HSP60 may Predict Good Pathological Response to Neoadjuvant Chemoradiotherapy in Bladder Cancer

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Background: Heat shock proteins (HSPs) play crucial roles in cellular responses to stressful conditions. Expression of HSPs in invasive or high-risk superficial bladder cancer was investigated to identify whether HSPs predict pathological response to neoadjuvant chemoradiotherapy (CRT).

Methods: Immunohistochemistry was used to assess expression levels of HSP27, HSP60, HSP70, HSP90 and p53 in 54 patients with invasive or high-risk superficial bladder cancer, prior to low-dose neoadjuvant CRT, followed by radical or partial cystectomy. Patients were classified into two groups (good or poor responders) depending on pathological response to CRT, which was defined as the proportion of morphological therapeutic changes in surgical specimens. Good responders showed morphological therapeutic changes in two-thirds or more of tumor tissues. In contrast, poor responders showed changes in less than two-thirds of tumor tissues.

Results: Using a multivariate analysis, positive HSP60 expression prior to CRT was found to be marginally associated with good pathological response to CRT ($P = 0.0564$). None of clinicopathological factors was associated with HSP60 expression level. In the good pathological responders, the 5-year cause-specific survival was 88%, which was significantly better than survival in the poor responders (51%) ($P = 0.0373$).

Conclusions: Positive HSP60 expression prior to CRT may predict good pathological response to low-dose neoadjuvant CRT in invasive or high-risk superficial bladder cancer.

Key words: chemo-urology – patho-molecular – prognostic factors – urologic-radiology – urologic-surgery

INTRODUCTION

Radical cystectomy is the gold standard treatment for muscle invasive bladder cancer. Although prognosis after surgery has improved, many patients still develop distant metastasis and finally die of the disease. However, bladder urothelial carcinomas are fairly sensitive to chemotherapy or radiotherapy, and recent reports suggest that neoadjuvant chemotherapy showed modest survival benefits (1). In addition, preservation of the bladder is possible in selected cases, with complete remission to pre-operative chemotherapy and/or radiotherapy (2,3). We previously performed low-dose neoadjuvant CRT to treat invasive or high-risk superficial bladder cancers, especially in elderly or high-risk patients, and obtained excellent outcomes (4). However, pre-operative treatments involve risks of possible delays in curative operation if tumors are not sensitive enough to CRT.

Exposure of cells to environmental stresses including heat shock, oxidative stress, heavy metals, or pathological conditions induces expression of heat shock proteins (HSPs) that function as molecular chaperones or proteases (5). Consequently, HSPs assist in recovery from stress either by repairing damaged proteins (protein refolding) or by degrading them, thus restoring protein homeostasis and promoting cell survival. Among heat shock proteins, HSP27, HSP60, HSP70 and HSP90 have been well investigated in clinical settings using samples of human cancers. HSP27 is associated with cellular differentiation including that of cancer cells (6). HSP60 is a mitochondrial protein involved in...
activation of apoptosis (7). HSP70 and HSP90 are known to act as anti-apoptotic factors (8,9). During carcinogenesis, HSPs change their expression levels, which can either increase or decrease (10). Recently, expression of HSP27, HSP60, HSP70 or HSP90 was demonstrated in human bladder cancers (11–13).

An association between expression of HSPs and response to chemotherapy and/or prognosis has been reported in different types of human malignancies. If pathological response to CRT was predicted by expression of HSPs, unnecessary treatments could be avoided in cases insensitive to CRT. Here, we retrospectively evaluated expression of heat shock proteins using biopsy samples from patients with invasive or high-risk superficial bladder cancer, prior to CRT; and investigated a possible association between HSP expression and pathological response to treatment.

MATERIALS AND METHODS

PATIENTS AND TREATMENT

The study population consisted of 54 patients with clinically invasive (T2-4N0M0) or high-risk superficial (T1 and G3) bladder cancer, who had undergone neoadjuvant low-dose CRT followed by cystectomy between March 1997 and December 2004. Patients were excluded if they could not be followed. Pathological diagnosis was established by transurethral cold biopsy prior to CRT. Clinical T stage was determined based on findings from biopsies and CT scans, according to the 1997 International Union Against Cancer classification (14). Histological grade was determined according to the classification by the Japanese Urological Association and the Japanese Society of Pathology (15).

Clinicopathological characteristics of patients are shown in Table 1. Median patient age was 68.5 years old (range, 45–83 years old). Thirty-five patients were male and 19 were female. Eighty per cent of tumors were classified as Grade 3. Clinical stages of the disease in patients were as follows: T1 in nine, T2 in 32, T3 in 11 and T4 in two patients.

All patients underwent pre-operative low-dose radiotherapy with concurrent low-dose cisplatin (CDDP). Pelvic irradiation was performed using a dose of 40 Gy over 4 to 5 weeks. Systemic (20 mg/body for 5 days) or intra-arterial (100 mg/body) administration of cisplatin was performed during weeks 1 and 4 of radiotherapy. Cisplatin dose was reduced depending on patient’s renal function or general condition.

Clinical response was evaluated by urinary cytology, CT scans and cystoscopy 1 month after CRT and classified as follows (15): complete response (CR), no visible tumor or negative biopsy and negative urinary cytology; partial response (PR), 50% or more reduction in tumor volume regardless of urinary cytology; no change (NC), less than 50% decrease or less than 25% increase in tumor volume regardless of urinary cytology. Radical cystectomy was performed in 43 cases. In 11 cases with advanced age or high risks, the bladder was preserved by partial cystectomy where the tumor was solitary and without carcinoma in situ.

Pathological responses to CRT were classified according to therapeutic changes including degree of necrosis or granulation or degenerative change or residual tumor volume (15): Ef. 3, all tumor tissues showed changes; Ef. 2, two-thirds or more but not all of tumor tissues changed; Ef. 1b, one-third or more but less than two-thirds of tumor tissues changed; Ef. 1a, less than a third of tumor tissues changed; Ef. 0, no apparent morphological therapeutic changes were observed. In this study, numbers of patients in each category were as follows: Ef. 3, n = 20; Ef. 2, n = 13; Ef. 1 (a and b), n = 18; Ef. 0, n = 3. Patients were further classified into two groups: good (Ef. 3 or 2) and poor responders (Ef. 1 or 0). Therefore, 33 patients were good responders and 21 patients were poor responders.

IMMUNOHISTOCHEMISTRY

Expressions of HSP27, HSP60, HSP70, HSP90 and p53 were analyzed by immunohistochemistry using TUR-biopsy specimens from main tumors before CRT. Primary antibodies against HSP27, HSP70, HSP90 and p53 (DO-1) were purchased from Novocastra Laboratories Ltd (Newcastle-upon-Tyne, UK). An anti-HSP60 antibody was
purchased from Chemicon International (Temecula, CA, USA). Five-μm paraffin-embedded sections were placed on poly-L-lysine-coated slides, deparaffinized in xylene and rehydrated. Sections were then incubated with 3% H₂O₂ in methanol to inhibit endogenous peroxidase. For epitope retrieval before immunostaining, slides were microwaved (550 W) for 15 min in 0.01 M citrate buffer, pH 6.0. Slides were incubated with primary antibodies in a humidity chamber for 1 h at room temperature (HSP27) or overnight at 4°C (HSP60, HSP70, HSP90 and DO-1). Dilutions for primary antibodies were: 1:20 for HSP27 and DO-1, 1:40 for HSP70, 1:50 for HSP60 and 1:200 for HSP90. Goat monoclonal antibodies were used as secondary antibodies. A standard avidin–biotin system was used for antibody localization. Sections were washed with phosphate buffered saline, before color reaction with 3,3′-diaminobenzidine-hydrogen peroxide, and counterstained with hematoxylin. Positive controls included breast cancer tissues for p53, HSP 27, HSP60 and HSP70, and endometrium tissues for HSP90.

EVALUATION OF STAINING PATTERNS

Sections were analyzed using light microscopy by two independent observers who had no knowledge of patient history or clinical behavior. For each section, all fields or at least 10 high power fields (at ×100 magnification) were randomly examined. Positive HSP expression was defined when the proportion of moderately or more intensely stained cells was ≥20% or greater. For p53 staining, 10% or greater nuclear reactivity was considered positive (16).

STATISTICAL ANALYSIS

Differences between the two groups were assessed using a χ² test or a Fisher’s exact test. Independence of variables was assessed using a logistic regression analysis. Cause-specific survival after cystectomy was analyzed using the Kaplan–Meier method and comparisons were performed using the log–rank test. Data were processed using the JMP 4.0.5J statistical software.

RESULTS

POSITIVE EXPRESSION OF HEAT SHOCK PROTEINS IN A SUBSET OF BLADDER CANCERS PRIOR TO CRT

For all HSPs, cytoplasmic staining was detected in tumor cells prior to CRT (Fig. 1). HSP expression levels varied between patients (Figs. 2 and 3). Proportions of patients with positive expression levels of each HSP were as follows: HSP27, 56%; HSP60, 30%; HSP70, 15%; and HSP90, 19%. Nuclear over-expression of p53 was observed in eight of 54 (15%) tumor samples.

HSP60 IS A POSSIBLE INDEPENDENT FACTOR OF PATHOLOGICAL RESPONSE

Associations between pathological response and pathological grade, clinical stage, or staining patterns were analyzed. Using a univariate analysis, positive expression of HSP60, HSP70 or HSP90 was found to be associated with good response to CRT (Table 2). However, using a multivariate analysis, positive expression of HSP60 was found to be a marginally independent factor (P = 0.0564) predicting good response to CRT (Table 3). Neither HSP27 nor p53 expression level was associated with response. Other factors.
including gender or age of patients were not associated with HSP60 expression.

**HSP60 Expression and Survival**

Overall survival and cause-specific survival of patients were analyzed by the Kaplan–Meier method (Figs. 4 and 5). In the good responders, the 5-year overall survival was 85%, which was significantly better than that in the poor pathological responders (46%) ($P = 0.0463$). In the good responders, the 5-year cause-specific survival was 88%, which was significantly better than that in the poor pathological responders (51%) ($P = 0.0373$). Although not statistically significant, there was a trend that survival of the positive HSP60 group was better than that of negative HSP60 group (Fig. 5). The 5-year overall survival was 89% in the positive HSP60 and 59% in the negative HSP60 ($P = 0.1599$). The 5-year cause-specific survival was 89% in the positive HSP60 and 64% in the negative HSP60 ($P = 0.2626$).

**Discussion**

Although this study involved a retrospective design, we demonstrated that pathological response to neoadjuvant CRT could be predicted by HSP60 expression in invasive or high-risk superficial bladder cancers prior to CRT. This is the first report demonstrating significance of HSP expression in clinical settings especially in CRT for the treatment of bladder cancers. In contrast to HSP70 and HSP90, which are thought to have anti-apoptotic roles, HSP60 has been suggested to act as a chaperone contributing to induction of apoptosis in tumors (17). In human esophageal cancer, apoptotic index of a tumor with positive HSP60 expression is significantly higher than that of a tumor with negative HSP60 expression. Our data further supported the pro-apoptotic nature of HSP60. Consistently, an increase in anti-HSP60 IgG level after intravesical BCG therapy may be significantly associated with a higher rate of recurrence in superficial bladder cancers (18).

**Table 2. Univariate analysis of pathological response to CRT according to clinicopathological factors**

<table>
<thead>
<tr>
<th>Pathological grade</th>
<th>Good (n = 33)</th>
<th>Poor (n = 21)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2</td>
<td>9</td>
<td>2</td>
<td>0.17</td>
</tr>
<tr>
<td>G3</td>
<td>24</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1(G3) or T2</td>
<td>26</td>
<td>14</td>
<td>0.20</td>
</tr>
<tr>
<td>T3 or T4</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(−)</td>
<td>28</td>
<td>18</td>
<td>0.93</td>
</tr>
<tr>
<td>(+)</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HSP27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(−)</td>
<td>14</td>
<td>10</td>
<td>0.71</td>
</tr>
<tr>
<td>(+)</td>
<td>19</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>HSP60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(−)</td>
<td>18</td>
<td>20</td>
<td>0.0017*</td>
</tr>
<tr>
<td>(+)</td>
<td>15</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HSP70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(−)</td>
<td>25</td>
<td>21</td>
<td>0.0172*</td>
</tr>
<tr>
<td>(+)</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HSP90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(−)</td>
<td>23</td>
<td>21</td>
<td>0.0043*</td>
</tr>
<tr>
<td>(+)</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. Multivariate analysis of pathological response to CRT according to clinicopathological factors**

<table>
<thead>
<tr>
<th>Factor</th>
<th>$P$ value</th>
<th>Odds ratio</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2 vs. G3</td>
<td>0.39</td>
<td>2.37</td>
<td>−1.09—3.02</td>
</tr>
<tr>
<td>T1(G3) or T2 vs. T3 or T4</td>
<td>0.40</td>
<td>1.95</td>
<td>−0.83—2.36</td>
</tr>
<tr>
<td>HSP60 (−) vs. (+)</td>
<td>0.0564*</td>
<td>0.10</td>
<td>−5.39 to −0.24</td>
</tr>
<tr>
<td>HSP70 (−) vs. (+)</td>
<td>0.93</td>
<td>0.01&gt;</td>
<td>−0.42</td>
</tr>
<tr>
<td>HSP90 (−) vs. (+)</td>
<td>0.93</td>
<td>0.01&gt;</td>
<td>−</td>
</tr>
</tbody>
</table>

HSP, heat shock protein; CRT, chemoradiotherapy.
*Statistically significant.

*Marginally independent.
Using our multivariate model, we showed that HSP60 was a possible independent factor for favorable pathological response. HSP60 expression level was not associated with pathological grade or clinical stage of the disease. Our data partly agreed with a previous report demonstrating that positive HSP60 expression was significantly associated with a lower tumor stage, whereas it was not significantly associated with tumor grade (12). Most of tumors analyzed in our study were invasive cancers, and this may explain discrepancies in results. In esophageal cancer, HSP60 expression level is scarcely associated with pathological grade or clinical stage of the tumor and this might further support our results (17).

We also evaluated the relationship between expression of p53 and pathological response because p53 over-expression was previously found to be associated with a mutant type, aggressive histology and poor outcomes in patients with bladder cancers (16,19). Our data suggested that p53 over-expression might not be correlated with pathological outcome. All tumors with no pathological response in our study showed p53-negative immunoreactivities, whereas lymph node metastases were seen in p53-positive tumors.

These observations agreed with recent reports showing that p53 was not significantly associated with pathological or clinical response or recurrence in CRT for bladder cancers (20,21). As expected, over-expression of p53 was not associated with HSP60 expression.

The 5-year overall and cause-specific survival of patients with good pathological response was significantly better than those of patients with a poor response as we reported previously (4). The findings are consistent with the reports demonstrating that complete remission after neoadjuvant chemotherapy and/or radiotherapy is a favorable marker for long-term survival of bladder cancer patients (2,3). There was a trend towards better survival in the HSP60 positive group although the results were not statistically significant. Based on the results of multivariate analysis, HSP60 could possibly be a predictor of survival with larger number of patients.

CONCLUSIONS

Positive HSP60 expression is a possible predictor of good pathological response to neoadjuvant CRT in invasive or high-risk bladder cancer. Because pathological response is the most reliable predictor of survival, it may be possible to identify patients most likely to benefit from neoadjuvant
CRT by evaluating HSP60 expression. This may contribute to better management of invasive or high-risk superficial bladder cancers by avoiding unnecessary side effects from chemotherapy and/or radiotherapy.

Conflict of interest statement
None declared.

References