Concomitant Overexpression of Heat-shock Protein 70 and HLA Class-I in Hepatitis C Virus-related Hepatocellular Carcinoma

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Abstract. Background: Human leukocyte antigen (HLA) class I expression is reportedly frequently reduced in cancer. We examined heat-shock protein (HSP) expression in hepatocellular carcinoma (HCC). Patients and Methods: HCV-related HCC was examined in 73 patients who had undergone hepatectomy, and the relationship between HSP70 and HLA class I expressions, clinicopathological factors and survival was evaluated. Results: Immunohistochemically positive results for HSP70 and HLA class I were seen in 67 (92%) and 43 cases (59%), respectively, while 38 patients (52%) were positive for both. Increased HSP70 immunoreactivity was significantly associated with high histological grade of tumor differentiation (p=0.0179), whereas reduced HLA class I expression was significantly associated with large tumor size (p=0.0082). No differences were apparent between disease-free and overall survival in regard to expression levels. Conclusion: These results suggest that HSP70 expression may be related to tumor differentiation and HLA class I loss may occur with tumor growth in HCV-related HCC.

Members of the heat-shock protein (HSP) family are molecular chaperones that are involved in numerous cellular functions, such as protein folding, transport, maturation and protection (1). HSPs have been classified into 7 major families according to the molecular size: HSP110, HSP90, HSP70, HSP60, HSP40, HSP28, HSP27 and the small HSP25. HSPs have been shown to regulate apoptosis and can thus be broadly categorized into two groups: anti-apoptotic and pro-apoptotic. HSP70 is an anti-apoptotic HSP. HSP70 overexpression thus allows cells to survive under various conditions, including carcinogenesis. In fact, overexpression of HSP70 has been reported in various human tumors (2-9). We have reported overexpression of 4 members of the HSP family in hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) samples by proteomic analysis (10). HSP70 is one of these 4 members and displayed the most increased levels in cancerous tissues compared to levels in corresponding noncancerous liver tissues by proteomic analysis.

Expression of human leukocyte antigen (HLA) class I molecules on the cell surface is generally accepted as critical for the recognition of tumor cells by cytotoxic T lymphocytes (CTLs), since CTLs recognize the endogenously processed antigenic peptides with HLA molecules expressed on the cell surface. Therefore, in cases where HSP70 was considered to be a tumor-associated antigen, levels of HLA class I molecule expression on the tumor cell surface may represent a determining factor for tumor clearance by CTL.

Taken together, cancer cells expressing HSP70 and HLA class I simultaneously may be attacked and killed by CTLs. Expression status of these two factors may thus be related to tumor growth and prognosis. The present study therefore evaluated the clinical significance of expression status of HSP70 and HLA class I in HCV-related HCC.

Patients and Methods

A total of 73 samples were obtained from 73 patients (55 men, 18 women; median age, 67.1 years) diagnosed with HCV-related HCC who underwent surgical liver resection between 2000 and 2006 at Yamaguchi University Hospital. The study protocol was approved by the Institutional Review Board for Human Use at Yamaguchi Hospital. Written informed consent was obtained from all patients prior to surgery. Sections from formalin-fixed,
paraffin-embedded tissues were used for immunohistochemical detection of HSP70 and HLA class I. Torigoe et al. recently generated a monoclonal antibody against HLA class I molecules, EMR8-5, allowing detection of HLA-A, -B, and -C antigens in formalin-fixed paraffin-embedded tissues (11). EMR8-5 was thus used in this study. In all instances, we compared expressions with the following clinicopathological parameters: histological grade, tumor number, tumor size, vascular invasion, intrahepatic metastasis, tumor status as primary or recurrent lesion and TNM stage. Clinicopathological parameters of the 73 patients were based on International Union against Cancer TNM classifications (12). Histological grade was determined on the predominant grade areas of selected specimens and classified into two groups: G1 (n=19) and G2/G3 (n=64). Tumor size was recorded as the maximum diameter of each specimen and classified based on the criteria of Yumoto et al. (13) as small (diameter <3 cm; n=25), or large (diameter ≥3 cm; n=48). TNM stage was classified as low (stage I + II; n=61), or high (stage III + IV; n=12). Serologically, all patients were positive for HCV antibody and negative for hepatitis B surface antigen. Clinical characteristics of the patients are shown in Table I. Histological examination of noncancerous lesions revealed normal liver in 1 case, liver fibrosis in 6, chronic hepatitis in 20, and cirrhosis in 39.

**Immunostaining of HSP70.** Immunostaining was performed using the dextran-polymer method. Formalin-fixed, paraffin-embedded samples were sectioned at 4 μm thickness. Sections were deparaffinized in xylene and ethanol, then placed in Target Retrieval Solution (pH 6.0; Dako, CA, USA). For antigen retrieval, sections were processed in a microwave oven at 95˚C for 20 min, then treated with 3% hydrogen peroxide for 5 min to block endogenous peroxidase activity and with Protein Block Serum-Free (Dako) for 10 min as a protein-blocking agent. Slides were incubated for 30 min at room temperature (RT) with polyclonal primary antibody (1:800; Dako). EnVision+ (Dako) was used as the secondary antibody for 30 min at RT. Reactive products were visualized by staining with 3,3’-diaminobenzidine (Dako) for 5 min, then counterstained with hematoxylin for 20 s.

**Interpretation of immunohistochemical results.** Staining of HSP70 and HLA class I was defined as reduced or non-reduced compared with the level of the non-HCC area. The distribution of positive staining for HSP70 and HLA class I was assessed according to the proportion of positive tumor cells as positive (≥10% ) or negative (<10% ).

**Statistical analysis.** Differences were analyzed using the χ² test or Fisher’s exact test. Disease-free survival (DFS) was estimated using Kaplan-Meier methods and compared with the log-rank test. Values of p<0.05 were accepted as statistically significant. All statistical analyses were performed using Statview 5.0 software (Abacus Concepts, Berkeley, CA, USA).

### Results

**Immunohistochemical expression of HSP70.** Immunohistochemically positive results for HSP70 were seen in 67 out of the 73 cases (92%). HSP70 preferentially localized to the cytoplasm, showing a granular pattern (Figure 1). In the non-HCC area, some HSP70-positive hepatocytes were detected with weak cytoplasmic staining.

**Immunohistochemical expression of HLA class I.** Immunohistochemically positive results for HLA class I were seen in 43 out of the 73 cases (59%). HLA class I was seen on the membrane of liver cancer cells. Membranous staining for HLA class I displayed a linear pattern, clarifying the borders between cells (Figure 2). Positive results for both HSP70 and HLA class I were seen in 38 cases (52%) (Table II).

Table I. Patient characteristics (n=73).

<table>
<thead>
<tr>
<th>Gender</th>
<th>Male/Female</th>
<th>Age (years)</th>
<th>No. of tumors</th>
<th>Tumor size (cm)</th>
<th>Primary HCC/recurrence</th>
<th>Venous invasion</th>
<th>Histological grade*</th>
<th>Stage**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55/18</td>
<td>67.1 (51-84)</td>
<td>42/31</td>
<td>4.0 (1.2-15)</td>
<td>59/14</td>
<td>27/46</td>
<td>G1/G2/G3</td>
<td>19/42/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low (I+II)/ high (III+IV)</td>
<td>61/13</td>
</tr>
</tbody>
</table>

*G1, G2 and G3 indicate well-, moderately, and poorly differentiated HCC, respectively. **Assessment based on TNM classification by the International Union against Cancer.

Table II. Expression of heat-shock protein (HSP)-70 and human leukocyte antigen (HLA) class I in HCV-related HCCs (n=73).

<table>
<thead>
<tr>
<th>HLA class I</th>
<th>HSP70</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

-, Negative expression; +, positive expression.
Immunoreactivities and clinicopathological findings. Increased HSP70 immunoreactivity was significantly associated with high histological grade of tumor differentiation ($p=0.0179$), whereas reduced HLA class I immunoreactivity was significantly associated with large tumor size ($p=0.0082$) (Table III).

Survival and DFS curves after surgery were compared between positive and negative expressions for the 51 patients who underwent curative hepatic resection. The number of cases with positive and negative expression respectively was 46 and 5 for immunohistochemical expression of HSP70, and 32 and 19 for HLA class I. No significant differences in DFS or overall survival were identified according to expression levels (Figures 3 and 4).

Discussion

This study examined the simultaneous expressions of HSP70 and HLA class I in HCV-related HCC samples. We found that HSP70 expression may be related to tumor differentiation and HLA class I loss may occur with tumor growth in HCV-related HCC. However, no relationship was identified between these expressions and prognosis or recurrence, even after combining the two factors.

Several studies have evaluated expression of HSP70 in HCC (7, 10, 14-19). Consistent with our findings, all studies have reported overexpression of HSP70 in HCC tissues. Chuma et al. (7) revealed significant overexpression of HSP70 in early HCC compared with precancerous lesions known as
adenomatous hyperplasia within nodule-in-nodule-type HCC. Furthermore, that study immunohistochemically confirmed that HSP70 protein levels are significantly higher in progressed HCC than in early HCC. This was supported by the present finding of a significant association between immunoreactivity for HSP70 and high histological grade of HCV-related HCC. Distinct infection patterns of hepatitis virus have been shown to substantially affect the molecular basis of HCC (20), raising the possibility that tumor HSP70 expression might be affected by a bias due to the viral pattern. In this regard, the report by

Figure 3. Comparison of A, disease-free survival (DFS) and B, overall survival (OS) between HCV-related HCC patients with positive and negative expression of HSP70. Open circles, patients with positive expression (n=46); closed circles, patients with negative expression (n=5). DFS log-rank test, p=0.25; OS log-rank test, p=0.98.

Figure 4. Comparison of A, DFS and B, OS between HCV-related HCC patients with positive and negative expression of HLA class-I. Open circles, patients with positive expression (n=32); closed circles, patients with negative expression (n=19). DFS log-rank test, p=0.49; OS log-rank test, p=0.94.
Tumor size and histological grade are closely related to progression of HCV-related HCC. Results thus strongly suggest that tumor HSP70 protein levels in HCV-related HCC samples, using a large sample set. The hepatitis B virus. In contrast, the present study only examined classification by the International Union against Cancer.

<table>
<thead>
<tr>
<th>Clinicopathological parameter</th>
<th>HSP70</th>
<th>p-Value</th>
<th>HLA class I</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological grade G1</td>
<td>4</td>
<td>15</td>
<td>0.0179</td>
<td>6</td>
</tr>
<tr>
<td>Histological grade G2/G3</td>
<td>2</td>
<td>52</td>
<td></td>
<td>24</td>
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<tr>
<td>Tumor size &lt;3 cm</td>
<td>3</td>
<td>22</td>
<td>0.396</td>
<td>5</td>
</tr>
<tr>
<td>Tumor size ≥3 cm</td>
<td>3</td>
<td>45</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>No. of tumors Single</td>
<td>5</td>
<td>37</td>
<td>0.182</td>
<td>16</td>
</tr>
<tr>
<td>No. of tumors Multiple</td>
<td>1</td>
<td>30</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Venous invasion Presence</td>
<td>1</td>
<td>26</td>
<td>0.282</td>
<td>14</td>
</tr>
<tr>
<td>Venous invasion Absence</td>
<td>5</td>
<td>41</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>IHM Primary</td>
<td>0</td>
<td>13</td>
<td>0.234</td>
<td>7</td>
</tr>
<tr>
<td>IHM Absence</td>
<td>6</td>
<td>54</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>HCC Primary</td>
<td>6</td>
<td>53</td>
<td>0.213</td>
<td>24</td>
</tr>
<tr>
<td>Recurrence</td>
<td>0</td>
<td>14</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Stage* Low (I+II)</td>
<td>6</td>
<td>55</td>
<td>0.257</td>
<td>24</td>
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<tr>
<td>Stage* High (III+IV)</td>
<td>0</td>
<td>12</td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

–, Negative expression; +, positive expression; IHM, intrahepatic metastasis; NS, not significant. *Assessment based on TNM classification by the International Union against Cancer.

Chuma et al. (7) included some HCC samples caused by hepatitis B virus. In contrast, the present study only examined HCV-related HCC samples, using a large sample set. The results thus strongly suggest that tumor HSP70 protein levels are closely related to progression of HCV-related HCC.

Most malignant tumor cells are known to be able to reduce expression of HLA class I on the cell surface to escape from immune surveillance (21). In this study, no significant association between immunoreactivities of HLA class I and histological grading of tumor differentiation was shown, but HLA class I immunoreactivity correlated negatively with tumor size. Various studies have examined expression of HLA class I in HCC (15, 22-30). Fujiwara et al. (15) reported that HLA class I expression in HCC was significantly reduced with reduced histological grading of tumor differentiation. The discrepancy between the present result and those of Fujiwara et al. might be partially attributable to differences in patients, concomitant liver disease, hepatitis virus infection pattern and methodological differences, including the antibody used.

Ciocca et al. (2) has reported that primary breast cancer patients with axillary lymph nodes showing high HSP70 expression on Western blot analysis have significantly shorter DFS than patients with low expression. In patients with malignant melanoma and colorectal cancer, survival is reportedly significantly longer for patients with high expression of HLA class I than for patients with low expression (31, 32). To the best of our knowledge, no previous studies have examined relationships between HSP70 or HLA class I expression level and DFS rates in HCC. This prompted us to investigate whether HSP70 or HLA class I expression could predict DFS in HCV-related HCC.

Unfortunately, these expression levels were not associated with DFS of patients with HCV-related HCC. The recurrence mode of HCC is complicated, so one possible cause of this failure might be the high frequency of metachronous carcinogenesis, i.e., multicentric occurrence. Other potential reasons include the short median duration of follow-up (708 days) and inadequate sample number.

HSP70 plays an important role as a chaperone of intracellular peptide antigens in cancer immunotherapy. Moreover, previous studies have shown that conditional overexpression of HSP70 in target cells enhances susceptibility to CTL (33) and transporter associated with antigen processing (TAP) function (34). Furthermore, Faure et al. (35) reported that three nonamer peptides, p380, p391 and p393, can raise CTL to recognize tumor cells overexpressing HSP70 in mice. The present study found that most HCV-related HCC cases were positive for HSP70 and half were also positive for HLA class I for this type of HCC, which represents 72.3% of HCCs in Japan (36). We thus suggest that HSP70 may represent a good molecular target for treatment of HCV-related HCC.

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References


