Heat Shock Proteins 27 and 70 Are Potential Biliary Markers for the Detection of Cholangiocarcinoma

Yasunori Sato,* Kenichi Harada,* Motoko Sasaki,* Takahiro Yasaka,† and Yasuni Nakanuma*

From the Department of Human Pathology,* Kanazawa University Graduate School of Medicine, Kanazawa; and the Division of Surgery,† Nagasaki Prefectural Kamigoto Hospital, Nagasaki, Japan

Cholangiocarcinoma often is diagnosed at an advanced stage. Thus, it is necessary to establish sensitive screening methods that would allow cholangiocarcinoma and preferably its precursor lesion [biliary intraepithelial neoplasia (BilIN)] to be detected. We sought to clarify the usefulness of heat shock protein (HSP) 27 and HSP70 as biomarkers of cholangiocarcinoma and have used immunohistochemical analyses of hepatolithiatic livers to characterize HSP27 and HSP70 expression during the multistep cholangiocarcinogenesis process. HSP27 and HSP70 were measured in serum and bile samples via enzyme-linked immunosorbent assay. In hepatolithiatic tissue, the expression of HSP27 and HSP70 was increased in BilIN as well as in invasive cholangiocarcinoma. The serum levels of HSP27 and HSP70 were not significantly different between the hepatolithiatic patients with and without cholangiocarcinoma. In contrast, the bile levels of HSP27 and HSP70 were increased significantly in the patients with cholangiocarcinoma compared with those in the patients with lithiasis. Combining the measurements of the bile levels of HSP27 and HSP70 increased their usefulness as biomarkers and the sum (HSP27 + HSP70) yielded the best sensitivity (90%) and specificity (100%). These results suggest that HSP27 and HSP70 could be used as biliary biomarkers for the detection of cholangiocarcinoma including BilIN. (Am J Pathol 2012, 180:123–130; DOI: 10.1016/j.ajpath.2011.09.010)

Cholangiocarcinoma often is diagnosed when it is at an advanced stage, and, hence, it displays a high mortality rate. Cholangiocarcinoma arising in the large bile ducts undergoes a multistep carcinogenesis process, and two types of precursor lesions have been proposed: biliary intraepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile duct (IPNB).1–4 The former is seen in the intrahepatic large bile ducts and extrahepatic bile ducts and is classified further into three grades based on atypia: BilIN-1 (low-grade lesions), BilIN-2 (intermediate-grade lesions), and BilIN-3 (high-grade lesions, carcinoma in situ).

BilIN is not uncommon in the intrahepatic large bile ducts in chronic biliary diseases such as hepatolithiasis.1–3 BilIN is a grossly unrecognizable lesion and is identifiable only on histologic sections, whereas IPNB forms a grossly visible mass, which is identifiable on radiologic images. BilIN usually is seen in the biliary epithelium around invasive cholangiocarcinoma and also incidentally is found in surgically resected specimens of hepatolithiasis. IPNB is far less common than BilIN, and the prognosis of IPNB is more favorable than that of conventional cholangiocarcinoma.4 Considering that cholangiocarcinoma often is diagnosed at an advanced stage, it is necessary to establish sensitive screening methods that would enable cholangiocarcinoma, and preferably BilIN, to be detected.

Heat shock proteins (HSPs) are stress proteins that are inducible in response to a wide variety of insults.5 Because they are powerful chaperones, their expression allows cells to survive otherwise lethal conditions. These cytoprotective effects are related to their ability to inhibit apoptosis.6 HSP27 and HSP70 may participate in oncogenesis because their overexpression and the consequent inhibition of apoptosis can increase the tumorigenic potential of cancer cells. In fact, higher than normal levels of HSP27 and HSP70 were detected in cholangiocarcinoma and pancreatic cancer tissues.7–9 HSP27 expression in cholangiocarcinoma tissues is associated with poor clinical outcome.7 In addition, HSP27 is a potential serum marker for pancreatic cancer.9

To date, the temporal expression of HSP27 and HSP70 during the multistep cholangiocarcinogenesis process...
and its availability as an indicator of cholangiocarcinoma have not been studied. The present study characterized the immunohistochemical expression of HSP27 and HSP70 in the livers of hepatolithiasis patients with or without BilIN and cholangiocarcinoma, and their serum and bile levels of HSP 27 and HSP70 were analyzed to elucidate their usefulness as biomarkers.

**Materials and Methods**

This human study was performed with the approval of the ethics committee of Kanazawa University Graduate School of Medicine.

**Tissue Preparation**

In this study, hepatolithiatic livers were used as a model of the multistep cholangiocarcinogenesis process. A total of 49 hepatolithiatic livers were retrieved from the liver disease files of our laboratory and affiliated hospitals. All cases were surgically resected. Twenty-four hepatolithiatic livers were associated with BilIN and/or invasive cholangiocarcinoma in the hilar and/or perihilar region, and the remaining 25 livers were not associated with neoplastic biliary epithelial lesions. The age and sex distribution of the patients is shown in Table 1. No cases of IPNB were included in this study. As a control, the hilar regions of normal/subnormal autopsy livers (n = 13) were used. The samples were fixed in 10% neutral formalin and embedded in paraffin. Then, 4-μm-thick, paraffin-embedded sections were prepared. One representative section from each case was used.

**Immunostaining**

After deparaffinization, antigen retrieval was performed by microwaving the sections in 10 mmol/L citrate buffer (pH 6.0) for the HSP70 immunostaining. The sections then were immersed in 0.3% hydrogen peroxidase in methanol for 20 minutes at room temperature to block endogenous peroxidase activity. After pretreatment with blocking serum (DakoCytomation, Glostrup, Denmark), the sections were incubated overnight at 4°C with primary antibodies against HSP27 (1:400, mouse monoclonal; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and HSP70/HSP72 (1:200, mouse monoclonal; Stressgen, Ann Arbor, MI). Then, the sections were incubated with a secondary antibody conjugated to peroxidase-labeled polymer using the HISTOFINE system (Nichirei, Tokyo, Japan). Color development was performed using 3,3'-diaminobenzidine tetrahydrochloride, and the sections were lightly counterstained with hematoxylin. Negative controls were produced by substituting the primary antibody for nonimmunized serum, which resulted in no signal detection.

**Histologic Assessment**

Semi-quantitative analysis of the immunostained sections was performed. Staining intensity was evaluated in a high-power field for the non-neoplastic and neoplastic biliary epithelia. At least five foci were examined in each section. The signal intensity was evaluated using the following grading system: - (negative), 1+ (mild to moderate), 2+ (marked).

**Enzyme-Linked Immunosorbent Assay**

The HSP27 and HSP70 levels of serum and bile samples were measured using enzyme-linked immunosorbent assay. The serum samples were obtained from 56 patients with hepatolithiatis, eight of whom had clinically detectable cholangiocarcinoma in the hilar region of their liver. Among these eight cholangiocarcinoma cases, surgical resection was performed in four cases, which resulted in noncurative resection, and the other four cases were not indicated for surgical resection because of disease progression. As controls, serum samples obtained from 16 healthy volunteers were used.

The bile samples were obtained from patients with cholecystolithiasis and/or choledocholithiasis (n = 10) and patients with cholangiocarcinoma (without lithiasis) (n = 10). The cholangiocarcinoma cases without lithiasis consisted of hilar (n = 6) and extrahepatic (n = 4) cholangiocarcinoma, and all of these cases presented with obstructive jaundice. The bile samples were obtained by percutaneous transhepatic cholangiographic drainage. The age and sex distributions of the patients from whom the serum and bile samples were obtained are summarized in Table 1.

HSP27 and HSP70 levels were measured using the HSP27 enzyme-linked immunoassay kit (Stressgen) and the HSP70 High Sensitivity EIA Kit (Stressgen), respectively, according to the manufacturer's instructions. Briefly, samples were added to a 96-well plate coated with a monoclonal antibody against HSP27 or HSP70 and incubated for the time indicated in the manufacturer's protocol at room temperature. After being washed, the plate was incubated with anti-HSP27 or anti-HSP70 antibody conjugated to horseradish peroxidase for 1 hour at room temperature. Color development was performed using a substrate solution, and absorbance was measured at 450 nm.

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**Table 1. Age and Sex Distribution of the Cases Studied**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>n</th>
<th>Age (years)</th>
<th>Sex (M:F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal/subnormal liver</td>
<td>13</td>
<td>62 ± 7</td>
<td>6.7</td>
</tr>
<tr>
<td>Hepatolithiasis</td>
<td>25</td>
<td>55 ± 10</td>
<td>8:17</td>
</tr>
<tr>
<td>Cholangiocarcinoma with</td>
<td>24</td>
<td>59 ± 10</td>
<td>7:17</td>
</tr>
<tr>
<td>hepatolithiiasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>60 ± 15</td>
<td>10:6</td>
</tr>
<tr>
<td>Hepatolithiasis</td>
<td>48</td>
<td>74 ± 10</td>
<td>26:22</td>
</tr>
<tr>
<td>Cholangiocarcinoma with</td>
<td>8</td>
<td>76 ± 10</td>
<td>4:4</td>
</tr>
<tr>
<td>hepatolithiiasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithiasis</td>
<td>10</td>
<td>61 ± 15</td>
<td>5:5</td>
</tr>
<tr>
<td>Cholangiocarcinoma</td>
<td>10</td>
<td>76 ± 10</td>
<td>6:4</td>
</tr>
<tr>
<td>without lithiasis</td>
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</table>

F, female; M, male.
Statistics
The data are expressed as the mean ± SD. Statistical significance was determined using the Mann–Whitney U test and the χ² test using Statview-J5.0 software (Abacus Concepts, Inc., Berkley, CA). P values of <0.05 were accepted as statistically significant. Receiver operating characteristic curves were constructed by plotting sensitivity versus 1 – specificity using Dr SPSS II software (version 11.01 J; SPSS Japan, Inc., Tokyo, Japan), and the area under the curve was calculated.

Results
Immunohistochemical Expression of HSP27 and HSP70
The results of immunohistochemical staining for HSP27 and HSP70 are shown in Figure 1. In the vast majority of normal livers, the immunohistochemical staining of HSP27 was faint or negligible, and that of HSP70 was weak in the biliary epithelium of the large bile ducts. Hepatocytes lacked positive signals for HSP27 and HSP70. Vascular smooth muscle cells, nerve fibers, and the microvessel endothelium constitutionally expressed HSP27, and the biliary epithelium of the small bile ducts also was positive for HSP70.

In hepatolithiasis, the epithelia of the large bile ducts and the neoplastic biliary epithelium showed various degrees of HSP27 and HSP70 expression (Figure 1). HSP27 was observed in the cytoplasm, whereas HSP70 was localized in the cytoplasm and/or the nuclei. Their expression tended to be intense in BilIN and invasive cholangiocarcinoma compared with that in the non-neoplastic, reactive biliary epithelium.

Semiquantitative Analysis of HSP27 and HSP70 Expression
The signal intensity of the immunohistochemical expression of HSP27 and HSP70 in the biliary epithelium was categorized into three grades, and a semiquantitative analysis was performed. The expression levels of HSP27 and HSP70 tended to be high in BilIN and invasive cholangiocarcinoma compared with those in the non-neoplastic biliary epithelium of the hepatolithiasis patients and the normal bile ducts (Figure 2, B and C).
Serum HSP27 and HSP70 Concentrations

The serum concentrations of HSP27 were 0.55 ± 0.49, 3.03 ± 2.52, and 3.71 ± 2.39 ng/mL in healthy controls (n = 16) and the patients with hepatolithiasis without cholangiocarcinoma (n = 48), and patients with hepatolithiasis with clinically detectable cholangiocarcinoma (n = 8), respectively (Figure 3A). Statistical analysis showed that the patients with hepatolithiasis with or without cholangiocarcinoma showed significantly higher serum levels of HSP27 than the healthy controls, but the difference between the hepatolithiatic groups with and without cholangiocarcinoma was not significant.

None of the healthy controls (n = 16) displayed detectable amounts of HSP70 in their serum, according to measurements taken using a commercially available EIA Kit (detection limit, 0.09 ng/mL; Stressgen) (Figure 3B). Of the hepatolithiasis patients without cholangiocarcinoma, 20 displayed serum HSP70 levels that exceeded the detection limit, whereas the other 28 cases remained below the detection limit (positive detection rate, 41.7%). Among the patients with cholangiocarcinoma with hepatolithiathis (n = 8), seven cases showed serum HSP70 values that were above the detection limit.

In four cases of hepatolithiasis, serum samples were obtained before and after the development of cholangiocarcinoma. In these four cases, the patients’ serum HSP27 levels tended to decrease after the development of cholangiocarcinoma (Figure 4A), whereas their serum HSP70 levels were increased or unchanged after the development of cholangiocarcinoma (Figure 4B).

Bile HSP27 and HSP70 Concentrations

Bile samples were obtained from patients with cholecystolithiasis and/or choledocholithiasis (n = 10) and chol-
angiocarcinoma patients without lithiasis ($n = 10$), and the HSP27 and HSP70 levels of these samples were examined. The HSP27 bile levels of the former and latter groups were $1.48 \pm 1.80$ and $8.52 \pm 8.97$ ng/mL, respectively, and a significant difference was observed between them (Figure 5A).

The bile HSP70 concentrations of the patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$) and cholangiocarcinoma without lithiasis ($n = 10$) were $3.41 \pm 3.04$ and $8.39 \pm 4.98$ ng/mL, respectively, and a significant difference was observed between them (Figure 5B). The bile HSP27 and HSP70 levels of the cholecystolithiasis and/or choledocholithiasis (Figure 6A) and cholangiocarcinoma without lithiasis patients (Figure 6B) showed almost parallel distributions, with several exceptions.

When the product and the sum of the values of HSP27 and HSP70 were calculated, both the product (HSP27 × HSP70) and sum (HSP27 + HSP70) were significantly higher in the cholangiocarcinoma patients without lithiasis than in those with cholecystolithiasis and/or choledocholithiasis (Figure 7, A and B).

A receiver operating characteristic curve was constructed for the bile HSP27 and HSP70 measurements. The resultant analysis showed that the sum of HSP27 and HSP70 was the best indicator of cholangiocarcinoma, producing a sensitivity of $90\%$ and a specificity of $100\%$. 

Figure 5. Bile HSP27 and HSP70 concentrations. The bile levels of HSP27 (A) and HSP70 (B) were examined in samples obtained from patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$), and patients with cholangiocarcinoma (without lithiasis) ($n = 10$). For both HSP27 and HSP70, the measured values were significantly higher in the cholangiocarcinoma patients than in the patients with cholecystolithiasis and/or choledocholithiasis. The black bars indicate mean values. **$P < 0.05$.

Figure 6. Comparison of bile HSP27 and HSP70 concentrations. The bile levels of HSP27 and HSP70 were compared between the patients with cholecystolithiasis and/or choledocholithiasis ($n = 10$) (A), and the patients with cholangiocarcinoma (without lithiasis) ($n = 10$) (B). The patients’ bile levels of HSP27 and HSP70 showed an almost parallel distribution in both groups with several exceptions.
at a cut-off value of 10.2 (Table 2). The area under the curve was calculated as 0.940.

**Discussion**

This study showed that HSP27 and HSP70 could be used as biliary markers for the detection of cholangiocarcinoma. Combining the bile values of HSP27 and HSP70 further increased their usefulness as biomarkers, and the sum (HSP27 + HSP70) yielded the best sensitivity (90%) and specificity (100%). In contrast, the levels of HSP27 and HSP70 in serum were not significantly different between the groups with and without cholangiocarcinoma. Immunohistochemical analysis showed that the expression of HSP27 and HSP70 was increased in BilIN as well as cholangiocarcinoma, indicating that the increased HSP27 and HSP70 bile levels found in these patients were caused by the local production and secretion of these molecules by the neoplastic epithelium.

Biliary tumor markers are considered to be secreted from bile and into the serum, possibly as a result of increasing biliary pressure owing to local obstruction triggered by the loss of cellular polarity. Therefore, the bile levels of HSP27 and HSP70 might reflect more accurately the local production of these molecules in the biliary tract than their serum levels.

The diagnostic values of the bile levels of carbohydrate antigen 19-9 and carcinoembryonic antigen have been investigated previously. The frequency of carbohydrate antigen 19-9 detection in bile from patients with both benign and neoplastic pancreaticobiliary tract diseases has been reported to range from 46% to 61%, and the associated specificity has been reported to range from 60% to 70%. Increased bile carcinoembryonic antigen levels also have been shown to predict cholangiocarcinoma with a sensitivity of 58% to 84% and a specificity of 33% to 84%. However, factors such as the presence of cholangitis, infection, and the sampling time (ie, before or after biliary drainage) have been shown to significantly influence the levels of carbohydrate antigen 19-9 and carcinoembryonic antigen and so the diagnostic value of bile carbohydrate antigen 19-9 and carcinoembryonic antigen levels is disputed.

In this study, it was unclear whether the patients without cholangiocarcinoma and/or BilIN had other disorders such as cholecystitis and pancreatitis. It should be noted that the absence of patients with these conditions could have increased the specificity of these markers and improved the area under the receiver operating characteristic curve. This was a potential limitation of the current study, which further studies will have to address.

Recently, *Wisteria floribunda* agglutinin–positive mucin 1 was identified as a novel biliary marker of cholangiocarcinoma that enables cholangiocarcinoma to be distinguished from benign diseases with a sensitivity of 90.0% and a specificity of 76.3%. The sensitivity of the bile HSP27 + HSP70 level in this study (90%) was comparable with that found in their study, but the specificity of the bile HSP27 + HSP70 level (100%) exceeded that of their study. More recently, it was shown that bile proteomic profiles can differentiate cholangiocarcinoma from benign biliary diseases. Thus, simultaneously testing the

### Table 2. Receiver Operating Characteristic Curve Analysis of HSP27 and HSP70 Bile Concentrations as Predictors of Cholangiocarcinoma

<table>
<thead>
<tr>
<th>Cut-off value</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP27 (ng/mL)</td>
<td>5.67</td>
<td>80</td>
<td>0.805</td>
</tr>
<tr>
<td>HSP70 (ng/mL)</td>
<td>17.9</td>
<td>80</td>
<td>0.825</td>
</tr>
<tr>
<td>HSP27 × HSP70</td>
<td>10.2</td>
<td>90</td>
<td>0.940</td>
</tr>
</tbody>
</table>

Bile samples from cholangiocarcinoma (*n* = 10) and lithiasis patients (*n* = 10) were analyzed.

AUC, area under the curve.
bile levels of several markers seems to be more useful for detecting cholangiocarcinoma.

A previous study showed that serum HSP27 was a useful marker for the detection of pancreatic cancer, displaying a sensitivity of 100% and a specificity of 84%. However, in this study, higher serum HSP27 levels were observed in patients with hepatolithiasis with or without cholangiocarcinoma than in the healthy controls, but the difference between the hepatolithiasis patients with and without cholangiocarcinoma was not significant. Similarly, the serum HSP70 levels of the hepatolithiatic patients with and without cholangiocarcinoma were not significantly different, but the positive detection rate was significantly higher among the patients with cholangiocarcinoma than among the hepatolithiasis patients without cholangiocarcinoma. Thus, the serum HSP70 levels could be a useful screening tool that could lead to further evaluations.

As shown in Figure 2, the immunohistochemical analysis showed that the expression levels of HSP27 and HSP70 were increased in the non-neoplastic, reactive biliary epithelia of several hepatolithiasis patients compared with those in the normal biliary epithelium. These results suggest that cholangitis caused by hepatolithiasis is associated with the induction of HSP27 and HSP70 expression in the biliary epithelium and might account for the increased serum HSP27 and HSP70 levels of the patients without clinically detectable cholangiocarcinoma. In addition, the possibility that hepatolithiatic patients with BilIN were included in the experimental groups is associated with the induction of HSP27 and HSP70 was induced in BilIN as well as invasive cholangiocarcinoma. HSP27 and HSP70 inhibit carcinoma cell apoptosis, and their overexpression is associated with resistance to treatment. The tumorogenic role of Fas/FasL in cholangiocarcinoma has been described, and it is suggested that this pathway is a potential molecular target of therapeutic strategies that aim to circumvent the mechanisms that regulate HSP27 and HSP70 expression have not been fully defined, they are potential targets of cholangiocarcinoma therapy.

In summary, this study showed that the expression of HSP27 and HSP70 was induced in BilIN as well as invasive cholangiocarcinoma, indicating that their induction is an early event in the development of cholangiocarcinoma and is associated closely with the multistep carcinogenesis process. Accordingly, the bile levels of HSP27 and HSP70 were significantly higher in patients with cholangiocarcinoma than in the control subjects, suggesting that HSP27 and HSP70 could be used as biliary biomarkers for the detection of cholangiocarcinoma. Because the expression of HSP27 and HSP70 was induced in BilIN, they also might be applicable to the detection of BilIN. These findings are preliminary, and a prospective trial needs to be conducted to determine the performance characteristics of an assay using these markers in clinical practice.

References


