Expression of heat shock proteins in premalignant and malignant urothelial lesions of bovine urinary bladder

Mariarita Romanucci · Daniela Malatesta ·
Andrea Ciccarelli · Laura Bongiovanni ·
Chiara Palmieri · Giuseppe Borzacchiello ·
Franco Roberto · Gennaro Altamura ·
Leonardo Della Salda

Received: 20 February 2012 / Revised: 5 April 2012 / Accepted: 9 April 2012 / Published online: 2 May 2012
© Cell Stress Society International 2012

Abstract Abnormal heat shock protein (HSP) levels have been observed in a number of human tumours, where they are involved in all hallmarks of cancer. Since bovine urothelial tumours share striking morphological and biochemical features with their human counterparts, the aim of this study was to evaluate the immunohistochemical levels of Hsp27, Hsp60, Hsp72, Hsp73 and Hsp90 in 28 normal bovine urinary bladders and 30 bovine papillomavirus-positive urothelial tumours (9 in situ carcinomas, 9 low-grade and 12 high-grade carcinomas) and adjacent premalignant lesions obtained from cows suffering from chronic enzootic haematuria, in order to investigate the role of these proteins in the process of urothelial carcinogenesis. A semi-quantitative method was used for the analysis of the results. Western blot analysis was also used to confirm HSP expression in normal controls. All investigated HSPs were expressed in normal bovine urothelium, showing characteristic patterns of immunolabelling throughout urothelial cell layers, which usually appeared to be conserved in urothelial hyperplasia and dysplasia. On the other hand, gradual loss of Hsp27 immunostaining resulted to be significantly associated with increasing histological grade of malignancy

($P<0.01$). As well, a significantly reduced immunosignal of Hsp73 and Hsp90 was observed in high-grade and low-/high-grade carcinomas, respectively ($P<0.01$). In contrast, Hsp60 ($P<0.01$) and Hsp72 ($P<0.05$) immunoreactivity appeared to be significantly increased both in premalignant and malignant lesions when compared to that observed in normal urothelium, thus suggesting an early involvement of these proteins in neoplastic transformation of urinary bladder mucosa.

Keywords Heat shock proteins · Chronic enzootic haematuria · Papillomavirus · Cattle · Urinary bladder · Tumour

Introduction

Heat shock proteins (HSPs), also known as stress proteins, are one of the most evolutionarily conserved classes of molecules and play a fundamental role in the maintenance of cellular homeostasis, under both physiological and stress conditions. The cytoprotective properties of HSPs rely on their primary functions as molecular chaperones in ‘protein holding’ and ‘protein folding’ (Calderwood et al. 2006). HSPs are classified into several families, named according to their approximate molecular weight, expressed in kilodalton, 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, including bladder cancer (Storm et al. 1993; Cardillo et al. 2000; Lebrecht et al. 2003b; Syrigos et al. 2003; Cappello et al.

M. Romanucci · D. Malatesta · L. Bongiovanni · C. Palmieri ·
L. Della Salda (✉)
Department of Comparative Biomedical Sciences,
Faculty of Veterinary Medicine, University of Teramo,
Teramo, Italy
e-mail: ldellasalda@unite.it

A. Ciccarelli
Department of Communication Sciences, University of Teramo,
Teramo, Italy

G. Borzacchiello · F. Roberto · G. Altamura
Department of Pathology and Animal Health,
University of Naples Federico II,
Naples, Italy

2006; EL-Meghawry EL-Kenawy et al. 2008), several 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 targets for cancer therapy (Ciocca and Calderwood 2005; Karapanagiotou et al. 2009). Preliminary studies in veterinary medicine have also demonstrated the presence of altered HSP expression in neoplasms suggesting a pattern of tumour development similar to the human counterpart (Kumaraguruparan et al. 2006; Romanucci et al. 2006, 2008). 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. However, to the best of our knowledge, no data are available concerning the expression of HSPs in animal models of urinary bladder tumours.

Spontaneously occurring urinary bladder tumours are common in dogs, relatively frequent in cats, but rare in all other species. Particularly in cattle, the incidence of bladder cancer accounts for 0.01–0.1 % of animals at slaughterhouse. However, the prevalence of bovine bladder neoplasia becomes extremely high in endemic areas where bracken fern (*Pteridium* spp.) grows, as a consequence of the prolonged ingestion of the carcinogenic and mutagenic toxins, mainly ptaquiloside, contained in this plant (Meuten 2002). Chronic bracken fern toxicity causes multiple tumours in the bovine bladder wall and haemorrhages in the bladder mucosa, characterising a syndrome termed ‘enzootic haematuria’ (Carvalho et al. 2006). A synergism between bracken carcinogens and bovine papillomavirus (BPV) type 2 is also believed to occur in bladder carcinogenesis in cattle, where the major BPV E5 oncoprotein plays a pivotal role in cancer development (Campo et al. 1992; Borzacchiello et al. 2003b; Borzacchiello and Roperto 2008; Roperto et al. 2008; Venuti et al. 2011). Since bovine urothelial tumours share striking morphological and biochemical features with their human counterparts (Ambrosio et al. 2001; Borzacchiello et al. 2001, 2003a, 2004; Roperto et al. 2005, 2007; Brun et al. 2008), a classification system similar to the 2004 World Health Organization morphological classification of human bladder tumours (Epstein et al. 2004; Lopez-Beltran et al. 2004; Lopez-Beltran and Montironi 2004; Reuter 2004; Fine et al. 2005; Montironi and Lopez-Beltran 2005) has been suggested to be appropriate for the classification of bovine bladder tumours (Roperto et al. 2009). The aims of this study were to evaluate the immunohistochemical levels and localization of different HSPs in normal bovine urothelium, as well as in malignant urothelial tumours and premalignant lesions of the urinary bladder obtained from cows suffering from chronic enzootic haematuria and known to express the BPV E5 oncoprotein, in order to establish whether HSP expression could be related to the histological growth pattern and/or grade of malignancy of the lesions, thus investigating the role of these proteins in the process of urothelial carcinogenesis.

Materials and methods

Histological examination

The study was carried out on 28 samples from normal bovine urinary bladders and 30 urothelial tumours and adjacent premalignant lesions (flat and papillary urothelial hyperplasia and urothelial dysplasia) collected from public slaughterhouses. Control animals came from lowlands where bracken is virtually absent, whereas tumour samples were obtained from cows suffering from chronic enzootic haematuria and coming from mountain areas where bracken fern is widely distributed. All tumour cases were supplied by the Department of Pathology and Animal Health, Naples University Federico II, Naples (Italy). Samples were fixed in 10 % neutral buffered formalin and processed routinely to paraffin wax. Sections were cut (5 µm) and stained with haematoxylin and eosin. The tumours were classified as described by Roperto et al. (2009) as carcinoma in situ (CIS, $n=9$), papillary low-grade urothelial carcinoma ($n=9$), papillary high-grade urothelial carcinoma ($n=6$), high-grade urothelial carcinoma with inverted (endophytic) growth pattern ($n=1$) and invasive high-grade urothelial carcinoma ($n=5$). These tumours were selected from a larger subset known to harbour BPV-2 DNA and were also known to be positive for the expression of BPV E5 protein (Borzacchiello et al. 2003b).

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were also processed using an immunohistochemical technique with specific antibodies (Abs) directed against Hsp27 (1:2,400, rabbit polyclonal, StressGen, Victoria, BC, Canada), Hsp60 (1:200, LK-1, mouse monoclonal, StressGen), Hsp72 (1:100, C92F3A-5, mouse monoclonal, StressGen), Hsp73 (1:500, 1B5, rat monoclonal, StressGen) and Hsp90 alpha/beta (stress-inducible and constitutively expressed isoforms of Hsp90, respectively) (1:2,500, AC88, mouse monoclonal, StressGen). Deparaffinized 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 and Rabbit (ready-to-use, Biospa) or Rabbit anti-Rat (1:100, DAKO, Copenhagen, Denmark) Abs and subsequently

detected using streptavidin–peroxidase (Biospa). Peroxidase activity was detected by 5-min application of 0.1 % hydrogen peroxide in 3–3'-diaminobenzidine (DAB) solution (D5905, Sigma-Aldrich, St. Louis, MO) 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).

Western blotting

For Western blotting, ten samples of normal bovine urinary bladders were immediately frozen and stored at -80°C until tested. In order to investigate the HSP expression only in the urinary bladder mucosa, the urothelium + lamina propria was dissected from the detrusor smooth muscle under a dissecting microscope (SMZ645, Nikon). The samples were then homogenised in lysis buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 % Triton X-100, 1 % IGEPAL and 0.1 % SDS (Sigma-Aldrich) to which protease inhibitor cocktail (1:100, Sigma-Aldrich) was added at the time of use. The insoluble fraction was separated by centrifugation ($12,000\times g$ for 5 min at 4°C). Proteins were quantified by Bradford assay and equal amounts of protein lysates (40 μg of protein) were boiled for 5 min in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), before being separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred from the gel onto polyvinylidene fluoride membranes using a Trans-Blot apparatus (Bio-Rad Laboratories) according to the manufacturer's instructions. Membranes were blocked with 5 % non-fat dried milk in TBS-0.05 % Tween 20 for 1 h, washed with TBS-0.05 % Tween and incubated with primary Abs directed against Hsp27 (1:5,000, rabbit polyclonal, StressGen), Hsp60 (1:1,000, LK-1, mouse monoclonal, StressGen), Hsp72 (1:1,000, C92F3A-5, mouse monoclonal, StressGen), Hsp73 (1:1,000, 1B5, rat monoclonal, StressGen) and Hsp90 α/β (1:1,000, AC88, mouse monoclonal, StressGen) in a blocking solution for 1 h and 30 min at room temperature. Mouse anti- β actin monoclonal antibody (1:200, C4, Santa Cruz Biotechnology, Santa Cruz, CA) was also used in order to confirm equal loading of proteins in each lane. Washed membranes were then incubated with a species-specific secondary biotinylated antibody for 1 h and 30 min at room temperature and proteins were finally visualised by incubation with an avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA) and DAB substrate. Densitometric analysis was performed using ImageJ software.

Statistical analysis

A semi-quantitative immunohistochemical assessment (– = absent immunolabelling; + = low (weak or focal, ≤ 50 % positive cells) immunolabelling; ++ = moderate and diffuse (> 50 % positive cells) immunolabelling; and +++ = strong and diffuse (> 50 % positive cells) immunolabelling) was made comparing normal and premalignant/malignant urothelial lesions and, in the latter, comparing HSP expression in different groups (hyperplasia/dysplasia, CIS, low-grade and high-grade carcinomas) by Fisher's exact test. Analyses were performed using the SPSS statistical software, with $P < 0.05$ considered as statistically significant.

Results

Results concerning immunohistochemical HSP levels in normal bovine urinary bladder, as well as in premalignant and malignant bovine urothelial lesions are summarised in Table 1. Even though variable degrees of inflammation in the lamina propria were detectable in most of the pathologic samples, HSP immunosignal in the urothelium appeared to be independent of the intensity of inflammation in the underlying connective tissue.

HSP expression in normal bovine urothelium

Similar patterns of HSP expression were observed in both urothelial cells of normal bovine urinary bladder and normal urothelium adjacent to the neoplastic lesions. Hsp27 showed a strong and diffuse cytoplasmic expression throughout all the urothelial cell layers. However, luminal cells frequently appeared to be negative (Fig. 1a). Hsp60 immunolabelling was particularly detectable on a narrow band corresponding to the luminal cell layer which showed an intense cytoplasmic granular expression, whereas all the other layers appeared to be negative or only faintly positive (Fig. 2a).

Hsp72 exhibited a moderate cytoplasmic, as well as an intense nuclear immunoreactivity in the basal and immediately suprabasal cells, whereas all the other layers showed an absent or only weak expression (Fig. 3a). Hsp73 and Hsp90 were intensely expressed in the cytoplasm of all the urothelial cells. An irregularly distributed intense nuclear positivity was also observed (Figs. 4a and 5a).

Western blot analysis confirmed the expression of all HSPs under study in normal bovine urinary bladder mucosa (Fig. 6). For each Hsp, densitometric analysis did not reveal significant differences of expression between the various samples examined.

Table 1 HSP expression in normal urothelium, hyperplastic/dysplastic urothelial lesions and carcinomas of bovine urinary bladder

Case no.	Histologic diagnosis	Score of immunohistochemical staining				
		Hsp27 (c)	Hsp60 (c)	Hsp72 (c/n)	Hsp73 (c/n)	Hsp90 (c/n)
–	Normal urothelium ^a	+++	+	+	+++	+++
–	Urothelial hyperplasia-dysplasia ^a	+++	++/+++	++(c) +++(n)	++/+++	+++
1	CIS	++	++	++(c) +++(n)	++	+++
2	CIS	++	+++	+	++	++(c) +++(n)
3	CIS	+++	+++	+	++	++(c) +++(n)
4	CIS	++	+++	++(c) +++(n)	++	+(c) ++(n)
5	CIS	++	++	+(c) ++(n)	+	++
6	CIS	+++	++	++	++(c) +(n)	+++
7	CIS	++	+++	+	+++ (c) +(n)	+++
8	CIS	+++	++	++	+(c) –(n)	+(c) ++(n)
9	CIS	+++	+++	+(c) ++(n)	++(c) +(n)	++(c) +++(n)
10	Papillary low-grade carcinoma	++	++	++	+(c) –(n)	++
11	Papillary low-grade carcinoma	++	+++	+	++	++
12	Papillary low-grade carcinoma	+++	+	+	+++	+(c) –(n)
13	Papillary low-grade carcinoma	–	++	++	+(c) –(n)	+
14	Papillary low-grade carcinoma	++	+++	++	++	–
15	Papillary low-grade carcinoma	++	+++	++(c) +++(n)	++	+
16	Papillary low-grade carcinoma	++	++	+	++(c) +++(n)	–
17	Papillary low-grade carcinoma	++	++	++	+++	+
18	Papillary low-grade carcinoma	++	++	+(c) ++(n)	++	+(c) –(n)
19	Papillary high-grade carcinoma	+	+++	++	++	+
20	Papillary high-grade carcinoma	–	+++	++	+(c) ++(n)	–
21	Papillary high-grade carcinoma	+	+++	++(c) +++(n)	+(c) –(n)	–
22	Papillary high-grade carcinoma	++	+	++(c) +++(n)	++	+(c) ++(n)
23	Papillary high-grade carcinoma	+	+++	+	+	+(c) ++(n)
24	Papillary high-grade carcinoma	+	+	+	+(c) –(n)	++(c) –(n)
25	Endophytic high-grade carcinoma	–	+++	++(c) +++(n)	++	–
26	Invasive high-grade carcinoma	+	+	++	+	++
27	Invasive high-grade carcinoma	–	+++	++(c) +++(n)	+	+(c) –(n)
28	Invasive high-grade carcinoma	+	+++	++(c) +++(n)	+(c) –(n)	+
29	Invasive high-grade carcinoma	+	++	++	+(c) –(n)	+
30	Invasive high-grade carcinoma	++	+++	++(c) +++(n)	+	++

– absent immunosignal, + weak or focal ($\leq 50\%$) immunosignal, ++ moderate and diffuse ($> 50\%$) immunosignal, +++ strong and diffuse ($> 50\%$) immunosignal, (c) cytoplasmic, (n) nuclear

^a Similar patterns of immunostaining were observed in the various samples examined

HSP expression in bovine hyperplastic/dysplastic urothelial lesions and CIS

HSP expression was investigated in hyperplastic/dysplastic urothelial lesions adjacent to all the urinary bladder tumours. As well, HSP expression was evaluated both in primary CIS and in CIS frequently involving the urothelium adjacent to papillary and invasive neoplasms.

In urothelial hyperplasia and dysplasia, Hsp27 immunolabelling appeared to be similar to that observed in normal bovine urothelium, whereas it usually appeared to be quite less intense, with the presence of negative cells irregularly distributed throughout cell layers in CIS (Fig. 1b). Hsp60

immunoreactivity appeared to be moderate to strong and irregularly distributed in all urothelial cell layers both in hyperplastic/dysplastic lesions and in CIS (Fig. 2b).

In urothelial hyperplasia and dysplasia, the intensity of Hsp72 immunosignal appeared to be similar to that observed in normal bovine urothelium, even though it was uniformly located in the cytoplasm and nucleus of a greater number of deeper layer cells. On the other hand, it usually acquired an irregular distribution pattern throughout cell layers in CIS, characterised by the presence of numerous cells exhibiting a moderate to intense cytoplasmic and/or nuclear immunoreactivity admixed with a minor number of urothelial cells

Fig. 1 Intense and diffuse cytoplasmic immunoreactivity of Hsp27 throughout all normal urothelial cell layers excluding luminal cells (**a**). A quite less intense immunosignal with presence of negative cells irregularly distributed throughout cell layers is observable in carcinoma in situ (**b**); a further reduction in intensity and distribution of cytoplasmic immunolabelling is also evident in low-grade (**c**) and especially high-grade urothelial carcinoma (**d**) (*bar*=25 μ m)

showing an absent to weak cytoplasmic immunolabelling (Fig. 3b).

As far as Hsp73 expression is concerned, both hyperplastic/dysplastic lesions and CIS showed a similar pattern of distribution throughout urothelial cell layers when compared to that observed in normal urothelium, even though cytoplasmic and/

or nuclear immunoreactivity resulted to be rather less intense (Fig. 4b). In urothelial hyperplasia and dysplasia, Hsp90 immunolabelling appeared to be comparable to that observed in normal bovine urothelium, whereas a quite reduction in intensity of cytoplasmic immunosignal was observed in CIS (Fig. 5b).

Fig. 2 Intense, cytoplasmic granular expression of Hsp60 detectable on a narrow band corresponding to the luminal cell layer in normal urothelium (**a**). Moderate to strong and widespread immunosignal of Hsp60 in carcinoma in situ (**b**), low-grade (**c**) and high-grade urothelial carcinoma (**d**) (*bar*=25 μ m)

Fig. 3 Moderate cytoplasmic and intense nuclear immunolabelling of Hsp72 in the basal and immediately suprabasal cells in normal urothelium (a). An increased number of nuclear positivities is observable in carcinoma in situ (b), low-grade (c) and high-grade urothelial carcinoma (d) (bar=25 μ m)

HSP expression in bovine low-grade and high-grade urothelial carcinomas

The expression of all HSPs under study appeared to be independent of the histological growth pattern (papillary, endophytic or invasive) of urothelial carcinomas. In particular, Hsp27 showed reduced intensity and distribution of

cytoplasmic immunoreactivity in malignant tumours, when compared to that observed in adjacent normal urothelium, hyperplastic/dysplastic lesions or CIS (Fig. 1c). This reduction was mainly evident in high-grade carcinomas, in which the expression was focally distributed and characterised by a predominantly weak intensity of immunolabelling (Fig. 1d). When observed, the highest intensity of immunosignal was

Fig. 4 Strong, diffuse cytoplasmic and irregularly distributed nuclear immunoreactivity of Hsp73 in normal urothelium (a), which appears to be reduced in its intensity and/or distribution in carcinoma in situ (b), low-grade (c) and especially high-grade (d) urothelial carcinoma (bar=25 μ m)

Fig. 5 Strong, diffuse cytoplasmic and nuclear immunoreactivity of Hsp90 in normal urothelium (**a**). A quite decreased intensity of cytoplasmic immunosignal is observable in carcinoma in situ (**b**); a further reduction in intensity and distribution of both cytoplasmic and nuclear immunolabelling is detectable in low-grade (**c**) and high-grade (**d**) urothelial carcinoma ($\text{bar}=25\ \mu\text{m}$)

usually detectable at the superficial layers of papillary tumours.

Hsp60 exhibited a diffuse cytoplasmic immunoreactivity, which was mainly moderate to strong in its intensity both in low- and high-grade carcinomas (Fig. 2c, d). Hsp72 demonstrated a variably intense and diffuse cytoplasmic and nuclear immunosignal in most of the tumour cases (Fig. 3c). However, widespread detection of nuclear immunolabelling was mainly evident in high-grade carcinomas

(Fig. 3d). As far as Hsp73 (Fig. 4c, d) and Hsp90 (Fig. 5c, d) are concerned, they evidenced a general reduction of intensity and distribution of both cytoplasmic and nuclear immunosignal in comparison to that observed in adjacent normal urothelium or hyperplastic/dysplastic lesions.

Comparison of HSP expression in normal urothelium and premalignant or malignant urothelial lesions

Hsp27 immunoreactivity did not significantly differ between normal urothelium and hyperplastic/dysplastic lesions, whereas a significant decrease of its expression was observed in urothelial tumours ($P<0.01$). In addition, when tumour groups (CIS, low-grade carcinomas and high-grade carcinomas) were compared, low to absent immunosignal was significantly associated with high-grade carcinomas ($P<0.01$).

A significant increase in Hsp60 expression ($P<0.01$), as well as in both cytoplasmic and nuclear ($P<0.05$) Hsp72 immunosignal, was noted in both hyperplastic/dysplastic

Fig. 6 Representative immunoblots of HSP expression in normal bovine urinary bladder mucosa

lesions and urothelial carcinomas, when compared to normal urothelium. However, no significant differences were observed when premalignant and malignant lesions were compared, as well as between the various histological tumour grades.

As far as Hsp73 was concerned, a significant reduction of both cytoplasmic and nuclear expression was observed in urothelial carcinomas, when compared to normal urothelium or premalignant lesions ($P < 0.01$). However, when tumour groups were considered, Hsp73 expression in CIS and low-grade carcinomas did not significantly differ from that observed in normal or hyperplastic/dysplastic urothelium, while low to absent immunostaining was significantly associated with high-grade carcinomas ($P < 0.01$). Likewise for Hsp90, even though in this case low to absent immunolabelling was significantly associated with both low- and high-grade carcinomas ($P < 0.01$), without significant differences between tumour grades.

Discussion

The results obtained in the present study demonstrate the immunohistochemical levels of several members of HSP families in normal bovine urinary bladder, as well as in premalignant and malignant bovine urothelial lesions. Particularly, as in human bladder (Lebret et al. 2003b), all investigated HSPs were expressed in normal bovine urothelium, showing characteristic patterns of immunolabelling throughout urothelial cell layers. In addition, these typical expression patterns frequently appeared to be conserved in urothelial hyperplasia and dysplasia, even though such lesions have been hypothesised to have a malignant potential in cattle (Roperto et al. 2009). On the other hand, all investigated HSPs exhibited, although not always reaching statistical significance, a quite modified expression in CIS, which is considered a malignant high-grade intraurothelial lesion that has been suggested to be able to progress to papillary or invasive urothelial tumours (Roperto et al. 2009). In particular, Hsp27 showed a reduction in intensity and distribution of immunosignal, which gradually became more evident in both papillary and invasive carcinomas, with low to absent signal also showing a significant association with high-grade tumours, thus suggesting that loss of Hsp27 expression can be a feature of bovine urothelial neoplastic transformation, related to increasing histological grade of malignancy. Hsp73 and Hsp90 immunoreactivity also resulted to be significantly decreased in urothelial carcinomas, even though their reduction of expression became significant when either low- or high-grade tumours for Hsp90, or only high-grade carcinomas for Hsp73, were considered. In this respect, loss of expression of these HSPs has been also observed in human bladder cancer (Lebret et

al. 2003b, 2007), although the role of HSPs in this kind of neoplasm appears to be rather controversial (Lebret et al. 2003a).

In contrast, Hsp60 immunolabelling, which was only detectable on luminal cells in normal bovine urothelium, showed a clear-cut increased distribution throughout urothelial cell layers both in premalignant and malignant urothelial lesions, without differences between histological tumour types or grades, thus suggesting that Hsp60 could exert a fundamental role in bovine urothelial carcinogenesis and the increase in its expression could be an early event during the process of neoplastic transformation of bovine urinary bladder mucosa. Hsp60 expression has been also extensively studied in human urinary bladder cancer (Lebret et al. 2003b; Cappello et al. 2006; Urushibara et al. 2007); however, unlike our results, the loss of expression of this protein has been usually detected, which appeared to be associated with poor prognosis (Lebret et al. 2003a), thus suggesting that Hsp60 could play different roles in bovine and human urothelial carcinogenesis. In this respect, since BPV-2 is recognised to be involved in the pathogenesis of urinary bladder neoplasia in cattle (Campo et al. 1992), it is important to underline that increased immunohistochemical levels of Hsp60, as well as Hsp70, have been also observed during human papillomavirus (HPV)-induced early cervical carcinogenesis (Cappello et al. 2002–2003; Castle et al. 2005), suggestive of stress responses to both the viral infection and an increased activation of the stress response throughout the progression of infection to precancer lesions (Castle et al. 2005). The upregulation of HSP expression also appeared to be correlated with the severity of cervical precancer lesions (Castle et al. 2005), and a further increase of Hsp60 expression was observed from preinvasive lesions to cancer (Cappello et al. 2002–2003). As far as Hsp70 is concerned, it has been also hypothesised that destabilisation of tumour suppressor protein p53 by oncogenic HPV E6 expression may result in upregulation of this Hsp (Castle et al. 2005), since p53 can repress transcription of the *hsp70* gene through the inhibition of CBF/HSP70, a transcription factor binding to the CCAAT box on the *hsp70* promoter (Agoff et al. 1993; Chae et al. 2005). Mutation of the *p53* gene may also reverse this effect with consequent transactivation of the *hsp70* promoter (Tsutsumi-Ishii et al. 1995). In addition, members of HSP70 family are involved in the regulation of p53 function in both normal and cancer cells (Zylicz et al. 2001), and high Hsp72 immunolabelling has been detected in HPV 16/18-induced anogenital skin lesions and squamous cell carcinomas, where altered p53 expression was expected to be present (Quenneville et al. 2002). In the present study, the increased Hsp72 immunostaining detectable in premalignant and malignant lesions could be in agreement with the aforementioned literature data, and even though p53 expression has not been so far investigated in bovine urothelial tumours, a strong nuclear p53 reaction, suggestive of gene

mutation or mechanisms influencing its stability, has been observed both in muscle-invasive urinary bladder hemangiosarcomas and in the dysplastic urothelium covering the endothelial tumours obtained from cows with enzootic haematuria (Carvalho et al. 2009).

In summary, the current study demonstrates the characteristic patterns of immunosignal of several HSPs in normal bovine urinary bladder, which appear to be altered during the process of urothelial carcinogenesis. Reduction of Hsp27 immunolabelling resulted to be significantly associated with increasing histological grade of malignancy, whereas increased immunoreactivity of Hsp60 and Hsp72 in both premalignant and malignant lesions suggests an early involvement of these proteins in neoplastic transformation of urinary bladder mucosa. Even though such results appear to be rather different from those achieved on the human counterpart, they seem to more closely reflect the data obtained from other studies carried out in humans concerning papillomavirus-induced tumorigenesis.

Acknowledgments We thank Fausto Cavalieri for sampling bovine urinary bladders and providing technical assistance.

References

- Agoff SN, Hou J, Linzer DI, Wu B (1993) Regulation of the human hsp70 promoter by p53. *Science* 259:84–87
- Ambrosio V, Borzacchiello G, Bruno F, Galati P, Roperto F (2001) Uroplakin expression in urothelial tumors in cows. *Vet Pathol* 38:657–660
- Borzacchiello G, Roperto F (2008) Bovine papillomaviruses, papillomas and cancer in cattle. *Vet Res* 39:45–63
- Borzacchiello G, Ambrosio V, Galati P, Poggiali F, Venuti A, Roperto F (2001) The pagetoid variant of urothelial carcinoma in situ of urinary bladder in a cow. *Vet Pathol* 38:113–116
- Borzacchiello G, Ambrosio V, Perillo A, Galati P, Roperto F (2003a) Cyclooxygenase-1 and -2 expression in urothelial carcinomas of urinary bladder in cows. *Vet Pathol* 40:455–459
- Borzacchiello G, Iovane G, Marcante ML, Poggiali F, Roperto F, Roperto S, Venuti A (2003b) Presence of bovine papillomavirus type 2 DNA and expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows. *J Gen Virol* 84:2921–2926
- Borzacchiello G, Ambrosio V, Leonardi L, Fruci R, Galati P, Roperto F (2004) Rare tumours in domestic animals: a lipid variant of urothelial carcinoma of the urinary bladder in a cow and a case of vesical carcinosarcoma in a dog. *Vet Res Commun* 28:273–274
- Brun R, Urraro C, Medaglia C, Russo V, Borzacchiello G, Roperto F, Roperto S (2008) Lymphoepithelioma-like carcinoma of the urinary bladder in a cow associated with bovine papillomavirus type-2. *J Comp Pathol* 139:121–125
- Calderwood SK, Khaleque MA, Sawyer DB, Ciocca DR (2006) Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem Sci* 31:164–172
- Campo MS, Jarrett WF, Barron R, O'Neil BW, Smith KT (1992) Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. *Cancer Res* 52:6898–6904
- Cappello F, Bellafiore M, Palma A, Marciano V, Martorana G, Belfiore P, Martorana A, Farina F, Zummo G, Bucchieri F (2002–2003) Expression of 60-kD heat shock protein increases during carcinogenesis in the uterine exocervix. *Pathobiology*, 70:83–88
- Cappello F, David S, Ardizzone N, Rappa F, Marasà L, Bucchieri F, Zummo G (2006) Expression of heat shock proteins HSP10, HSP27, HSP60, HSP70, and HSP90 in urothelial carcinoma of urinary bladder. *J Cancer Mol* 2:73–77
- Cardillo MR, Sale P, Di Silverio F (2000) Heat shock protein-90, IL-6 and IL-10 in bladder cancer. *Anticancer Res* 20:4579–4583
- Carvalho T, Pinto C, Peleteiro MC (2006) Urinary bladder lesions in bovine enzootic haematuria. *J Comp Pathol* 134:336–346
- Carvalho T, Naydan D, Nunes T, Pinto C, Peleteiro MC (2009) Immunohistochemical evaluation of vascular urinary bladder tumors from cows with enzootic haematuria. *Vet Pathol* 46:211–221
- Castle PE, Ashfaq R, Ansari F, Muller CY (2005) Immunohistochemical evaluation of heat shock proteins in normal and preinvasive lesions of the cervix. *Cancer Lett* 229:245–252
- Chae HD, Yun J, Shi DY (2005) Transcription repression of a CCAAT-binding transcription factor CBF/HSP70 by p53. *Exp Mol Med* 37:488–491
- Ciocca DR, Calderwood SK (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress Chaperones* 10:86–103
- EL-Meghawry EL-Kenawy A, El-Kott AF, Hasan MS (2008) Heat shock protein expression independently predicts survival outcome in schistosomiasis-associated urinary bladder cancer. *Int J Biol Markers* 23:214–218
- Epstein JL, Amin MB, Reuter VR (2004) Bladder biopsy interpretation. Lippincott Williams & Wilkins, Philadelphia
- Fine SW, Humphrey PA, Dehner LP, Amin MB, Epstein JI (2005) Urothelial neoplasms in patients 20 years or younger: a clinicopathological analysis using the World Health Organization 2004 bladder consensus classification. *J Urol* 174:1976–1980
- Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, Cheetham ME, Chen B, Hightower LE (2009) Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones* 14:105–111
- Karapanagiotou EM, Syrigos K, Saif MW (2009) Heat shock protein inhibitors and vaccines as new agents in cancer treatment. *Expert Opin Investig Drugs* 18:161–174
- Kumaraguruparan R, Karunakaran D, Balachandran C, Manohar BM, Nagini S (2006) Of humans and canines: a comparative evaluation of heat shock and apoptosis-associated proteins in mammary tumors. *Clin Chim Acta* 365:168–176
- Lebret T, Watson RW, Fitzpatrick JM (2003a) Heat shock proteins: their role in urological tumors. *J Urol* 169:338–346
- Lebret T, Watson RW, Molinié V, O'Neill A, Gabriel C, Fitzpatrick JM, Botto H (2003b) Heat shock proteins HSP27, HSP60, HSP70, and HSP90: expression in bladder carcinoma. *Cancer* 98:970–977
- Lebret T, Watson RW, Molinié V, Poulain JE, O'Neill A, Fitzpatrick JM, Botto H (2007) Hsp90 expression: a new predictive factor for BCG response in stage Ta-T1 grade 3 bladder tumours. *Eur Urol* 51:161–166
- Lopez-Beltran A, Montironi R (2004) Non-invasive urothelial neoplasms: according to the most recent WHO classification. *Europ Urol* 46:170–176
- Lopez-Beltran A, Sauter G, Gasser T, Hartmann A, Schmitz-Dräger BJ, Helpap B, Ayala AG, Tamboli P, Knowles MA, Sidransky D, Cordon-Cardo C, Jones PA, Cairns P, Simon R, Amin MB, Tyczynski JE (2004) Infiltrating urothelial carcinoma. In: Eble JN, Sauter G, Epstein JI, Sesterhenn IA (eds) World Health Organization classification of tumours. Pathology and genetics of tumours of the urinary system and male genital organs. IARC, Lyon, pp 93–97
- Meuten DJ (2002) Tumors of the urinary system. In: Meuten DJ (ed) Tumors in domestic animals, 4th edn. Iowa State Press, Ames, pp 509–546

- Montironi R, Lopez-Beltran A (2005) The 2004 WHO classification of bladder tumors: a summary and commentary. *Int J Surg Pathol* 13:143–153
- Quenneville LA, Trotter MJ, Maeda T, Tron VA (2002) p53-dependent regulation of heat shock protein 72. *Br J Dermatol* 146:786–791
- Reuter VE (2004) Non-invasive papillary urothelial carcinoma, high grade. In: Eble JN, Sauter G, Epstein JI, Sesterhenn IA (eds) *World Health Organization classification of tumours. Pathology and genetics of tumours of the urinary system and male genital organs*. IARC Press, Lyon, pp 117–118
- Romanucci M, Marinelli A, Sarli G, Della Salda L (2006) Heat shock proteins expression in canine malignant mammary tumours. *BMC Cancer* 6:171
- Romanucci M, Bastow T, Della Salda L (2008) Heat shock proteins in animal neoplasms and human tumours—a comparison. *Cell Stress Chaperones* 13:253–262
- Roperto S, Ambrosio G, Borzacchiello G, Galati P, Paciello O, Russo V, Roperto F (2005) Bovine papillomavirus type-2 (BPV-2) infection and expression of uroplakin IIIb, a novel urothelial marker, in urinary bladder tumors of cows. *Vet Pathol* 42:812–818
- Roperto S, Borzacchiello G, Casellato R, Galati P, Russo V, Sonnino S, Roperto F (2007) Sialic acid and GM3 ganglioside expression in papillomavirus-associated urinary bladder tumours of cattle with chronic enzootic haematuria. *J Comp Pathol* 137:87–93
- Roperto S, Brun R, Paolini F, Urraro C, Russo V, Borzacchiello G, Pagnini U, Raso C, Rizzo C, Roperto F, Venuti A (2008) Detection of bovine papillomavirus type 2 (BPV-2) in the peripheral blood of cattle with urinary bladder tumours: possible biological role. *J Gen Virol* 89:3027–3033
- Roperto S, Borzacchiello G, Brun R, Leonardi L, Maiolino P, Martano M, Paciello O, Papparella S, Restucci B, Russo V, Salvatore G, Urraro C, Roperto F (2009) A review of bovine urothelial tumours and tumour-like lesions of the urinary bladder. *J Comp Pathol* 142:95–108
- Storm FK, Mahvi DM, Gilchrist KW (1993) Hsp-27 has no diagnostic or prognostic significance in prostate or bladder cancers. *Urology* 42:379–382
- Syrgios KN, Harrington KJ, Karayiannakis AJ, Sekara E, Chatziyianni E, Syrigou EI, Waxman J (2003) Clinical significance of heat shock protein-70 expression in bladder cancer. *Urology* 61:677–680
- Tsutsui-Ishii Y, Tadokoro K, Hanaoka F, Tsuchida N (1995) Response of heat shock element within the human hsp70 promoter to mutated p53 genes. *Cell Growth Differ* 6:1–8
- Urushibara M, Kageyama Y, Akashi T, Otsuka Y, Takizawa T, Koike M, Kihara K (2007) Hsp60 may predict good pathological response to neoadjuvant chemoradiotherapy in bladder cancer. *Jpn J Clin Oncol* 37:56–61
- Venuti A, Paolini F, Nasir L, Corteggio A, Roperto S, Campo MS, Borzacchiello G (2011) Papillomavirus E5: the smallest oncoprotein with many functions. *Mol Cancer* 10:140
- Zylicz M, King FW, Wawrzynow A (2001) Hsp70 interactions with the p53 tumour suppressor protein. *EMBO J* 20:4634–4638