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Original Article

Biological characteristics of heat shock protein 90 in human liver cancer cells

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Abstract: Primary hepatic cancer refers to a malignant tumor that enables lethal cancero-metastasis. And hepatocellular carcinoma (HCC) is a most common cancer, accounting for around 75% of all cases. However, effective screening and diagnostic methods of HCC are limited currently. Heat shock protein 90 (HSP90), a sensitive biomarker, is found marked elevation in various malignancies. Thus, potential association between HSP90 expression and pathological onset of HCC is needed to be investigated. In current human study, plasma samples of advanced HCC patients were collected for biochemical assays, and cancer, non-cancer tissues from biopsy were stained immunohistochemically. In cell culture study, a human HepG2 cell line was subjected to a group of assays followed by HSP90 and inhibitor treatments. As results, the clinical data of HCC patients resulted in abnormal altered levels of serous molecules when compared to diagnostical references. However, these enzymatic changes showed no statistical significance. Significantly, plasma contents of HSP90 in HCC patients were elevated in comparison with those in HCC-free adults ($P<0.01$). As shown in immunofluorescence stains, hepatocellular HSP90-labeled cells in alpha fetoprotein (AFP)-positive and negative HCC sections were obviously expressed. In cell culture data, HSP90-induced HepG2 cells resulted in increased cell proliferation, and proliferating cell nuclear antigen (PCNA)-, B-cell lymphoma 2 (Bcl-2)-positive cells ($P<0.05$). In addition, HSP90 inhibitor-treated HepG2 cells showed effectively reduced cell growth, and PCNA-, Bcl-2-positive cell counts. Taken together, our current findings demonstrate that hepatocellular HSP90 may be positively involved in development of HCC, and it is likely a potential biomarker for monitoring advanced HCC.

Keywords: Hepatocellular carcinoma, HSP90, cell proliferation, biomarker

Introduction

HCC is a clinical common tumor, marked by chronic liver dysfunction and hepatic failure [1]. The pathogenetic factors of HCC are most closely associated with hepatitis infection, toxins exposure (such as aflatoxin, alcohol). Epidemiologically, the vast majority of HCC is found in Asia, because the infection of hepatitis B virus is prevalent. Once epigenetic alterations and mutations occur, HCC will develop and change the cellular signals, eventually resulting in apoptotic resistance and uncontrolled proliferation [2, 3]. Most cases of HCC are related to a high mortality, because advanced HCC is hard to detected in early diagnosis. Some of HCC patients may be without evident symptoms during initial tumor examination [4]. Clinically, the medical assessment of HCC is commonly used with blood test and imaging assay. In addition, neoplastic biopsy is necessary to validate the combined diagnosis via histopathologic confirmation [5]. In current clinical biochemical test, AFP is a common biomarker for HCC diagnosis, and it is one of standard references for many years. However, serum AFP content may be negative for around 20% of all HCC patients, marked by reduced diagnostic application [6, 7]. Therefore, pursuing effective and accurate HCC biomarker has becoming urgent task in this scientific topic. HSP90, a functional chaperone protein, activates the regulatory proteins essential for oncogenesis through inducing intracellular PI3K/AKT signaling pathway [8]. Additionally, some promising Hsp90 inhibitors are presently undergoing clinical trials against diversified cancers. SNX-2112 is a target Hsp90 inhibitor with

broad-spectrum, high-efficiency, low-toxic, and its functional mechanisms are inducing cell apoptosis, cell cycle arrest, and activating endoplasmic reticulum unfolded protein response (UPR) [9, 10]. Based on the literature analyses, we proposed that peripheral and hepatocellular HSP90 biomolecule may be associated with HCC progression, and abnormal HSP90 release in HCC cells is likely to be potential strategy for monitoring HCC. In our present study, human and cell culture studies were designed to validate the scientific hypothesis.

Materials and methods

Human design

All HCC patients were collected from Department of Oncology, Liuzhou Worker's Hospital (Liuzhou, China) during 2017 to 2018. These thirty cases were medically diagnosed as advanced HCC through a number of serological, pathological, and imaging tests before the first round of therapy. The diagnostic identification of advanced HCC was referenced by issued directions [11]. Further, other ten health HCC-free adults were set as controls, in addition to any possible liver diseases. This study was approved by the Ethics Committee of Liuzhou People's Hospital, and consent has been obtained from participants for performing experiments. Blood samples were harvested via ulnar vein, and plasma was prepared for biochemical testing by using an automatic analyzer (H7170S, Hitachi, Japan). Measurement of plasma HSP90 content was tested by using an enzyme linked immunosorbent assay kit (Shanghai Elisa Biotech, China). In addition, HCC samples were isolated by biopsy, and the samples were fixed with 10% neutral formalin prior to further pathological examination and histiocytic staining.

Cell culture and CCK8 assay

HepG2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)-high glucose medium (Solarbio, Beijing, China) contained 10% fetal bovine serum (Sijiqing Biotech, Hangzhou, China), 100 U/mL penicillin and 100 μg/mL streptomycin (Solarbio, Beijing, China), and were kept in a 5% CO_2 incubator. Cell proliferation or toxicity test was evaluated by cell counting kit (CCK8, Nanjing Institute of Bioengineering, China) according to manual's instructions. Other separate passages of HepG2 cells were seeded at a density of 2×10^5 cells/cm^2 in a 6-well plate for 48 hours treatments of dosed HSP90 (200, 400 pg/mL, Shanghai Elisa Biotech, China) and SNX-2112 inhibitor (20 nM, Beyotime Biotechnology, China). And then the cells were subjected to immunofluorescence staining and positive cell analysis.

Immunohistochemistry protocols

Formalin-fixed and paraffin-embedded HCC sections were dewaxed and permeabilized, followed by 5% blocking with bovine serum albumin (BSA) for 2 hours. After being washed with phosphate buffer saline (PBS)/0.5% tween 20 several times, the sections were incubated with diluted primary antibodies of alpha-fetoprotein (AFP), chromogranin (CgA), cluster of differentiation (CD34), Glypcian-3 (Gly3), cytokeratin-19 (CK19), Antigen Ki-67 (Ki-67) (1:50-1:100, Maixin Biotech, Fuzhou, China) for overnight at 4°C, followed by further incubation of corresponding secondary antibodies (1:100, Maixin Biotech, Fuzhou, China). In addition, the complexes were chromogenic by using a dye of diaminobenzidine (DAB, Boster, Wuhan, China), followed by being counterstained with hymatoxylin on nuclei before further imaged and assayed [12, 13].

Immunofluorescence procedures

As reported previously [14, 15], the HepG2 cells were seeded on a sterilized cover glass in a 6-well plate before HSP90, SNX-2112 treatments. After 48 hours exposure, the cells were fixed with 4% paraformaldehyde (Sigma, USA) for at least 20 min. The cells were pre-permeabilized by use of PBS/0.1% triton-100 buffer. The cover glasses were blocked with 5% BSA for at least 1 hour, followed by incubation with diluent primary antibodies of HSP90, AFP (1:100, Beyotime Biotechnology, China) on HCC samples, and proliferating cell nuclear antigen (PCNA), B-cell lymphoma 2 (Bcl-2) (1:100, Boster, China) on HepG2 cells, overnight at 4°C, and further re-incubation with Alexa-Fluor-conjugated secondary antibodies (1:200, Abcam, USA). Cellular nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Abcam, USA) prior to imaged and analyzed.

Table 1. Preliminary clinical data of advanced HCC patients

Biochemical parameters	HCC cases	Diagnostic reference
Sexes (F/M)	4/26	-
Age (year)	55.3±12.96	-
TBIL (μmol/L)	38.82±68.02	0-21
DBIL (μmol/L)	24.83±51.57	0-6
IBIL (μmol/L)	8.81±9.35	1.71-15
ALT (U/L)	41.05±22.89	0-38
AST (U/L)	80.77±73.41	0-40
ALP (U/L)	233.28±148.37	40-150
GGT (U/L)	213.71±173.98	0-50
TBA (μmol/L)	24.85±29.68	0-15
TP (g/L)	67.69±12.29	62-83
ALB (g/L)	32.09±8.06	35-55
AFP (ng/mL)	330.02±533.4	0-25
GLU (mmol/L)	5.09±1.29	1.7-8.3
Urea (mmol/L)	6.99±7.62	53-106
Cr (μmol/L)	75.45±22.62	150-420
UA (μmol/L)	284.85±161.94	0.57-1.03
β2-MG (mg/L)	4.28±0.84	-
CYS-C (mg/L)	1.35±0.34	-
CK-NAC (U/L)	33±18.38	-
CK-MB (U/L)	22±15.56	-
LDH-L (U/L)	246±86.08	-
α-HBDH (U/L)	242.67±18.5	-
K (mmol/L)	3.59±0.22	-
Na (mmol/L)	130.25±2.99	-
Cl (mmol/L)	94±3.65	-
Ca (mmol/L)	2.06±0.07	-
WBC (10^9/L)	9.23±5.87	4-10
NEUT%	68.83±21.29	0.37-0.72
LY%	14.67±11.84	0-0.06
MONO%	8.91±3.62	0-0.01
EO%	2.07±2.65	0-0.14
BASO%	0.56±0.47	3-5.5
NEUT (10^9/L)	10.68±16.82	110-150
LY (10^9/L)	1.94±2.55	0-0.7
MONO (10^9/L)	0.88±0.59	1.5-7
EO (10^9/L)	0.22±0.4	0-0.4
BASO (109/L)	0.05±0.03	0-0.1
RBC (10^{12}/L)	4.09±0.8	0.2-0.5
HGB (g/L)	111±21.93	1-3.7

Abbreviation: F, female; M, male; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; TBA, total bile acid; TP, total protein; ALB, albumin; GLB, globulin; AFP, alpha fetoprotein; GLU, glucose; NEUT%, neutrophil ratio; LY%, lymphocyte ratio; MONO%, monocyte ratio; EO%, eosinophil ratio; BASO%, basophil ratio; NEUT, neutrophil count; LY, lymphocyte count; MONO, monocyte count; EO, eosinophil count; BASO, basophil count; RBC, red blood cell; HGB, hemoglobin.

Statistical analysis

All data are expressed as mean ± standard deviation. Statistical assays were conducted by using statistical product and service solutions 19.0 (Chicago, IL, USA). Data were assayed by one-way analysis of variance followed by Duncan's multiple test in CCK-8 tests, and the Student's t test was used in ELISA data of HSP90 contents in plasms samples. A *P*-value less than 0.05 was set as statistical significance.

Results

Demographic data of advanced HCC patients

In the human study, advanced HCC cases included 4 women and 26 men with an average age of 55.3±12.96 years. As highlighted in **Table 1**, the clinically serological results showed marked abnormal changed levels of liver functional molecules, AFP, and immune cell ratio when compared to clinical references. However, these parameter alterations were inapparent statistical significance because of evident variance, especially in AFP (a HCC biomarker).

Intrahepatic clinicopathologic findings of advanced HCC patients

To biologically examine hepatocellular pathological phenotypes, the HCC sections were implemented immunohistochemically. As results, representative HCC case with AFP-positive detection resulted in positively expressed cytoplasmic proteins of AFP, CgA, Gly3, and epithelial proteins of CD34, CK19, as well as nuclear proliferative proteins of Ki-67. In AFP-negative HCC case, the positive expressions of these targeting proteins were detected in the liver cells (**Figure 1**).

Physio-pathological characterization of hepatocellular HSP90 of advanced HCC patients

In order to further assess the bioactivity of HSP90 on advanced HCC patients, enzyme-linked immunosorbent assay (ELISA) test and immunofluorescence stain were conducted respectively. As compared to that in HCC-free controls, AFP-negative HCC patients exhibited elevated HSP90 contents in plasma samples with significant statistical significance ($P< 0.05$). As shown in immunofluorescence stain-

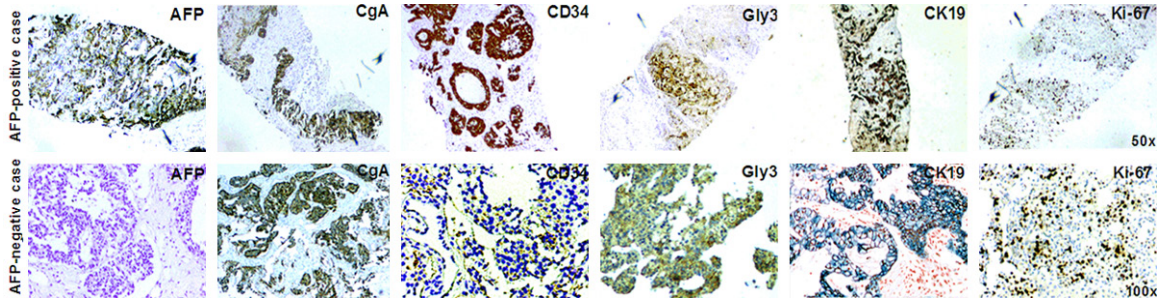

Figure 1. Representational clinicopathologic micrograph of advanced HCC patients. Immunohistochemically, AFP-positive HCC section showed visible cytoplasmic expressions of AFP, CgA, Gly3, epithelial positive cells of CD34, CK19, and nuclear positive cells of Ki-67 under an optical microscope. As shown in AFP-negative HCC section, these positive cells of target proteins were observed in liver cells with similar immunophenotypes.

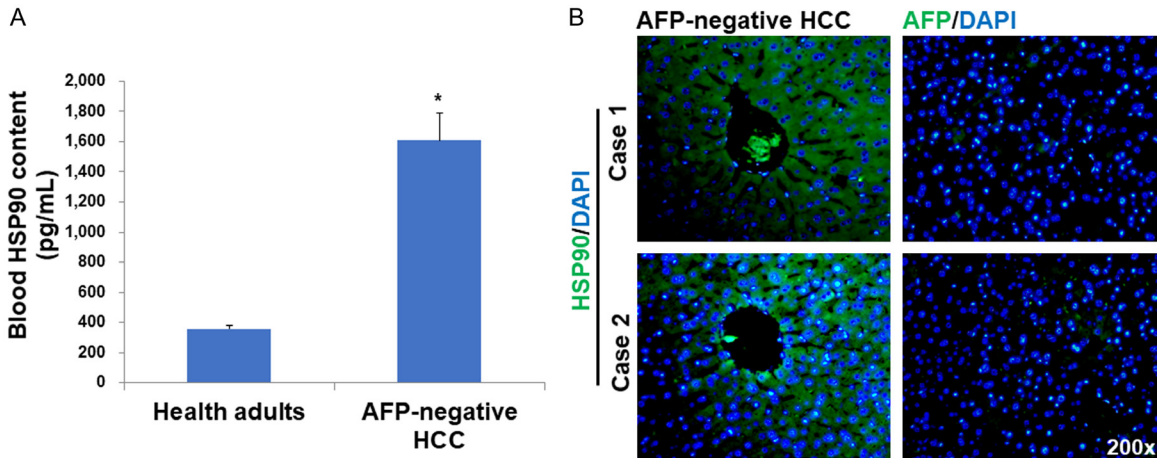

Figure 2. Determination of hepatic HSP90 of advanced HCC patients. In comparison with health adults, AFP-negative HCC cases resulted in increased HSP90 contents in plasma samples by using an ELISA kit (A). In further immunofluorescence-stained observation, AFP-negative HCC sections showed elevated count of HSP90-labeled cells when compared to those in AFP-positive sections of HCC (B).

ing, AFP-negative HCC sections had increased count of HSP90-labeled cells in comparison with those in AFP-positive HCC case (**Figure 2**).

HSP90 induced cell proliferation of human cancer cells

To validate the potent bioeffects of HSP90 on cell growth, a human liver cancer line, HepG2 was used to the further experiment. As results, CCK-8 assayed data showed that marked effect on cell viability was observed in HSP90-dosed treatments ($P<0.05$). However, HSP90-specific inhibitor resulted in suppressed cell proliferation ($P<0.05$). Further, we assessed whether the HSP90 affected the expression of key protein in cell differentiation and growth. As highlighted in immunofluorescent staining, a dose-dependent up-regulation of PCNA-, Bcl-2-labeled proteins were observed among the solvent control and HSP90-dosed groups, while the significant reduced changes in the cellular contents of PCNA and Bcl-2 were showed in HSP90-specific inhibitor treatment (**Figure 3**).

Discussion

HCC is the common typed liver tumor, accounting for approximate 75% of all primary liver tumors [16]. In medical inspection, about 30% of HCC cases will be diagnosed as negative outcome through referencing AFP's range. As revealed in clinical practice, low-diagnostic rate of HCC, especially in early stage, is partly because of marked elevation of blood levels of AFP are also detected in some conditions of high-risk liver diseases, suggesting AFP-related poor accuracy for HCC diagnosis connected

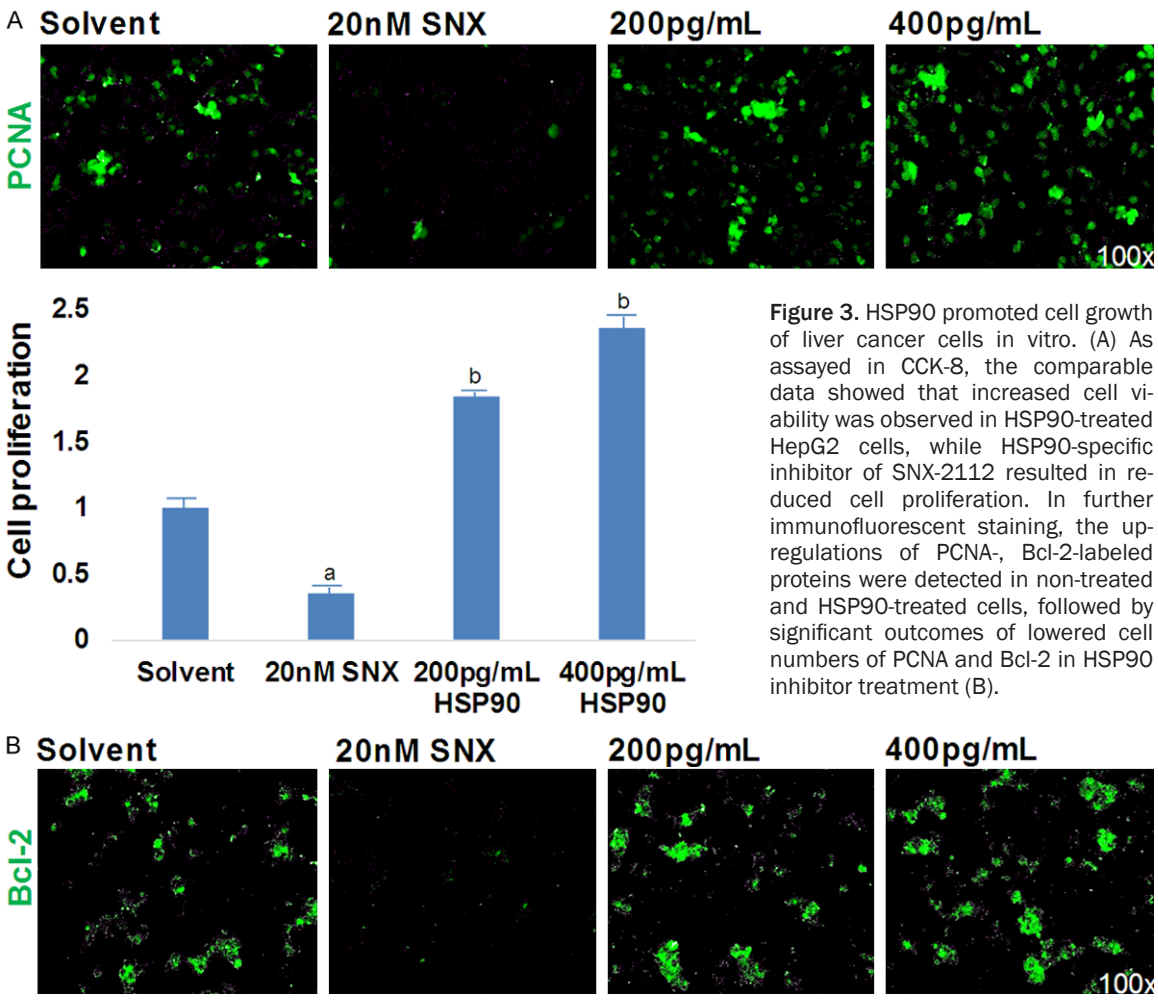

Figure 3. HSP90 promoted cell growth of liver cancer cells in vitro. (A) As assayed in CCK-8, the comparable data showed that increased cell viability was observed in HSP90-treated HepG2 cells, while HSP90-specific inhibitor of SNX-2112 resulted in reduced cell proliferation. In further immunofluorescent staining, the up-regulations of PCNA-, Bcl-2-labeled proteins were detected in non-treated and HSP90-treated cells, followed by significant outcomes of lowered cell numbers of PCNA and Bcl-2 in HSP90 inhibitor treatment (B).

with disease's complexity and heterogeneity [17, 18]. In current human study, blood contents of these HCC patients showed visible data-based standard deviation. And AFP-positive and negative HCC sections also exhibited additional positive biomarkers for clinicopathological diagnosis. Therefore, the current clinical data indicated that blood AFP may be inefficient as a non-invasive biomarker for HCC diagnosis. Therefore, to further screen the specific and sensitive biomarker for HCC warrants to be investigated. Interestingly, we found HSP90 is induced by elevated expression levels in blood and liver samples of HCC, even in AFP-negative cases. These findings demonstrate that abnormal expression of peripheral and intrahepatic HSP90 is likely be related to HCC development, characterized with promising clinical application.

Hsp90 is an essential molecular chaperone that modulates the functional cell signaling in mammality. Activation of intracellular HSP90 is triggered by exogenous stimuli, such as oxidative stress, inflammatory reaction, and immune insult [19, 20]. These durative stresses are associated with tumor-based growth, invasion, and activated HSP90 phenotype induces oncogenesis and metastasis [21]. Thereby, HSP90 is likely to be an important cancer target. As results, hepatocellular up-regulation of HSP90 was notably detected in advanced HCC sections of our current clinical data. Therefore, positive expression of HSP90 exerts a key clinical implication in cancer treatment, and it can function as a biological molecule in predicting HCC progression.

HSP90 inhibitor is a powerful chemical that specifically inactivates the activity of Hsp90, resulting in suppression of various types of malignancies [22]. In underlying molecular mechanism, the inactivation of Hsp90 results in the intracellular destruction of functional

Akt, insulin-like growth factor 1 receptor (IGF1R) and Kit proteins with the result of inhibiting cell survival and proliferation [23]. SNX-2112 is a potent Hsp90 inhibitor, and it induces marked cell death in human cancer cells, thereby being an effective targeted therapeutic agent. PCNA is a deoxyribonucleic acid (DNA) clamp that controls DNA replication and repair, cell survival [24]. Bcl-2 is a mitochondrial protein that regulates cell apoptosis, marked by over-expression of anti-apoptotic Bcl-2 in cancer cells [25]. The resultant data from cell culture study showed increased cell proliferation, elevated expressions of PCNA, Bcl-2 in HSP90-dosed HepG2 cells were detected dose-dependently. Instead, HSP90-specific inhibitor reversed these changes markedly. Therefore, the present experiments indicated that HSP90 played potent physiological function of promoting cancer cells growth in a dose-dependent way. In addition, endogenous HSP90 may be assessed in cancers other than HCC, and the HSP90 over-expression is linked to the developed pathology of HCC.

Conclusions

To sum up, our current findings disclose elevated expression of HSP90 in blood and liver samples of AFP-positive and negative HCC patients, marked by more specific than these non-sensitive blood biomarkers. In addition, HSP90, a candidate biomolecule, may function as a sensitive biomarker for screening HCC.

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Disclosure of conflict of interest

None.

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