

Increased glyceraldehyde-3-phosphate dehydrogenase expression indicates higher survival rates in male patients with hepatitis B virus-associated hepatocellular carcinoma and cirrhosis

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Abstract. Elevated expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been reported in different human malignancies. To understand its role in hepatitis B virus (HBV) infection-associated hepatocellular carcinoma (HCC), the expression of GAPDH was quantitatively measured in a cohort of 72 male HCC patients without preoperative treatment, all with evidence of chronic HBV infection. Using C-terminal banding protein 1 (CTBP1) or hypoxanthine phosphoribosyltransferase 1 (HPRT1) as reference genes, the level of GAPDH mRNA in tumor tissue was found to be significantly higher compared with that in paired non tumor tissues ($P=0.0087$ for CTBP1; $P=0.0116$ for HPRT1). Accordingly, compared with the non-tumor tissue, 37.5% (27/72) of patients' tumor tissues had a more than 2-fold increase of GAPDH

expression. Furthermore, following knockdown GAPDH expression via siRNA transient transfection, HepG2 cells exhibited enhanced resistance to cytosine arabinoside (IC_{50} , 308.28 μ M vs. 67.68 μ M in the control; $P=0.01$). Notably, higher GAPDH expression was significantly associated with lower liver fibrosis score ($P=0.0394$) and a tendency towards higher survival rates for patients with HCC. To the best of our knowledge, the present study is the first study to report that the elevated expression levels of GAPDH in HCC tumor tissue may be relevant to an improved fibrosis score and survival probability in male patients with HBV infection; however, the underlying mechanism requires further investigation.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-associated mortality in males and the sixth leading cause in females worldwide (1). In addition, HCC is ranked as the second leading cause of cancer-related mortality in China (2). Over 50% of the annual HCC cases reported worldwide are diagnosed in the Chinese population (3), while hepatitis B virus (HBV) infection is responsible for 80% of all HCC causes in China (4). While a number of studies have revealed that HBV infection is the key pathogenic factor for the development of HCC, male gender and cirrhosis have also been found to be independent risk factors for the occurrence of HCC (5-7). However, few studies exist on patients presenting all these risk factors.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was initially considered to be an essential glycolytic enzyme that is expressed in all prokaryotic and eukaryotic organisms. GAPDH plays a major role in cellular metabolism, converting glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (8). In addition, studies have demonstrated that GAPDH is involved in certain important physiological functions, including the transport of transfer RNA (9), translational control (10) and binding with viral RNAs (11,12). Furthermore, increased GAPDH expression has been previously reported in renal

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Abbreviation: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CTBP1, C-terminal banding protein 1; HPRT1, hypoxanthine phosphoribosyltransferase 1; araC, cytosine arabinoside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

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cell carcinoma (13), lung cancer (14), breast cancer (15), prostate carcinoma (16) and HCC (17). A number of studies have demonstrated that the elevated expression of GAPDH is correlated with chemotherapy-induced DNA damage response (18,19). The induction of cell cycle arrest in p53-proficient carcinoma cells through GAPDH abrogation indicates that GAPDH-depleting agents may have a cytostatic effect in cancer cells (20). In addition, higher intranuclear GAPDH expression has been found to be correlated with higher cell sensitivity to mercaptopurine treatment in human leukemia cell lines (21). Therefore, investigating the underlying mechanism resulting in aberrant expression of GAPDH in the development of HCC in patients presenting the aforementioned risk factors is essential.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is a powerful tool used to detect the level of gene expression in certain tissues under normal and disease conditions (22,23). The authors of the present study have previously used RT-qPCR to analyze the stability of six candidate genes in paired tumor and non-tumor tissues from 33 male with untreated HBV-associated HCC and cirrhosis, using the geNorm and NormFinder software (24). C-terminal banding protein 1 (CTBP1) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) were demonstrated to be evidently more stable compared with GAPDH, while CTBP1 was found to have the most stable gene expression (24). The present study aimed to investigate whether GAPDH expression was aberrantly altered in the tumors of male HCC patients with chronic HBV infection. The clinical significance was also addressed.

Materials and methods

Patient information and sample collection. HCC tumor and paired non-tumor tissue samples were obtained from 72 untreated male patients, suffering from HBV-associated HCC with cirrhosis. The patients underwent hepatectomy at the Henan Cancer Hospital (Zhengzhou, China) between July 2009 and October 2010. The selected HCC patients met all the following criteria: seropositive for HBV surface antigen (HBsAg) or HBV DNA-positive in tumor tissues; received no chemotherapy prior to surgery; male gender; and suffered from liver cirrhosis. Liver cirrhosis and HCC were evaluated by two pathologists independently. The Ishak scoring system was used to assess fibrosis stage (25). Samples were collected from the tumor and paired non-tumor tissues following hepatectomy and the specimens were snap-frozen in liquid nitrogen. The age range of the 72 patients was 34-76 years (mean age, 51.25 ± 9.91 years). The Institutional Review Board of Peking University (Beijing, China) approved all the procedures in the present study. Written informed consent was obtained from the patients/patients' families prior to their participation.

Analysis of GAPDH mRNA expression levels in tumor and non-tumor tissues from HCC patients using RT-qPCR. The tissue specimens were ground in liquid nitrogen and homogenized in TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) using a mortar. Total RNA was extracted with TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. Genomic DNA contamination was removed by on-column digestion using the RNase-free DNase kit (Takara

Bio Inc., Otsu, Japan). The concentration of the isolated total RNA was calculated by measuring the absorbance (A) at 260 and 280 nm using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). A260/A280 ratio of >1.90 and 28S/18S ratio of ≥ 1.7 were the threshold values for inclusion of the RNA samples in this study. The integrity of the RNA samples was confirmed by electrophoresis on a 1% agarose gel. First-strand cDNA was synthesized using a random primer and the RevertAid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions. The primers used in the RT-qPCR assays of GAPDH were designed using the Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). The Roche LightCycler 480 detection system (Roche Diagnostics GmbH, Mannheim, Germany) was used for RT-qPCR analysis. The reactions were performed in a final volume of 20 μ l, containing 10 μ l of SYBR[®] Green master mix (Roche Diagnostics GmbH), 0.5 μ l of each 10 μ M primer (500 nM), 1 μ l cDNA and 8 μ l nuclease-free sterile water. All the standard solutions and samples were analyzed in triplicate on 96-well reaction plates. The cycling conditions were set as follows: 10 min template denaturation at 95°C, 40 cycles of denaturation at 95°C for 30 sec and elongation at 72°C for 30 sec. Melting-curve analysis was performed following RT-qPCR and the baseline and cycle threshold values (Ct values) were automatically determined for all the plates using the Roche LightCycler 480 software. A Ct value difference between triplicates of ≤ 1 was considered as acceptable and was used to calculate the average Ct values. Genes exhibiting an >2 -fold increase ($2^{-\Delta Ct} > 2$) or an <0.5 -fold decrease ($2^{-\Delta Ct} < 0.5$) in their expression levels were considered to be differentially expressed, whereas genes with an ≤ 2 -fold increase or ≥ 0.5 -fold decrease ($0.5 \leq 2^{-\Delta Ct} \leq 2$) were defined as equally expressed, as previously reported (26).

Construction of GAPDH expression vectors. The 1026 bp GAPDH cDNA containing the entire open reading frame was amplified using RT-qPCR and cloned into the expression vector, pIRES2-EGFP (Clontech Laboratories, Inc., Palo Alto, CA, USA). The GAPDH primers used were as follows: sense, 5'-CCGGAATTCATGGGGAAGGTGAAGG-3'; and anti-sense, 5'-GACGTCGACTTACTCCTTGGAGGCCATG-3'. The GAPDH-expressing vector, pGAPDH-IRES2-EGFP, was constructed and confirmed by automated sequencing, in order to establish the direction of cloning and determine whether the sequence was correct.

HepG2 and PLC/PRF/5 cell cultures, drug treatment and viability assay. HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA), while PLC/PRF/5 cells (American Type Culture Collection) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. In this study, the stock solutions of 10^4 μ M epirubicin (Pfizer, New York, NY, USA) or 10^6 μ M araC (Sigma-Aldrich, St. Louis, MO, USA) were used, which were dissolved in phosphate-buffered saline and stored at -20°C.

Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; Promega Corporation,

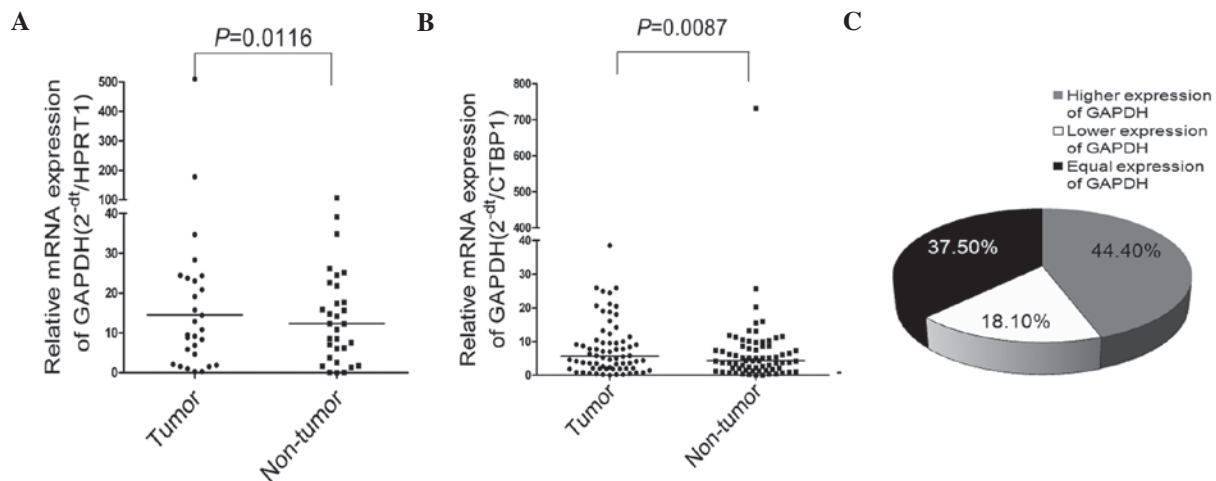


Figure 1. Relative expression levels of GAPDH in paired tumor and non tumor tissues, (A) in 33 patients, using hypoxanthine phosphoribosyltransferase 1 (HPRT1) as the reference gene, and (B) in 72 patients, using C-terminal banding protein 1 (CTBP1) as the reference gene. (C) Distribution of relative GAPDH expression levels in 72 patients. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Madison, WI, USA]. HepG2 cells (7,000 cells/well) and PLC/PRF/5 cells (8,000 cells/well) were plated into 96-well plates and cultured for two days with various drug concentrations (10^{-3} - $10 \mu\text{M}$ epirubicin or $5\text{-}5 \times 10^4 \mu\text{M}$ araC). The IC_{50} values, representing the half maximal inhibitory concentration, were calculated using the GraphPad Prism 5.0a software (GraphPad Software Inc., San Diego, CA, USA) software. To evaluate the effect of GAPDH expression on drug resistance, the cells were treated with araC or epirubicin at 48 h after transfection.

The cells were seeded in six-well plates or in 60-mm dishes and grown for 24 h before transfection. Plasmids and small interfering RNA (siRNA) molecules were transfected into the cells using Lipofectamine™ 2000 transfection reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. A GAPDH siRNA kit (#NM-002046) and scrambled control siRNA were purchased from Sigma-Aldrich. The primer sequences used were as follows: GAPDH siRNA sense, 5'-GGUUUACAUGUCCAAUAUdTdT-3', and anti-sense, 5'-AUAUUGGAACAUGUAAACCDdT-3'; siRNA negative control sense, 5'-UUCUCCGAACGUGUCACGUTT-3', and anti-sense, 5'-ACGUGACACGUUCGGAGAATT-3'. Initially, western blot analysis was performed to validate the expression of GAPDH in the transfected plasmid (27).

The primary antibodies used in the present study included mouse p21 [MBL(K0081-3); 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA], mouse GAPDH [MBL(M171-3); 1:3,000 Santa Cruz Biotechnology, Inc.] and rabbit β -actin (sc-1616; 1:1,000; Santa Cruz Biotechnology, Inc.). The cultured cells were collected and the proteins were lysed in RIPA buffer (Applygen Technologies Inc., Beijing, China). Protein samples (30 μg each) were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels, electrophoresed and transferred onto nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden). Briefly, membranes were blocked with 5% dried milk in phosphate-buffered saline (PBS) for 2 h followed by incubation with the primary antibodies for 2 h. After 3 washes with PBS containing 0.1% Tween-20, the membranes were incubated with the IRDye® 680 goat anti-rabbit

(#926-32221; 1:8,000; LI-COR Biosciences, Cambridge, UK) and IRDye® 680 goat anti-mouse (#926-32220; 1:8,000; LI-COR Biosciences) secondary antibodies for 1 h at room temperature. Protein-antibody complexes were visualized using the secondary antibodies conjugated with Cy5.5 (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) and the LI-COR Odyssey® IR Imaging system (LI-COR Biosciences). Subsequently, siRNA-transfected cells were treated with araC or epirubicin at 48 h after transfection and MTT assay was performed to measure the cell viability.

Statistical analyses. Student's t-test or Wilcoxon signed-rank test were used for statistical analyses with the SAS software (version 9.1; SAS Institute Inc., Cary, NC, USA). A Kaplan-Meier survival curve was generated to analyze the patients' survival rates following surgery. $P \leq 0.05$ was considered to indicate a statistically significant difference. The results are expressed the mean \pm standard error of mean of three independent experiments.

Results

Significantly increased GAPDH expression levels in tumor tissues when compared with non-tumor tissues. Using the HPRT1 housekeeping gene as a reference gene, the mRNA expression levels of GAPDH were detected in 33 out of the 72 cases. Compared with non-tumor tissues, significantly higher GAPDH expression was observed in the tumor tissues ($P=0.0116$; Fig. 1A). In order to validate this observation in the 72 patients with HCC, CTBP1 was used as the reference gene, since it has been previously demonstrated to be the most stable gene among six candidate housekeeping genes (24). The relative expression of GAPDH was measured in the 72 HCC cases using RT-qPCR. The results of the Wilcoxon signed-rank test demonstrated that GAPDH expression was significantly increased in the HCC tumor tissues when compared with the non-tumor tissues ($P=0.0087$; Fig. 1B). In total, 37.5% of cases (27/72 patients) presented increased expression of GAPDH ($2^{-\Delta\text{Ct}} > 2$), 18.1% of cases (13/72 patients) presented

Table I. Patient characteristics according to the expression levels of GAPDH in tumor tissues.

Characteristics	Increased group (n=27), n	Non-increased group (n=45), n	P-value
Age			0.9036
<50 years	13	21	
≥50 years	14	24	
Abdominal dropsy			0.9374
Yes	5	8	
No	22	37	
Portal vein cancerous thrombus			0.5118
Complete	7	15	
Incomplete	20	30	
Preoperative AFP value			0.7873
≤20 μg/l	7	13	
>20 μg/l	20	32	
Liver cirrhosis			0.0394
I-III	21 (46.7%)	24	
IV-VI	6 (22.2%)	21	

Liver fibrosis score was evaluated by two pathologists independently and the Ishak scoring system was used. Higher expression of GAPDH was significantly associated with a lower liver fibrosis score ($P=0.0394$). AFP, α -fetoprotein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

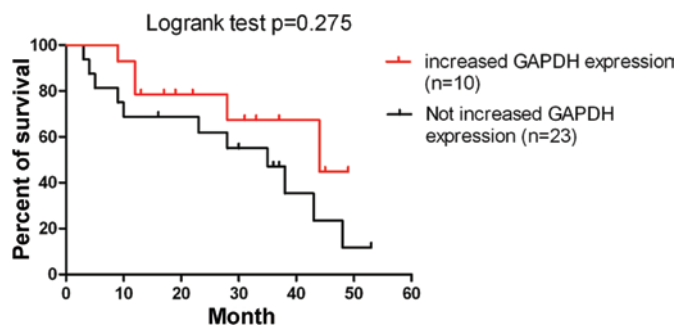


Figure 2. Survival rates (%) of HCC patients with increased or non-increased GAPDH expression. A clear tendency for higher survival rates of the HCC patients is observed in the increased GAPDH expression group; however, the difference was not statistically significant ($P=0.275$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

reduced expression of GAPDH ($2^{-\Delta C_t} < 0.5$) and 44.4% of cases (32/72 patients) presented unchanged expression of GAPDH ($0.5 \leq 2^{-\Delta C_t} \leq 2$). Fig. 1C shows the distribution of the different expression levels of GAPDH detected in the HCC patients.

In order to investigate whether there was an association between the mRNA levels of GAPDH and the clinical characteristics, the patients were further divided into the increased ($n=27$) and non-increased expression group ($n=45$), which included the cases presenting reduced and unchanged expression levels of GAPDH. Statistical analysis revealed that the increased expression group was associated with a lower score of liver fibrosis (Ishak score I-III vs. IV-VI; 46.7%, vs. 22.2%; $P=0.0394$; Table I) (25). This indicated that increased GAPDH expression was significantly associated with a lower liver fibrosis score. However, other clinicopathological characteristics, including age, abdominal dropsy, preoperative

α -fetoprotein value and portal vein cancerous thrombus, did not induce a statistically significant difference on the expression of GAPDH ($P>0.05$; Table I).

To investigate whether an association exists between the elevated GAPDH expression and patient survival rates, Kaplan-Meier survival analysis was conducted and patients with increased GAPDH expression presented higher overall survival rates; however, no statistically significant difference was observed ($P=0.275$; Fig. 2).

Knockdown of GAPDH expression results in reduced cell sensitivity to chemotherapy with araC in HepG2 cells, but not in PLC/PRF/5 cells. HepG2 and PLC/PRF/5 cells were initially transiently transfected with GAPDH siRNA (siGAPDH) or siRNA NC. Subsequently, the sensitivity of each cell line to araC or epirubicin was assayed individually. Notably, GAPDH expression knockdown was found to significantly alter the HepG2 cell chemotherapy sensitivity to araC (308.28 μM in the siGAPDH group, vs. 67.68 μM in the siRNA NC group; $P=0.01$), but not to epirubicin (10.37 μM in the siGAPDH group, vs. 8.96 μM in the siRNA NC group). However, GAPDH knockdown did not significantly alter the PLC/PRF/5 cell chemotherapy sensitivity to araC (4719.05 μM in the siGAPDH group vs. 6834.30 μM in the siRNA NC group) or epirubicin (6.15 μM in the siGAPDH group vs. 13.99 μM in the siRNA NC group; Fig. 3A).

To investigate the underlying mechanism, the protein level of p21, which is a cyclin-dependent kinase (CDK) inhibitor, was further evaluated by Western blot analysis using the p21-specific antibody. However, no statistically significant difference was observed in the protein levels of p21 between cells transfected with siGAPDH or siRNA NC. In addition, p21 expression in PLC/PRF/5 cells was below the detectable levels (Fig. 3B).

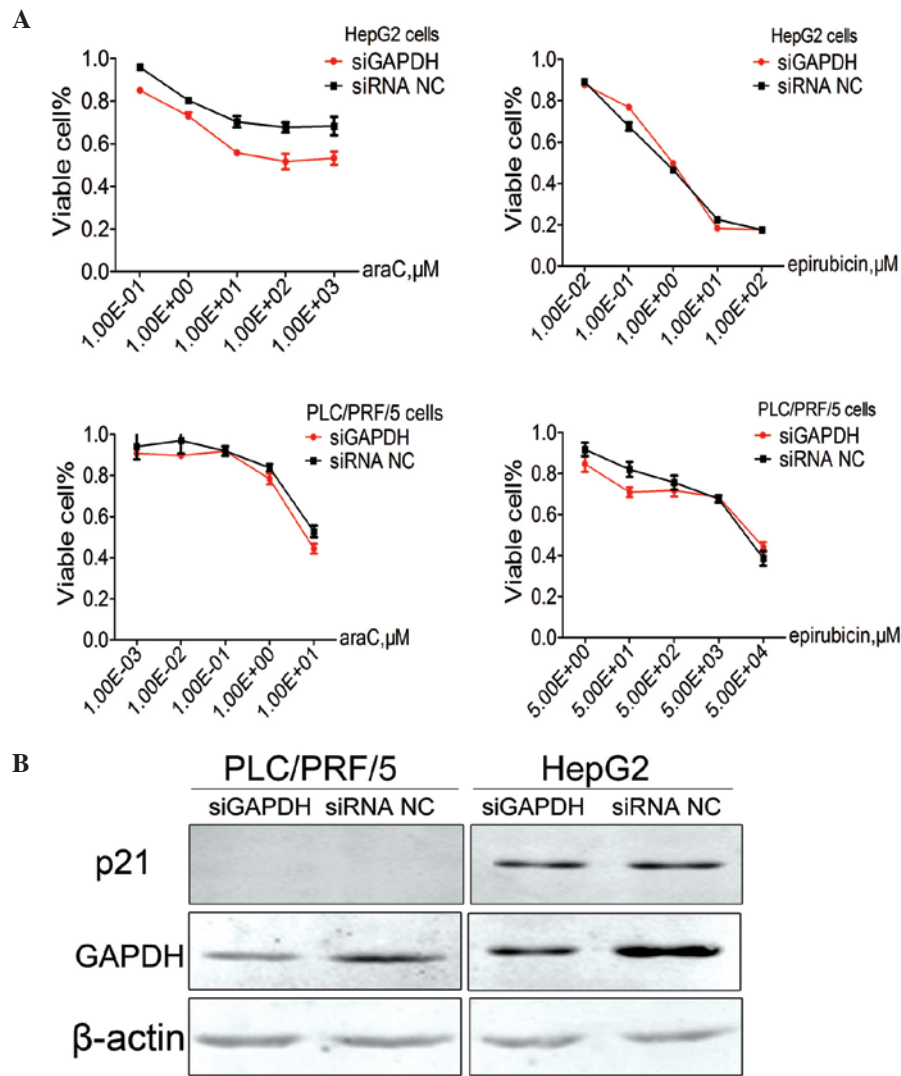


Figure 3. GAPDH expression knockdown resulted in reduced cell chemotherapy sensitivity in HepG2 cells, but not in PLC/PRF/5 cells, upon treatment with araC. (A) HepG2 cells (above) and PLC/PRF/5 cells (below) were transiently transfected with GAPDH siRNA (siGAPDH) and siRNA NC. Next, the cells were treated with araC or epirubicin and the absorbance was measured using MTT. Knockdown of GAPDH significantly alter HepG2 cells chemotherapy sensitivity to araC ($P=0.01$), but did not alter cellular sensitivity to epirubicin ($10.37 \mu\text{M}$ vs. $8.96 \mu\text{M}$). However, GAPDH expression knockdown did not alter cellular sensitivity of PLC/PRF/5 to treatment with araC or epirubicin. The data are expressed as the mean \pm standard deviation. (B) siRNA knockdown of HepG2 and PLC/PRF/5 cells following transient transfection with GAPDH siRNA (siGAPDH) and siRNA NC. p21 protein levels were measured; however, no statistically significant differences were observed in the p21 protein levels between the siGAPDH- and siRNA NC-transfected HepG2 cells. For PLC/PRF/5 cells, the expression of p21 was not sufficiently high to detect. araC, cytosine arabinoside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

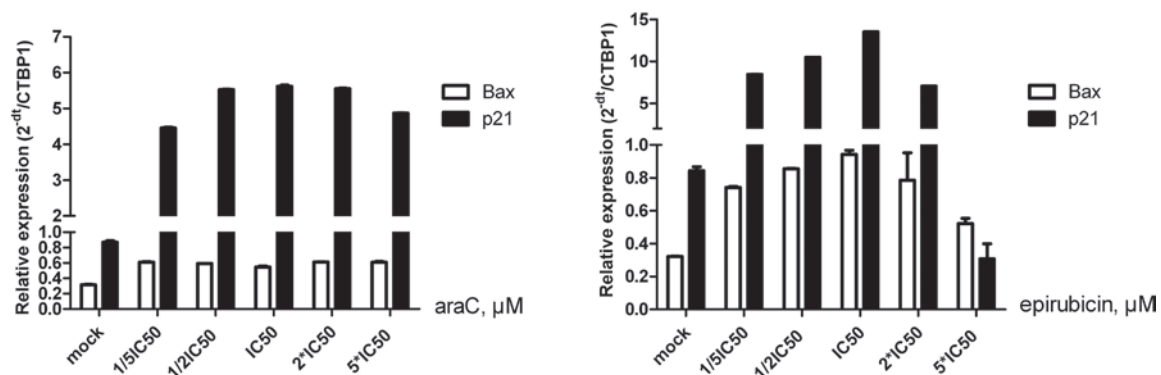


Figure 4. mRNA expression levels of p21 and Bax in HepG2 cells treated with araC or epirubicin. HepG2 cells were treated with five different concentrations of araC (left) or epirubicin (right) for 48 h. Subsequently, the mRNA expression levels of p21 and Bax were detected using reverse transcription-quantitative polymerase chain reaction. The data represent the results of three independent experiments. Compared with the control group, the mRNA expression of p21 was increased with increasing concentrations of araC or epirubicin; however, it decreased between the doses 2xIC₅₀ and 5xIC₅₀. The expression of Bax increased more than twice and was slightly altered upon araC treatment, while for epirubicin, it increased about four times until the 2xIC₅₀ dose, and then decreased. araC, cytosine arabinoside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Bax, B-cell lymphoma 2-associated X protein.

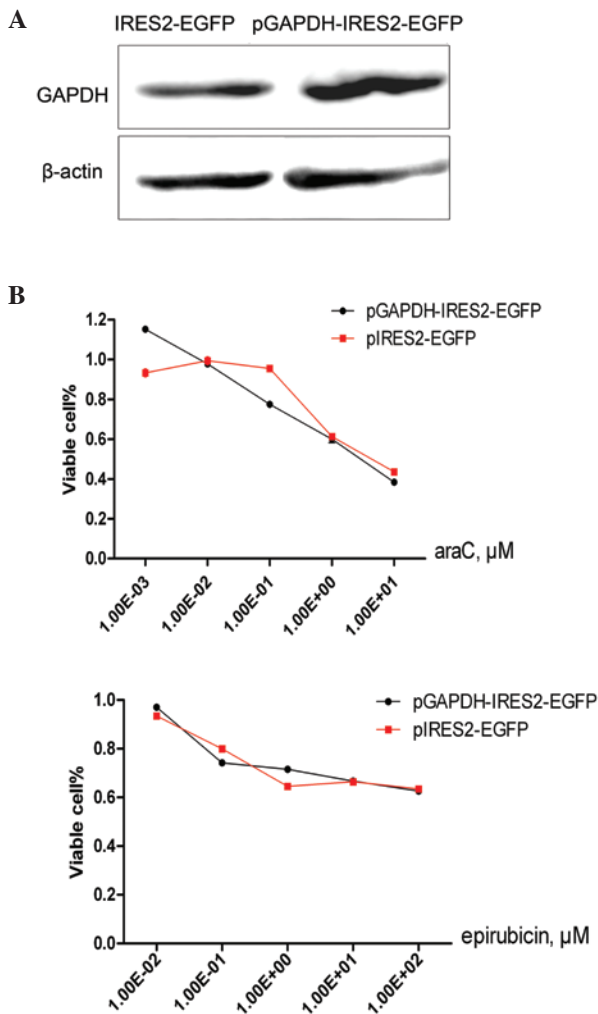


Figure 5. GAPDH overexpression did not alter HepG2 cellular sensitivity to araC and epirubicin. (A) Following transfection of HepG2 cells with GAPDH for 48 h, GAPDH protein levels were measured using 12% SDS-PAGE. (B) The cellular sensitivity of HepG2 cells (with overexpressed GAPDH) to araC or epirubicin was detected by MTT. Overexpression of GAPDH did not alter HepG2 cellular sensitivity to araC and epirubicin. araC, cytosine arabinoside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

In order to investigate the effect of araC on HepG2 cellular sensitivity at the mRNA level of p21 or B-cell lymphoma 2-associated X protein (Bax), the expression levels of these genes were detected following treatment with five different concentrations of araC or epirubicin for 48 h. Compared with the control group (mock), the mRNA expression levels of p21 increased with increasing concentrations of araC or epirubicin; however, a decrease was observed between $2 \times IC_{50}$ and $5 \times IC_{50}$ doses. By contrast, the mRNA expression of Bax following araC treatment was slightly altered. Following epirubicin treatment, the mRNA expression of Bax increased until the IC_{50} dose and then decreased (Fig. 4).

GAPDH overexpression did not alter HepG2 cellular sensitivity to chemotherapy with araC or epirubicin. Knockdown of GAPDH expression did not alter p21 protein level expression. Therefore, the effect of the overexpression of GAPDH on HepG2 cell sensitivity to araC or epirubicin was investigated. The HepG2 cells were initially transiently transfected with 2μ g pGAPDH-IRES2-EGFP or pIRES2-EGFP

and the transfection was validated by western blot analysis (Fig. 5A). Subsequently, the transfected HepG2 cells were treated with araC or epirubicin for 24 h. As opposed to the expected result, the GAPDH overexpression was not found to alter the cellular sensitivity to araC (2.11μ M vs. 2.11μ M) or epirubicin treatment (23.48μ M vs. 21.01μ M; Fig. 5B).

Discussion

Since the implementation of the national HBV vaccine immunization program in China, the overall rate of the population carrying the HBsAg declined from 9.75% in 1992 to 7.18% in 2006 (28). However, a previous study has estimated that 93 million individuals of the Chinese population are infected with chronic HBV, which may eventually result in significant public health problems in the future (29). Patients with HBV infection that is accompanied by cirrhosis are in high risk of hepatocarcinogenesis. In addition, evidence revealed that males are more prone to HCC development, compared with females (3).

GAPDH has been widely used as a reference gene in RT-qPCR analysis performed in HCC cells; however, elevated mRNA and protein expression levels of GAPDH in HCC patients have been reported in a number of studies (30,31). These studies investigated patients with HBV-associated HCC, hepatitis C virus-associated HCC and HCC without viral hepatitis infection history. Therefore, it is essential to investigate the expression of GAPDH in male patients with HBV-associated HCC and cirrhosis.

The group of the present study has previously demonstrated the presence of aberrant GAPDH expression in male patients with HBV-associated HCC and cirrhosis (24). In addition, GAPDH expression was found to be the one of the most unstable genes among the six housekeeping gene investigated, which was consistent with a previous study on HBV-associated HCC (32).

In the present study, ~56% of tumor tissue samples exhibited differential expression of GAPDH in tumor tissues (including 37.5% of samples with increased expression and 18.1% with decreased expression) compared with non-tumor tissues, with an >2-fold difference. A large number of studies have demonstrated that GAPDH is involved in multiple basic cellular metabolism functions, as observed by the altered GAPDH expression levels in tumor tissues (9-12). Therefore, in the present study, HCC patients were divided into the increased and non-increased GAPDH expression groups. Notably, increased expression of GAPDH was found to be significantly associated with a lower liver fibrosis score ($P=0.0394$); to the best of our knowledge, the present study is the first to report this in male patients with HBV-associated HCC and cirrhosis. These results are in agreement with the findings of a previous study comparing healthy controls and patients with hepatitis B or C virus, which identified that GAPDH expression levels were significantly increased in patients with cirrhosis and the presence of HCC was closely associated with high GAPDH levels (33).

To demonstrate whether the aberrant expression of GAPDH affects the survival rates of HCC patients within a period of 60 months, survival curve analysis was performed. Notably, patients with increased GAPDH expression exhibited

a tendency of improved survival rates; however, no statistically significant differences were observed ($P=0.275$) and, to the best of our knowledge, no similar studies has been conducted.

Numerous studies have indicated that GAPDH is involved in apoptosis and elevated GAPDH expression has been observed in HCC (17) and other cancer cells (13-16). Tumorigenesis has been hypothesized to be due to the occurrence of increased cell necrosis compared with apoptosis, which supports the observation of the present study that patients with increased GAPDH expression present higher survival rates.

Enrolling a group of patients with HBV-associated HCC but without history of cirrhosis as the control group may have provided valuable information in the present study. However, only a few cases without cirrhosis were detected in >500 HBV-associated HCC cases, and therefore these were not included in the present study. A similar observation was identified by Obata *et al*, reporting that HCC was developed only in 23% of patients who were HBsAg-positive and suffered from cirrhosis and in only 5.9% of patients who were HBsAg-negative and presented liver cirrhosis (34).

Although several studies have indicated that GAPDH is involved in chemotherapy-induced DNA damage response, the specific mechanism of GAPDH in HCC chemotherapy treatment remains unclear (18,19). araC is considered to inhibit the proliferation of cells through incorporation into the DNA during replication (35), by inhabiting the DNA synthesis and arresting cell division; however, it does not disturb the RNA synthesis (36). Previous results have demonstrated that araC-induced apoptosis of cerebellar granule cells involves the expression of GAPDH and p53, while, similar to Bax, GAPDH is upregulated by p53 following exposure to the apoptotic insult (37).

In the experiments of the current study, knockdown of GAPDH expression significantly altered the sensitivity of p53-proficient HepG2 cells to araC chemotherapy, but not to epirubicin chemotherapy. However, the knockdown of GAPDH expression in the p53 mutation of PLC/PRF/5 cells did not alter the cellular chemotherapy sensitivity to araC or epirubicin. This is supported by the results of previous studies, which identified that araC-induced apoptosis was p53-dependent (38). In the present study, upon transfection of HepG2 cells with siGAPDH, the cell proliferation arrest in GAPDH-depleted cells occurred through the p53-induced expression of p21. In order to investigate the specific mechanism through which the GAPDH expression knockdown in HepG2 cells induced resistance to araC treatment, the protein levels of CDK inhibitor, p21waf1/cip1, were further investigated in the two cell lines. The expression of p21waf1/cip1 in PLC/PRF/5 cells was found to be undetectable at the protein level; therefore, its effect on chemotherapy in PLC/PRF/5 cells was not evaluated.

The DNA synthesis inhibitor epirubicin eliminates cancer cells mainly via inducing G2/M arrest and apoptosis (39,40). The results of the present study demonstrated that, in epirubicin treated HepG2 cells, significant upregulation of the mRNA expression levels of cell cycle progression inhibitor, p21waf1/cip1, and pro-apoptosis Bax was detected (Fig. 4). However, in araC-treated HepG2 cell, the expression of Bax was slightly altered, while the expression of p21waf1/cip1 was significantly upregulated. These differences were also

observed in Fig. 3A, where epirubicin treatment significantly higher HepG2 cell apoptosis with increasing concentration.

In conclusion, to the best of our knowledge, this study is the first to report that elevated GAPDH expression in tumor tissues may be involved in the development of fibrosis. In addition, a tendency towards higher survival rates was observed for male patients with HBV-associated HCC patients and cirrhosis; however, the underlying mechanism remains unclear. Furthermore, increased GAPDH expression may enhance the sensitivity of HCC cells to antimetabolite chemotherapy.

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