TITLE PAGE

Heat shock protein 90 is a potential therapeutic target in cholangiocarcinoma

Authors: Tomoki Shirota[1,2], Hidenori Ojima[3], Nobuyoshi Hiraoka[3], Kazuaki Shimada[4], Hirofumi Rokutan[1], Yasuhito Arai[1], Yae Kanai[3], Shinichi Miyagawa[2] and Tatsuhiro Shibata[1, 5]

Department and institution: 1 Division of Cancer Genomics, National Cancer Center Research Institute, Tokyo, Japan; 2 Division of surgery, Shinshu University School of Medicine, Nagano, Japan; 3 Division of Molecular Pathology, National Cancer Center Research Institute, Tokyo, Japan; 4 Hepatobiliary and Pancreatic Surgery Division, National Cancer Center Hospital, Tokyo, Japan; 5 Laboratory of Molecular Medicine, Human Genome Center. The institute of Medical Science, the University of Tokyo, Tokyo, Japan.

Running title: HSP90 expression concerned with cholangiocarcinoma

Keywords: cholangiocarcinoma; heat shock protein 90; NVP-AUY922;

immunohistochemistry.

Corresponding author contact information:

Tatsuhiro Shibata

Division of Cancer Genomics, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo, 104-0045, Japan

Tel: 81-3-3547-5137

FAX: 81-3-3545-3567

Email address: tashibat@ncc.go.jp

Conflict of interest: No potential of conflict of interest to disclose.

Funding: This work has supported in part by the Foundation for Promotion of Cancer Research (FPCR), Japan (To T. shirota), and by Grants-in-Aid from the Ministry of Health, Labour, Japan (Health and Labour Sciences Research Expenses for Commission and Applied Research for Innovative Treatment of Cancer), National Cancer Center Research and Development Funds (26-A-5) (To T. Shibata) and Grant-in-Aid for Scientific Research (C) Grant Number 26460501 (To H. Ojima). T. Shirota is a recipient

of a Research Resident Fellowship from the FPCR.

Word count: 4054 characteristics.

Total number of figures and tables: 5 figures and 1 table.

Abbreviations: CC, Cholangiocarcinoma; HSP90, heat shock protein 90; IHCC, intrahepatic cholangiocarcinoma; EHCC, extrahepatic cholangiocarcinoma; IHC, immunohistochemistry; CI, Confidence Interval; FBS, fetal bovine serum; $IC_{50,}$ the 50% inhibitory concentration;

Abstract

Cholangiocarcinoma (CC) 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 post-translational 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 CC. Immunohistochemical expression of HSP90 was assessed retrospectively in 399 CC cases and 17 human CC cell lines, along with the effect of a small-molecule HSP90 inhibitor (NVP-AUY922) on CC tumor growth and angiogenesis in human CC 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 CC cells *in vitro*. Furthermore, treatment of CC xenograft-bearing mice with NVP-AUY922 significantly inhibited growth at doses far below the maximum tolerated dose. HSP90 overexpression is a prognostic marker for CC. HSP90-targeted therapy may be an option for a subset of CC.

Introduction

Cholangiocarcinoma (CC) 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 pathological 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 CC 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 post-translational 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 E et al and Song CH et al reported that overexpression

of HSP90 was associated with poor outcome in breast carcinoma patients (14, 15). Chen et al reported association between HSP90 expression and clinicopathological characteristics and prognosis in patients with IHCC (16); however, no study has examined the associations between HSP90 expression and EHCC. To improve our understanding of the clinical significance of HSP90 in CC, 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 CC cell lines.

Materials and Methods

Patients

A total of 399 patients with CC 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 pathological 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 33-84 years (median, 65 years) and the follow-up period ranged from 3.7-253.2 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, Glostrup, Denmark). 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 deparaffinised in xylene and rehydrated through a graded ethanol series (50-100%) and endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide solution for 30 min. Antigen retrieval was performed by heating in 0.01 M citrate buffer in a

pressure cooker at 121℃ for 10min. The sections were then incubated for 1 hour at room temperature with mouse anti-HSP90 (1:500 dilution; Santa Cruz, USA), anti-human Ki67 (1:100 dilution; Chemicon, Temecula, CA, USA), anti-human HER2 (1:50 dilution; Dako, Glostrup, Denmark). After a washing in PBS, the sections were exposed to Envision+ Dual link reagent for 30 min at room temperature and then visualized using 3,3'-Diaminobenzidine tetrahydrochloride as the chromogen. Finally, the tissue sections were counterstained with haematoxylin.

Evaluation of immunohistochemistry results

All sections were evaluated by three researchers (T. Shirota, H. Ojima, and T. Shibata) who were blinded to the clinical and pathological data. Differences in interpretation were resolved by consensual agreement. HSP90 expression in CC was compared with that in the bile ducts. Nuclear or cytoplasmic staining of CC 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 each areas, so as to appraise the lesion as a

whole. Receiver-operator characteristic (ROC) curve analysis was performed for predictive variables and an explorative cut-off 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 percent Confidence Interval (CI): 0.59 to 0.70). The cut-off 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 TGBC24TKB cells were purchased from RIKEN BioResource Center or from the Japanese Collection of Research Bioresources. TKKK, TGBC24TKB, and HuCCT1 were derived from IHCC, and OZ was derived from EHCC. All cell lines were derived from Japanese patients. The originally established 13

CC cell lines and HuCCT1 were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin. TGBC24TKB, TKKK, and OZ were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 μg/ml penicillin. TKKK, HuCCT1, OZ and TGBC24TKB cells were authenticated by RIKEN BioResource Center or from the Japanese Collection Research Biorsources 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, NCC5, NCC-CC6-1, NCC-CC6-2, NCC-BD3, NCC-BD4-1 and NCC-BD4-2 were not authenticated by authors. TKKK, HuCCT1, OZ and TGBC24TKB 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, NCC5, 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 3rd generation and passed in culture for no more than 4 months after being thawed from authentic stocks. The culture media was replaced every2-3 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 (Basel, Switzerland). For the *in vitro* experiments, stock solutions of NVP-AUY922 (10 mM) were prepared in 100% DMSO and stored at -20℃. 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 mM lactic acid or 2.5% ethanol, 20% 50 mM 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-tetra zolium, inner salt (MTS) assay using the CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 3000 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 μM). After 72 hour, 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 ± S.D.

Western blot analysis investigating molecular effects of NVP-AUY922 *in vitro*

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 5 times the 50% inhibitory concentration (IC_{50}) and then harvested at the indicated time points. Briefly, sub-confluent cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, a complete protease inhibitor cocktail (Roche, Basel, Switzerland) and a phosphate inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) at 4℃ for 30 min. Equal amounts (40 μg) of cell extract were then electrophoresed, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and

immunoblotted with the following antibodies (all purchased from Cell Signaling Technology, Danvers, Ma, USA): 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 (New Jersey, USA) and ß-actin (PM053, rabbit polyclonal) was purchased from MBL (Aichi, Japan). 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 (Tokyo, Japan). A total of 8×10^6 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 mm^3. 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 haematoxylin-eosin and then stained for Ki67 (a proliferation marker), HSP90, and HER2 to examine the histological effects of NVP-AUY922.

Statistical analysis

The associations between the IHC results and clinicopathological factors were assessed using the χ^2 and Fisher's 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's 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 stage, which are composed of other factors. Between-group comparisons of response to NVP-AUY922 (tumor volume, proliferation index, and HSP90 expression) were estimated using one-way analysis of variance (ANOVA) followed by Dunnett's 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., Chicago, Illinois, USA).

Results

Associations between HSP90 expression and clinicopathological characteristics

HSP90 staining was localized in both the nucleus and cytoplasm of CC cells (Figure 1). HSP90 positivity (determined as in the Methods section) 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 clinicopathological factors is shown in Tables 1A and 1B (IHCC and EHCC, respectively). Increased expression HSP90 in IHCC showed a significant association with macroscopic type ($P < 0.001$), histological classification ($P = 0.002$), intrahepatic metastasis ($P = 0.008$), 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 biological significance of HSP90, we microscopically examined positive cases in detail and compared their expression with histological 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 was 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 (Figure 2).

The results of multivariate analysis following univariate analysis regarding overall survival and tumor recurrence are shown in 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) as independent prognostic factors for overall survival in IHCC. HSP90 expression was also an independent prognostic factor (HR, 2.236; 95% CI, 1.553-3.216; P <0.001) for overall survival in EHCC, along with lymph node metastasis (HR, 2.135; 95% CI, 1.461-3.120; P< 0.001). Multivariate analysis also identified HSP90 expression as a significant risk factor for tumor recurrence in IHCC (HR, 1.821; 95% CI, 1.238-2.679: P =0.002), along with preoperative CEA level (HR, 1.711; 95% CI, 1.314-2.774: P =0.012), preoperative CA19-9 level (HR, 1.984; 95% CI, 1.314-2.998: P

=0.001), intrahepatic metastasis (HR, 2.361; 95% CI, 1.546-3.334; P <0.001), and lymph node metastasis (HR, 2.234; 95% CI, 1.497-3.334; P <0.001). HSP90 expression (HR, 1.930; 95% CI, 1.344-2.772; P <0.001) and lymph node metastasis (HR, 1.986; 95% CI, 1.374-2.871; P<0.001) were independent risk factors for tumor recurrence in EHCC cases.

HSP90 expression and anti-proliferative activity of NVP-AUY922 *in vitro*

We next examined the effect of NVP-AUY922 on CC cell proliferation and performed IHC analysis of HSP90 expression in 17 CC cell lines (Supplementary Table S3). One of the 11 IHCC cell lines was positive for HSP90 (9.1%) whereas 2/6 EHCC cell lines were positive (33.3%). The IC_{50} values for NVP-AUY922 in the IHCC cell lines ranged from 9-950 nM, whereas that in the EHCC cell lines ranged from 16-42 nM (Supplementary Table S3), indicating a difference in sensitivity between IHCC and EHCC cell lines. Comparing HSP90 expression with drug sensitivity judged by IC_{50} of NVP-AUY922 in CC cell lines, cell lines, which are positive of HSP90 staining, were all sensitive to NVP-AUY922, while cell lines which are negative 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) (Figure 3) and examined the expression of HSP90 client proteins, particularly those associated with the growth of CC (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 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 (Figure 4).

Anti-proliferative 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)

(Figure 5A). Both the proliferation index (Figure 5B) and the expression of HSP90 in tumor tissues were significantly lower in the NVP-AUY922-treated groups (Figure 5C).

Discussion

This study has reported HSP90 expression in the largest cohort of CC reported so far, and demonstrated that increased HSP90 expression was significantly associated with decreased overall and disease free survival (Supplementary Figure 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 et al. found that HSP90 plays an important role in cellular differentiation, and Milicevic et al. 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 non-ansamycin HSP90 inhibitor was provided by Astex Pharmaceuticals (Cambridge, United Kingdom) 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 Gl et al 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 anti-proliferative 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 (Figure 5B). The antitumor effect of NVP-AUY922 in this model appears to be mediated by inhibiting tumor cell proliferation. This data suggested that there was a correlation HSP90 expression and proliferation of tumor cell. Recently Chen et al. reported that NVP-AUY922 has potent cytotoxic effects against CC cells, and reduces cancer cell motility *in vitro* (23). When, we examined the association between HSP90 expression and NVP-AUY922-sensitivity in CC 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 CC. However, some CC 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, TKKK, an NVP-AUY922 refractory cell line, showed highly active EGFR signaling (high pEGFR, pHER and pAKT) and elevated HER2 expression

at the baseline compared to 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 CC 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) (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 CC models *in vivo* (12).

In conclusion, the results of the present study show that HSP90 expression is an independent prognostic factor for CC. The preclinical results reported herein also suggest that NVP-AUY922 may have potential utility as a post-operative adjuvant

therapy for these tumors.

Acknowledgements

National Cancer Center Biobank is supported by the National Cancer Center Research and Development Fund, Japan. Novartis (Basel, Switzerland) provided NVP-AUY922 at no cost.

References

1. Hezel AF, Zhu Ax. Systemic therapy for biliary tract cancers. Oncologist 2008; 13: 415-23.

2. Aljiffry M, Abdulelah A, Walsh M, Pelekian K, Alwayn I, Molinari M. Evidence-based approach to cholangiocarcinoma: a systemic review of the current literature. J Am Coll Surg 2009; 208: 134-47.

3. Blechacz B, Gores GJ. Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. Hepatology 2008; 48: 308-21.

4. Sia D, Tovar V, Moeini A, JM Llovet. Intrahepatic cholangiocarcinoma: pathogenesis and rationale for molecular therapies. Oncogene 2013; 32: 4861-70.

5. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer 2010; 10: 537-49.

6. Workman P, Burrows F, Neckers L, Rosen N. Drugging the cancer chaperone HSP90 combinatorial therapeutic exploitation of oncogene addiction and tumor stress. Ann N Y Acad Sci 2007; 1113: 202-16.

7. Sekimoto T, Oda T, Pozo FM, Murakumo Y, Masutani C, Hanaoka F, et al. The molecular chaperone Hsp90 regulates accumulation of DNA polymerase eta at replication stalling sites in UV-irradiated cells. Mol Cell. 2010: 37; 79-89.

8. Wainberg ZA, Anghel A, Rogers AM, Desai AJ, Kalous O, Conklin D, et al. Inhibition of HSP90 with AUY922 induces synergy in HER2-amplified trastuzumab-resistant breast and gastric cancer. Mol Cancer Ther 2013; 12: 509-19.

9. Garon EB, Finn RS, Hamidi H, Dering J, Pitts S, Kamranpour N, et al. The HSP90 inhibitor NVP-AUY922 potently inhibits non-small cell lung cancer growth. Mol Cancer Ther 2013; 12: 890-900.

10. Ueno T, Tsukuda K, Toyooka S, Ando M, Takaoka M, Soh J, et al. Strong anti-tumor effect of NVP-AUY922, a novel HSP90 inhibitor, on non-small lung cancer. Lung Cancer 2012; 76: 26-31.

11. Bao XH, Takaoka M, Hao HF, Fukazawa T, Yamatsuji T, Sakamura K, et al. Antiproliferative effect of the HSP90 inhibitor NVP-AUY922 is determined by the expression of PTEN in esophageal cancer. Oncol Rep 2013; 29: 45-50.

12. Moser C, Lang SA, Hackl C, Wagner C, Scheiffert E, Schlitt HJ, et al. Targeting HSP90 by the novel inhibitor NVP-AUY922 reduces growth and angiogenesis of pancreatic cancer. Anticancer Res 2012; 32 (7): 2551-62.

13. Okui T, Shimo T, Hassan NM, Fukazawa T, Kurio N, Takaoka M, et al. Antitumor effect of novel HSP90 inhibitor NVP-AUY922 against oral squamous cell carcinoma. Anticancer Res 2011; 31: 1197-204.

14. Pick E, Kluger Y, Giltane JM, Moeder C, Camp RL, Rimm DL, et al. HSP90 expression is associated with decreased survival in breast cancer. Cancer Res. 2007; 67: 2932-7.

15. Song CH, Park SY, Eom KY, Kim JH, Kim SW, Kim JS, et al. Potential prognostic value of heat-shock protein 90 in the presence of phosphatidylinositol-3-kinase overexpression or loss of PTEN, in invasive breast cancers. Breast Cancer Res. 2010; 12(2): R20.

16. Chen MH, Chiang KC, Cheng CT, Huang SC, Chen YY, Chen TW, et al. Antitumor activity of the combination of an HSP90 inhibitor and a PI3K/mTOR dual inhibitor against cholangiocarcinoma. Oncotarget 2014; 5: 2372-89.

17. Yoshikawa D, Ojima H, Iwasaki M, Hiraoka N, Kosuge T, Kasai S, et al. Clinicopathological and prognostic significance of EGFR, VEGF, and HER2 expression in cholangiocarcinoma. Br J Cancer 2008; 98: 418-25.

18. Miyamoto M, Ojima H, Iwasaki M, Shimizu H, Kokubu A, Hiraoka N, et al. Prognostic significance of overexpression of c-Met oncoprotein in cholangiocarcinoma. Br J Cancer 2011; 105, 131-8.

19. Hamilton SR, Aaltonen LA. World Health Organization Classification of Tumours: pathology & genetics: Tumours of the Digesitive System. Lyon: 2000; IARCPress.

20. Sobin LH, Gospodarowicz MK, Wittekind C. TNM Classification of Malignant Tumors. 7th ed. New York: 2009; Wiley-Blackwell.

21. Lim So, Park SG, Yoo JH, Park YM, Kim HJ, Jang KT, et al. Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and dysplastic nodules. World J Gastroenterol. 2005; 11: 2072-9.

22. Ojima H, Yoshikawa D, Ino Y, Shimizu H, Miyamoto M, Kokubu A, et al. Establishment of six new human biliary tract carcinoma, cell lines and identification of MAGEH1 as a candidate biomarker for predicting the efficacy of gemcitabine treatment. Cancer Sci 2010; 101: 882-8.

23. Chen MH, Lin KJ, Yang WL, Kao YW, Chen TW, Chao SC, et al. Gene expression-based chemical genomics identifies heat-shock protein 90 inhibitors as potential therapeutic drugs in cholangiocarcinoma. Cancer 2013; 119: 293-303.

24. Zhu AX, Hezel AF. Development of molecularly targeted therapies in biliary tract cancers: reassessing the challenges and opportunities. Hepatology 2011; 53: 695-704.

25. Milicevic Z, Bogojevic D, Mihailovic M, Petrovic M, Krivokapic Z. Molecular characterization of hsp90 isoforms in colorectal cancer cells and its association with tumour progression. Int J Oncol 2007; 32: 1169-78.

26. Yamada T, Hashiguchi A, Fukushima S, Kakita Y, Umezawa A, Maruyama T, et al. Function of 90 kDa heat shock protein in cellular differentiation of human embryonal carcinoma cells. In Vitro Cell Dev Biol Anim 2000; 36: 139-46.

27. Kim YS, Alarcon SV, Lee S, Lee MJ, Glaccone G, Neckers L, et al. Update on HSP90 in clinical trial. Curr Top Med Chem 2009; 9: 1479-92.

28. Graham B, Curry J, Smyth T, Fazal L, Feltell R, Harada I, et al. The heat shock protein 90 inhibitor, AT13387, displays a long duration of action in vitro and in vivo in non-small cell lung cancer. Cancer Sci. 2012; 103: 522-527.

29. Chan KC, Ting CM, Chan PS, LoMC, Lo KW, Curry JE, et al. A novel Hsp90 inhibitor AT13387 induces senescence in EBV-positive nasopharyngeal carcinoma cells and suppresses tumor formation. Mol Cancer. 2013; 12: 128. Doi: 10. 1186/1476-4598-12-128.

30. Shapiro Gl, Kwak E, Dezube BJ, Yule M, Ayrton J, Lyons J, et al. First-in-human phase I dose escalation study of second-generation non-ansamycin HSP90 inhibitor, AT13387, in patients with advanced solid tumors. Clin Cancer Res. 2015; 21: 87-97.

31. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. Nat Rev Cancer 2005; 5: 761-72.

32. Basso AD, Solit DB, Chiosis G, Giri B, Tsichlis P, Rosen N. Akt forms an

intracellular complex with Hsp90 and Cdc37 and is destabilized by inhibitors of Hsp90 function. J Biol Chem 2002; 277: 39858-66.

33. Jiao Y, Ou W, Meng F, Zhou H, Wang A. Targeting HSP90 in ovarian cancers with multiple receptor tyrosine kinase coactivation. Mol Cancer 2011; 10: 125.

34. Scaltriti M, Serra V, Normant E, Guzman M, Rodriguez O, Lim AR, et al. Antitumor activity of the HSP90 inhibitor IPI-504 in HER2-positive trastuzumab-resistant breast cancer. Mol Cancer Ther 2011; 10: 817-24.

Figure legends

Figure 1. HSP90 expression in tumor sections. (A)HSP90 is expressed in tumor cells (T) but not in the non-cancerous 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).

Abbreviations: HSP90, heat shock protein 90; IHC, immunohistochemistry;

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 ± S.D.

Abbreviations: IHCC, intrahepatic cholangiocarcinoma; EHCC, extrahepatic cholangiocarcinoma; HSP90, heat shock protein 90;

Figure 3. The anti-proliferative effects of NVP-AUY922 against CC cell lines *in vitro*. NCC-BD3 and NCC-CC4 cells were sensitive to NVP-AUY922 (IC_{50}s, 21 nM and 27 nM, respectively), whereas TKKK cells were considered refractory (IC_{50}, 950 nM). *Closed circles*, NCC-CC4-2: *closed triangles*, TKKK: and *closed squares*,

NCC-BD3. The horizontal axis indicates the concentration of NVP-AUY922 (μM), and the vertical axis shows the cell line/control ratio. All data are expressed as the mean ± S.D.

Abbreviations: CC, cholangiocarcinoma; $IC_{50,}$ the 50% inhibitory concentration;

Figure 4. Effects of NVP-AUY922 on activation of signaling cascades. NVP-AUY922 inhibited the phosphorylation of AKT and MAPK in all cell lines tested, whereas phosphorylation of STAT3 was inhibited in NCC-BD3 and NCC-CC4-2 (sensitive to NVP-AUY922) cell but not in TKKK (refractory to NVP-AUY922) cells.

Figure 5. Anti-tumor effects of NVP-AUY922 *in vivo*. (A) 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 the controls (saline). *Closed circles*, 50 mg/kg: *closed squares*, 25 mg/kg: and *closed triangles*, control. (B) The proliferation index (measured according to Ki-67 expression) decreased in all NVP-AUY922-treated groups. (C) HSP90 expression was significantly lower in the NVP-AUY922-treated group. Box plots present that upper bar means 90th and 10th percentile, box means between 25th and 75th percentile, and the line in the box means median.

Abbreviations: ANOVA, analysis of variance; HSP90, heat shock protein 90;

Figure 1

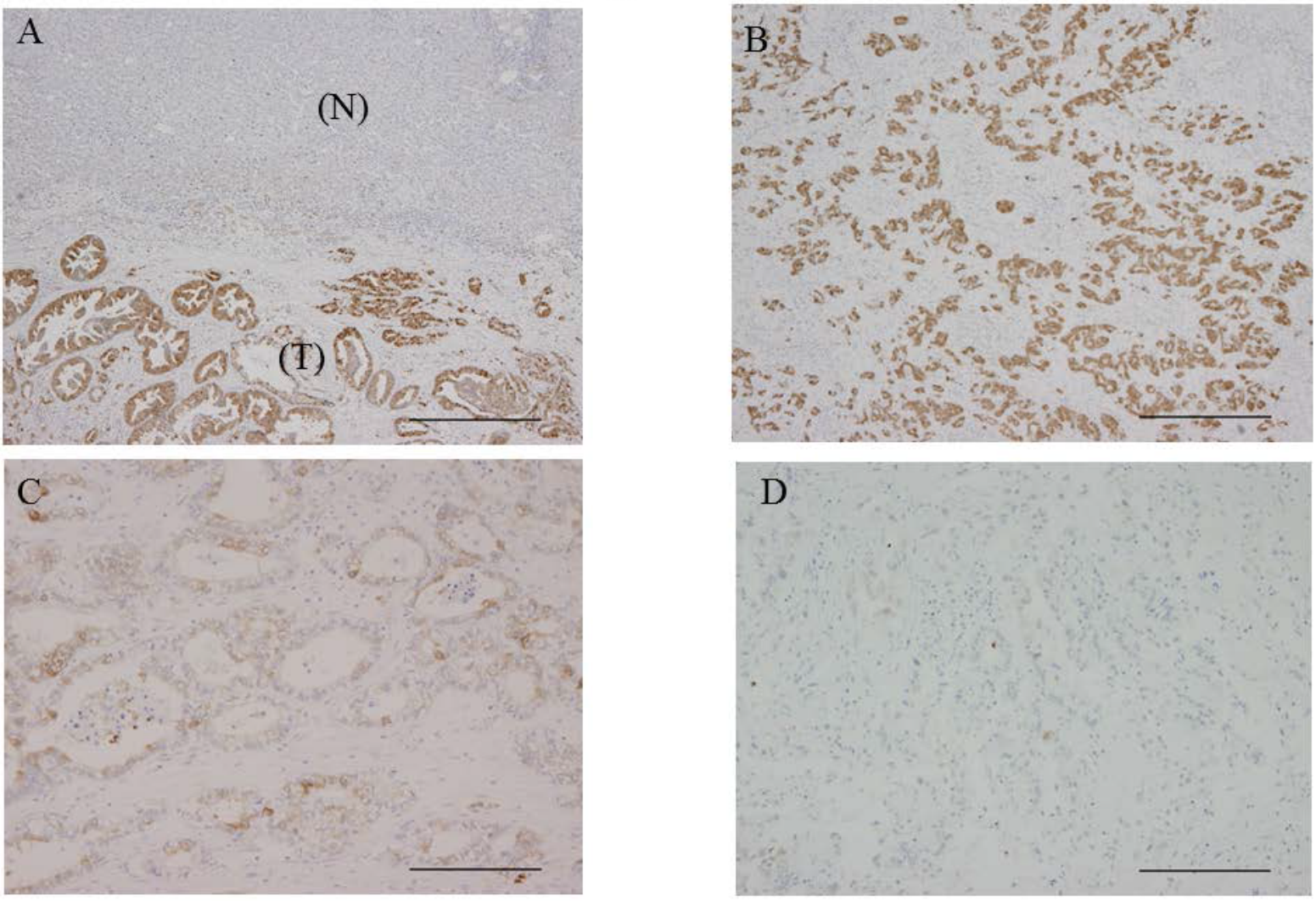


Figure 2

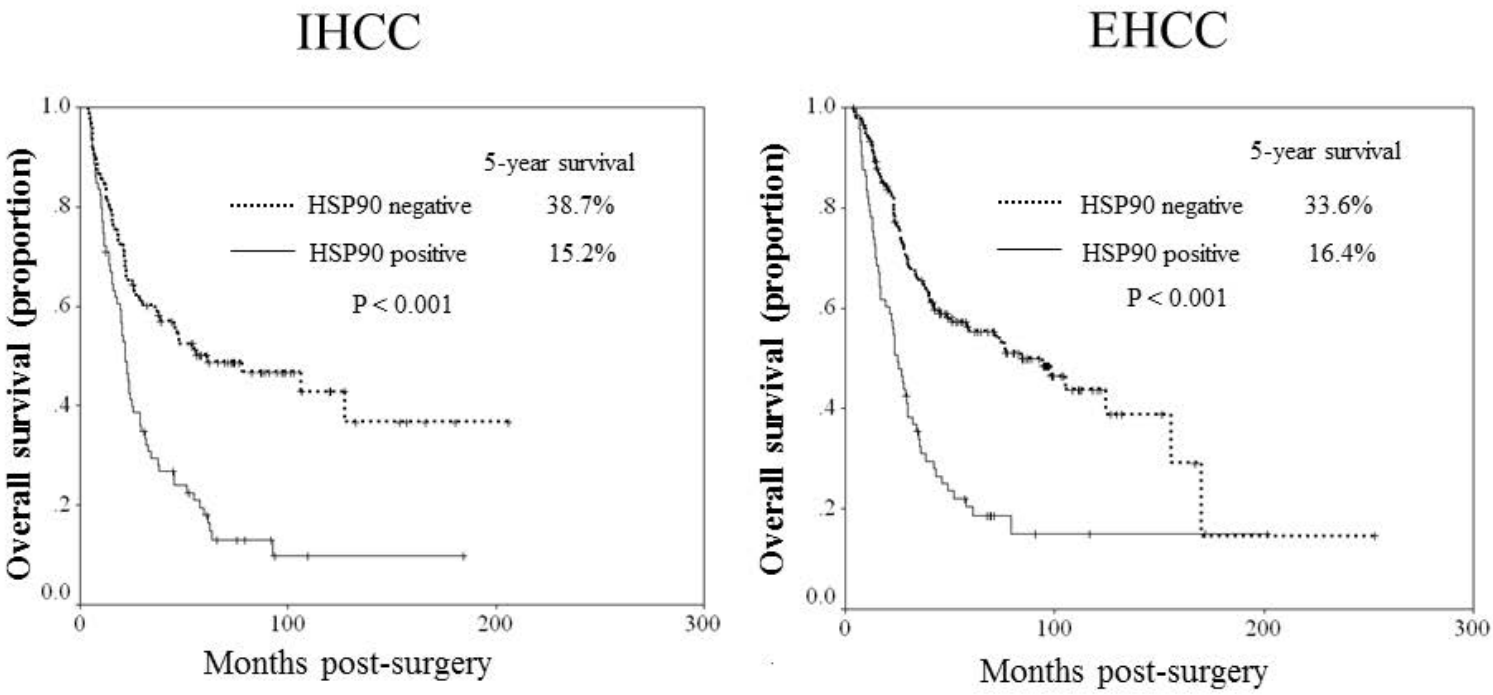
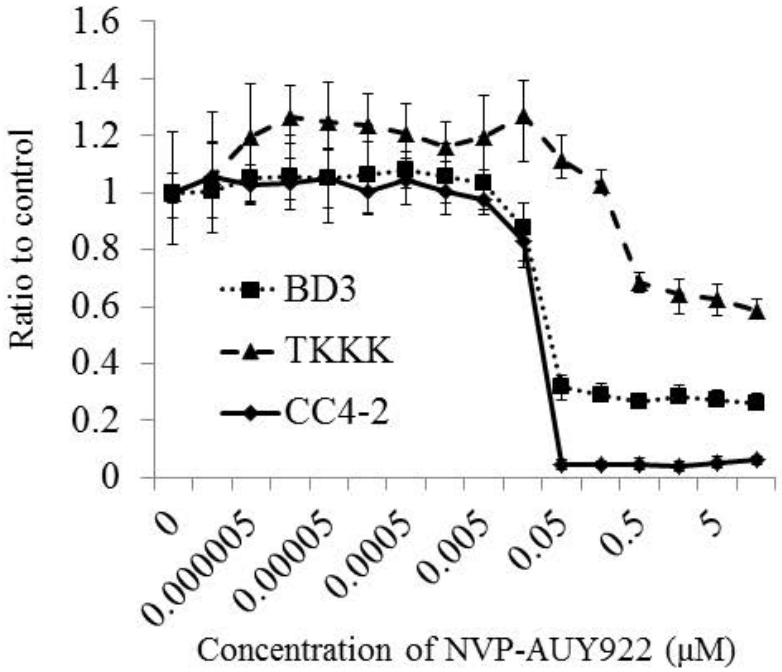

IHCC
EHCC
Overall survival (proportion)
1.0
.8
.6
.4
.2
0.0
0
100
200
300
Months post-surgery
5-year survival
HSP90 negative
38.7%
HSP90 positive
15.2%
P < 0.001
5-year survival
HSP90 negative
33.6%
HSP90 positive
16.4%
P < 0.001

Figure 3



Cell line	IC50 (nM/l)	Sensitivity
TKKK	950	Resistant
BD3	21	Sensitive
CC4-2	27	Sensitive

Figure 4

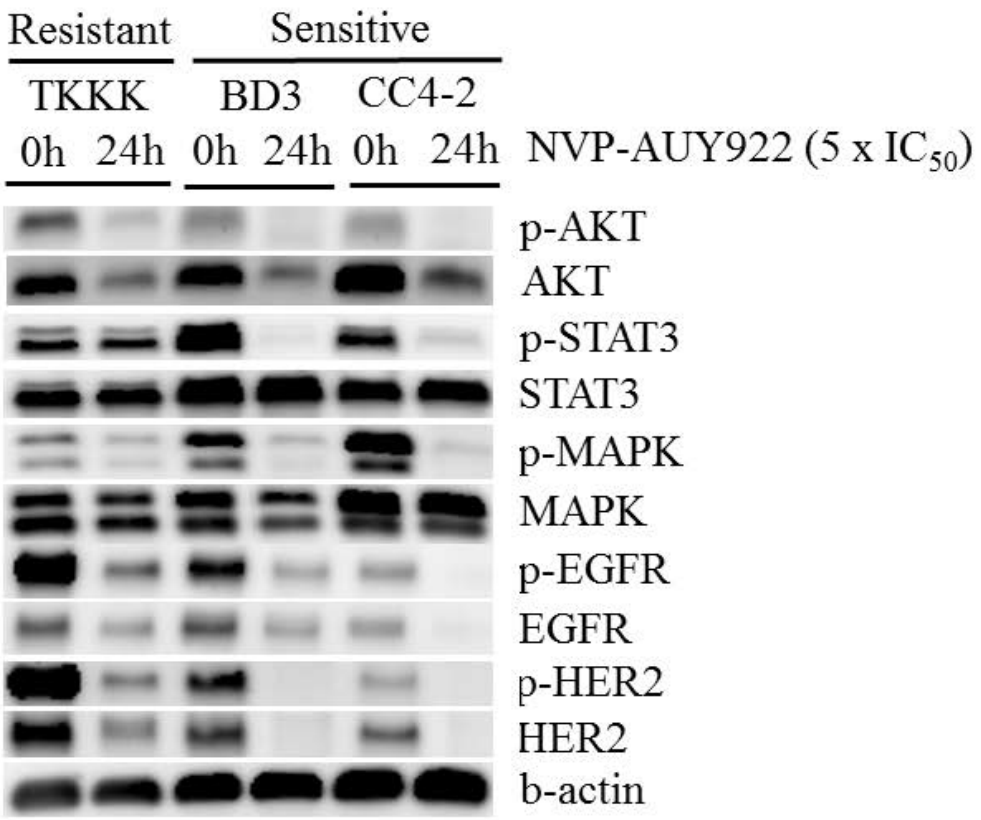
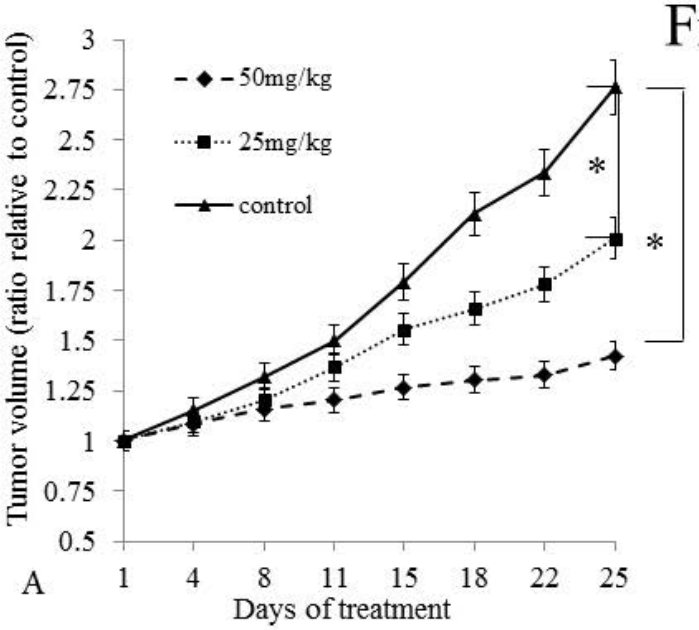

Resistant
Sensitive
TKKK
BD3
CC4-2
0h 24h 0h 24h 0h 24h
NVP-AUY922 (5 x IC50)
p-AKT
AKT
p-STAT3
STAT3
p-MAPK
MAPK
p-EGFR
EGFR
p-HER2
HER2
b-actin

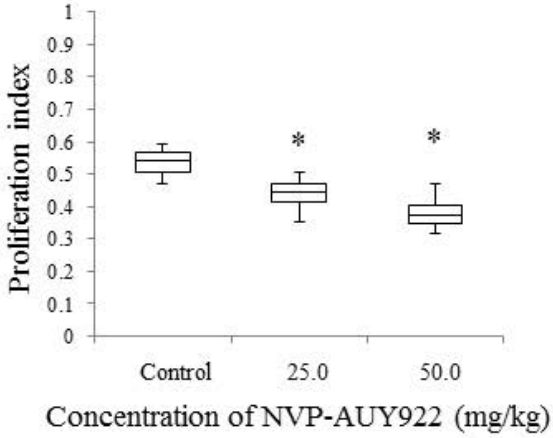
Figure 5



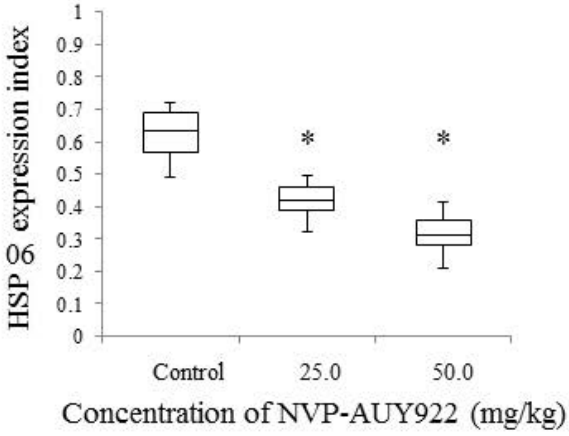
A

* P<0.05: vs control group (ANOVA and Dunnett's test)

B. Ki-67:proliferation index



C. HSP90 expression



06

Table 1A. Associations between HSP90 expression and clinicopathological factors in IHCC

IHCC characteristics	HSP90 negative (n=98)	HSP90 positive (n=79)	P value
Median age (range)	64.0 (35-84)	66.0 (41-84)	0.171
Sex			0.384
Male	64	49	
Female	34	30	
Preoperative CEA level			0.381
≤ 5.0 U/ml	75	58	
> 5.0 U/ml	23	21	
Preoperative CA19-9 level			0.159
≤ 37 U/ml	38	24	
> 37 U/ml	59	54	
Average tumor size			0.826
cm (range)	5.1 (0.5-15.0)	5.2 (1.4-15.0)	
Macroscopic type			< 0.001 *
non mass forming	24	3	
mass forming	74	76	
Histological classification (differentiated)			0.002 *
mucinous and well	28	9	
moderately	69	66	
poorly	0	4	
Portal vein invasion			0.482
negative	25	19	
positive	73	60	
Hepatic vein invasion			0.248
negative	57	41	
positive	41	38	
Lymphatic invasion			0.055
negative	66	43	
positive	32	36	
Venous invasion			0.471
negative	55	43	
positive	43	36	
Perineural invasion			0.525
negative	46	38	
positive	51	41	
Intrahepatic metastasis			0.008 *
negative	79	50	
positive	19	29	
Bile duct margin			0.063
negative	69	46	
positive	29	33	
UICC pathologic tumor status			0.013*
T1	10	1	
T2	41	38	
T3	8	15	
T4	39	25	
Lymph node metastasis			0.032 *
negative	70	45	
positive	28	34	
UICC pathologic stage status			0.007 *
I	10	1	
II	30	20	
III	4	12	
IVA + IVB	54	46	
c-MET			0.304
negative	43	44	
positive	8	5	
EGFR			0.308
negative	40	31	
positive	13	14	
VEGF			0.232
negative	25	17	
positive	28	28	

* showed that significantly different ($P < 0.05$)

HSP90 heat shock protein 90, *IHCC* intrahepatic cholangiocarcinoma, *UICC* union for international cancer control, *EGFR* epidermal growth factor receptor, *VEGF* vascular epithelial growth factor.

Legend of Table 1A: HSP90 in IHCC showed a significant association with macroscopic type ($P < 0.001$), histological classification ($P = 0.002$), intrahepatic metastasis ($P = 0.008$), pathologic tumor status ($P = 0.013$), lymph node metastasis ($P = 0.032$), and pathologic stage ($P = 0.007$).

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

EHCC characteristics	HSP90 negative (n=149)	HSP90 positive (n=73)	P value
Median age (range)	66.0 (33-84)	66.0 (43-80)	0.961
Sex			0.707
Male	110	53	
Female	39	20	
Preoperative CEA level			0.337
≤ 5.0 U/ml	132	63	
> 5.0 U/ml	16	10	
Preoperative CA19-9 level			< 0.001 *
≤ 37 U/ml	71	17	
> 37 U/ml	77	56	
Average tumor size			0.707
cm (range)	4.1 (0.6-11.5)	4.2 (1.2-10.0)	
Macroscopic type			0.118
non polypoid	122	65	
polypoid	27	8	
Histological classification (differentiated)			0.106
papillary and well	55	17	
moderately	74	46	
poorly	18	10	
Depth of tumor invasion			0.129
Within fm	14	3	
Beyond fm	135	70	
Lymphatic invasion			0.199
negative	88	38	
positive	61	35	
Venous invasion			0.358
negative	89	41	
positive	60	32	
Perineural invasion			0.358
negative	56	13	
positive	91	60	

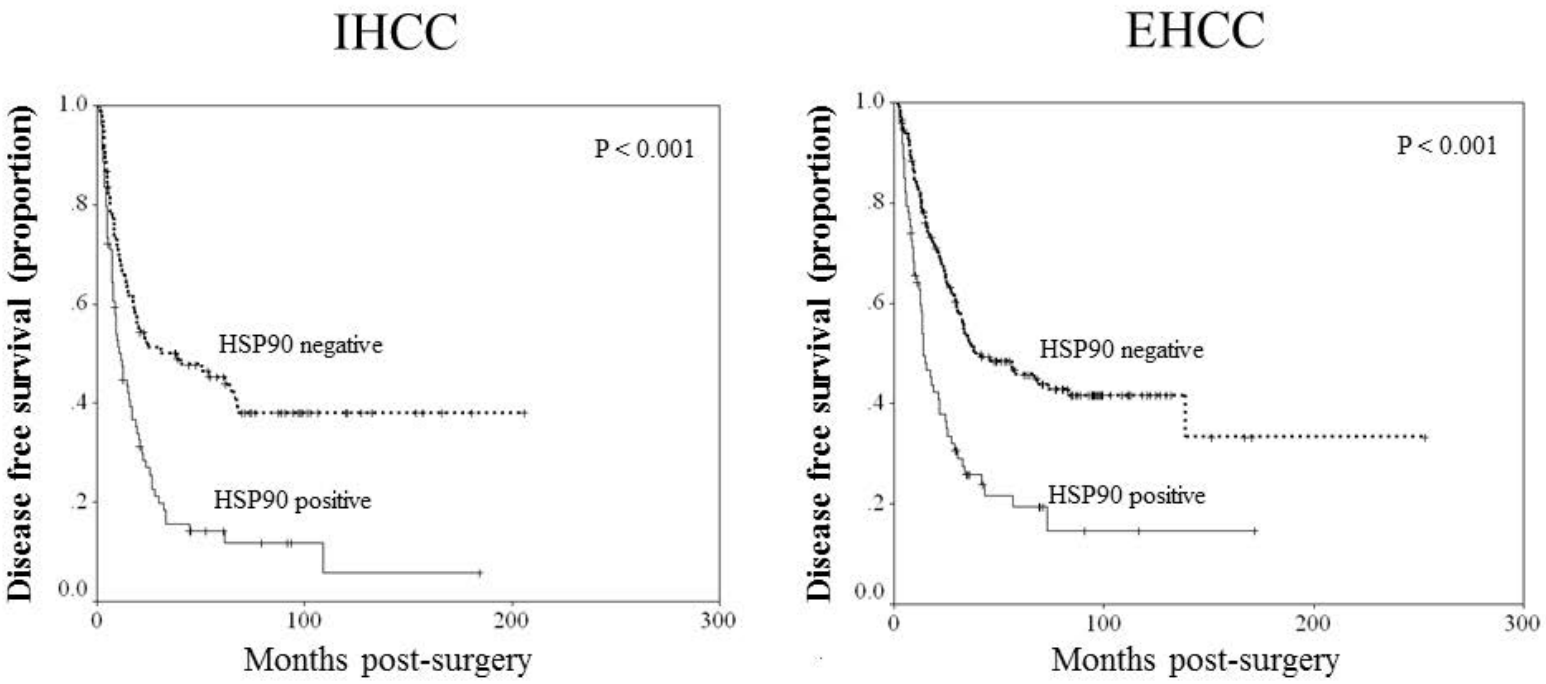
EHCC characteristics	HSP90 negative (n=149)	HSP90 positive (n=73)	P value
Bile duct margin			0.512
negative	56	27	
positive	92	46	
Other organ invasion			0.431
negative	54	25	
positive	92	48	
UICC pathologic tumor status			
T1	14	3	0.195
T2	29	15	
T3	90	42	
T4	14	13	
Lymph node metastasis			0.053
negative	84	32	
positive	65	41	
UICC pathologic stage status			0.089
IA + IB	32	12	
IIA + IIB	101	46	
III	14	14	
IVA + IVB	0	1	
c-MET			0.425
negative	74	37	
positive	13	8	
EGFR			0.450
negative	69	38	
positive	13	9	
VEGF			0.443
negative	31	14	
positive	54	28	
HER2			0.275
negative	79	37	
positive	6	5	

* showed that significantly different ($P < 0.05$)

HSP90 heat shock protein 90, *EHCC* extrahepatic cholangiocarcinoma, *fm* fibromascular layer, *UICC* union for international cancer control, *EGFR* epidermal growth factor receptor, *VEGF* vascular epithelial growth factor, *HER2* human epidermal growth factor receptor 2

Legend of Table 1B: HSP90 expression was associated only with preoperative CA19-9 level ($P < 0.001$) in EHCC.

Supplementary Figure S1



Legend of Supplementary Figure S1: HSP90 expression was significantly associated with decreased disease free survival in patients with IHCC (P <0.001) and EHCC (P <0.001).

Supplementary Table S1.

Multivariate analysis following univariate analysis regarding to overall survival in IHCC

	Overall survival			Disease free survival		
	HR	95% CI	P value	HR	95% CI	P value
Preoperative CEA level	1.499	0.938-2.394	0.090	1.711	1.314-2.774	0.012 *
Preoperative CA19-9 level	2.016	1.324-3.067	0.001 *	1.984	1.314-2.998	0.001 *
Intrahepatic metastasis	2.093	1.370-3.198	0.001 *	2.361	1.546-3.607	< 0.001 *
Lymph node metastasis	2.328	1.543-3.512	< 0.001 *	2.234	1.497-3.334	< 0.001 *
HSP90 expression	1.859	1.258-2.747	0.002 *	1.821	1.238-2.679	0.002 *

* showed that significantly different (P < 0.05)

IHCC intrahepatic cholangiocarcinoma, *HR* hazard ratio, *CI* confidence intervals, *HSP90* heat shock protein 90,

Legend of Supplementary Table S1: In IHCC cases, multivariate analysis identified HSP90 expression (P =0.002), preoperative CA19-9 level (P =0.001), intrahepatic metastasis (P =0.001), and lymph node metastasis (P <0.001) as independent prognostic factors for overall survival.

Supplementary Table S2.

Multivariate analysis following univariate analysis regarding to overall survival in EHCC

	Overall survival			Disease free survival		
	HR	95% CI	P value	HR	95% CI	P value
Preoperative CA19-9 level	1.291	0.862-1.934	0.215	1.240	0.847-1.816	0.268
Other organ invasion	1.400	0.933-2.100	0.104	1.324	0.890-1.969	0.166
Lymph node metastasis	2.135	1.461-3.120	< 0.001 *	1.986	1.374-2.871	< 0.001 *
HSP90 expression	2.236	1.555-3.216	< 0.001 *	1.930	1.344-2.772	< 0.001 *

* showed that significantly different (P < 0.05)

EHCC extrahepatic cholangiocarcinoma, *HR* hazard ratio, *CI* confidence intervals, *HSP90* heat shock protein 90,

Legend of Supplementary Table S2: In EHCC cases multivariate analysis identified HSP90 expression (P <0.001) and lymph node metastasis (P <0.001) as independent prognostic factors for overall survival and disease free survival in EHCC.

Supplementary Table S3.

IC_{50} inhibition values for NVP-AUY922 in CC cell lines (including IHC evaluation of HSP90 expression and doubling time)

		Doubling time (hours)	AUY922		HSP90
Cancer type	Cell lines		Sensitivity	IC_{50} (nM)	Expression
IHCC	NCC-CC1	56.8	resistant	120	negative
	NCC-CC3-1	39.2	resistant	320	negative
	NCC-CC3-2	38.2	resistant	870	negative
	NCC-CC4-1	41.8	sensitive	9	negative
	NCC-CC4-2	30.9	sensitive	27	positive
	NCC-CC5	27.4	resistant	200	negative
	NCC-CC6-1	30.8	sensitive	32	negative
	NCC-CC6-2	55.8	sensitive	29	negative
	TKKK	30.8	resistant	950	negative
	TGBC24TKB	66.7	sensitive	42	negative
	HuCCT1	34.9	sensitive	30	negative
EHCC	NCC-BD1	30.9	sensitive	16	negative
	NCC-BD2	41.1	sensitive	17	negative
	NCC-BD3	62.6	sensitive	21	positive
	NCC-BD4-1	50.3	sensitive	25	positive
	NCC-BD4-2	47.9	sensitive	37	negative
	OZ	44.2	sensitive	23	positive

IC_{50} the 50% of inhibitory concentration, *IHC* immunohistochemistry, *HSP90* heat shock protein 90, *CC* cholangiocarcinoma, *IHCC* intrahepatic cholangiocarcinoma, *EHCC* extrahepatic cholangiocarcinoma

Legend of Supplementary Table S3: There was no significant difference between doubling time and HSP90 expression on cholangiocarcinoma cell lines. There was no significant difference with association the doubling time of cell lines and HSP90 expression both IHCC (P=0.4142, Fisher's exact test) and EHCC (P=0.4279, Fisher's exact test).