025
Humeral	Non-gnathic cranial	0.036
Antebrachial	Non-gnathic cranial	0.009
Radial	Non-gnathic cranial	0.01
DATJ	Non-gnathic cranial	0.048
Tibial	Non-gnathic cranial	0.027
Appendicular	Mandibular	0.026
Forelimb	Mandibular	0.024
Hindlimb	Mandibular	0.039
Humeral	Mandibular	0.044
Antebrachial	Mandibular	0.012
Radial	Mandibular	0.014
DATJ	Mandibular	0.016
Tibial	Mandibular	0.016

DATJ, distal to the antebrachiocarpal or tarsocrural joint.
* 1 had the higher MI of the two sites.

The parameters chosen in the present study as components of the tumour grading system (mitotic index, degree of necrosis, degree of pleomorphism) are generally considered important in determining tumour behaviour. The hierarchy of their importance, however, is unknown and it is uncertain as to which prognostic factor should be weighted more heavily. In this study, the degree of nuclear pleomorphism, although useful in helping to determine the tumour grade, did not appear to have prognostic merit, as most osteosarcomas were highly pleomorphic. Unlike pleomorphism, however, the tumour grade, tumour score, mitotic index and degree of necrosis were significantly associated with a number of clinicopathological parameters, including the metastatic status, age of the animal, tumour location, proximal or distal status, histopathological subtype, and compound or simple status. Young animals differed from old animals in having osteosarcomas of higher grade, score and mitotic index.

Primary tumours which had metastasized were of a significantly higher grade than that of non-metastatic osteosarcomas. This important association, which has not been demonstrated previously, supports the thesis that tumour grading may have particular prognostic value in individual osteosarcoma cases.

Appendicular osteosarcomas are believed to be more aggressive than axial ones (Heyman *et al.*, 1992). This was supported by the present study, which showed that appendicular osteosarcomas had a higher metastatic rate and tumour grade, although the differences were statistically significant only with regard to the mitotic index. However, the differences observed between the various tumour sites in respect of the tumour grade, score, mitotic index and degree of necrosis were more pronounced. Antebrachial, tibial or rib tumours, tumours distal to the

Table 6
Mean and standard deviation values of tumour grade (TG), tumour score (TS), mitotic score (MI), necrotic score (degree of necrosis) (NS) and degree of pleomorphism (Pleom) for the histopathological subtypes of primary osteosarcoma

Subtype	*No. cases*	*TG*	*TS*	*MI*	*NS*	*Pleom*
Osteoblastic productive	73	2.10 ± 0.81	6.68 ± 1.86	2.74 ± 1.06	1.60 ± 0.95	2.38 ± 0.66
Osteoblastic min.prod.	18	2.25 ± 0.68^{a}	6.94 ± 1.29^{b}	3.06 ± 1.14	1.35 ± 1.12	2.31 ± 0.57
Chondroblastic	17	2.25 ± 0.77	7.38 ± 1.82^{c}	2.88 ± 1.15	1.75 ± 0.93	2.69 ± 0.48
Telangiectatic	9	2.11 ± 0.60	6.78 ± 1.48	2.67 ± 1.12	1.67 ± 1.23	2.44 ± 0.53
Fibroblastic	7	1.5 ± 0.83^{a}	$5.5 \pm 1.64^{b,c}$	2.00 ± 0.89	1.17 ± 1.47	2.33 ± 0.82
Giant cell rich	3	2.25 ± 0.95	7.33 ± 2.08	2.75 ± 1.50	2.00 ± 1.00	2.25 ± 0.50
Multipatterned	3	2.00 ± 0.00	6.33 ± 0.58	2.00 ± 1.00	1.67 ± 1.53	2.67 ± 0.58
Poorly differentiated	2	1	5	2.50 ± 0.71	0	3.00 ± 0.00

Osteoblastic min.prod., Osteoblastic minimally productive.
[a,b,c]Entries with the same superscript letter differed significantly ($P < 0.05$).

antebrachiocarpal or tarsocrural joint, and appendicular tumours overall were of a significantly higher grade, score or mitotic index than cranial tumours. Rib osteosarcomas were characterized by a degree of necrosis significantly higher than that at most other sites. Finally, osteosarcomas arising in distal metaphyses appeared at a younger age and were of significantly higher grade than osteosarcomas arising in proximal metaphyses.

The relevance of classifying osteosarcomas into subtypes, which has been questioned by some authors, remains unresolved (Misdorp and Hart, 1979; Straw, 2000). A particularly favourable prognosis was reported for canine fibroblastic osteosarcoma (Misdorp and Hart, 1979), but Grundmann *et al.* (1995) pointed out that this observation lacked statistical significance. In the present study, the statistically significant differences in tumour grade and score between a number of subtypes support the view that subtypes represent distinct clinicopathological entities and underline the validity of the scheme we used to classify osteosarcomas into subtypes. As it would appear that tumour grade is related to malignant potential, future studies should examine the relation between tumour grade and survival time.

In keeping with this study, Kirpensteijn *et al.* (2002) found a tumour grading system to be of prognostic value in canine osteosarcoma, showing an association with survival time. However, no association was shown between tumour grade and histopathological subtype or metastatic status. Furthermore, most osteosarcomas were high grade and only 4% were grade I, in contrast to the present study, in which 35% of tumours were grade III, 37% grade II and 28% grade I. The grading system used was somewhat different from ours, in that the quantity of tumour matrix and tumour cell density were also assessed; tumours were still classified into three tumour grades, but the parameters assessed were not added up to give a grade.

A previous study on mandibular osteosarcomas (Straw *et al.*, 1996), which employed the same grading system as that used here, and a study on osteosarcomas of flat or irregular bones (Owen, 1980; Hammer *et al.*, 1995), found that the grading systems used showed no prognostic significance.

Histopathological grading was of prognostic significance in human osteosarcoma (Grundmann *et al.*, 1995). Meister *et al.* (1979) classified 60 human osteosarcomas into three histological subtypes and graded them on the basis of mitotic index, osteoid formation, presence of multinucleated giant cells, and tumour necrosis. Although there was no statistically significant correlation between tumour grade and incidence of metastases or survival rate, most grade III osteosarcomas metastasized, and differed from grade I and II tumours in being associated with a shorter survival time. The authors suggested grading osteosarcomas, in addition to TNM (tumour, node, metastasis) staging, and found histological grading more applicable than classification into subtypes. The Japanese Orthopaedic Association modified a TNM system proposed by the International Union against Cancer (UICC), taking into account serum alkaline phosphatase concentrations and grading osteosarcomas on the basis only of their mitotic index, and showed a correlation between survival rate and stage (Fukuma *et al.*, 1997).

When applied, the classification of osteosarcoma into grades is at present based solely on histopathological grounds. Loukopoulos *et al.* (2003a, b, 2004) examined the relationship of the tumour grade with molecular prognostic indicators for canine osteosarcoma, such as the p53 tumour suppressor gene protein—a known prognostic indicator in human osteosarcoma (Lonardo *et al.*, 1997)—and matrix metalloproteinases (MMPs), which are proteolytic enzymes that play an important role in metastasis. A strong correlation was demonstrated between p53 immunohistochemical expression and a range of clinicopathological parameters in osteosarcoma, including the tumour grade, as defined in this study, and its constituent parameters (Loukopoulos *et al.*, 2003b). Osteosarcomas with a high tumour grade, mitotic index or degree of pleomorphism had significantly higher p53 expression than did low grade osteosarcomas (Loukopoulos *et al.*, 2003b). Similarly, total and pro-MMP-9 production levels, as measured by gelatin zymography, showed a correlation with the tumour grade of osteosarcomas (Loukopoulos *et al.*, 2003a). Total and pro-MMP-2 values were also markedly, but not significantly, higher for grade III than for grade I or II osteosarcomas. Furthermore, only high-grade osteosarcomas produced the 62 kDa form of active MMP-2. Taken together, these features further support the findings presented here. Combined with molecular, immunohistochemical or other studies on the pathobiology of osteosarcoma, such results may lead to a more detailed grading system in the future.

A limitation of retrospective studies is that clinical data on survival and disease-free intervals are often lacking, because many owners elect euthanasia for their dog shortly after osteosarcoma has been diagnosed. An important future aim remains the construction of tumour profiles that may be based on a number of features (age, tumour grade, tumour location, and expression of p53 and MMP) and will predict tumour behaviour for individual patients, rather than for patient subpopulations.

Acknowledgments

The authors thank Drs D. Spearritt, N. Sullivan, G.J. Sternberg, S. Chapman, C.F. Chandler and T. Poutahidis for providing case material.

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Received, November 23rd, 2005
Accepted, November 14th, 2006