

Research Paper

GP73 level determines chemotherapeutic resistance in human hepatocellular carcinoma cells

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Abstract

Objective GP73 is a new hepatocellular carcinoma (HCC) marker, which is highly expressed in hepatocellular carcinoma and closely relates to prognosis. This study was to investigate the effects of GP73 on cellular proliferation, apoptosis, oxaliplatin (OXA) resistance and secretory clusterin (sCLU) of HCC cells.

Materials and Methods Western blot and immunofluorescence was used to detect the expression of GP73 in 8 types of commonly used HCC cell lines. Drug resistance was induced by increasing concentration gradient method. The drug-resistant human HCC cell lines underwent GP73 overexpression or inhibition. Flow cytometry were used to detect the proliferation and apoptosis of HCC cell lines. The changes of sCLU were detected by enzyme-linked immunosorbent assay (ELISA).

Results The expression of GP73 in MHC-97H cells was the highest and in Hep3B cells the lowest. The expression of GP73 was found further elevated in OXA-resistant MHC-97H cells. After the knockdown of GP73 in OXA-resistant 97H cells, the IC₅₀ of OXA decreased and the ability of cell proliferation decreased significantly. After over-expression of GP73 in OXA-resistant Hep3B cells, the IC₅₀ of OXA increased and the cell proliferation ability increased, showing that GP73 is critical for OXA resistant in HCC cell lines; No significant change of sCLU level in GP73 overexpressed Hep3B and GP73 blocked MHCC-97H were identified.

Conclusion The expression level of GP73 is critical for the resistance of OXA in HCC cell lines.

Key words: hepatocellular carcinoma; GP73; chemotherapy; drug resistance; OXA

Introduction

Primary hepatocellular carcinoma (PHC) is the sixth most commonly occurring cancer in the world and the third largest cause of cancer mortality [1]. The incidence of PHC is 37.5% in China [2]. Most PHC patients are hepatitis B carrier and have liver cirrhosis background. They are often diagnosed at late stage,

lacking operation opportunities. Thus, ablation or TACE treatment of PHC patients with systemic chemotherapy is commonly used in palliative treatment.

Oxaliplatin (OXA) is the third-generation platinum analog, which is cell cycle non-specific drug

and has been shown efficacy against many tumor cell lines, including some that are resistant to cisplatin and carboplatin. It is currently considered as the most effective chemotherapy drugs for PHC patients. Like other platinum-based compounds, OXA exerts cytotoxic effect mainly through DNA damage. OXA induces DNA lesion crosslinks formation, blocks DNA and mRNA synthesis [3]. In the treatment of advance PHC patients, OXA has achieved a certain effect. However, the efficacy of chemotherapy contained OXA to liver cancer are only 8.15% to 15.6% [3, 4]. The efficacy of OXA on PHC did not show encouraging results and chemotherapeutic resistance has become a huge obstacle in cancer treatment. Therefore, the mechanism of chemotherapeutic resistance in HCC requires further studies.

Golgi protein-73 (GP73) was originally detected in hepatocytes of patients with hepatocellular carcinoma caused by adenovirus. It also expresses in epithelial cells such as stomach, spleen and colon. In the normal hepatobiliary system, GP73 only expresses in adult bile duct epithelium while liver cells do not express it [5]. However, GP73 is highly expressed in HCC cells, regardless of whether viruses' infection exist or not [6]. Secretory GP73 is also found in serum of patients with HCC. Some studies suggested that GP73 serum levels can assist HCC diagnosis and prediction, showing even better efficacy and accuracy than AFP [7, 8]. Additionally, the increase of GP73 after treatment predicts a higher tumor recurrence rates in HCC [9]. Moreover, the expression level of GP73 in hepatocellular carcinoma is related to tumor size, tumor invasiveness, and tumor differentiation [10]. Using cDNA microarray, Takeyasu katada *et al* found that GP73 gene highly expressed in high-resistant TE4 esophageal cancer cells and the sensitivity of TE4 cells to cisplatin was significantly increased after GP73 inhibition [11]. Thus, we hypothesize that GP73 may be involved in the chemotherapeutic resistance of HCC. However, no report about GP73 and chemo-resistance of HCC is found. The aim of this study was to investigate the effects of the manipulation of GP73 on OXA resistance of HCC cell lines.

Methods

Cell culture

Human normal liver cell line and human hepatic carcinoma cell line (HepG2, Bel7402, Bel7404, SMMC7721, SNU739) were purchased from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (CAS). After being thawed, resuscitated and passaged, the cells were kept in RPMI 1640 medium containing 10% fetal

bovine serum and cultured in 37 °C, 5% CO₂ saturated humidity cell incubator.

Antibodies, Western blots and Immunofluorescence

The antibodies used in this study were as follows: mouse-derived anti-GP73 antibody (Proteintech); the secondary antibodies in this study were derived from Jackson ImmunoResearch. The expression of GP73 was detected by Western blotting (1: 800 dilution). The secondary antibody was either mouse anti-IgG or rabbit anti-IgG (1: 8000 dilution). An enhanced chemiluminescent agent, supplied by Millipore Corporation, is used for immunoreaction detection. For immunofluorescence, Alexa Fluor® 488 Donkey Anti-Rabbit IgG (Invitrogen, No: A21206, 1:1000) and Alexa Fluor® 555 Donkey Anti-Goat IgG (Invitrogen, No: A21432, 1:1000) were applied as secondary antibodies. Experiments were conducted based on standard protocols.

Establishment of OXA-resistant Hepatocellular Carcinoma Cell Line

The drug resistance was induced by increasing the concentration gradient. First, the IC₅₀ of the cells was detected by CCK method, and the highest concentration of OXA was the concentration determined by IC₅₀. After 2 μM of OXA was selected as the starting concentration, the culture medium was changed to a culture medium containing OXA for 48 hours and the culture was continued until cell death was observed. After the first round incubation, the drug concentration was gradually increased to 4, 6, 8, 10, 30 and 60 μM. After rounds of induction, drug-resistant cell lines were frozen for the following experiments.

Establishment of stable cell lines in which GP73 was overexpressed or knocked down

The siRNA sequence was the following: Si RNA 1: 5'-agggaacgtgcttggttaa-3'; Si RNA 2: 5'-gaatagaa gaggtcaccaa-3'. Lentiviral vectors encoding shRNA were designed based on the sequences of siRNA to knock down GP73 expression (GP73-KD). These vectors were constructed by Hanyin Co. (Shanghai, China). The recombinant lentiviruses (KD) and negative control (NC) lentivirus were prepared and titered to 10⁹ transfection units (TU)/mL. To obtain stable cell lines, cells were seeded in six-well plates and infected with virus and polybrene on the following day. Positive clones were selected with puromycin for 14 days to establish the stable cell lines. The lentiviruses expressing the GP73 sequence (OE) and the negative control lentivirus (Vector) were also constructed by Hanyin Co. (Shanghai, China).

GP73-OE and control stable cell lines were then established in a similar way. The efficiency of GP73 knockdown and overexpression were confirmed by qRT-PCR.

CCK method

5×10^3 cells in 200 μ l culture medium were inoculated into each well of 96-well plate. After cell adherence, the medium was changed to different concentrations of OXA (8-256 μ M) contained RPA1640 and further incubation for 72 hours. Then, 10 μ l of MTT solution was added to each well. After further incubation for 1 hour, the absorbance at 450 nm was measured by a microplate reader. IC₅₀ was calculated by software, and the multiple of drug resistance and reversal of drug resistance after GP73 manipulation were calculated (drug-resistant multiples = IC₅₀ of drug-resistant cells/IC₅₀ of parent cells; drug-resistant reversal multiple = IC₅₀ before reversal drug resistance/ IC₅₀ after reversal drug resistance).

Fluorescence-activated cell sorting (FACS)

The reagents used were as follows: Annexin V-FITC, Propidium Iodide (BD Biosciences, USA). The cells were digested with 0.25% trypsin without EDTA and washed with PBS. After resuspending the cells, Annexin V-FITC and propidium iodide were added and mixed well. Cells were incubated in dark room for 15 minutes and analyzed by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA)

The reagents used were as follows: human CLU elisa kit (MLBIO Co, Shanghai, China). CLU expression test was performed strictly according to the instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism7 software (standard version, California, USA). A two-sided probability value less than 0.05 was considered statistically significant. Unless stated, data are presented as mean \pm SD in the figures. A Student t test was performed to compare the data.

Results

The expression of GP73 in HCC cell lines

The expression of GP73 in 8 HCC cell lines and normal liver cell line L02 was shown by both western blot and immunofluorescence (Fig 1A and B), they consistently revealed that GP73 expressed highest in MHCC-97H cells and lowest in Hep3B cells. The expression of GP73 in normal liver cell line L02 is obviously lower than other liver cancer cell lines as shown by immunofluorescence. After resistance

induction, the expression of GP73 in OXA-resistant MHCC-97H cells (MHCC-97H-OXA) was obviously higher than that in parental MHCC-97H cell lines (Fig 1C and D). To address the question about expression difference in normal and cancerous liver cells, we showed some representative figures from Human Protein Atlas (www.proteinatlas.org). It clearly demonstrated that GP73 express preferentially in liver cancer cells (Fig 1E).

The correlation between GP73 level and OXA resistance of HCC cells

The IC₅₀ of MHCC-97H cell line was 39.62 μ M. After induction of drug resistance, the IC₅₀ of MHCC-97H-OXA cells was 412.7 μ M, and drug-resistance multiple was 10.42. GP73 was further knocked down in MHCC-97H-OXA cells, resulting to a decreased IC₅₀ (218.5 μ M of MHCC-97H-OXA-GP73-sh4 and 166.1 μ M of MHCC-97H-OXA-GP73-sh5 respectively). Drug resistance reversal multiple of MHCC-97H-OXA-GP73-sh4 is 1.91 and MHCC-97H-OXA-GP73-sh5 is 2.48. The IC₅₀ difference between MHCC-97H-OXA and GP73 blocked MHCC-97H-OXA cell lines was statistically significant (Figure 2A).

The IC₅₀ of Hep3B cell line was 17.69 μ M. After induction of drug resistance, The IC₅₀ of Hep3B-OXA was 64.64 μ M, and the drug-resistance multiple was 3.65. After GP73 overexpression, the IC₅₀ of Hep3B-OXA-GP73-OE was 162.3 μ M and the drug-resistance reversal multiple was 0.398. There was significant difference in the IC₅₀ among these three different Hep3B lines (Fig 2B).

GP73 and the proliferation of HCC cell

GP73 expression is successfully depleted in Sh4 and Sh5 groups (Fig 3A, left, middle). Importantly, the proliferation of MHCC-97H-GP73-sh4 and MHCC-97H-GP73-sh5 obviously decreased when compared with normal control (NC) cell line. On the fifth day, the differences of proliferation of each cell line was statistically significant ($P < 0.001$, Fig. 3A, right).

The proliferation of GP73 overexpressed Hep3B cells (Hep3B-GP73-OE) was much higher than that of the parental cell lines (Hep3B). On the fifth day, the proliferation ability difference was statistically significant ($p < 0.01$, Fig 3B).

OXA-resistant cells (MHCC-97H-OXA) were established by concentration-elevation method (OXA, 500 μ M, 48h). The proliferation of MHCC-97H-OXA cells was the highest. When GP73 was knocked down after the induction of drug resistance, the proliferation ability of the MHCC-97H-OXA-GP73-sh4 and MHCC-97H-OXA-GP73-sh5 were much lower than that of the MHCC-97H cells. On the fifth day, the

proliferation ability of each cell line was statistically significant ($P < 0.001$, Fig 3C, left).

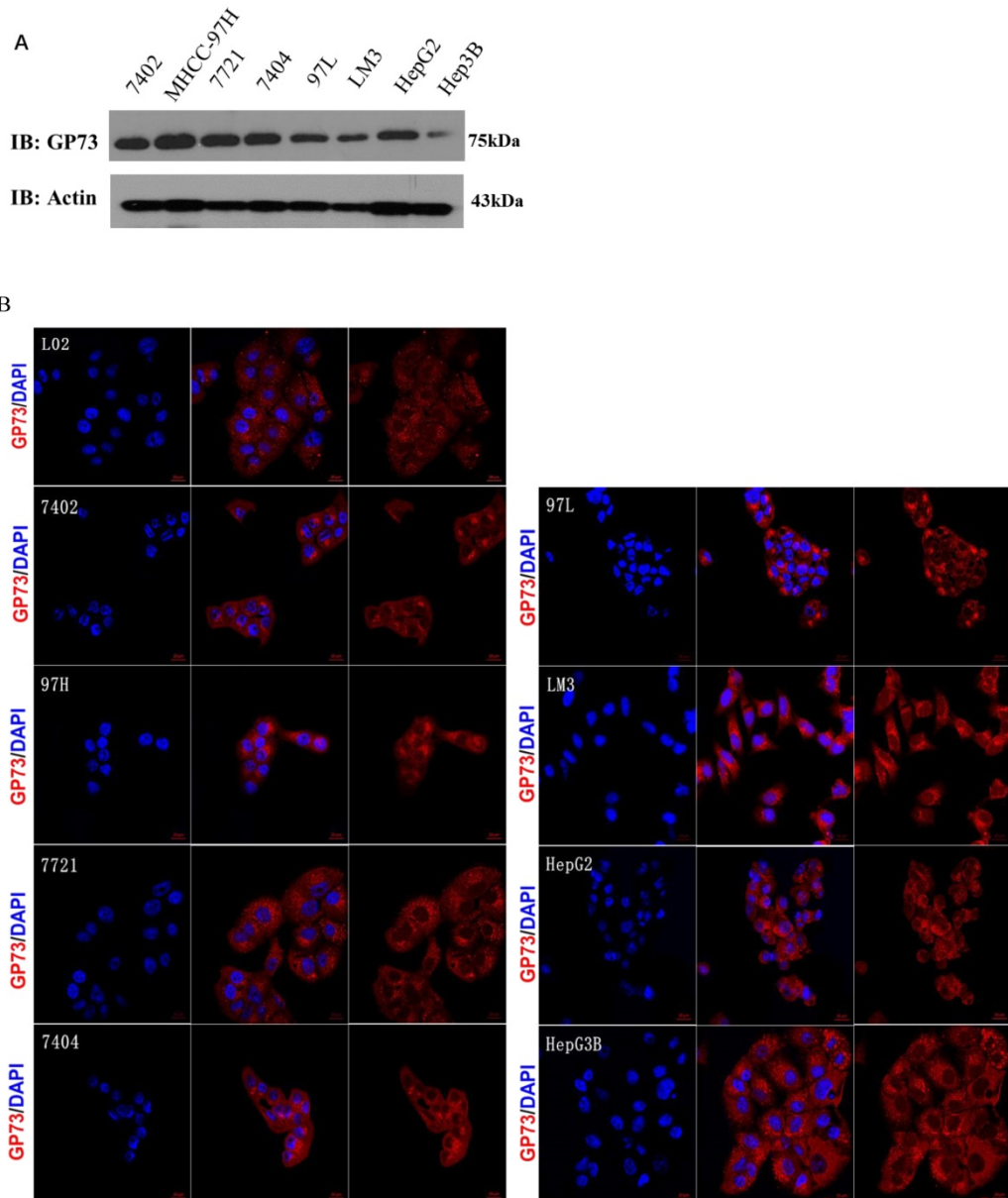
The proliferation of OXA resistant Hep3B-OXA cells was shown to be significantly higher than that of Hep3B cells ($P < 0.05$, Fig 3C, right). Overexpression of GP73 resulted in a higher proliferation ability in Hep3B-OXA-GP73-OE cells. On the fifth day, the proliferation ability of each cell line was statistically significant ($p < 0.01$, Fig 3C, right).

GP73 and apoptosis of HCC cell lines

As shown in Fig 4A, the knockdown of GP73 in MHCC-97H cells (MHCC-97H-GP73-sh4 and MHCC-97H-GP73-sh5 leads to increased apoptosis rates ($p < 0.001$, Fig 4A, left). As shown in Fig 4A, the apoptosis rates of GP73 over-expressed cells (Hep3B-GP73-OE) was significantly lower than

that of the parent Hep3B cells ($p = 0.008$, Fig 4A, right).

In order to study the effects of GP73 on the anti-apoptotic ability of drug-resistant HCC cell lines. After treatment with OXA (500 μ M) for 72 h, the apoptosis of different types of 97H cell line was examined. The results showed that MHCC-97H-OXA possessed lowest apoptosis rates. MHCC-97-OXA-GP73-sh5 and MHCC-97-OXA-GP73-sh4 showed higher apoptosis rate than that of MHCC-97H-OXA cells ($p < 0.01$, Figure 4B, left). The apoptosis rate of the Hep3B was the highest when compared with OXA resistant cell lines. After GP73 over expression, the apoptosis rate was further decreased when compared with OXA resistant Hep3B cells ($p < 0.01$, Fig 4B, right).



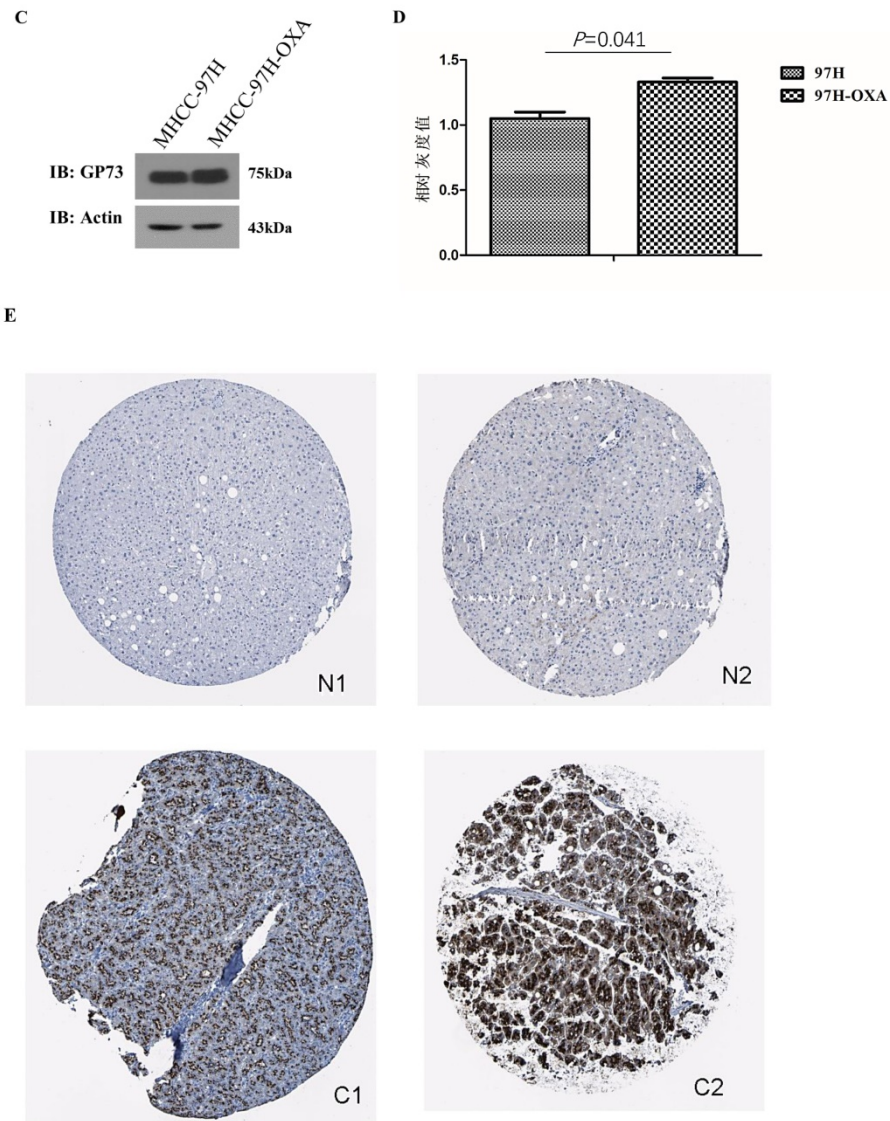


Figure 1. The expression of GP73 in liver cancer cell lines and liver tissues. The expression of GP73 in 8 HCC cell lines and normal liver cell line L02, Scale bar = 20 μm (Figure 1A, 1B); Expression of GP73 in HCC cell lines before and after OXA induction MHCC-97H-OXA was the cell line after induction of OXA resistance on MHCC-97H (Figure 1C, 1D). Representative figures of GP73 expression by IHC from Human Protein Atlas (www.proteinatlas.org). N: normal tissue; C: cancer tissue (Fig 1E).

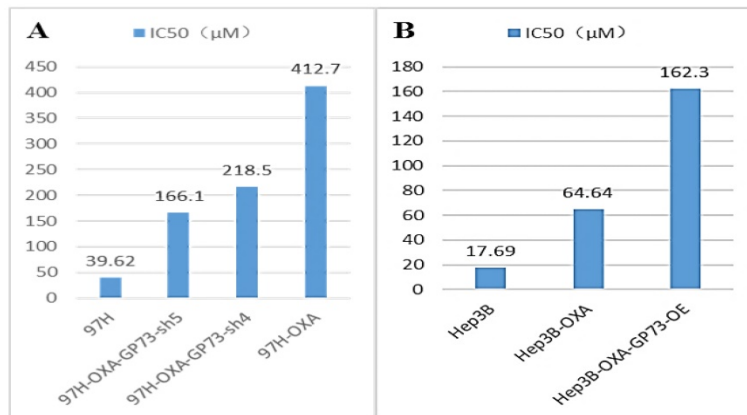


Figure 2. The comparison of the IC50 (μM) before and after the intervention of the HCC cell line. 97H-OXA was the cell line after induction of MHCC-97H resistance; 97H-OXA-GP73-sh4 and 97H-OXA-GP73-sh5 were the cell lines that knock-out of GP73 gene after OXA resistance induction of MHCC-97H cell; Hep3B-OXA was the Hep3B cells line induced by OXA resistance; Hep3B-OXA-GP73-OE was the cell line over-expressed GP73 after induction of OXA resistance of Hep3B cells.

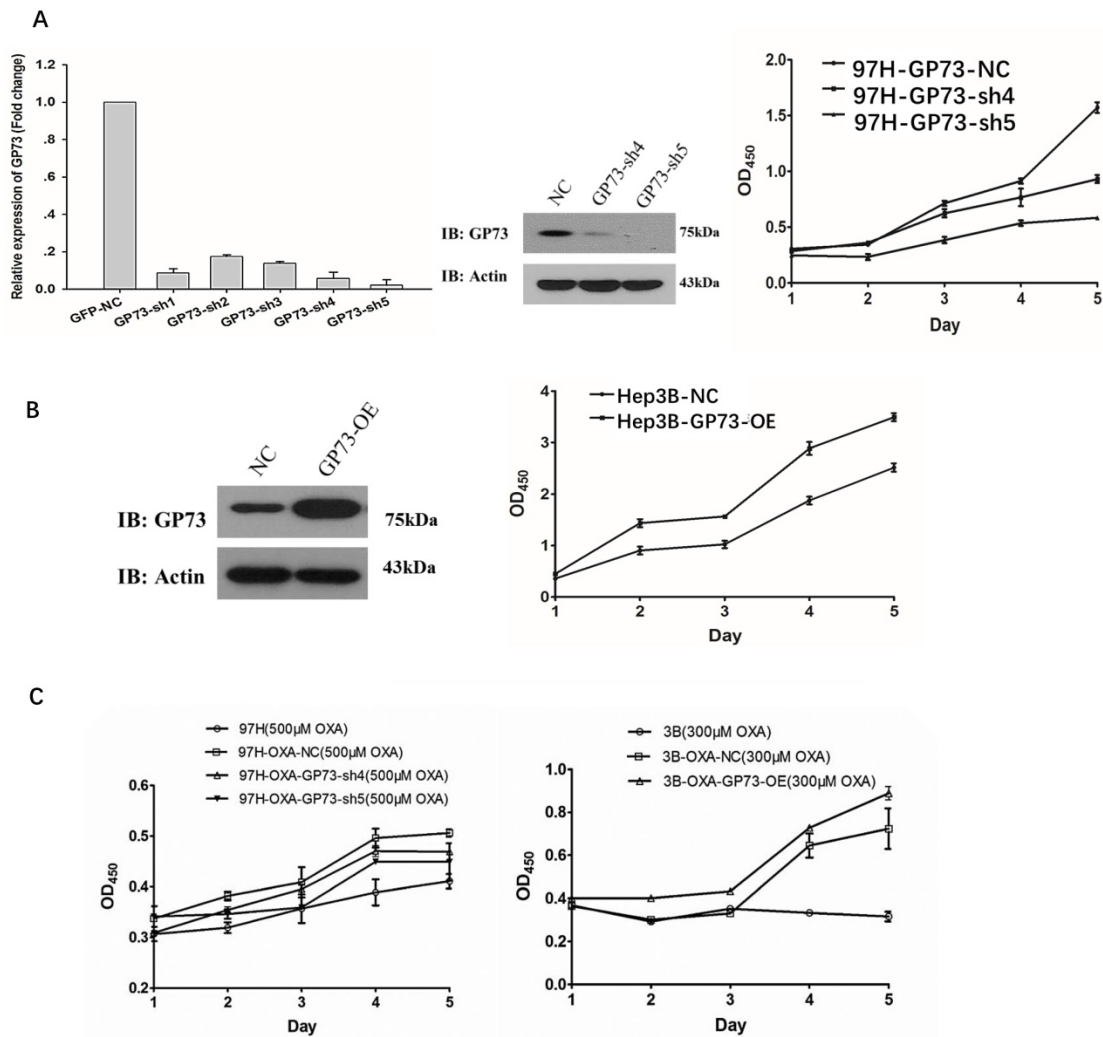


Figure 3. GP73 and the proliferation activity. GP73 and the proliferation activity of MHCC-97H cells (Figure 3A). GFP-NC was the GP73 negative control MHCC-97H cells; GP73-sh1 to sh5 were the MHCC-97H cells that GP73 gene were knocked down, respectively. **GP73 and The proliferation activity of Hep3B cells** (Figure 3B, left) GP73-OE was the Hep3B cells that GP73 gene over-expressed; Hep3B-NC was the negative control Hep3B cells. **GP73 and proliferation activity of OXA-resistance cell** (Fig 3C, $P < 0.01$) 97H-OXA was the cell line after induction of MHCC-97H resistance; 97H-OXA-nc is 97H-OXA cell as negative control; 97H-OXA-GP73-sh4 and 97H-OXA-GP73-sh5 were the cell lines with knocked down GP73 expression on the basis of 97H-OXA cells. Hep3B-OXA-nc was the negative control; Hep3B-OXA-GP73-OE was the cell line over-expressed GP73 after induction of OXA resistance of Hep3B cells.

The relationships between GP73 and sCLU

Secretory clusterin (sCLU) is thought to be an inhibitor of apoptosis, which can inhibit the apoptosis of HCC cells by interacting with GRP78 and caspase-9, and it is closely associated with HCC drug resistance to OXA and other chemotherapeutics. We detected sCLU level by Elisa as mentioned in Method section. We found that the manipulation of GP73 (overexpression or knockdown) have no effects on sCLU in both MHCC-97H and Hep3B cell lines. There was no significant difference in the concentration of sCLU secreted by different types of OXA resistant HCC cell lines (Fig 4D).

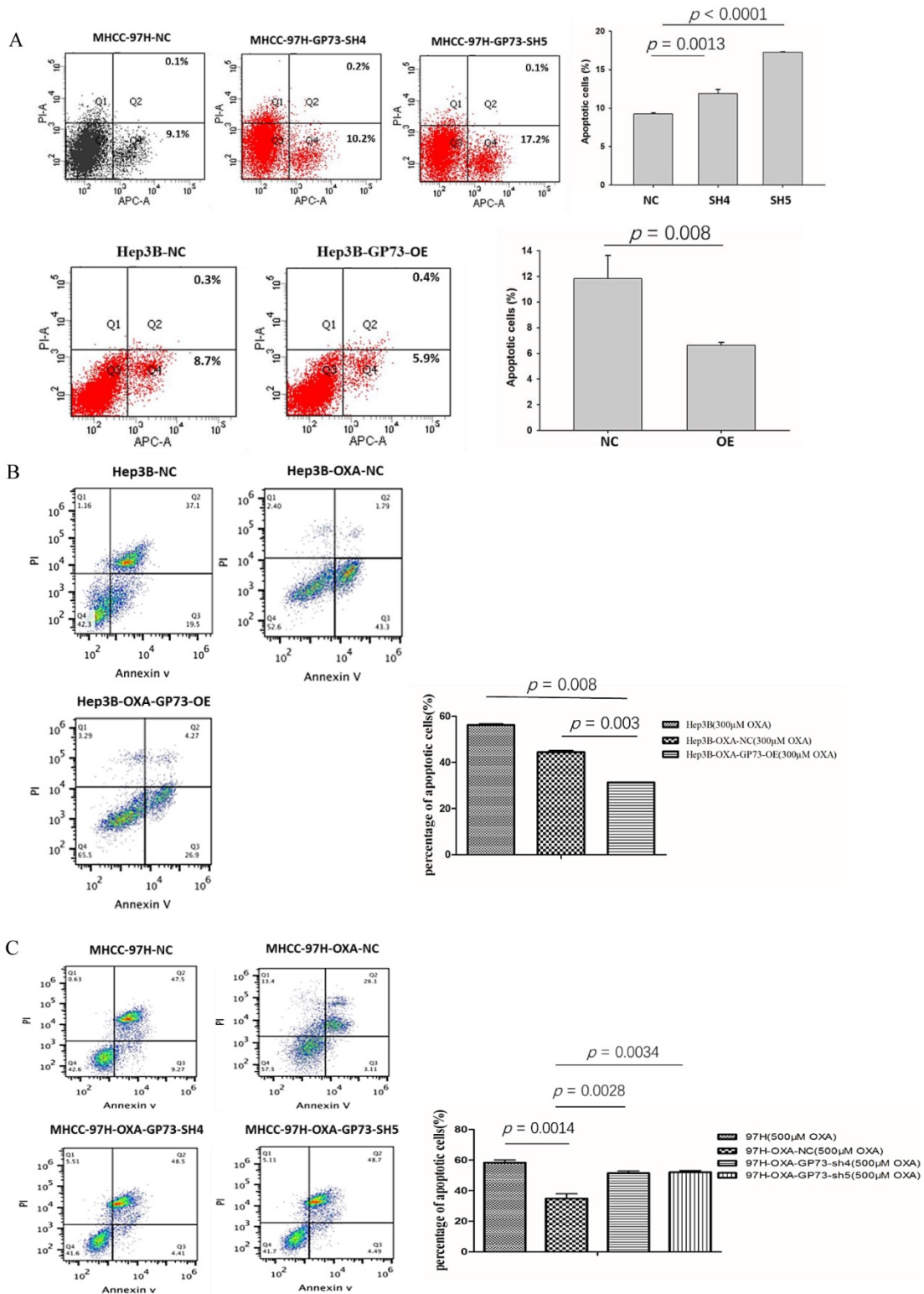
Discussion

GP73 is a transmembrane glycoprotein with a molecular weight of 73 kDa. GP73 is high expressed in

a variety of tumors, such as in liver cancer, lung adenocarcinoma, prostate cancer and seminoma [12-15]. It is worth noting that in serum and liver tissue of patients with HCC, both virus infected and non-viral caused (alcoholic liver disease and autoimmune hepatitis), GP73 levels were significantly increased [6]. GP73 is considered as a valuable diagnostic marker of liver cancer in recent years. It has been shown that soluble GP73 in serum is derived from the Golgi apparatus. The amino terminus is a hydrophobic end, mainly located in the Golgi apparatus, constituting a transmembrane region and a signal peptide cleavage site. However, the carboxyl terminus is located outside the Golgi apparatus and consists of a continuous sequence of fourteen alkanoyl groups, five glycosylation sites, an acidic domain and a coiled-coil structure. These unique structures are the basis for GP73 to participate in the pathophysiology of

intracellular signaling, phosphorylation and dephosphorylation of proteins, and to give full scenario to its biological function [16, 17]. At present, it is known that the carboxyl terminal of G73 is the main functional domain since the truncation of carboxyl terminus of GP73 shortened the survival time of the transgenic mice and severely damaged

the development of the kidney and liver system and the epithelial function [18]. GP73 participates in many physiological processes, such as immune regulation, cell adhesion and cell apoptosis [19]. Therefore, we speculate that the high expression of GP73 has important biological functions in the development and chemotherapeutic resistance of HCC.



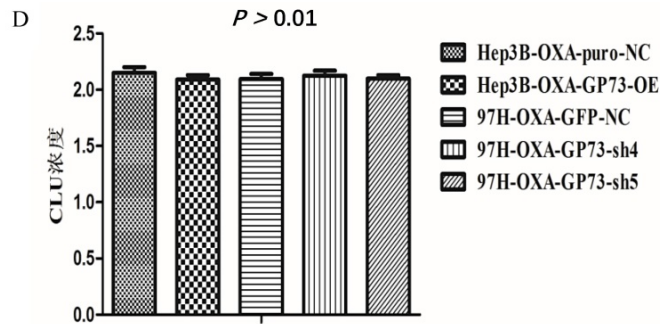


Figure 4. GP73 and the apoptosis activity. GP73 and the apoptosis of MHCC-97H cell (Figure 4A, upper) and Hep3B cell (Figure 4A, lower). NC was the MHCC-97H cell as negative control; SH4 (97H-OXA-GP73-sh4) and SH5 (97H-OXA-GP73-sh5) were the cell lines with knocked down GP73 expression on the basis of MHCC-97H; NC (Hep3B-NC) was the Hep3B cell as negative control; OE (Hep3B-GP73-OE) was the cell line with over-expressed GP73. **GP73 and the apoptosis of MHCC-97H OXA-resistance cell (Figure 4B) Hep3B OXA-resistance cell (Figure 4C) after treatment with OXA.** MHCC-97H-NC was the MHCC-97H cell as negative control; MHCC-97H-OXA was OXA resistant; 97H-OXA-GP73-sh4 and 97H-OXA-GP73-sh5 were the cell lines with knocked down GP73 expression on the basis of MHCC-97H-OXA; Hep3B-NC was the Hep3B cell as negative control; Hep3B-OXA was the Hep3B cells line induced with OXA resistance; Hep3B-OXA-GP73-OE was the cell line with over-expressed GP73 after induction of OXA resistance of Hep3B cells. **The expression of GP73 and the level of sCLU in OXA resistant HCC cell lines (Figure 4D).** Hep3B-OXA was the Hep3B cells line induced by OXA resistance; Hep3B-OXA-puro-NC was the Hep3B-OXA cell as negative control; Hep3B-OXA-GP73-OE was the cell line with over expressed GP73 after induction of OXA resistance of Hep3B cells; 97H-OXA-GP73-NC was the MHCC-97H cell after induced OXA resistance; 97H-OXA-GP73-sh4 and 97H-OXA-GP73-sh5 were the cell lines with knocked down GP73 expressions after OXA resistance induction of MHCC-97H cell line.

In this study, we investigated the effects of GP73 on the proliferation, apoptosis, and resistance to OXA in hepatic carcinoma cells from vitro (cell culture). The results of the following two aspects revealed that the expression level of GP73 directly affects the proliferation, apoptosis and drug resistance of HCC cells: (1) The expression level of GP73 changed the proliferation and apoptosis of HCC cells; (2) The expression level of GP73 changed the IC_{50} , proliferation activity and apoptosis of OXA resistant HCC cells, affecting the sensitivity of HCC cells to OXA.

Zhang *et al* reported that GP73 silence not only inhibited the proliferation of hepatocellular carcinoma cells, but also induced apoptosis [20]. Our study also showed that knock down of GP73 in MHCC-97H cells and over-expression of GP73 in Hep3B cells significantly changed the proliferation and apoptosis of HCC cells. OXA inhibits the proliferation and promote apoptosis of hepatic carcinoma cells [21, 22]. One of the main mechanisms of tumor cell resistance to chemotherapy is cancer cells can resist and escape chemotherapy-induced apoptosis [23]. We successfully constructed the OXA-resistant hepatic carcinoma cells in vitro and found that the expression level of GP73 in MHCC-97H resistant cell line was significantly higher than that in non-resistant cell line, suggesting that GP73 expression level correlated with drug resistance. Knock down of GP73 in MHCC-97H drug-resistance cells decreased its IC_{50} significantly. GP73 overexpression in Hep3B cells showed opposite phenomenon. In addition, the proliferation and apoptosis of HCC cell lines treated with different concentration of OXA and manipulation of GP73 were detected. The results showed that GP73 positively

correlated with proliferative activity in the cell lines and negatively correlated with the apoptosis, indicating that GP73 directly affects the drug sensitivity of OXA. sCLU is considered to be an inhibitor of apoptosis by interacting with GRP78, caspase-9 and other factors [24, 25]. sCLU increase the resistance of HCC cells to chemotherapy by interacting with AKT and Bcl-2 / Bax pathway [25]. In pancreatic cancer, prostate cancer and other tumors, also observed increased sCLU expression, and increased resistance of tumor cells to chemotherapeutic drugs, while blocking the expression of sCLU protein increased the sensitivity of chemotherapy [26, 27]. To analyze the relationship between GP73 and sCLU, we detected the expression of sCLU in OXA resistant HCC cell lines with different GP73 expression levels. We failed to observe any significant change of sCLU levels between groups, suggesting that GP73 might regulate apoptosis in a sCLU independent pathway. However, this study only analyzed the expression level of sCLU protein, the interaction between sCLU and GP73 as well as the functional changes of sCLU needs further studies.

In conclusion, we demonstrated that GP73 is involved in the regulation of cellular proliferation, apoptosis and OXA resistance in HCC cells in vitro. GP73 can be a potential therapeutic target.

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Authors' contributions

Jia-Zhou Ye analyzed the data and wrote the manuscript; Rong Liang and Yan Lin designed the study and revised the manuscript; Chun-ling Yuan, Jin-yan Zhang, Zhi-Hui Liu and Yong-Qiang Li performed all the experiments and collected all the data; Shu-mei Yan, Hui-ni Wu and Xiao-Ling Luo analyzed the data.

Competing Interests

The authors have declared that no competing interest exists.

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