

# Expression of Heat Shock Proteins HSP10, HSP27, HSP60, HSP70, and HSP90 in Urothelial Carcinoma of Urinary Bladder

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**AIM:** Heat shock proteins (HSPs) have essential roles in a number of pathophysiological conditions including carcinogenesis and represent a group of novel molecular markers in cancer management. The aim of this study is to explore the expression status of HSPs in bladder urothelial carcinoma (BUC) patients.

**METHODS:** The immunohistochemical staining of HSP10, HSP27, HSP60, HSP70, and HSP90 were done in 46 vesical BUC patients with different grades (G) and stages (T). Statistical analyses were performed to determine whether there was any correlation between tumoral HSP expression and both G and T of tumors.

**RESULTS:** We found a significant correlation between high grading ( $G \geq 2$ ) and tumor tissues positive for HSP10 and HSP90 staining ( $P < 0.001$ ). Moreover, the positive immunostaining of HSP70 was significantly linked with G3 tumors ( $P < 0.001$ ), but HSP27 or HSP60 expression was not related with the G level of tumor. There was a significant correlation between higher T stages ( $T > 1$ ) and tumor tissues expressing HSP70 ( $P < 0.001$ ); in contrast, the number of tumor cells having HSP60 expression was significantly decreased in  $T > 1$  tumors ( $P < 0.005$ ). No relationship was found between tumoral T status and HSP10, HSP27 and HSP90 expression. Finally, we found a significant correlation between the high-graded ( $G \geq 2$ ) neoplasms and the percentage of tumoral cells positive for HSP10, HSP27, HSP70, or HSP90 ( $P < 0.005$ ).

**CONCLUSION:** This is the first report to show the presence of HSP10 in bladder cancer tissues, with its expression correlated to the tumoral grading. These data may also be valuable for developing new molecular anti-cancer therapies.

## Keywords:

bladder cancer  
carcinogenesis  
clinical outcome  
molecular markers

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## Introduction

Urothelial carcinoma of bladder (BUC<sup>2</sup>) is the fifth most common malignancy in Western countries [1]. It accounts for 5% of all diagnosed cancers and the peak of incidence is between the fifth and seventh decades. The medical assessment of BUC includes clinical and histological diagnoses with the grading (G) and staging (T) of primary tumour by the pathological materials obtained through endoscopy and/or bladder resection [2,3]. The therapeutic approach for individual patients is currently guided by histology-based prognostic factors, although more recently, a number of experimental and clinical studies are exploring novel molecular predictors.

### The significance of the researches of heat shock proteins

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<sup>2</sup>Abbreviations: BUC, urothelial carcinoma of bladder; T, tumor stage; G, tumor grade; HSP, heat shock protein.

(HSPs) in clinical management of tumoral patients is now expanding. These molecules play a major role in protecting cells against damage in stress conditions, but they also have essential roles under a number of physiological conditions [4]. Experimental data suggest that they may suppress apoptosis and thus promote tumorigenesis [5]. Nevertheless, some HSPs may have a pro-apoptotic action as well as play important roles in the immune response against cancer [6].

HSPs are a family of highly conserved proteins, firstly described over 40 years ago [7]. HSP10, HSP60 and HSP90 are constitutively expressed in mammalian cells, while HSP27 and HSP70 are induced by several stresses [5]. HSPs have different functions inside normal cells, such as prevention of the aggregation of denatured polypeptides [8], interorganelar transport [5], and antigen processing and presentation [9]. Moreover, most of them have been proposed to regulate apoptosis. For example, HSP27, HSP70 and HSP90 were considered as antiapoptotic, since they were able to bind to some pro-apoptotic molecules, including cytochrome *c* and Apaf [10]. In contrast, HSP10 and HSP60 were suggested as

proapoptotic [11], although the exact role(s) of the latter has still not been fully elucidated [12].

The clinical significance of HSP expression in bladder cancers has not been entirely investigated yet. Storm *et al.* were the first to examine the diagnostic and prognostic significance of HSP27 expression, finding that it did not provide a predictive biologic marker in some genitourinary tumors, among them bladder cancer [13]. More recently, Syrigos *et al.* have shown that HSP70 was frequently overexpressed in vesical tumoral cells and it could be a useful biochemical marker [14]. Leuret *et al.* have demonstrated that high levels of HSP90 and a low expression of HSP60 might have a prognostic relevance in patients with bladder carcinoma, having HSP60 a practical use as a prognostic marker to identify patients for whom local treatment would be insufficient [15].

In the present study, we investigate the expression of five HSPs, specifically HSP10, HSP27, HSP60, HSP70, and HSP90, in a series of bladder BUC with different G and T. Our data partially agree with previous results, some other is new.

## Materials and Methods

### Specimen collection

We collected biopsies from 46 consecutive BUC patients of our pathological archives during a period of 24 months. All tumors were primary. The G and T of these cases were determined according to the World Health Organization/International Society of Urological Pathology (WHO/ISUP) guidelines [16] and are shown in Table 1. Although eight patients with invasive disease subsequently underwent a cystectomy in the same hospital, we used the first removed biopsies for our experiments; we obtained follow-up data at one-year from only 3 out of 8 cases and these patients were free of local recurrence. We have not had any follow-up data from the other patients. All the tissues were formalin-fixed and paraffin-embedded for pathologic diagnoses. Moreover, we collected 10 samples of normal vesical mucosa from our anatomical archives. The latter were Bouin-fixed and paraffin-embedded. We obtained sections with a thickness of 4-5  $\mu$ m from all specimens for immunohistochemistry.

### Immunohistochemistry

Immunohistochemistry (IHC) was performed by a streptavidin-biotin complex method using LSAB2 kit (DAKO Co., Carpinteria, CA, USA). The used primary antibodies included anti-HSP10 (dilution 1:400, Cat. No. SPA-110, StressGen Biotechnologies Co., Victoria, BC, Canada), HSP27 (dilution 1:200, Cat. No. SC-13132, Santa Cruz Biotechnology, Santa Cruz, CA, USA), HSP60 (dilution 1:400, Cat. No. H4149, Sigma Co., Saint Louis, MI, USA), HSP70 (dilution 1:200, Cat. No. SC-24, Santa Cruz Biotechnology) and HSP90 (dilution 1:200, Cat. No. SC-13119, Santa Cruz Biotechnology). After deparaffination and rehydration, the sections were incubated for 10 min with protein blocking agent (DAKO). Subsequently, the primary antibodies were added to the sections. Non-immune rabbit serum was used for negative controls, and appropriate positive controls were done simultaneously.

Table 1: Tumoral stages and grades of studied BUC patients, determined according to the WHO/ISUP classification

	Ta/Tis*	T1	> T1	Total
G1	12	8	4	24
G2	6	6	2	14
G3	2	4	2	8
Total	20	18	8	46

\*15 of them were Ta and 5 were Tis.

Diaminobenzidine was used as chromogen and hematoxylin aqueous formula was used as counterstaining. The IHC results were assessed by two independent observers (F. Cappello and F. Rappa).

### Quantitative analysis of immunohistochemistry

The tissue expression of each HSP was quantified by a computer-assisted image analysis system Colourvision 1.7.6 (Improvision, Coventry, UK). For each biopsy, the tissue was systematically assessed, in two non-serial sections, on basis of red, green and blue (RGB) colour balance. In the beginning of each session, the image analysis system was standardised using the same section of tissue stained for the HSP to ensure reproducibility of analysis. The digitised image of the standard section was used to interactively sample an example of the positive staining and the system was then allowed to select all the pixels of the same RGB colour balance (i.e., positive staining) within the image. The area of the examined tissue was then delineated interactively and the percentage of positive staining within the tissue was determined; the colour balance and perceptual staining value was recorded for future sessions. In the beginning of each subsequent session, the image analyser was calibrated using this section and adjusted to within  $\pm 5\%$  of the original pixel reading. Once the system had been calibrated using the 'standard' slide, the test sections were analysed, using the same parameters. Each tissue section was analysed on two separate occasions by two independent observers (F. Cappello and F. Rappa), and mean values of the duplicate observation data were analysed using Mann Whitney U test. A value of  $P < 0.05$  was considered significant.

### Statistical analyses

We analysed the significance of the data using the Student *t* test ( $P < 0.05$ ). A one-way analysis of variance was used to determine the correlation between: (a) tumoral expression of HSPs and tumoral G, (b) tumoral expression of HSPs and tumoral T, and (c) the number of HSPs-expressing tumoral cells in each tumor and tumoral G.

## Results

### Expression of HSPs in normal uroepithelium and BUC

We found a variable number of tumors positive for each HSP (Figure 1 and Table 2). Most of the cases were positive for HSP27 (91.3%), while HSP60 and HSP70 (21.7%) resulted to have the smallest amount of expression. In particular, all HSPs were present at the cytoplasmic level in tumor cells of BUC with different grades. Interstitial stroma was commonly negative. Additionally, a variable number of normal vesical mucosa expressed HSPs in the cytoplasm above all in the superficial layers of uroepithelium.

### Correlation between expression of HSPs and malignancy of BUC

We researched a correlation between tumor HSP expression and tumoral G, and we obtained a significant correlation between highly graded ( $G \geq 2$ ) neoplasms and tumoral HSP10 and HSP90 expression ( $P < 0.001$ , Table 3). Moreover, the presence of HSP70 expression was significantly linked to tumors with  $G = 3$  ( $P < 0.001$ , Table 3). In contrast, the BUC tissues positive for HSP27 or HSP60 staining did not show any relationship with G (Table 3).

On the other hand, the expression of HSP60 was significantly decreased in BUC tissues with  $T > 1$  ( $P < 0.005$ , Table 4). Indeed, in low-staged tumors, there were more cases expressing HSP60 than in high-staged ones. In addition, a significant correlation was found between high T tumors ( $T > 1$ ) and HSP70 expression ( $P < 0.001$ , Table 4). No correlation

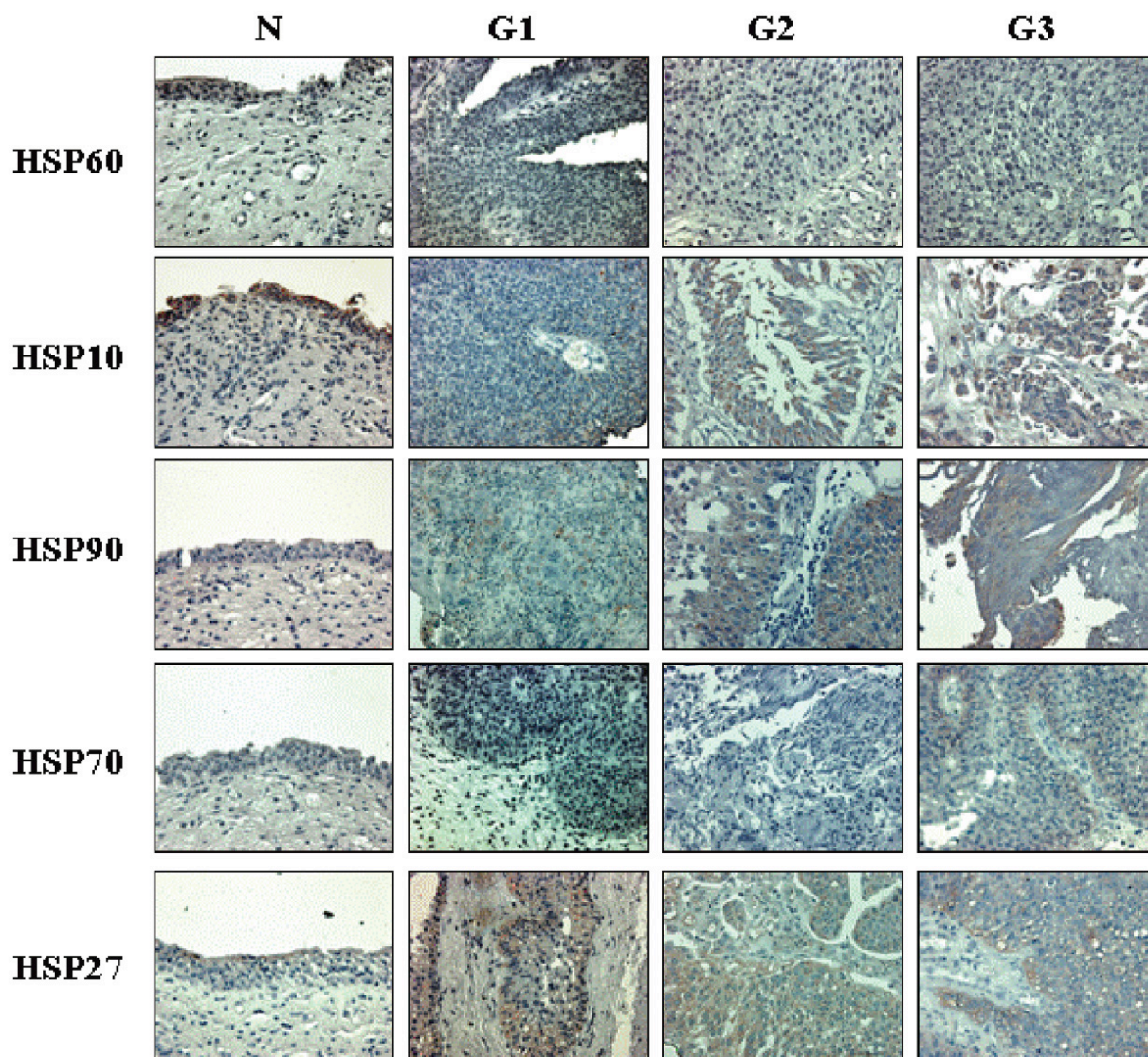


Figure1: Panel of representative pictures of the IHC results of HSPs. The number of tumor tissues positive for each HSP was variable. Positive immunostaining was noted at the cytoplasm of tumor cells. And also a variable number of normal specimens expressed HSPs in cytoplasm, above all in the superficial layers. N: normal urothelium; G1, G2 and G3: BUC with different grades of differentiation. Original magnification: 40 x.

Table 2: Number and percentage of the normal bladder and BUC specimens with IHC positive for HSPs

	HSP10	HSP27	HSP60	HSP70	HSP90
Normal bladder	5/10 (50%)	10/10 (100%)	3/10 (30%)	3/10 (30%)	7/10 (70%)
BUC	32/46 (69.6%)	42/46 (91.3%)	10/46 (21.7%)	10/46 (21.7%)	34/46 (73.9%)

Table 3: Relationships between tumoral expression of HSPs and tumoral grading (G) in BUC

	HSP10	HSP27	HSP60	HSP70	HSP90
N	5/10 (50%)	10/10 (100%)	3/10 (30%)	3/10 (30%)	7/10 (70%)
G1	10/24 (41.7%)	20/24 (83.3%)	4/24 (16.7%)	2/24 (8.3%)	12/24 (50%)
G2	14/14 (100%) <sup>a</sup>	14/14 (100%)	4/14 (28.6%)	2/14 (14.3%)	14/14 (100%) <sup>a</sup>
G3	8/8 (100%) <sup>a</sup>	8/8 (100%)	2/8 (25%)	6/8 (75%) <sup>b</sup>	8/8 (100%) <sup>a</sup>

<sup>a</sup> $P < 0.001$  if  $G \geq 2$  vs.  $G = 1$ .

<sup>b</sup> $P < 0.001$  if  $G = 3$  vs.  $G \leq 2$ .

**Table 4: Relationships between tumoral expression of HSPs and tumoral staging (T) in BUC**

	HSP10	HSP27	HSP60	HSP70	HSP90
N	5/10 (50%)	10/10 (100%)	3/10 (30%)	3/10 (30%)	7/10 (70%)
Ta/Tis	14/20 (70%)	18/20 (90%)	6/20 (30%)	2/20 (10%)	14/20 (70%)
T1	12/18 (66.7%)	16/18 (88.9%)	4/18 (22.2%)	2/18 (11.1%)	14/18 (77.8%)
> T1	6/8 (75%)	8/8 (100%)	0/8 (0%) <sup>a</sup>	6/8 (75%) <sup>b</sup>	6/8 (75%)

<sup>a</sup> $P < 0.005$  if  $T > 1$  vs.  $T \leq 1$ .<sup>b</sup> $P < 0.001$  if  $T > 1$  vs.  $T \leq 1$ .**Table 5: Relationships between the grading (G) of BUC and the ratio of HSP-positive tumor cells in BUC tissues**

	HSP10	HSP27	HSP60	HSP70	HSP90
N	5% (0-10%)	10% (0-20%)	1% (0-2%)	5% (0-10%)	10% (0-20%)
G1	15% (0-20%)	45% (20-90%)	1% (0-2%)	15% (0-25%)	20% (0-30%)
G2	85% (60-95%) <sup>a</sup>	90% (75-100%) <sup>a</sup>	1% (0-3%)	80% (60-95%) <sup>a</sup>	90% (60-100%) <sup>a</sup>
G3	95% (65-100%) <sup>a</sup>	95% (80-100%) <sup>a</sup>	1% (0-2%)	90% (60-100%) <sup>a</sup>	95% (80-100%) <sup>a</sup>

<sup>a</sup> $P < 0.005$  if  $G \geq 2$  vs.  $G = 1$ .

between the expression of HSP10, HSP27 or HSP90 and T levels of BUC was noted.

#### Correlation between HSP-positive tumor cell number and BUC grading

We analysed if the existence of a statistical correlation between the tumoral G and the number of tumor cells positive for each HSP immunostaining. We found a significant association of the high grades ( $G \geq 2$ ) of BUC with the percentage of tumor cells positive for HSP10, HSP27, HSP70, and HSP90, but not for HSP60 ( $P < 0.005$ , Table 5).

#### Discussion

The study of the levels of HSPs in several tumours is a topic of growing interest. An increased expression of HSP27 has been reported in a number of tumours, such as breast carcinoma [17], endometrial cancer [18] and leukemia [19]. High levels of HSP70 have been detected in osteosarcoma [20] and renal tumor cells [21]. In addition, HSP90 has been shown to be overexpressed in lung cancer [22] and breast carcinoma [23]. In recent studies, we researched the expression status of HSP60 and HSP10 during exocervical [24,25], colonic [25,26] and prostatic carcinogenesis [27], discovering that their expression increased from normal through dysplastic to neoplastic tissues. Other studies confirmed our results [28,29]. Commonly, high expression of HSPs has been associated with cancer metastases, poor prognoses and resistance to chemotherapy and radiation therapy [30-33].

At the present time, only few researches have been focusing on HSP expression in BUC. The first of these papers focused on the diagnostic and prognostic significance of HSP27 in a series of vesical (as well as prostatic) cancers [13]. The authors did not find any correlation between the expression of HSP27 and (i) the degree of histologic differentiation, (ii) the T stage, (iii) lymph node involvement, (iv) local tumor recurrence, (v) tumor metastases, and (vi) survival, and therefore concluded that tumor HSP27 overexpression had neither a diagnostic nor prognostic significance. More recently, another study investigated the expression of HSP90 (as well as IL-6 and IL-10) in a series of superficial and invasive bladder cancers [34]. Interestingly, it was found that HSP90 was expressed in 92.9% of examined tumors; moreover, the invasive samples contained significantly higher levels of HSP90 than the superficial ones.

The authors concluded that this protein could have independent functional roles, also representing an immunogenic target for antitumoral response.

The above described results were confirmed by Leuret *et al.* who studied the presence and expression of HSP27, HSP60, HSP70 and HSP90 in a wide series of bladder malignancies [15]. In particular, they looked for the existence of a correlation between HSP expression and tumoral T and G, other than prognosis. Although they did not find any correlation of the expression of HSP27 and HSP60 with tumoral T, the HSP60 and HSP90 levels were found to associate with the outcome of the patients with superficial bladder carcinoma. They stated that loss of HSP60 expression, together with persistent HSP90 overexpression, might have a prognostic relevance in BUC management. Finally, Syrigos *et al.* researched the expression of HSP70 in a series of BUC, finding that this molecule was frequently overexpressed in advanced grades of bladder cancer and suggesting its role as biochemical marker [14].

In the present work, we studied the expression of five HSPs in a series of BUC tissues. We have shown that HSP10 but not HSP60 increased from normal through dysplastic to neoplastic specimens. Down-regulation of HSP60 in bladder carcinogenesis was a novel but not utterly original result. It was reported that 39 out of 42 (> 90%) urothelial cancer tissues exhibited HSP60 expression from a mean of 77% of cancer cells [15], whereas in our series we found that only ~20% of BUC patients had HSP60 expression from < 2% of cancers cells (Table 2). This discordance could be due to the biological variability of the tumours present in different series. Nevertheless, in agreement with Leuret [15], we observed that HSP60 expression significantly decreased in the tumors with  $T > 1$  and we consider the latter as the most notable data. Recently, we were the first to notice a similar down-regulation of HSP60 level during bronchial carcinogenesis. HSP60 was present and expressed by basal and columnar elements of normal and hyperplastic bronchial mucosae, while gradually disappeared ongoing towards airway metaplasia, dysplasia and cancer [35]. We supposed that the HSP60 levels during carcinogenesis may depend on the expression of other proteins probably involved in the pro-apoptotic pathways [36], but we are still far from recognizing the underlying molecular mechanisms.

We presently also detected a small number of cases positive for HSP70. This HSP70 expression was significantly associated with the neoplasms with a higher T status ( $T > 1$ ),

in accordance with the results obtained by Syrigos and colleagues [14]. In addition, we indicated the presence of a correlation between the tumoral G and the number of tumour cells positive for four HSPs. The significant correlation was demonstrated between the high-grading ( $G \geq 2$ ) neoplasms and the ratio of tumour cells positive not only for HSP70 and HSP90, as already reported [14,15,34], but also for HSP10 and HSP27 (Table 5).

Noteworthy, the obvious IHC result for HSP10, as also demonstrated elsewhere [25,27,29], suggest that this protein may have a diagnostic and prognostic marker role in BUC management.

In conclusion, our present data confirm that tumoral HSP90 and HSP70 expression is correlated with tumoral T status as already shown by Lebret [15] and Syrigos [14], while HSP60 expression is inversely correlated with tumoral T in accordance with Lebret [15]. We showed a correlation between the tumoral G and the number of tumour cells positive for HSP27; nevertheless, we did not find any correlation between the ratio of HSP27-positive tumor cells and the tumor status with both high G and high T. Since this result was not in agreement with the study of Storm [13], perhaps due to the phenotypic variability of the tumors, our suggestion is to reconsider this datum in a wider series. The present report is the first paper to study the HSP10 expression in bladder carcinoma tissues. It would be interesting to consider the value of this tumoral HSP10 expression in the clinical follow-up of BUC, but we have not yet these data. We also hypothesise that the mitochondrial chaperones HSP60 and HSP10, which are functionally related in normal cells, have distinct roles in BUC pathogenesis because of their different expression status in BUC tissues.

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