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Abstract Abnormal levels of heat shock proteins have been observed in a number of human neoplasms and demonstrate prognostic, predictive and therapeutic implications. Since osteosarcoma (OSA) in dogs provides an important model for the same disease in humans, the aim of this study was to evaluate the immunohistochemical expression of Hsp27, Hsp72, Hsp73 and Hsp90 in 18 samples of canine appendicular OSA, in relation to histological grade and overall survival (OS), in order to investigate their potential prognostic, predictive and/or therapeutic value. A semiquantitative method was used for the analysis of the results. Hsp27, Hsp73 and Hsp90 showed a variably intense, cytoplasmic and nuclear immunoreactivity that was not associated with histological type or grade. On the other hand, a high percentage of Hsp72 immunostaining was significantly associated with grade III ($P<0.01$) and a lack of immunolabelling was significantly correlated to a longer OS ($P=0.006$). Neoplastic emboli were occasionally positive for Hsp27, faintly immunoreactive for Hsp72 and intensely immunolabelled by Hsp73 and Hsp90. In conclusion, absence of Hsp72 immunosignal appears to be associated with a favourable prognosis whilst the widespread Hsp90 immunoreactivity detected in all tumour cases as well as in neoplastic emboli, suggests this protein could be targeted in the therapy of canine OSA, and likewise in its human counterpart.

Keywords Heat shock proteins. Osteosarcoma . Animal model, Dog

Introduction

Heat shock proteins (HSPs), also known as stress proteins, are one of the most evolutionarily conserved classes of molecule and play a fundamental role in the maintenance of cellular homeostasis, under both physiological and stress conditions, acting as 'molecular chaperones' (Calderwood et al. 2006). HSPs can be classified into several families, named according to their approximate molecular weight, expressed in kilodaltons, even though new guidelines for the nomenclature of the human HSP families have been proposed (Kampinga et al. 2009). A growing body of evidence suggests that HSPs are implicated in all phases of cancer from proliferation, impaired apoptosis and sustained angiogenesis to invasion and metastasis (Calderwood et al. 2006). Since abnormal HSP levels have been observed in a wide range of human tumours, a number of studies have been carried out in order to determine whether these proteins could be used as diagnostic, prognostic and/or predictive markers or represent new molecular targets for cancer therapy (Ciocca and Calderwood 2005; Karapanagiotou et al. 2009). In particular, several studies have been performed to investigate the expression and prognostic significance of HSPs in human osteosarcoma (OSA; Trieb et al. 1998; 2000; Uozaki et al. 2000; Ozger et al. 2009; Moon et al. 2010). In addition, during the last decade, researches have been focused on the potential usefulness of Hsp90 inhibitors as novel treatment approaches of cancer (Porter et al. 2010), including OSA (Gazitt et al. 2009), particularly in the context of childhood sarcomas (Bagatell et al. 2005; 2007). HSPs expression has also been demonstrated in preliminary studies carried out on canine neoplasms (Kumaraguruparan et al. 2005; Romanucci et al. 2005; 2006; Carrasco et al. 2011), which show an abnormal expression pattern similar to that observed in human neoplasms (Kumaraguruparan et al. 2005; Romanucci et al. 2005; 2006). These parallel findings underline the relevance of studying the multiple roles of HSPs in carcinogenesis in animal models as an additional source of information for clinical cancer research. In fact spontaneously occurring tumours in dogs provide an important model for human cancer biology studies and therapeutic strategies, and OSA is considered one of the canine

malignancies of most interest as it shows striking similarities in tumour biology and behaviour to its human counterpart (Mueller et al. 2007). However, to the best of our knowledge, no data are available concerning HSP expression in canine OSA tissues.

The aim of this study was to evaluate the immunohistochemical expression and localization of different HSPs in canine OSA and to establish whether this expression could be related to histological tumour grade and overall survival (OS). This study also aimed to shed further light on the role of these proteins in the pathogenesis of canine OSA and therefore their potential prognostic, predictive and/or therapeutic value.

Methods

Histological examination

The study was carried out on surgical samples from 18 canine appendicular OSA. All tumour cases were supplied by the Department of Animal Pathology, Faculty of Veterinary Medicine, University of Turin (Italy). Samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Histological diagnosis was established using haematoxylin and eosin stained slides, according to WHO guidelines (Slayter et al. 1994), while histological grade was determined according to the system proposed by Kirpensteijn et al. (2002).

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were also processed using an immunohistochemical technique with a variety of specific antibodies (Abs) namely, anti-Hsp27 (1:300, rabbit polyclonal, StressGen, Victoria, BC, Canada), anti-Hsp72 (1:50, C92F3A-5, mouse monoclonal, StressGen), anti-Hsp73 (1:100, 1B5, rat monoclonal, StressGen), anti-Hsp90 (1:600, AC88, mouse monoclonal, StressGen) Abs.

Deparaffined and rehydrated sections were incubated in 3% hydrogen peroxide in absolute methanol for 45 min to inhibit endogenous peroxidase activity, then rinsed in 0.05 M Tris-buffered saline (TBS), pH 7.6, for 5 min. Antigen retrieval was performed by heat-treatment in citrate buffer at pH 6 in a microwave oven for 5 min (three cycles). To reduce non-specific binding, slides were incubated in normal goat serum (Biospa, Milan, Italy) for 10 min at room temperature before overnight incubation with primary Ab in a humidified chamber at 4°C. After rinsing with TBS, immune complexes were treated at room temperature for 10 min with secondary biotinylated Goat anti-mouse/rabbit (ready-to-use, Biospa) or Rabbit anti-Rat (1:100, Dako, Copenhagen, Denmark) Abs and subsequently detected using strepta-vidin peroxidase (Biospa). Tissue peroxidase activity was detected by 5 min application of 0.1% hydrogen peroxide in 3,3'-diaminobenzidine solution (D5905, Sigma-Aldrich, St Louis, Missouri) and followed by counterstaining with Mayer's Haematoxylin for 1 min before rinsing, dehydrating and mounting.

A negative control was performed in all instances by omitting the primary Ab and incubating tissue sections with TBS and/or with an irrelevant Ab directed against an unrelated antigen such as rabbit anti-human von Willebrand factor polyclonal Ab or mouse anti-human desmin monoclonal Ab (Dako, Glostrup, Denmark).

Clinical follow-up

Survival data concerning the dogs were supplied by the Department of Animal Pathology, Faculty of Veterinary Medicine, University of Turin (Italy). All the dogs included in the present study had no evidence of metastasis at presentation and a histologically confirmed appendicular OSA. In all cases work-up included history, physical exam, complete blood count, serum biochemical profile, urinalysis and abdominal ultrasound. Limb (LL and AP views) and chest (LL, right and left, and DV views) radiographic evaluation was performed to examine respectively features and extention of the tumour and presence of lung metastasis; computed tomography was performed in case of suspicious lung radiographs.

Regional lymph nodes were aspirated and cytologically examined when enlarged. Initial diagnosis was attempted by fine needle aspiration and cytology but, for a more specific identification of the tumour type, a peroperative biopsy was obtained in all cases using a Jamshidi needle and submitted to histopathology. For a more precise tumour characterization, histopathology was again per-formed on the entire tumoural specimen postoperatively. All dogs included in the present study were surgically treated (amputation or limb sparing) before receiving adjuvant chemotherapy using doxorubicin (30 mg/m^2, four to five administrations, 21 days apart) or cisplatin (70 mg/m^2, four to five administrations, 21 days apart) as a single agent or a combination of cisplatin and doxorubicin (four cycles, 21 days apart, each cycle consisting of cisplatin 50 mg/m^2 at day 1 and doxorubicin 15 mg/m^2 at day 2). Local tumour control (surgery) was followed by chemotherapy administration since it has been proved that adjuvant chemotherapy can improve survival in dogs with appendicular OSA, even though no protocol has been demonstrated to be superior (Dernell et al. 2007). Therefore, the protocol was decided based on both literature data and owner's decision (Straw et al. 1991; Berg et al. 1995; Chun et al. 2005). Besides, histopatho-logical tumour grade never influenced the choice of the chemotherapeutic protocol adopted. Canine patients were clinically and radiographically examined every 3 months during the first year after the conclusion of chemotherapy and then every 6 months for a minimum of 2 years. For the animals that died of tumour-related causes within the 2 year period, OS was considered the days between surgery and death, whilst for dogs that survived >2 years, OS was the number of days from surgery to the last clinical examination. OS data in relation to chemotherapeutic protocol, histological type and grade are shown in Table 1.

Statistical analysis

A semiquantitative immunohistochemical assessment (absent, no positive cells; low, >0 to <10% positive cells; moderate, >10 to <50% positive cells; high, >50% positive cells) was made and Fisher's exact test was used to compare HSP expression with histological grades. Kaplan-Meier analysis was used to estimate survival, and the significances of the differences were determined by the log-rank test. For this purpose, the cases were grouped according to absence/presence of immunostain-ing or to the expression score as follows: <10% positive cells (absent+low semiquantitative evaluation) versus >10% positive cells (moderate+high semiquantitative evaluation) or <50% positive cells (absent/low+moderate)

	Histological type	Post-surgical treatment	Overall survival (days)
Grade I			
No. 1	Fibroblastic osteosarcoma	Doxorubicin	1,033
No. 2	Productive osteoblastic osteosarcoma	Cisplatin/doxorubicin	449
No. 3	Fibroblastic osteosarcoma	Cisplatin/doxorubicin	1,250
No. 4	Nonproductive osteoblastic osteosarcoma	Cisplatin/doxorubicin	1,089
Grade II			
No. 5	Chondroblastic osteosarcoma	Cisplatin/doxorubicin	168
No. 6	Mixed osteosarcoma	Cisplatin/doxorubicin	553
No. 7	Mixed osteosarcoma	Doxorubicin	503
No. 8	Poorly differentiated osteosarcoma	Doxorubicin	715
No. 9	Fibroblastic osteosarcoma	Cisplatin/doxorubicin	496
No. 10	Chondroblastic osteosarcoma	Cisplatin/doxorubicin	2,209
No. 11	Teleangectatic osteosarcoma	Cisplatin/doxorubicin	261
No. 12	Productive osteoblastic osteosarcoma	Cisplatin/doxorubicin	391
Grade III			
No. 13	Productive osteoblastic osteosarcoma	Cisplatin/doxorubicin	169
No. 14	Productive osteoblastic osteosarcoma	Cisplatin/doxorubicin	242
No. 15	Nonproductive osteoblastic osteosarcoma	Doxorubicin	335
No. 16	Giant cell type osteosarcoma	Cisplatin	211
No. 17	Productive osteoblastic osteosarcoma	Cisplatin/doxorubicin	200
No. 18	Giant cell type osteosarcoma	Cisplatin/doxorubicin	203

Table 1. Tumour grade, histological type, post-surgical treatment and overall survival of the cases included in the present studysemiquantitative evaluation) versus >50% positive cells (high semiquantitative evaluation). Analyses were per-formed using the SPSS statistical software, and the conventional 5% level was used to define statistical signi ficance.

Results

HSP expression in canine OSA

Hsp27 appeared to be weakly to moderately expressed in most tumour cases with a predominantly cytoplasmic localization, rarely nuclear (Fig. 1); only one case was negative. A strong cytoplasmic immunolabelling of multinucleated giant cells was found independently of the immunoreactivity of the surrounding tumour cells (Fig. 1, inset). Neoplastic emboli were not consistently positive, although they reflected the expression observed in the primary tumour.

Hsp72 immunolabelling differed among the various samples examined in intensity of immunosignal and percentage of positive cells; the highest signal was usually noted in tumour cells surrounding necrotic areas. Both cytoplasmic and nuclear localizations were detected, although nuclear signal was more frequent (Fig. 2). Multinucleated giant cells appeared negative whilst lymphatic emboli were negative or only faintly positive (Fig. 2, inset), independently of the immunoreactivity of the primary tumour. Four cases were negative for Hsp72.

Hsp73, as well as Hsp90, exhibited a widespread, moderate to high, cytoplasmic and nuclear positivity in all tumours examined (Figs. 3-4). An intense and diffuse

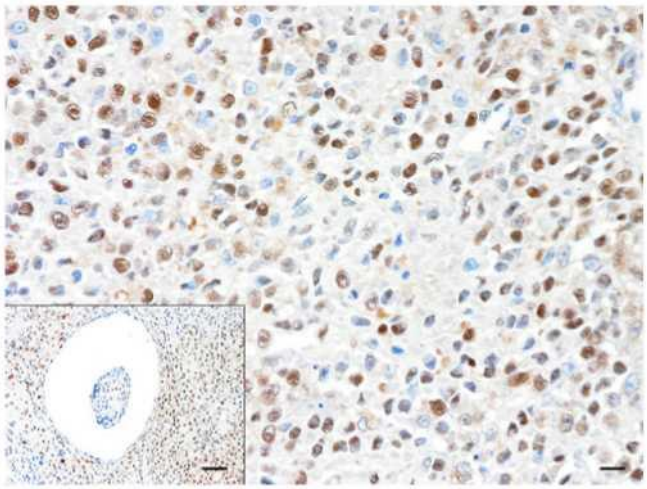

Fig. 2 Nonproductive osteoblastic osteosarcoma: intense Hsp72 immunosignal predominantly located in the nucleus of tumour cells *(bar=10 \an). Inset* absence of immunolabelling in a lymphatic embolus *(bar*= 60 |j.m)

reactivity in lymphatic emboli was also observed (Fig. 4, inset). Multinucleated giant cells showed a characteristic multifocal intense nuclear immunosignal for Hsp73 (Fig. 3, inset) but were inconsistently positive for Hsp90, with either cytoplasmic or nuclear localization.

No significant differences in HSP immunoexpression were found in relation to the histological type of OSA.

Relationship between HSP expression and tumour grades

The expression of Hsp27, Hsp73 and Hsp90 did not appear to be related to histological grade. High levels of Hsp72 immunostaining, on the other hand, were significantly associated to high-grade (grade III) OSA (P=0.009). Expression scores of the different HSPs under study in relation to histological grades are illustrated in Fig. 5.

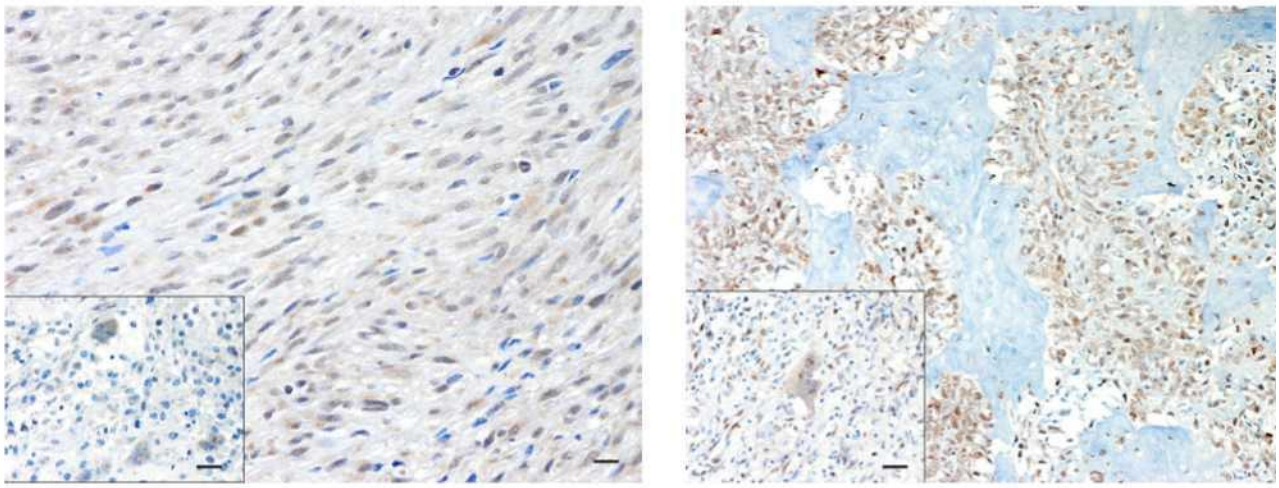

Fig. 1 Fibroblastic osteosarcoma: moderate Hsp27 immunoreactivity with diffuse cytoplasmic and scattered nuclear localization *(bar*= 10 |j.m). *Inset* intense and diffuse positivity in the cytoplasm of multinucleated giant cells *(bar=30 \m\)*

Fig. 3 Productive osteoblastic osteosarcoma: intense, diffuse, cytoplasmic and nuclear Hsp73 immunoreactivity *(bar=22* |xm). *Inset* scattered, strong nuclear positivities associated with low cytoplasmic immunosignal in a multinucleated giant cell *(bar=30 \an)*

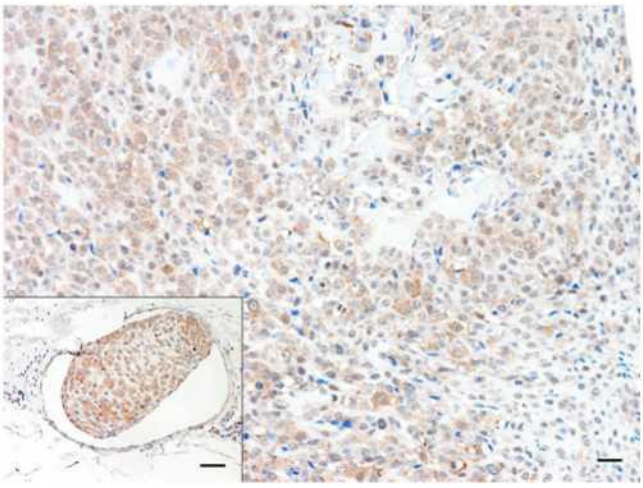

Fig. **4** Productive osteoblastic osteosarcoma: intense and diffuse cytoplasmic Hsp90 immunolabelling in primary tumour cells as well as in a lymphatic embolus *(inset) (bar=22* |j.m; *inset bar*= 60 |j.m)

Relationship between HSP expression and OS

Survival analysis revealed that absence of Hsp72 immunosignal was significantly associated to a longer post-surgical OS (P=0.006) when compared with cases exhibiting Hsp72 immunoreactivity. However, a significant association with OS was not found when Hsp72 expression scores were taken into consideration, although its lower expression levels (<10%) indicated a trend toward a prolonged OS *(P*= 0.058; Fig. 6). No significant relationship was found between Hsp27, Hsp73 and Hsp90, and OS.

Discussion

This study demonstrates the immunohistochemical expression of several members of the major HSP families in canine OSA. Similar studies have been carried out in human OSA (Trieb et al. 1998; Uozaki et al. 2000; Ozger et al. 2009; Moon et al. 2010). However, results published in literature are conflicting: Hsp27, Hsp60 and Hsp70 over-expression have been related to poor prognosis by some studies (Uozaki et al. 2000) whilst others have failed to demonstrate any significant influence on the survival rate in human OSA (Ozger et al. 2009; Moon et al. 2010). The results of the present study do not show a significant relationship between the expression scores of the different HSPs evaluated and OS, although absence of Hsp72 immunosignal appeared to be related to a longer post-surgical OS (P=0.006), with also a tendency toward a prolonged OS for its lower expression levels (P=0.058), and a significant association was found between high Hsp72 immunodetection and high-grade OSA. Given the poor prognostic significance of high histological grade in canine OSA (Kirpensteijn et al. 2002), the present results suggest a similarly poor prognostic value for Hsp72. A significantly higher Hsp70 immunohistochemical expression, with respect to low grade central OSA, has also been detected in human conventional high-grade OSA (Moon et al. 2010). Although current data suggest an association between high Hsp70 immunodetection and poor prognosis in both human and canine OSA, a correlation between the presence of Hsp72 expression and a better response to neoadjuvant chemotherapy has been observed in human OSA patients (Trieb et al. 1998). However, to the best of our knowledge, no information concerning its possible influence on adjuvant chemotherapy is available. The results of the present study would suggest that absent/low Hsp72 immunolabelling in primary OSA could be predictive of a better response to postoperative chemotherapy, regardless of the type of chemotherapeutic choice, since no protocol has been demonstrated to be superior in improving survival of dogs with appendicular OSA (Dernell et al. 2007), thus not affecting the evaluation of the relationship between HSP expression and OS. Notwithstanding this, the role of Hsp72 in the response to adjuvant chemotherapy should be investigated further in a larger subset of metastatic OSA, also in light of its faint to absent immunosignal observed in lymphatic neoplastic emboli, since adjuvant treatments are mainly aimed at controlling the metastatic disease, which may not show the same pattern of expression with respect to the primary tumour (Cardoso et al. 2001). Likewise for Hsp27, whose over-expression has been associated with resistance to cisplatin and doxorubicin in several cancer cell lines (Garrido et al. 1997; Yamamoto et al. 2001; Qian et al. 2006), even though no literature data concerning OSA cells are available and our results do not suggest any influence of Hsp27 expression on the response to these chemotherapeutic agents. On the other hand, Hsp27 has been hypothesized to be involved in drug resistance of OSA cells to zoledronic acid (Morii et al. 2010), whose adjuvant potential has been highlighted in several studies carried out in animal models of OSA, although its efficacy against pulmonary metastasis remains controversial (Ory et al. 2005; Dass and Choong 2007; Labrinidis et al. 2010).

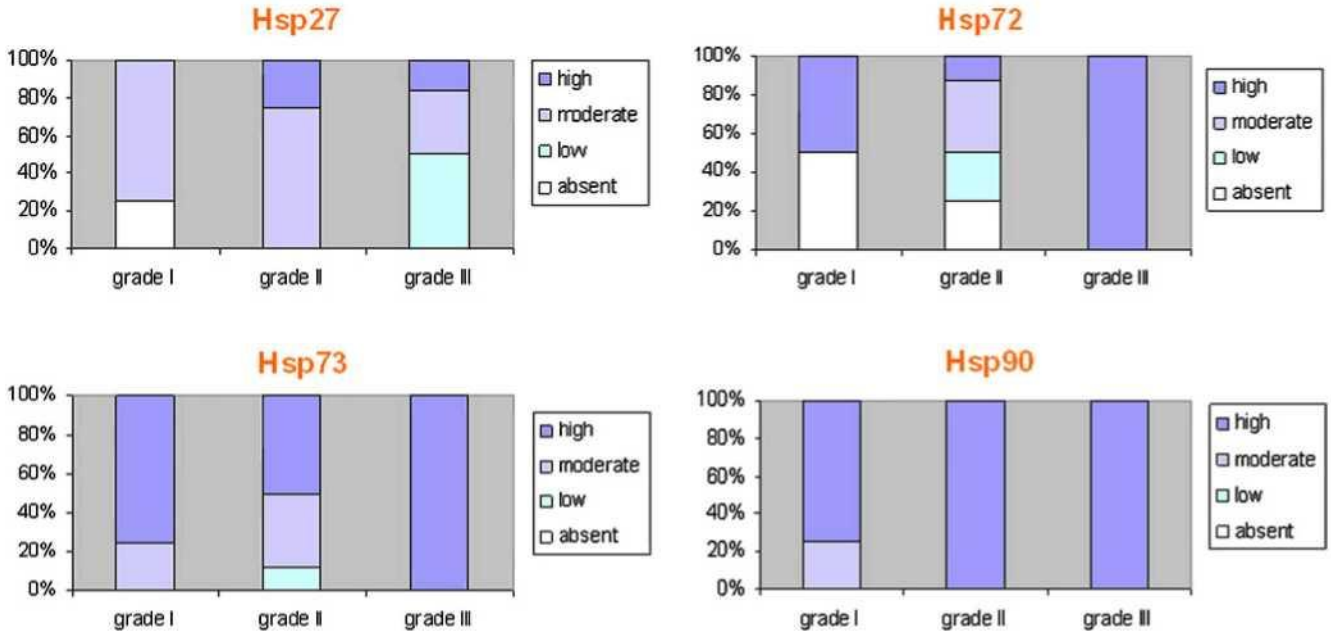

Fig. 5 The figure shows the percentage of expression of each Hsp (absent, low, moderate, high) in the different histological grades of canine osteosarcoma. Hsp27, Hsp73 and Hsp90 expression did not appear to be related to histological grade whereas high percentage of Hsp72 immunostaining was significantly associated with high-grade (grade **III)** osteosarcoma (P=0.009)

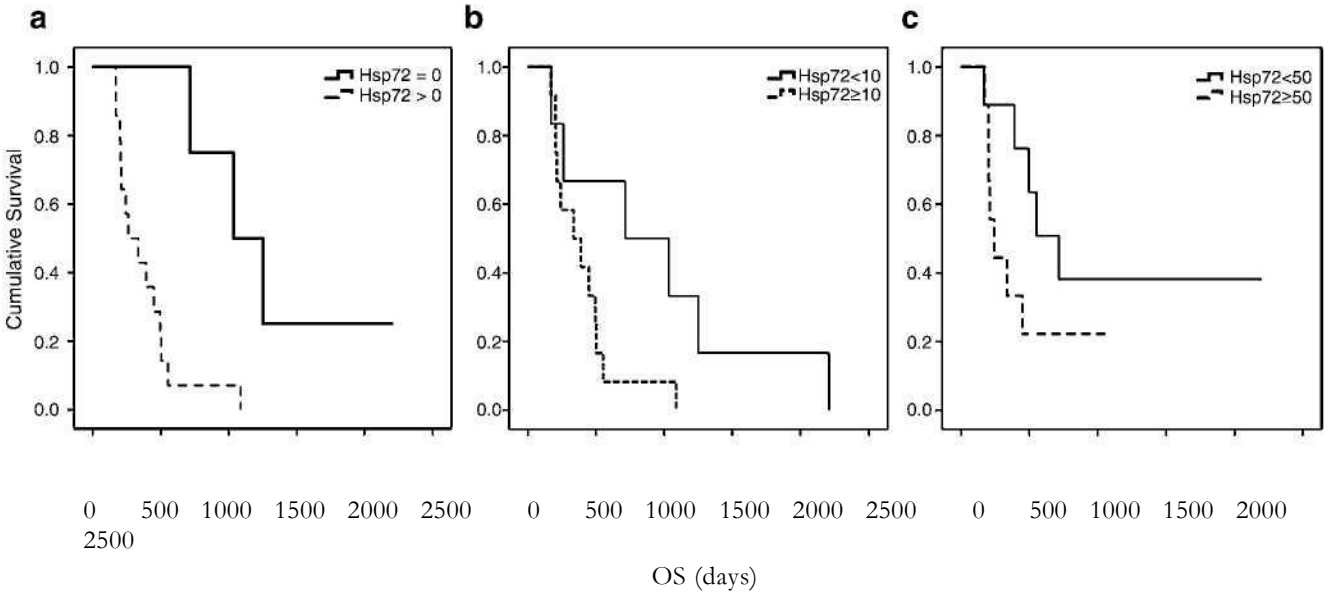

Fig. 6 Kaplan-Meier plots showing influence on survival of Hsp72 b-c Hsp72 expression scores did not show a significant relation to OS immunoexpression: a Absence of Hsp72 immunodetection appeared (P=0.058-P=0.112) to be significantly associated to a longer post-surgical OS (P=0.006);

Both Hsp73 and Hsp90 were the most abundantly expressed proteins in canine OSA. These findings appear to be in line with the participation of the constitutive member of the HSP70 family in the regulation of protein folding, assembly and degradation, as well as in the protection of cellular proteins from the damage caused by cellular stress like hypoxia, thus possibly conferring proliferative advantage on tumour cells. Our results concerning Hsp90 correspond to its role as a specialized molecular chaperone, responsible for folding numerous oncogenic proteins and thus leading to an 'addiction' to this Hsp by cancer cells (Trepel et al. 2010). Information regarding its expression in human OSA tissues is limited (Uozaki et al. 2000; Ozger et al. 2009), although several studies have highlighted the possible effectiveness of Hsp90-binding agents, particularly 17-allylamino-17-deme-thoxygeldanamicin (17-AAG), in the targeted therapy of this kind of tumour, especially in the context of childhood sarcomas (Bagatell et al. 2005; 2007; Gazitt et al. 2009). In addition, McCleese et al. (2009) have

recently demonstrated the selective cytotoxicity of the novel Hsp90 inhibitor STA-1474 for human and canine OSA cell lines. However, to the best of our knowledge, HSP expression in canine OSA samples has not been investigated. The results of this study suggest the involvement of Hsp90 in the pathogenesis of canine OSA although no association seems to exist between its expression and tumour prognosis, similarly to humans (Uozaki et al. 2000; Ozger et al. 2009). Lastly, the widespread expression of this protein observed both in primary tumours and in neoplastic emboli strongly high-lights the possibility of using a canine model for further testing of Hsp90-targeted cancer therapy.

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