Heat Shock Protein 90 Is a Potential Therapeutic Target in Cholangiocarcinoma

Tomoki Shirota1,2, Hidenori Ojima3, Nobuyoshi Hiraoka3, Kazuaki Shimada4, Hirofumi Rokutan1, Yasuhiro Araki1, Yae Kanai3, Shinichi Miyagawa2, and Tatsuhiro Shibata1,5

Abstract

Cholangiocarcinoma is an aggressive malignancy with a poor prognosis, with no effective therapy other than surgical resection. Heat shock protein 90 (HSP90) is a key component of a multi-chaperone complex involved in the posttranslational folding of a number of client proteins, many of which play essential roles in tumorigenesis. Here, we attempted to clarify its prognostic significance and potential utility as a therapeutic target in cholangiocarcinoma. Immunohistochemical expression of HSP90 was assessed retrospectively in 399 cholangiocarcinoma cases and 17 human cholangiocarcinoma cell lines, along with the effect of a small-molecule HSP90 inhibitor (NVP-AUY922) on cholangiocarcinoma cell growth and angiogenesis in human cholangiocarcinoma cell lines and xenografts. The positivity of HSP90 was 44.6% in intrahepatic cholangiocarcinoma (IHCC) and 32.8% in extrahepatic cholangiocarcinoma (EHCC), respectively. HSP90 expression was significantly associated with the 5-year survival rate for IHCC (P < 0.001) and EHCC (P < 0.001). HSP90 inhibition showed potent antiproliferative activity and reduced growth-associated signaling in human cholangiocarcinoma cells in vitro. Furthermore, treatment of cholangiocarcinoma xenograft-bearing mice with NVP-AUY922 significantly inhibited growth at doses far below the maximum-tolerated dose. HSP90 overexpression is a prognostic marker for cholangiocarcinoma. HSP90-targeted therapy may be an option for a subset of cholangiocarcinoma.

Introduction

Cholangiocarcinoma is a highly malignant invasive carcinoma arising through malignant transformation of cholangiocytes of the epithelial bile ducts. It is a heterogeneous malignancy that comprises two different pathologic entities: intrahepatic cholangiocarcinoma (IHCC), which arises from the bile ducts in the liver, and extrahepatic cholangiocarcinoma (EHCC), which involves hilar bile ducts and the extrahepatic biliary tree. Most patients with cholangiocarcinoma are diagnosed at the advanced disease, and curative surgical resection is the only therapy; however, recurrence is common (1). The incidence and mortality rates for IHCC are increasing worldwide, reflecting the poor survival associated with this neoplasia (2–4).

The molecular chaperone, heat shock protein 90 (HSP90), plays an important role in the posttranslational maturation and activation of many critical oncogenic client proteins that are essential for facilitating malignant transformation and promoting the survival, growth, and invasive potential of cancer cells (5, 6). HSP90 expression was associated with proliferating cell nuclear antigen, which is known as one of proliferation marker (7). Increased expression of HSP90 occurs in a range of solid tumors, including breast, gastric (8), non–small-cell lung (9, 10), esophageal (11), pancreatic (12), and oral carcinomas (13). Especially, Pick and colleagues (14) and Song and colleagues (15) reported that overexpression of HSP90 was associated with poor outcome in breast carcinoma patients. Chen and colleagues (16) reported association between HSP90 expression and clinicopathologic characteristics and prognosis in patients with IHCC; however, no study has examined the associations between HSP90 expression and EHCC. To improve our understanding of the clinical significance of HSP90 in cholangiocarcinoma, the primary aim of this study is to clarify the frequency of HSP90 expression in clinical cases, and we further assessed the activity of an HSP90 inhibitor (NVP-AUY922; Novartis) using in vitro and in vivo models of cholangiocarcinoma cell lines.

Materials and Methods

Patients

A total of 399 patients with cholangiocarcinoma were examined. The patients had undergone surgery and been diagnosed histologically as having adenocarcinoma of the bile duct, except for cancer of gallbladder and ampulla of Vater, at the National Cancer Center Hospital, Tokyo, between February 1990 and December 2009. Patients with other malignancies or had died within 4 weeks after surgery were excluded. Clinical and pathologic data were obtained from the medical records of the patients. The correlation between HSP90 expression and that of other
receptor tyrosine kinases (EGFR, HER2, VEGF, or c-MET) was also examined by reviewing data pertaining to the overexpression of these molecules in the same cohort (17, 18).

Of the 399 patients enrolled, 276 were male and 123 were female. The age range was 33 to 84 years (median, 65 years) and the follow-up period ranged from 3.7 to 253.2 months (median, 33.5 months). The cases were divided into two groups, IHCC and EHCC, in accordance with the TNM classification of malignant tumors [defined by the Union for International Cancer Control (UICC)] and the World Health Organization Histological Classification of Tumors (19, 20). There were 177 cases of IHCC and 222 cases of EHCC. Peri-hilar EHCC and distal EHCC were classified as EHCC due to the difficulty in categorizing this cancer based on the origin of the cystic duct. Tumor recurrence was defined as tumor growth in any site of the body after the operation, which was diagnosed clinically, radiologically, or pathologically (most diagnoses were made by computed tomography and ultrasonography). Only tumor death was used for analysis. The research protocol was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan. All patients gave written informed consent for inclusion in this study.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded sections of tumors and cell lines. Sections were stained for HSP90 using a polymer-based method (Envision + Dual Link System-HRP; Dako). Serial sections (4-μm thick) cut from representative paraffin-embedded samples were prepared on silicone-coated slides, and sections cut through the maximum tumor diameter were selected for IHC analysis. Briefly, sections were deparaffinized in xylene and rehydrated through a graded ethanol series (50%–100%) and endogenous peroxidase activity was blocked by incubation in 0.01 mol/L citrate buffer (pH 6.0). Heat-induced antigen retrieval was performed by heating in 0.01 mol/L citrate buffer at 121°C for 10 minutes. The sections were then incubated for 1 hour at room temperature with mouse anti-HSP90 (1:50 dilution; Dako). After a washing in PBS, the sections were then incubated for 1 hour at room temperature with mouse anti-human Ki67 (1:100 dilution; Chemicon), anti-human HER2 (1:50 dilution; Dako). After a washing in PBS, the sections were exposed to Envision + Dual link reagent for 30 minutes at room temperature and then visualized using 3,3′-Diaminobenzidine tetrahydrochloride as the chromogen. Finally, the tissue sections were counterstained with hematoxylin.

Evaluation of IHC results

All sections were evaluated by three researchers (T. Shirota, H. Ojima, and T. Shibata) who were blinded to the clinical and pathologic data. Differences in interpretation were resolved by consensual agreement. HSP90 expression in cholangiocarcinoma was compared with that in the bile ducts. Nuclear or cytoplasmic staining of cholangiocarcinoma that showed stronger intensity than that in the normal bile ducts was considered positive (21). The intensity of HSP90 staining was scored as: 0, complete absence of nuclear or cytoplasmic staining; 1+, faint and partial nuclear or cytoplasmic staining; 2+, strong and complete staining. In all cases, five fields (×100 magnification) were assessed and a minimum of 100 cancer cells were evaluated in the designated areas, so as to appraise the lesion as a whole. Receiver-operator characteristic (ROC) curve analysis was performed for predictive variables and an explorative cutoff value was determined by seeking the most optimal conformation of high sensitivity and specificity values, while maintaining the lowest likelihood ratio of a negative test and the highest likelihood ratio of a positive test. The areas under the ROC curve was 0.643 [95% confidence interval (CI), 0.59–0.70]. The cutoff value was defined as a score of “2+” in 20% of tumor cells. For each section, staining was assessed as negative (an average of 0%–19% of cells were scored as 2+), or positive (an average of >20% of cells were scored as 2+).

Cell lines

All cell lines tested were established at the National Cancer Center Research Institute (22). NCC-CC1, NCC-CC3-1, NCC-CC3-2, NCC-CC4-1, NCC-CC4-2, NCC-CC5, NCC-CC6-1, and NCC-CC6-2 cells were derived from human IHCC, and NCC-BD1, NCC-BD2, NCC-BD3, NCC-BD4-1, and NCC-BD4-2 were derived from human EHCC. TKKK, HuCCT1, OZ, and TGC24TKB cells were purchased from RIKEN BioResource Center or from the Japanese Collection of Research Bioresources. TKKK, TGC24TKB, and HuCCT1 were derived from IHCC, and OZ was derived from EHCC. All cell lines were derived from Japanese patients. The originally established 13 cholangiocarcinoma cell lines and HuCCT1 were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin. TGC24TKB, TKKK, and OZ were maintained in Dulbecco’s modified Eagle medium supplemented with 10% FBS, 100 μg/mL penicillin. TKKK, HuCCT1, OZ, and TGC24TKB cells were authenticated by RIKEN BioResource Center or from the Japanese Collection Research Bioresources before purchase by the standard short tandem repeat DNA typing methodology. NCC-CC1, NCC-CC3-1, NCC-CC3-2, NCC-CC4-1, NCC-BD1, and NCC-BD2 were authenticated by authors by DNA microarray and quantitative RT-PCR (22). Otherwise, NCC-CC4-2, NCC-5, NCC-CC6-1, NCC-CC6-2, NCC-BD3, NCC-BD4-1, and NCC-BD4-2 were not authenticated by authors. TKKK, HuCCT1, OZ, and TGC24TKB cells were purchased in 2010. NCC-CC1, NCC-CC3-1, NCC-CC3-2, NCC-CC4-1, NCC-BD1, and NCC-BD2 were established in 2010, and NCC-CC4-2, NCC-5, NCC-CC6-1, NCC-CC6-2, NCC-BD3, NCC-BD4-1, and NCC-BD4-2 were established in 2011.

All cells were expanded and frozen in multiple vials after third generation and passed in culture for no more than 4 months after being thawed from authentic stocks. The culture medium was replaced every 2 to 3 days. The confluent cells were subcultured by splitting them at 1:3 ratios. Cell lines were routinely tested for mycoplasma contamination.

Drug and formulation

NVP-AUY922 was kindly provided by Novartis. For the in vitro experiments, stock solutions of NVP-AUY922 (10 mmol/L) were prepared in 100% DMSO and stored at −20°C. Immediately prior to use, the stock solution was diluted to the required concentration in culture medium. For the in vivo studies, the free base of NVP-AUY922 was dissolved in a solution comprising 60 mmol/L lactic acid or 2.5% ethanol, 20% 50 mmol/L tartaric acid, and 77.5% (5% glucose in water containing 1% Tween-80) vol/vol. An optimized NVP-AUY922 salt with high solubility in aqueous solution was formulated in 5% glucose in water and was administered by intraperitoneal injection. Fresh solutions were used for each administration.
Cell proliferation assay
The sensitivity of cells to NVP-AUY922 was estimated in the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay using the CellTiter 96 Aqueous One Solution Reagent (Promega) according to the manufacturer’s instructions. Briefly, 3,000 cells were suspended in 100 μL of culture medium supplemented with 10% FBS and added to the wells of a 96-well culture plate. Each well was then treated with various concentrations of NVP-AUY922 (0–10 μmol/L). After 72 hours, 20 μL of CellTiter 96 Aqueous One Solution Reagent was added to each well and the absorbance was read at 490 nm. The experiment was conducted in triplicate and repeated three times. All data were calculated as a ratio to control, which means a ratio of absorbance in each concentration of NVP-AUY922 treatment relative to that in the negative control, and presented as mean ± SD.

Western blot analysis investigating molecular effects of NVP-AUY922
Western blot analysis was performed to examine the effect of NVP-AUY922 on cell signaling intermediates and predictors of in vitro sensitivity. NCC-BD3, NCC-CC4-2, and TKKK cells were treated with NVP-AUY922 at five times the 50% inhibitory concentration (IC50) and then harvested at the indicated time points. Briefly, subconfluent cells were lysed in a buffer containing 10 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 150 mmol/L NaCl, a complete protease inhibitor cocktail (Roche), and a phosphate inhibitor cocktail (Nacalai Tesque) at 4°C for 30 minutes. Equal amounts (40 μg) of cell extract were then electrophoresed, transferred to polyvinylidene difluoride membranes (Millipore), and immunoblotted with the following antibodies (all purchased from Cell Signaling Technology): anti-AKT (#2967, mouse monoclonal), anti-phospho-AKT (#4060, rabbit polyclonal), anti-EGFR (#4267, rabbit polyclonal), anti-phospho-EGFR (#3777, rabbit polyclonal), anti-HER2 (#4290, rabbit polyclonal), anti-phospho-HER2 (#2243, rabbit polyclonal), anti-phospho-STAT3 (#9145, rabbit polyclonal), anti-MAPK (#4695, rabbit polyclonal), anti-phospho-MAPK (#9106S, mouse monoclonal). Anti-STAT3 (610189, mouse monoclonal) was purchased from BD Transduction Laboratories and β-actin (PM053, rabbit polyclonal) was purchased from MBL. All antibodies were diluted and used in accordance with the manufacturer’s instructions.

Subcutaneous xenograft models
All animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation and conducted in accordance with the Guideline for Animal Experiments of the National Cancer Center (Tokyo, Japan).

Eight-week-old female BALB/c-nu/nu athymic mice were purchased from CLEA. A total of 8 × 10⁶ of NCC-BD3 cells were suspended in 0.2 mL of culture medium (without FBS) and injected subcutaneously into the right flank of each mice. Tumor growth was measured twice per week. Tumor volume was calculated using the following formula: (short diameter)² × (long diameter)/2. Mice were randomized and assigned into treatment and control groups (n=10 per group). Intraperitoneal injections of NVP-AUY922 (50 mg/kg, twice a week; or 25 mg/kg, twice a week) or saline (control) began when tumor volume exceeded 20 mm³. Treatment continued for at least 4 weeks. All mice were sacrificed at the end of study period and tumors removed completely. The tumor volume was calculated and the tumors were sectioned through the maximum diameter. Half were fixed in 10% formalin, and paraffin-embedded, and stained with hematoxylin-eosin and then stained for Ki67 (a proliferation marker), HSP90, and HER2 to examine the histologic effects of NVP-AUY922.

Figure 1.
HSP90 expression in tumor sections. A, HSP90 is expressed in tumor cells (T) but not in the noncancerous bile duct (N). B–D, representative IHC images showing the intensity of HSP90 expression [images at higher magnification (>100 magnification) than A (<40 magnification)]. Staining was scored as follows: 2+ (B), 1+ (C), and 0 (D).
Table 1A. Associations between HSP90 expression and clinicopathologic factors in IHCC

<table>
<thead>
<tr>
<th>IHCC characteristics</th>
<th>HSP90 Positive (n = 98)</th>
<th>HSP90 Negative (n = 79)</th>
<th>P</th>
<th>IHCC characteristics</th>
<th>HSP90 Positive (n = 98)</th>
<th>HSP90 Negative (n = 79)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>64.0 (35–84)</td>
<td>66.0 (41–84)</td>
<td>0.171</td>
<td>Perineural invasion</td>
<td>Negative</td>
<td>46</td>
<td>38</td>
</tr>
<tr>
<td>Male</td>
<td>64</td>
<td>49</td>
<td>0.384</td>
<td>Negative</td>
<td>Positive</td>
<td>51</td>
<td>41</td>
</tr>
<tr>
<td>Preoperative CEA level</td>
<td>75 (5.0 U/mL)</td>
<td>58 (5.0 U/mL)</td>
<td>0.381</td>
<td>Negative</td>
<td>Positive</td>
<td>79</td>
<td>50</td>
</tr>
<tr>
<td>Preoperative CA19-9 level</td>
<td>38 (≤5.0 U/mL)</td>
<td>24 (&gt;5.0 U/mL)</td>
<td>0.159</td>
<td>Negative</td>
<td>Positive</td>
<td>69</td>
<td>46</td>
</tr>
<tr>
<td>Average tumor size</td>
<td>5.1 (0.5–15.0)</td>
<td>2.1 (1.4–15.0)</td>
<td>&lt;0.001</td>
<td>UICC pathologic tumor status</td>
<td>Negative</td>
<td>29</td>
<td>33</td>
</tr>
<tr>
<td>Macroscopic type</td>
<td>Non-mass forming</td>
<td>3</td>
<td>0.826</td>
<td>T1</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mass forming</td>
<td>74</td>
<td>76</td>
<td>0.002</td>
<td>Lymph node metastasis</td>
<td>70</td>
<td>45</td>
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</tr>
<tr>
<td>Histologic classification (differentiated)</td>
<td>28 (Mucinous and well differentiated)</td>
<td>9 (Moderately differentiated)</td>
<td>&lt;0.001</td>
<td>Negative</td>
<td>Positive</td>
<td>28</td>
<td>34</td>
</tr>
<tr>
<td>Poorly</td>
<td>0</td>
<td>4</td>
<td>0.007</td>
<td>UICC pathologic stage status</td>
<td>Negative</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Hepatic vein invasion</td>
<td>Negative</td>
<td>19</td>
<td>0.482</td>
<td>II</td>
<td>30</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>73</td>
<td>60</td>
<td>0.248</td>
<td>IVA + IVB</td>
<td>54</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td>Negative</td>
<td>42</td>
<td>0.055</td>
<td>EGFR</td>
<td>Positive</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Positive</td>
<td>32</td>
<td>36</td>
<td>0.471</td>
<td>VEGF</td>
<td>Negative</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>Venous invasion</td>
<td>Negative</td>
<td>43</td>
<td>0.436</td>
<td>Positive</td>
<td>Positive</td>
<td>28</td>
<td>28</td>
</tr>
</tbody>
</table>

NOTE: HSP90 in IHCC showed a significant association with macroscopic type (P = 0.001), histologic classification (P = 0.002), intrahepatic metastasis (P = 0.006), pathologic tumor status (P = 0.013), lymph node metastasis (P = 0.032), and pathologic stage (P = 0.007).

*Significantly different (P < 0.05).

Statistical analysis

The associations between the IHC results and clinicopathologic factors were assessed using the χ² and Fisher exact tests. Cumulative survival rates and survival curves were calculated using the Kaplan–Meier method and the log-rank test was performed for the comparison of survival curves. A Cox proportional hazard model was used to estimate the hazard ratio (HR) and the 95% CI for each outcome (death and recurrence). Multivariate analyses were performed using the factors identified as risk factors for each outcome by univariate analyses, without UICC pT and UICC pN status. Between-group comparisons of response to NVP-AUY922 (tumor volume, proliferation, and HSP90 expression) were estimated using one-way analysis of variance (ANOVA) followed by Dunnett post hoc test. All P values were two-sided and the significance level was set at P < 0.05. All statistical analyses were performed using the Statistical Package for the Social Sciences (version 20.0; SPSS Inc.).

Results

Associations between HSP90 expression and clinicopathologic characteristics

HSP90 staining was localized in both the nucleus and cytoplasm of cholangiocarcinoma cells (Fig. 1). HSP90 positivity (determined as in Materials and Methods) was observed in 79 of the 177 IHCCs (44.6%), and in 73 of the 222 EHCCs (32.8%).

The relationship between HSP90 expression and clinicopathologic factors is shown in Table 1A and 1B (IHCC and EHCC, respectively). Increased expression HSP90 in IHCC showed a significant association with macroscopic type (P = 0.001), histologic classification (P = 0.002), intrahepatic metastasis (P = 0.006), pathologic tumor status (P = 0.013), lymph node metastasis (P = 0.032), and pathologic stage (P = 0.007). On the other hand, HSP90 expression was associated only with preoperative CA19-9 level (P < 0.001) in EHCC. To elucidate the biologic significance of HSP90, we microscopically examined positive cases in detail and compared their expression with histologic components. HSP90 tended to be expressed in the poorly differentiated component, which is characterized by infiltration in IHCC with significantly difference (Table 1A). No significant association was observed between HSP90 expression and that of any other client proteins (c-MET, EGFR, and VEGF).

HSP90 expression is an independent prognostic factor for both IHCC and EHCC

The 5-year survival rates for patients with IHCC and EHCC were 28.2% and 32.0%, respectively. The 5-year survival for IHCC patients with HSP90-positive and HSP90-negative tumors were 15.2% and 38.7% (P < 0.001), and those for EHCC patients were 16.4% and 33.6% (P < 0.001), respectively (Fig. 2).

The results of multivariate analysis following univariate analysis regarding overall survival and tumor recurrence are shown in

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Supplementary Table S1 (IHCC) and Supplementary Table S2 (EHCC). Multivariate analysis identified HSP90 expression (HR, 1.859; 95% CI, 1.258–2.747; P = 0.002), preoperative CA19-9 level (HR, 1.449; 95% CI, 1.324–3.067; P = 0.001), intrahepatic metastasis (HR, 2.093; 95% CI, 1.370–3.198; P = 0.001), and lymph node metastasis (HR, 2.328; 95% CI, 1.543–3.512; P < 0.001).

Table 1B. Associations between HSP90 expression and clinicopathologic factors in EHCC

<table>
<thead>
<tr>
<th>EHCC characteristics</th>
<th>HSP90</th>
<th>P</th>
<th>HSP90</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (n = 149)</td>
<td>Positive (n = 73)</td>
<td></td>
<td>Negative (n = 149)</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>66.0 (33–84)</td>
<td>66.0 (43–80)</td>
<td>0.961</td>
<td>Bile duct margin</td>
</tr>
<tr>
<td></td>
<td>Male 110</td>
<td>53</td>
<td>0.707</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Female 39</td>
<td>20</td>
<td></td>
<td>Other organ invasion</td>
</tr>
<tr>
<td>Preoperative CEA level</td>
<td>≤5.0 U/mL 132</td>
<td>63</td>
<td>0.337</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>&gt;5.0 U/mL 16</td>
<td>10</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Preoperative CA19-9 level</td>
<td>≤37 U/mL 71</td>
<td>17</td>
<td>&lt;0.001*</td>
<td>T1</td>
</tr>
<tr>
<td></td>
<td>&gt;37 U/mL 77</td>
<td>56</td>
<td></td>
<td>T2</td>
</tr>
<tr>
<td>Average tumor size</td>
<td>c (range)</td>
<td>4.1 (0.6–11.5)</td>
<td>4.2 (1.2–10.0)</td>
<td>0.707</td>
</tr>
<tr>
<td></td>
<td>Nonpolyploid</td>
<td>122</td>
<td>65</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Polypoid</td>
<td>27</td>
<td>8</td>
<td>Positive</td>
</tr>
<tr>
<td>Histologic classification (differentiated)</td>
<td>0.106</td>
<td></td>
<td></td>
<td>UICC pathologic stage status</td>
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<td></td>
<td>Papillary and well</td>
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<td>17</td>
<td>IA + IB</td>
</tr>
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<td></td>
<td>Moderately</td>
<td>74</td>
<td>46</td>
<td>IIA + IIB</td>
</tr>
<tr>
<td></td>
<td>Poorly</td>
<td>18</td>
<td>10</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Depth of tumor invasion</td>
<td>0.129</td>
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<td>IVA + IVB</td>
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<td></td>
<td>Within fm</td>
<td>14</td>
<td>3</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Beyond fm</td>
<td>135</td>
<td>70</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>Lymphatic invasion</td>
<td>0.199</td>
<td></td>
<td>EGFR</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>88</td>
<td>38</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>61</td>
<td>35</td>
<td>Positive</td>
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<td>Venous invasion</td>
<td>0.358</td>
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<td>VEGF</td>
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<td></td>
<td>Negative</td>
<td>89</td>
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<td>Positive</td>
<td>60</td>
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<td></td>
<td>Perineural invasion</td>
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<td>HER2</td>
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<td>Negative</td>
<td>56</td>
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</tr>
<tr>
<td></td>
<td>Positive</td>
<td>91</td>
<td>60</td>
<td>Positive</td>
</tr>
</tbody>
</table>

NOTE: HSP90 expression was associated only with preoperative CA19-9 level (P < 0.001) in EHCC.

*aSignificantly different (P < 0.05).

Supplementary Table S1 (IHCC) and Supplementary Table S2 (EHCC). Multivariate analysis identified HSP90 expression (HR, 1.859; 95% CI, 1.258–2.747; P = 0.002), preoperative CA19-9 level (HR, 1.449; 95% CI, 1.324–3.067; P = 0.001), intrahepatic metastasis (HR, 2.093; 95% CI, 1.370–3.198; P = 0.001), and lymph node metastasis (HR, 2.328; 95% CI, 1.543–3.512; P < 0.001).

Figure 2. Survival curves of overall survival for IHCC and EHCC patients stratified according to HSP90 expression (Kaplan–Meier method). The outcome for HSP90-positive IHCC and EHCC cases was significantly worse than that for HSP90-negative cases (P < 0.001 and P < 0.001, respectively; log-rank test). All data are expressed as the mean ± SD.

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However, whereas the phosphorylation of STAT3 was inhibited in the NCC-BD3 and NCC-CC4-2 cell lines (both sensitive to NVP-AUY922), it was not inhibited in the TKKK cell line (refractory to NVP-AUY922). NVP-AUY922 treatment led to a reduction in AKT, pAKT, EGFR, pEGFR, HER2, and pHER2 protein expression in all three cell lines (Fig. 4).

**Discussion**

This study has reported HSP90 expression in the largest cohort of cholangiocarcinoma reported so far, and demonstrated that HSP90 expression and antiproliferative activity of NVP-AUY922 in vitro

We next examined the effect of NVP-AUY922 on cholangiocarcinoma cell proliferation and performed IHC analysis of HSP90 expression in 17 cholangiocarcinoma cell lines (Supplemental Table S3). One of the 11 IHCC cell lines was positive for HSP90 (9.1%) whereas two of six EHCC cell lines were positive (33.3%). The IC50 values for NVP-AUY922 in the IHCC cell lines ranged from 9 to 950 nmol/L, whereas that in the EHCC cell lines ranged from 16 to 42 nmol/L (Supplementary Table S3). HSP90 expression in NCC-BD3 xenografts compared with that of control (saline; Fig. 5A). Both the proliferation index (Fig. 5B) and the drug sensitivity judged by IC50 in vivo xenograft model (Table S3). The IC50 values for NVP-AUY922 in cholangiocarcinoma cell lines were positive for HSP90 staining were not always refractory to NVP-AUY922. To further evaluate the downstream effect of HSP90 inhibition, we selected three cell lines (NCC-BD3 and NCC-CC4-2, which were HSP90-positive and NVP-AUY922-sensitive, and TKKK, which was HSP90-negative and refractory to NVP-AUY922; Fig. 3) and examined the expression of HSP90 client proteins, particularly those associated with the growth of cholangiocarcinoma (23, 24). We examined changes of EGFR and HER2 protein expression, and the phosphorylation status of their downstream molecules (AKT, MAPK, and STAT3) in the three cell lines after NVP-AUY922 treatment. NVP-AUY922 inhibited the phosphorylation of AKT and MAPK in all three cell lines. However, whereas the phosphorylation of STAT3 was inhibited in the NCC-BD3 and NCC-CC4-2 cell lines (both sensitive to NVP-AUY922), it was not inhibited in the TKKK cell line (refractory to NVP-AUY922). NVP-AUY922 treatment led to a reduction in AKT, pAKT, EGFR, pEGFR, HER2, and pHER2 protein expression in all three cell lines (Fig. 4).

**Antiproliferative activity of NVP-AUY922 in vivo**

Based on the in vitro result, we selected NCC-BD3 to construct xenograft model and then used this model to examine the effect of NVP-AUY922 in vivo. Treatment with NVP-AUY922 (2 × 50 mg/kg/week or 2 × 25 mg/kg/week) led to a significant reduction in the growth of NCC-BD3 xenografts compared with that of control (saline; Fig. 5A). Both the proliferation index (Fig. 5B) and the expression of HSP90 in tumor tissues were significantly lower in the NVP-AUY922-treated groups (Fig. 5C).
increased HSP90 expression was significantly associated with decreased overall and disease-free survival (Supplementary Fig. S1) in patients with IHCC and EHCC. HSP90 expression was often higher in poorly differentiated IHCC. HSP90 expression in poorly differentiated IHCC cases with metastasis was higher than that in well-to-moderately differentiated cases without metastasis, indicating that overexpression of HSP90 is related to progression and metastasis. This finding is supported by previous reports (25, 26). Yamada and colleagues (26) found that HSP90 plays an important role in cellular differentiation, and Milicevic and colleagues (25) reported that elevated expression of HSP90 in cases of metastatic colorectal cancer is often associated with more invasive and poorly differentiated components with metastasis.

HSP90 is a potential drug target; indeed, a number of HSP90 inhibitors are in clinical trials (27). AT13387, a nonansamycin HSP90 inhibitor provided by Astex Pharmaceuticals, was reported to suppress cell growth of various cancer types (non–small-cell lung carcinoma, and nasopharyngeal carcinoma) by previous reports (28, 29). No previous study reported the effectiveness of AT13387 for cholangiocarcinoma cell lines; however, Shapiro and colleagues reported a phase I study of AT13387 for solid tumors, including one cholangiocarcinoma patient. AT13387 administered once or twice weekly showed an acceptable safety profile and demonstrated evidence of target engagement (30). Here, we found that NVP-AUY922 had potent antiproliferative effects and reduced growth-associated signaling both in vitro and in vivo. Ki-67 was significantly decreased in all NVP-AUY922–treated groups of NCC-BD3 xenograft models (Fig. 5B). The antitumor effect of NVP-AUY922 in this model appears to be mediated by inhibiting tumor cell proliferation. These data suggested that there was a correlation HSP90 expression and proliferation of tumor cell. Recently Chen and colleagues reported that NVP-AUY922 has potent cytotoxic effects against cholangiocarcinoma cells, and reduces cancer cell motility in vitro (23). When, we examined the association between HSP90 expression and NVP-AUY922-sensitivity in cholangiocarcinoma cell lines, and we found that all HSP90-positive cell lines were sensitive to NVP-AUY922. This suggests that HSP90 expression of IHC may be a potential positive predictive marker for NVP-AUY922-sensitivity in cholangiocarcinoma. However, some cholangiocarcinoma cells showing low expression of HSP90 were also sensitive, suggesting that diversities of downstream targets regulated by HSP90 (e.g., STAT3) could influence the responsiveness to NVP-AUY922 treatment. Alternatively, TKK, an NVP-AUY922 refractory cell line, showed highly active EGFR signaling (high pEGFR, pHER, and pAKT) and elevated HER2 expression at the baseline compared with the sensitive cell lines. Therefore, activation level of these downstream signaling could also be associated with the drug sensitivity. HSP90 inhibitors have been used to interfere with a broad range of oncogenic signaling components in tumor cells (31). We found that NVP-AUY922 treatment inhibited the phosphorylation of AKT, STAT3, and MAPK in cholangiocarcinoma cells; all of these molecules are important signaling components for cell proliferation and angiogenesis. AKT requires a functional HSP90/CDC37 complex to remain stable; however, NVP-AUY922 induces ubiquitination of AKT, a process that targets it to the proteasome where it is degraded (32). Inhibiting HSP90 downregulates the phosphatidylinositide-3-kinase pathway, which is due (at least in part) to the degradation of AKT and its upstream effectors (e.g., EGFR and HER2; refs. 33, 34). Moreover, a previous study shows that NVP-AUY922 has an antiangiogenic effect on pancreatic cancer cells. As a functional consequence of these multifactorial effects on cancer...
cells, as well as on the tumor microenvironment, HSP90 targeting by NVP-AUY922 translates into potent inhibition of both tumor growth and vascularization in cholangiocarcinoma models in vivo (12). In conclusion, the results of the present study show that HSP90 expression is an independent prognostic factor for cholangiocarcinoma. The preclinical results reported herein also suggest that NVP-AUY922 may have potential utility as a postoperative adjuvant therapy for these tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T. Shirota, H. Ojima, T. Shibata
Development of methodology: T. Shirota, T. Shibata
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Shirota, H. Ojima, N. Hiroaka, K. Shimada, Y. Ara, T. Shibata
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Shirota, H. Ojima, H. Ojima, K. Rokutan, Y. Kanai, T. Shibata
Writing, review, and/or revision of the manuscript: T. Shirota, H. Ojima, T. Shibata

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Tomoki Shirota, Hidenori Ojima, Nobuyoshi Hiraoka, et al.


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