A Promising Serum Autoantibody Marker, Anti-Heat Shock Protein 90α, for Cholangiocarcinoma

Sirintip Boonjaraspyino1,2, Amornrat Juasook3, Thidarut Boonmars2,4*, Ratchadawan Aukkanimart2,4, Atit Silsirivanit2,6, Watcharin Loilome2,6, Praneet Sirira2,5, Zhiliang Wu7, Panaratana RatanaSuwan8

Abstract

The present study was designed to investigate cholangiocarcinoma (CCA) antibodies in hamster serum. Hamster CCA cell lines were processed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. A candidate biomarker was confirmed by immunoprecipitation and western blot, and was further analyzed using ELISA and sera from normal control hamsters, hamsters with opisthorchiasis and hamsters with various stages of CCA, as well as from CCA patients and healthy individuals. One candidate marker was identified as HSP90α, as indicated by a high level of anti-HSP90α in hamster CCA sera. It was found that the levels of anti-HSP90α were specifically elevated in the sera of hamsters with CCA compared with other groups and progressively increased with the clinical stage. At the cut-off point of 0.4850 on the receiver operating characteristic curve, anti-HSP90α could discriminate CCA from healthy control groups with a sensitivity of 76.2%, specificity of 71.4% and total accuracy 75.5%. In the present study, we have shown that anti-HSP90α may be a potential useful serum biomarker to discriminate CCA cases from healthy persons.

Keywords: Serum autoantibody marker - anti-heat shock protein 90α - liver fluke - cholangiocarcinoma

Introduction

Cholangiocarcinoma (CCA) is a carcinoma arising from the epithelium of intrahepatic and extrahepatic bile ducts. The highest prevalence of CCA is in northern Thailand, an endemic area of the liver fluke, *Opisthorchis viverrini* (OV), infestation (IARC, 2011; Sithithaworn et al., 2014), a serious medical problem in this region and one that is causally linked to the development of CCA (IARC, 2011). CCA is an incurable and rapidly lethal disease if the tumors cannot be removed completely. In non-resectable hilar CCA patients, the overall median and 1-, 3-, and 5-year survival rates were 13 months and 52, 12 and 7%, respectively (Ruys et al., 2012). The median overall survival rate for resected patients is 18.9 months versus 5.0 months for patients not resected (Schiffman et al., 2011). Patients may receive palliative chemotherapy or radiation therapy, but these are not effective for treatment of late-stage disease (Friman, 2011). Unfortunately, CCA is asymptomatic in its early stages and first diagnosis is usually at an advanced stage (Khan et al., 2012). Therefore, the prognosis of CCA is poor and survival time is short (Schiffman et al., 2011; Khan et al., 2012; Ruys et al., 2012).

In order to find candidate biomarkers, hamster CCA cell lines were analyzed using one-dimensional SDS-PAGE, followed by identification of protein using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and ELISA assay.

Materials and Methods

Human ethics

Human sera were provided from the Liver Fluke and Cholangiocarcinoma Center, Khon Kaen University and approved by the Ethics committee Khon Kaen University based on the principles of the Declaration of Helsinki and ICH-CCP (HE551236).

Parasite preparation

Fresh water fish were bought from the local market in Khon Kaen Province, northeast Thailand, an area endemic for opisthorchiasis. The fresh fish were digested using 0.25% pepsin-1.5% HCl solution, incubated at 37°C for

*For correspondence: bthida@kku.ac.th; boonmars@yahoo.com
1 h, filtered through 1000, 300, 250 and 106 µm sieves and metacercariae were recovered via sedimentation in normal saline in a sedimentation jar. OV metacercariae, which have a double-walled cyst, oval shape, and oral and ventral suckers, could be clearly seen under a dissecting microscope. Each hamster was infected with 50 metacercariae by intragastric intubation.

Animals

Syrian hamsters, 6 weeks old, from the Animal Unit, Faculty of Medicine, Khon Kaen University, were divided into three groups (5 hamsters per group) as follows: group 1; uninfected (control); group 2; OV-infected (OV) and group 3; infected with OV and administered NDMA (OVN). Hamsters were sacrificed at 1, 2, 3 and 6 months post-infection. Whole blood (3 ml, by heart puncture) was collected separately from each hamster at the time of sacrifice and the sera kept at -20°C for immune-western blotting assay. CCA masses collected from livers of group 3, hamsters were used to establish hamster CCA cell lines. All work was conducted with the approval of the Khon Kaen University Animal Ethics Committee (AEKKU51/2555).

Administration of NDMA for induction of cholangiocarcinoma

NDMA (final concentration 12.5 ppm in drinking water) was administered every day from day 1 of the experiment for 2 months for inducing cholangiocarcinoma (Juasook et al., 2013).

Establishment of hamster CCA cell lines

Primary cholangiocarcinoma Syrian hamster cell lines were cultured in 10% Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B (antibiotic-antimycotic, Invitrogen). Cells were maintained in an incubator with humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cell culture techniques were performed using standard protocols (Boonjaraspinyo et al., 2012b).

Hamster CCA cell line protein extraction

Cells were allowed to grow until 80-90% confluent and were subsequently harvested using trypsin digestion and then washed three times with phosphate buffered saline. Approximately 3x10⁶ cells were then spun down in a 1.5 ml microcentrifuge tube (4°C, 10 minutes, 220g), 500 µl of PBS added to the pellets in the 1.5 ml centrifuge tube and the cells disrupted on ice using an ultra-sonicator (Vibra Cell™, Inc, USA). Following centrifugation, the supernatant was collected and the elution buffer and the process repeated. The two 50 µl supernatant complexes were washed with 0.5 ml of the immunoprecipitation buffer, followed by centrifugation for 2-3 min in a microcentrifuge and the supernatant discarded. This washing procedure was repeated at least six times. The bound antigen-antibody complex from the immobilized protein A or G was eluted by incubation for five min with 50 µl of the elution buffer. After centrifugation, the supernatant was collected and the gel incubated (five minutes) with another 50 µl of the elution buffer and the process repeated. The two 50 µl supernatant samples were combined and immediately adjusted to a physiological pH by addition of a suitable, more concentrated buffer such as 1.0 M Tris, pH 7.5 (10 µl of this buffer to 100 µl of the supernatant was usually sufficient). The eluted fraction was desalted. The sample was then ready for gel electrophoresis.

Gel electrophoresis and immune-western blotting using antibodies

Approximately 3x10⁶ cells were then spun down in a 1.5 ml microcentrifuge tube (4°C, 10 minutes, 220g), 500 µl of PBS added to the pellets in the 1.5 ml centrifuge tube and the cells disrupted on ice using an ultra-sonicator (Vibra Cell™, Inc, USA). Following centrifugation, the supernatant was transferred to a new 1.5 ml centrifuge tube and the protein concentration measured using a spectrophotometer at 280 nm. Tubes were then kept at -80°C until use.

Gel electrophoresis and immune-western blotting using hamster CCA lysate and OV sera

15 µg of whole cell lysate with an equal volume of 2x electrophoresis sample buffer was boiled for 2-3 min. Up to 10 µl of lysate was loaded into each well of duplicate 12% polyacrylamide gels of 0.75 mm thickness (Amersham™ ECL™ Gel 12% (GE Healthcare Bio-Science, Sweden). One gel was stained with Coomassie blue (AppliChem, Germany) and the other was used to transfer proteins to a nitrocellulose membrane (Bio-Rad Laboratories, USA) using an electro-blotting apparatus according to the manufacturer’s protocols. The Coomassie blue-stained gel was dried and kept for gel digestion. The nitrocellulose membrane on which proteins from the other gel were blotted was blocked against non-specific binding using 0.5% skim milk at 4°C overnight in a covered box. The membrane was then washed three times for five min each with TBST (10mM Tris, 154mM NaCl, pH7.5 plus 0.1% Tween 20). The membrane was then incubated with each OV serum (dilution of 1:100 in 0.5% skim milk) at 1, 2 and 3 month(s) and OVN serum at 1, 2, 3 and 6 months at 37°C for 1.5h and then washed three times for five min each with TBST. The membrane was then incubated with a 2nd antibody, hamster IgG peroxidase (Rabbit Envision (Dako North America, Inc.) at a dilution of 1:10000 in 0.5% skim milk at 37°C for 1.5h and finally washed three times for five min each with TBS and developed using the ECL Gel Box (GE Healthcare Bio-Science, Sweden). Photos were taken using Molecular Imager™ Gel Doc™ XR (Bio-Rad Laboratories, Inc.).

Gel electrophoresis and immune-western blotting for anti-HSP90α

Preparation of immunoprecipitation: In brief, hamster CCA antigen was solubilized in 50 µl of immunoprecipitation buffer and a molar excess of hamster sera or HSP90α added to the protein solution containing the antigen of interest. The volume of the sample was adjusted to 0.2 ml with immunoprecipitation buffer and the sample incubated overnight at 4°C. An appropriate amount of immobilized protein A or G was added to the antigen-antibody complex and the sample incubated with gentle mixing for 2h at room temperature. The immobilized protein A or G-bound complexes were washed with 0.5 ml of the immunoprecipitation buffer, followed by centrifugation for 2-3 min in a microcentrifuge and the supernatant discarded. This washing procedure was repeated at least six times. The bound antigen-antibody complex from the immobilized protein A or G was eluted by incubation for five min with 50 µl of the elution buffer. After centrifugation, the supernatant was collected and the gel incubated (five minutes) with another 50 µl of the elution buffer and the process repeated. The two 50 µl supernatant samples were combined and immediately adjusted to a physiological pH by addition of a suitable, more concentrated buffer such as 1.0 M Tris, pH 7.5 (10 µl of this buffer to 100 µl of the supernatant was usually sufficient). The eluted fraction was desalted. The sample was then ready for gel electrophoresis.
of each group using TRIZOL.

Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The isolated RNA was treated with DNase I (RNase-Free DNase, Fermentas Inc., Ontario, Canada) in the presence of ribonuclease inhibitor (Fermentas Inc., Ontario, Canada). The treated RNA was extracted with phenol/ chloroform, precipitated with ethanol, and dissolved in RNase-free water. Reverse transcription was performed using M-MLV Reverse transcriptase (Fermentas Inc., Ontario, Canada) according to the manufacturer’s instructions. In brief, 3 µg of total RNA from each sample was placed in a new tube and 1 µl of oligo(dT) 18 primers added followed by DEPC-treated water to 12 µl. The sample was gently mixed, spun down, and incubated at 70°C for 5 minutes. On ice, the following were added: 5X reaction buffer 4 µl; ribonuclease inhibitor 1 µl, 10 mM dNTP mix 2 µl. Each tube was mixed gently and incubated at 37°C for 5 minutes. After that, 1 µl of M-MLV reverse transcriptase was added. The reaction was incubated at 42°C for 60 min and then inactivated by heating at 70°C for 10 min (Boonmars et al., 2011).

Statistical analyses

We used SPSS software version 17.0 (SPSS Inc., USA) to conduct all statistical comparisons. The difference between two independence groups were analyze by Student’s t-test. One-way ANOVA was performed for compare means of three or more samples group. Two-tailed P values of 0.05 or less were considered significant. Receiver operating characteristic (ROC) analysis was used to detect the optimal cut-off points (i.e. those with the highest total accuracy) for separating CCA from other tested groups.

Results

1D gel electrophoresis and immunowestern blotting from hamster sera

Figure 1 shows the result of Coomassie blue-stained gels using hamster CCA lysate and membrane-transferred hamster CCA lysate incubated with hamster sera. A cross reaction was found at about 64-98 kDa in all serum groups but the intensity of the band differed. Intensity was very low when normal hamster serum was used and increased
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with duration of the OV infection, 1, 2 and 3 month(s) post infection. For the CCA hamster model (OV plus NDMA), the cross-reaction band was more intense, especially at 3 and 6 months.

Protein identification and gene expression in hamster CCA model

Coomassie blue-stained gels (Figure 2) were estimated bands with nitrocellulose membrane band and cut for protein analysis. Protein identification was differentially expressed in hamster CCA cells. Ions score is $-10^\log (P)$, where P is the probability that the observed match is a random event. Individual ions scores $>34$ indicate identity or extensive similarity ($p<0.05$). These differentially expressed protein bands were furthermore identified by in-gel trypsin digestion and the subsequent MALDI-TOF MS and Peptide mass matching identified two different protein products that were differentially expressed in hamsters with CCA. The identifications were based on protein sequence coverage 48% matched peptide matching peptides, numbers statistically necessary for a confident match (Figure 3) and Table 1. Of the two proteins (HSP90α and elongation factor 2), only HSP90α was confirmed (Figure 4) and further studied. HSP90α gene expression was observed in all groups (Figure 5), normal control, OV alone and OVN but the expression was different in intensity due to upregulation in OVN at three months of CCA development. Relative to uninfected controls, HSP90α expression in OV infected hamsters was 2-5 fold greater and 2-14 fold greater in the OVN groups. The highest expression was observed at 2 months in both the OV and OVN groups and was statistically significantly higher in the OVN group ($P<0.001$). At 6 months, the HSP90α expression was lower than at 2 M in both groups but still higher than uninfected normal controls. In the OVN group, expression was significantly

Table 1. Proteins identified as Differentially Expressed in the Hamster CCA Cell Line

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Protein name</th>
<th>PI</th>
<th>Mr</th>
<th>MOWSE score</th>
<th>Matches</th>
<th>Sequence coverage (%)</th>
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<td>49</td>
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<td>45</td>
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<tr>
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<td>Heat shock protein HSP 90-alpha</td>
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<td>1022</td>
<td>57</td>
<td>41</td>
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<tr>
<td>EF2_MOUSE</td>
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<td>649</td>
<td>29</td>
<td>24</td>
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<td>1.67</td>
<td>96205</td>
<td>629</td>
<td>29</td>
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</tr>
</tbody>
</table>

*PI=the isoelectric point; Mr= average mass of the protein ; MOWSE score=Molecular Weight Search is a protein identification method using molecular weight of peptides

Figure 1. Immuno-western blotting of normal hamster (Normal), Opisthorchis viverrini infected for 1 month (OV1M), O. viverrini infected for 2 months (OV2M), O. viverrini infected for 3 months (OV3M), O. viverrini infected and NDMA administered for 1 month (OVN1M), O. viverrini infected and NDMA administered for 2 months (OVN2M), O. viverrini infected and NDMA administered for 3 months (OVN3M) and O. viverrini infected and NDMA administered for 6 months (OVN 6M)

Figure 2. Gel Electrophoresis and Selected Band for Protein Identification (Arrow)

Figure 3. Amino Acid Sequences of Peptides from Acrylamide Gels. There was 48% coverage shown in bold red is HSP90 α

Figure 5. Relative expression of HSP90α in hamster CCA cells. Expression was detected as a band at 98 kDa with three bands of 90.1, 90.2, and 90.3 kDa in the OVN group. Expression was statistically significantly higher (P<0.001) in the OVN group compared to normal controls.
higher (P<0.001) than in the OV group.

**Anti-HSP90α antibody levels in human and hamster sera assayed using ELISA**

Evaluation of antibody to HSP90α was performed using ELISA in human and hamster sera using the ROC curve analysis of serum antibody to HSP90α for discriminating CCA patients from healthy individuals (Figure 6). The area under the curve (AUC) was 0.7925 (95% confidence interval, 0.6198 to 0.9652), and the cut off value of the serum HSP90α optical density (OD) level was 0.4850 U ml\(^{-1}\) (sensitivity, 76.19%; specificity, 71.43%; LR+, 2.67).

In the hamster model, the HSP90α levels were determined at 1, 2, 3 and 6 month(s) p.i. in the three groups (Figure 7). The range of antibody to HSP90α in normal hamster sera at these time points was 0.115-0.155 U ml\(^{-1}\). The levels of anti-HSP90α in the group OV and OVN were higher than in normal hamster controls at 3 and 6 months. The mean serum level of anti-HSP90α increased from 0.115 in normal controls to 0.191 at 3 months and the highest was 0.206 at 6 months p.i. in the group infected with *O. viverrini* and given NDMA.

![Figure 4. Gel Electrophoresis and Western Blotting Analysis to Confirming the Anti-HSP90 α in Serum](image)

![Figure 5. HSP 90 α Gene Expression. *statistically significant difference (P<0.05)](image)

![Figure 6. ROC Curve Analysis of Serum Antibody to HSP90α for Discriminating CCA Patients from Healthy Individuals](image)

![Figure 7. Levels of Serum Antibody to HSP90α in the three Groups of Hamsters (normal control, *O. viverrini* Infection and Infection Plus NDMA;OVN) at Various Stages (1, 2, 3 and 6 Month(s) p.i.). The anti-HSP90α serum level was presented as the mean±SEM from five hamsters in each group. OVN was trend to higher than normal](image)

![Figure 8. Levels of Serum Antibody to HSP90α in CCA Patients Compared with Healthy Controls. *P-value < 0.05, determined using Student’s t-test.](image)
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CCA patients had a mean of 0.659±0.035 and ranged from 0.344 to 1.233. The anti-HSP90α levels in CCA patient sera were significantly higher than in healthy control sera by 1.5 fold (p=0.024, t-test).

The comparisons of serum levels of anti-HSP90α in people with O. viverrini infection, perportal fibrosis, CCA patients and healthy volunteers are shown in Figure 9. The serum levels of anti-HSP90α in O. viverrini infections patients were 0.504±0.043 U ml⁻¹ and ranged from 0.277 to 0.726 U ml⁻¹. Sera from perportal fibrosis patients were 0.545±0.031 U ml⁻¹ and ranged from 0.307 to 0.783 U ml⁻¹. The serum anti-HSP90α levels in people with O. viverrini infection, perportal fibrosis and CCA patients were higher than in healthy control serum (1.1, 1.2, and 1.5 fold, respectively—Figure 9). Anti-HSP90α levels in CCA patients (0.659±0.035) were higher than in patients with cirrhosis (0.538±0.039), hepatitis (0.598±0.089), and HCC CCA (0.642±0.076) but lower than in patients with colon cancer (0.811±0.052). However, there was no significant difference between patients with CCA and these diseases (Figure 10).

Discussion

The present study is the first report to show that the serum marker, anti-HSP90α, increased significantly in a time-dependent manner during CCA development, suggesting that anti-HSP90α is a promising diagnostic and or prognostic biomarker for CCA in the hamster model. HSPs are induced during heat shock and by other stimuli, such as infections, inflammation, oxidative stress and growth factors (Kim and Yenari, 2013; Tomcik et al., 2014). These proteins correct the conformation of misfolded proteins and helps incorrigibly misfolded proteins to be removed (Hong et al., 2013). HSPs provide either the stability or the proteasomal degradation of selected proteins under stress conditions thus contributing to cell survival (Jego et al., 2013). HSPs have been classified according to their molecular weight (Jego et al., 2013). HSP90 is an important chaperone that interacts with and refolds its client proteins in a cycle that is driven by the binding and hydrolysis of ATP (Patel et al., 2011). In normal condition, HSPs have been shown to act as intercellular signaling molecules, which merits their inclusion among hormones, cytokines, and growth factors (Khalil et al., 2011). Normal cells, HSP90 is response to tissue injury from the environmental insults, including heat, hypoxia, UV, gamma-irradiation, reactive oxygen species (ROS), injury-released growth factors but tumor cells also recognize the usefulness of HSP90 in tissue invasion and metastasis (Li et al., 2012). HSP90 is implicated in maintaining the conformation, stability, activity and cellular localization of several key oncogenic client proteins that are involved in signal transduction pathways leading to the proliferation, cell-cycle progression, apoptosis and angiogenesis (Messaud et al., 2011). HSP90 is over-expressed in cancer cells and several of its client proteins are signaling oncoproteins that represent nodal points in multiple oncogenic signaling pathways (Hong et al., 2013). The anti-apoptotic action of HSP90 is also reflected by its capacity to interact with Akt/PKB, TNF-receptor signaling and NF-kB (Khalil et al., 2011). As mention above, HSP90α was observed in the normal cells and several tumors but there is no report of anti-HSP90α in the sera of any cancers, even in animal models. Interestingly, this is the first report to show anti-HSP90α profiles in hamster CCA and in sera of CCA patients together with HSP90α gene expression profiles in hamster CCA tissues and tissues from CCA patients (Boonjaraspinyo et al., 2012a).

The western blot of CCA hamster model showed a gradual increase in levels of anti-HSP90α in OVN at 3 and 6 months, similar to the ELISA results that showed antibody to HSP90α in CCA hamster sera was elevated at 3 and 6 months in the OVN group. For CCA patients, a previous study showed that gene expression of HSP90α was up-regulated in 76% of patients (Boonjaraspinyo et al., 2012a), consistent with findings of the present study, in which the anti-HSP90α levels in patient sera significantly increased 1.5 fold compared to healthy volunteers (p=0.024, t-test). This evidence seems to suggest that if tissue has a high level of HSP90α, there should be a high level of anti-HSP90α in serum as well.

The ELISA analysis of sera from patients with various other liver diseases showed that anti-Hsp90α levels were slightly increased in O. viverrini infection and perportal fibrosis patients (1.1 and 1.2 fold, respectively) but not significantly so when compared with healthy volunteers. At the cut-off point of 0.4850 on the receiver operating characteristic curve, anti-HSP90α could discriminate CCA
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from healthy control groups with a sensitivity of 76.19%, specificity of 71.43% and total accuracy 75.51%. The serum antibody levels to HSP90α in CCA patients was higher than in cirrhosis, hepatitis, and HCC CCA, but lower than in colon cancer, and the difference was not significant. This finding was supported by the elevated HSP90α expression levels in colon carcinomas with one third of tumors (Drecoll et al., 2014).

In conclusion, anti-HSP90α is potentially a serological biomarker for hamster CCA. This autoantibody is present at concentrations related to the stage of CCA development. The serum level of anti-HSP90α, in combination with other markers, may be valuable for early detection of CCA and for prediction of the stage of the disease.

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