

Document heading doi: 10.1016/S1995-7645(14)60335-7

HSP90 and SIRT3 expression in hepatocellular carcinoma and their effect on invasive capability of human hepatocellular carcinoma cells

Ming Gao¹, Xiao-Ping Geng^{2*}, He-Ping Xiang¹¹Department of Emergency Surgery, Second Affiliated Hospital of Anhui Medical University, Hefei 230601, China²Department of General Surgery, Second Affiliated Hospital of Anhui Medical University, Hefei 230601, China

ARTICLE INFO

Article history:

Received 15 January 2015

Received in revised form 20 February 2015

Accepted 15 March 2015

Available online 20 April 2015

Keywords:

HSP90

SIRT3

EMT

Cancer stem cell

Liver cancer

ABSTRACT

Objective: To explore expression of HSP90, SIRT3 in liver cancer tissue and its effect on liver cancer cell invasion ability. **Methods:** Moderate expression of HSP90 in SMMC-7721, HepG2, LO2 and Hep-3B cell lines were screened, which was validated by RT-PCR. Over-expression of HSP90 cell line and lentivirus packaging HSP90-RNAi were established, which was validated by RT-PCR and western blot. The level of epithelial-mesenchymal transition (EMT) related gene was detected by western blot. The percentage of cancer stem cells was assayed by flow cytometry. **Results:** RT-PCR demonstrated the highest expression of HSP90 mRNA in SMMC-7721 cells, the lowest expression of HSP90 mRNA in Hep3B and LO2 and the moderate expression of HSP90 mRNA in Hep-G2. Therefore, HepG2 was selected as a follow-up experiment cell lines. Compared with the blank control group, expression of HSP90 in HSP overexpression group was increased obviously, and expression of HSP90 in HSP90 shRNA group was significantly decreased, which indicated successful establishment of HSP overexpression and shRNA group. The apoptotic cell in hsp-siRNA group was higher than the blank control group, while the HSP overexpression group showed opposite results. Western blot results showed transfection HSP promoted cells EMT transformation, up-regulated the level of E-cadherin, and down-regulated the level of Vimentin; meanwhile, shRNA group showed opposite results. **Conclusions:** Carcinoma HepG2 cell transfected high expression of HSP can promote the transformation of EMT, improve the expression of Vimentin, reduce the expression of E-cadherin, and inhibit apoptosis of cancer stem cells, which improve the invasive ability of cancer of the liver cells. While hsp-siRNA group presents opposite results. In summary, the expression of HSP is closely related to the occurrence, development and invasion of cancer of the liver tissue.

1. Introduction

Normal epithelial cells have a typical “apical-basal polarity”, wherein, tight and adherent junctions exist between cells. The basal surface is connected to the basement membrane. Epithelial cells lose the typical epithelium structure and acquire the capacity

to move around and invade, then become mesenchymal like cells. This process is called epithelial-mesenchymal transition(EMT)[1]. During the process of EMT transdifferentiation, the expressions of characteristic proteins of cells are changed a lot. The expression of characteristic proteins of epithelial cells (E-cadherin) is decreased, however, the expression of characteristic proteins of mesenchymal-like cells (Vimentin, α -smooth muscle actin) is increased[2,3]. EMT process is completed when epithelial cell basal surface degrades and cells migrate away from epithelial layer. As the processing of profound studies on tumor, it was found that the growth and differentiation of malignant tumors are closely related to stem cells, and tumors originated in the stem cells were furthermore presumed, the proposal of cancer stem cell concept (CSC) was also

*Corresponding author: Xiao-Ping Geng, Doctoral Supervisor, Chief Physician, Department of General Surgery, Second Affiliated Hospital of Anhui Medical University, Hefei 230601, China.

Tel: 13956010132

E-mail: genfjxiaox@163.com

Foundation project: It is supported by Department of Scientific Research Projects in Anhui Province (No. 09020304021).

put forward[1,4]. Whereafter, tumor stem cells have been isolated from acute myeloid leukemia, breast cancers and brain tumors (such as astrocytic tumors)[5,6]. Therefore, this study studied on the heat shock protein (HSP) HSP90 and SIR3, both induced epithelial cell mesenchymal transdifferentiation and enhanced tumor stem cell characteristics, so as to promote the occurrence and development of liver cancer, and provide a theoretical reference value for the subsequent treatment of liver cancer.

2. Materials and methods

2.1. Cell culture

Cell line SMMC-7721, HepG2, LO2 and Hep3b were purchased from American Type Culture Collection (ATCC). Cells were placed in DMEM containing 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μ g/mL), then cultured in the 5% CO₂ incubator at constant temperature 37 °C. Packaging cell line GP2-293 and retroviral vector pBABE-puro were a gift from Professor Mouri Ran of the University of California, Berkeley.

2.2. Detection of HSP90 expression in different liver cancer cell lines by RT-PCR

Total RNA were extracted according to trizol (Invitrogen) kit instructions, the whole extracting process was RNAase free. Primers were designed as follows, *HSP90* gene upstream primer sequence: 5'-GGGGGATCCCcagctatgaactctctcc-3'; downstream primer sequence: 5'-GGGGTGCACctacattgccaagagcct-3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal reference, upstream primer sequence: 5'-TGACTTCAACAGCGACACCCA-3'; downstream primer sequence: 5'-CACCTGTGCTGTAGCCAAA-3'. cDNA was synthesized by reverse transcription with RT-PCR one step method kit and amplified by PCR, 5 μ L amplified products were performed 2% agarose gel electrophoresis. Electrophoretic bands were measured with ultraviolet spectrophotometer and photographed.

2.3. Construction of target gene HSP90 over expression cell line

Human HSP90-HA cDNA was cloned into pBABE-puro expression vector. Construction of stabilized expressed cell lines was completed by retroviral vector infection method. When packaging cell line GP2-293 grew to 60 percent, the liposome infection protocol (liposome 2000, Invitrogen, USA) was used to transfect 5 μ g of pBABE-puro or pBABE-puro-HSP90-HA and 5 μ g of pCMV-VSVG. After transfection for 48 hours, the virus supernatant was collected and centrifuged at 3 000 g for 3 minutes. The supernatant was transferred to a new clean eppendorf tube and filtered through a 0.45 μ m filtrate membrane to obtain experimental viral particles. Supernatant was used to infect target cells for 24 h, then placed into selective medium containing 2 mg/mL puromycin. HSP90-HA cell lines or empty vector cell lines were verified by RT-PCR in vivo

experiment and Western blot.

2.4. Target gene HSP90-RNAi lentiviral packaging

Optimal interference sequence designed by Ambion's software which based on target gene was adopted. *HSP90* (siRNA target sequence of HSP90 is TGATGTAAGTTCTGAGTGTGAGCAGTGGAAAGCTATTCAGAA) and control (siRNA sequences is TGACATGATAAATACTCTCT, without interference suppression on the expression of human gene). Target fragment was annealed by 3' and 5' single strand and digested by *AgeI* and *EcoR I* (New England Biolabs, NEB) restriction enzymes, then it was connected to the pGCSIL-GFP vector. After sequencing identification, pGCSIL-GFP-HSP90 and pGCSIL-GFP-vector was used to transfect Gp2-293 packaging cells by liposomes 2000. The supernatant was collected after 48 h and filtered through a 0.45 μ m filtrate membrane, then Polybrene was add into it until the final concentration was 8 μ g/mL. Supernatant was used to infect target cells for 24 h, then placed into new medium for another 24 h. After 48 h infection, cells were collected by centrifugation and resuspended in medium containing 0.5 μ g/mL puromycin. The infected cells were isolated by the limiting dilution method. After cells were cultured for 2 weeks, resistant monoclonies were selected for amplification culture, and HSP90 protein expression was detected by western.

2.5. Detection of EMT-related gene mRNA levels by real-time quantitative PCR

Total RNA were extracted according to trizol (Invitrogen) kit instructions, the whole extracting process is RNAase free. EMT markers associated genes including *E-cadherin* and *vimentin* mRNAs levels were amplified by real-time quantitative RT-PCR with SYBR Green method. *GAPDH* was taken as an internal reference, and then normalization processing was performed for data. Three biological checks was conducted in each sample. Primer sequences were as follows: *E-cadherin*, forward primer: 5'-TGCCAGAAAATGAAAAGG-3', reverse primer 5'-GTGTATGTGGCAATGCGTTC-3'; *vimentin*, forward primer: 5'-GAGAACTTTGCCGTTGAAGC-3', reverse primer: 5'-GCTTCCTGTAGGTGGCAATC-3' and so on.

2.6. Western blot detection

Western blot method was described as shown in the literature[7]: After cell lysis, cell lysis solution was obtained and SDS-PAGE gel electrophoresis was conducted. Immunohistochemical analysis was performed with anti-E-cadherin and vimentin for further experimental analysis to determine the expression levels of each gene in different cell samples.

2.7. Tumor cell stemness by flow cytometry

CD44⁺/CD24 cell subsets separation were detected by fluorescein isothiocyanate-labeled CD44 antibody (BD Biosciences) and PE-labeled CD24 antibody (BD Biosciences). After cells were labeled,

FACSCalibur flow cytometer (BD Biosciences) was used to detect the CD44/CD24 marker. The number of CD44⁺/CD24⁻ cell subsets of hepatocellular carcinoma cell lines was analyzed further.

3. Results

3.1. HSP90 gene expression of four strains of hepatoma cell lines

Real-time quantitative PCR showed that *HSP90* expression in smmc-7721 strain was the highest, whereas *HSP90* expression in Hep3B and LO2 were lower, in which *HSP90* expression in HepG2 were under medium level and more consistent with further study.

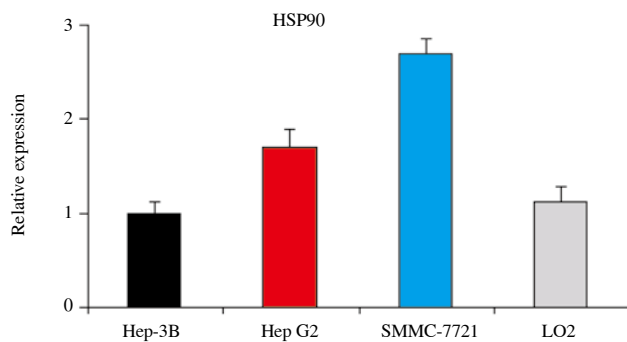


Figure 1. Expressions of HSP90 in HepG2.

3.2. HSP90 overexpression and shRNA lentiviral packaging result identification

As shown in Figure 2, HSP expression was determined by real-time quantitative PCR and Western blot. Compared with the control group, HSP expression in the HSP90 overexpression group was significantly increased, whereas HSP expression in the HSP90 shRNA group was significantly reduced, which indicated the overexpression group and shRNA group were successfully established.

group, suggesting that the expression of HSP can suppress the apoptosis of tumor cells.

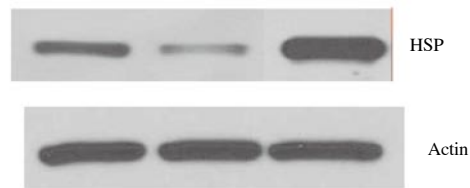
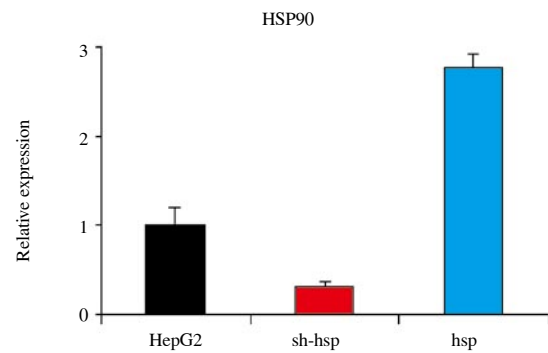


Figure 2. HSP90 overexpression and shRNA lentiviral packaging result identification.

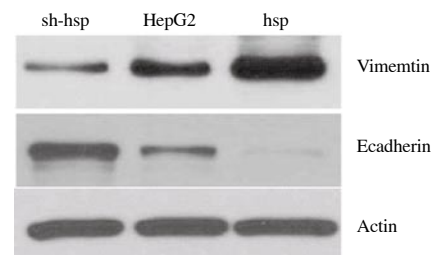


Figure 3. EMT associated markers protein detection.

3.3. EMT associated markers protein detection

The results showed that cells EMT transformation was promoted after HSP transfection. E-cadherin protein expression was down-regulated, whereas vimentin was expression up-regulated. Meanwhile in SH-HSP group, due to the interference of HSP expression in cells, E-cadherin protein was upregulated, whereas vimentin expression was down-regulated (Figure 3).

3.4. Cancer stem cells percentage by flow cytometry

The number of apoptosis cells in HSP-siRNA-treated group was significantly increased, with the ratio reached 20.52%, whereas the ratio of apoptosis in the control group was 5%. Meanwhile, the apoptosis rate in HSP overexpression group was reduced to 5.6%, which was significantly decreased when compared with the control

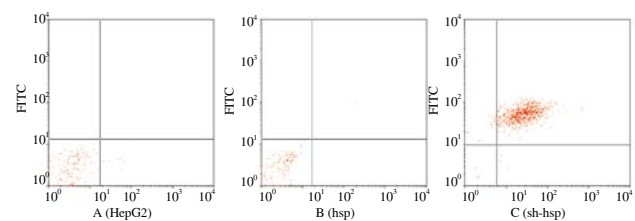


Figure 4. Cancer stem cells percentage by flow cytometry.

4. Discussion

HSP is induced by various stressor such as high temperature, anoxia, heavy metal poisoning, infection, starvation, trauma, metabolic poisons and so on, the *HSP* gene was activated and there are a group of highly expressed and highly conserved proteins. HSP widely exists in people, animals, microorganisms and plant cells[8]. As a molecular chaperone, the main function of HSP is to participate in the processes of protein folding, subunits composition, intracellular transport and protein degradation, to regulate the activity and function of the target protein, but it is not involved in the composition of HSP target protein[9].

HSP90 is one of the most important proteins of heat shock protein family. As a molecular chaperone, HSP90 is involved in regulating and maintaining the conformation and function of a variety of proteins within cells. It plays an important role in regulating cell growth, differentiation and apoptosis, etc[10]. It can be combined with a variety of signaling proteins and stabilize their activity, and thus participate in the regulation of important activity of cellular processes[11]. Currently there are more than 100 kinds of HSP90 substrate proteins[12], accumulating evidences showed that many of HSP90 is closely related to tumor invasion and metastasis. Studies have shown that HSP90 inhibition can inhibit HGF / SFMET signaling pathway, decline the invasive ability and migration ability[13]. Recent studies have shown that after the inhibition of HSP90, the invasion and metastasis of EGF-induced thyroid cancer can be inhibited[14].

SIRT3 is an extracellular matrix protein, which is highly expressed in many malignant tumors, and is closely related to tumor invasion and metastasis. SIRT3 has been reported to promote tumor stem cells stemness and inhibit the differentiation of adipose-derived stem cells, but the relationship between promoting metastasis and the characteristics of mesenchymal stem cell as the pluripotent stem cell is still not clear[15]. We found that SIRT3 can give stem cell characteristics to human hepatocellular carcinoma epithelial cells and liver cancer cells, and has a mesenchymal-like characteristics. At the same time, SIRT3 is highly expressed in bone marrow-derived and its differentiated adipocytes, osteoblasts and chondrocytes. After the over-expression of SIRT3 protein or rescheduled SIRT3 protein, the human hepatocellular carcinoma epithelial cells and liver cancer cells can differentiate to multiple directions like MSCs.

In animal experiments, human hepatocellular carcinoma epithelial cells with SIRT3 overexpression can promote the tumor formation and pulmonary metastasis of human hepatocellular carcinomas cancer cells in mice[16]. Therefore, SIRT3 protein can promote human hepatocellular carcinoma epithelial cells and liver cancer cells to obtain the potential of differentiate to multiple directions of some mesenchymal stem cells, and promote tumor growth and metastasis.

Therefore, this study discusses the development of liver cancer from the heat shock protein HSP90 and SIR3's synergistic effect on inducing epithelial cell mesenchymal transdifferentiation and enhancing characteristics of cancer stem cells. We detected HSP90 and SIR3 expressions in different liver cancer cell lines by modern molecular biology techniques such as real-time quantitative PCR and Western blot method, and further use the genes up-regulated and down-regulated technology to study the role of HSP90 in the development and progression of HCC EMT. The results showed that HSP90 can be used as an indicator to determine the biological behavior of malignant liver cancer and predict the risk of metastasis. Therefore, inhibited HSP90 expression and activity can provide new approach for blocking liver cancer cell metastasis in clinical and provide new method in anti-tumor invasion and metastasis.

Conflict of interest statement

We declare that we have no conflict of interest.

References

- [1] Scheel C, Weinberg RA. Cancer stem cells and epithelial-mesenchymal transition: concepts and molecular links. *Semin Cancer Biol* 2012; **22**(5-6): 396-403.
- [2] Ding L, Zhang Z, Shang D, Cheng J, Yuan H, Wu Y, et al. Alpha-smooth muscle actin-positive myofibroblasts, in association with epithelial-mesenchymal transition and lymphogenesis, is a critical prognostic parameter in patients with oral tongue squamous cell carcinoma. *J Oral Pathol Med* 2014; **43**(5): 335-343.
- [3] Yang J, Hu P, Zhou M, Huang J, Liu M. Construction of retroviral vector carrying Twist gene and its induction of epithelial-mesenchymal transition in human mammary epithelial cells. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2013; **29**(9): 905-909.
- [4] Chang CJ, Chao CH, Xia W, Yang JY, Xiong Y, Li CW, et al. p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs. *Nat Cell Biol* 2011; **13**(3): 317-323.
- [5] Brown RL, Reinke LM, Damerow MS, Perez D, Chodosh LA, Yang J, et al. CD44 splice isoform switching in human and mouse epithelium is essential for epithelial-mesenchymal transition and breast cancer progression. *J Clin Invest* 2011; **121**(3): 1064-1074.
- [6] Katsuno Y, Lamouille S, Derynck R. TGF-beta signaling and epithelial-mesenchymal transition in cancer progression. *Curr Opin Oncol* 2013; **25**(1): 76-84.
- [7] Bao B, Wang Z, Ali S, Kong D, Banerjee S, Ahmad A, et al. Overexpression of FoxM1 leads to epithelial-mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells. *J Cell Biochem* 2011; **112**(9): 2296-2306.
- [8] Joly AL, Wettstein G, Mignot G, Ghiringhelli F, Garrido C. Dual role of heat shock proteins as regulators of apoptosis and innate immunity. *J Innate Immun* 2010; **2**(3): 238-247.
- [9] Basha E, O'Neill H, Vierling E. Small heat shock proteins and alpha-crystallins: dynamic proteins with flexible functions. *Trends Biochem Sci* 2012; **37**(3): 106-117.
- [10] Garrido C, Paul C, Seigneuric R, Kampinga HH. The small heat shock proteins family: the long forgotten chaperones. *Int J Biochem Cell Biol* 2012; **44**(10): 1588-1592.
- [11] Kaul G, Thippeswamy H. Role of heat shock proteins in diseases and their therapeutic potential. *Indian J Microbiol* 2011; **51**(2): 124-131.
- [12] Miot M, Reidy M, Doyle SM, Hoskins JR, Johnston DM, Genest O, et al. Species-specific collaboration of heat shock proteins (Hsp) 70 and 100 in thermotolerance and protein disaggregation. *Proc Natl Acad Sci U S A* 2011; **108**(17): 6915-6920.
- [13] Bachleitner-Hofmann T, Sun MY, Chen CT, Liska D, Zeng Z, Viale A, et al. Antitumor activity of SNX-2112, a synthetic heat shock protein-90 inhibitor, in MET-amplified tumor cells with or without resistance to selective MET Inhibition. *Clin Cancer Res* 2011; **17**(1): 122-133.
- [14] Musiani D, Konda JD, Pavan S, Torchiario E, Sassi F, Noghero A, et al. Heat-shock protein 27 (HSP27, HSPB1) is up-regulated by MET kinase inhibitors and confers resistance to MET-targeted therapy. *FASEB J* 2014.
- [15] Mihaylova MM, Sabatini DM, Yilmaz OH. Dietary and metabolic control of stem cell function in physiology and cancer. *Cell Stem Cell* 2014; **14**(3): 292-305.
- [16] Simic P, Zainabadi K, Bell E, Sykes DB, Saez B, Lotinun S, et al. SIRT1 regulates differentiation of mesenchymal stem cells by deacetylating beta-catenin. *EMBO Mol Med* 2013; **5**(3): 430-440.