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Original Article

HRP-3 protects the hepatoma cells from glucose deprivation-induced apoptosis

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Abstract: Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. It is important for HCC cells to resist to apoptosis caused by adverse energy pressure in microenvironment during the HCC tumorigenesis. HRP-3, a member of hepatoma-derived growth factor (HDGF)-related proteins (HRP) family, was shown to be highly up-regulated in HCC tissues and play an important role in HCC pathogenesis based on our previous research. The aim of the study was to investigate the HRP-3's role in HCC cells endurance against energy pressure. Method: The HRP-3 expression level in primary rat hepatocytes and human HCC cell lines were examined when changing the extracellular glucose concentration. To assess biological function of HRP-3 during glucose deprivation, HRP-3 stable knockdown and control clones of SMMC-7721 and SK-hep1 were constructed for further analysis including cellular morphology observation, apoptotic sub G1 peak analysis and the mTOR-mediated phosphorylation of S6K1 detection in the absence of glucose. Results: Expression level of HRP-3 protein was highly up-regulated both in primary rat hepatocytes and HCC cells as prolonging the stimulation of glucose deprivation. Both morphology and sub-G1 phase analyses indicated that stable knockdown of HRP-3 sensitized HCC cells to glucose deprivation-induced cell apoptosis. Furthermore, silence of HRP-3 prevented the de-phosphorylation of S6K1 induced by glucose deprivation, which was an essential molecular event for HCC cell survival in energy pressure. Conclusions: We propose that glucose deprivation-induced HRP-3 up-regulation potentially plays a major role in protecting HCC cells against apoptosis caused by energy pressure.

Keywords: Hepatocellular carcinoma, HRP-3, glucose deprivation, apoptosis

Introduction

Hepatocellular carcinoma (HCC), ranks as the sixth fatal cancer for female and second for male [1], is one of the most common malignant tumors worldwide. Based on GLOBOCAN estimates, about 782500 new HCC cases and 745500 million deaths occurred in 2012 worldwide and about half of them occurred in China [1].

In the initial stage of carcinogenesis, tumor cells must overcome the temporary nutrient poor and hypoxia microenvironment due to the lack of microvascular and the quick increase of consumption of nutrition and energy caused by the rapidly proliferation of carcinoma cells, which is generally acknowledged in medicine, especially in oncology [2]. Resistance of energy pressure differ the hepatocellular carcinoma tissue from normal tissue and bring the cancer cells to modulate angiogenesis by promoting proliferation and migration in endothelial cells in response to the temporary nutrient poor microenvironment.

A sufficient glucose supply facilitates rapid cell growth through the generation of intermediates that are required for the synthesis of essential cellular components. The mammalian target of rapamycin (mTOR), a member of the family of phosphatidylinositol kinase like kinases (PIKK) [3], is able to sense environmental signals from nutrients (amino acids and energy) and functions as a rheostat to regulate the rate of cell growth and cell proliferation by regulating pro-

tein biosynthesis [4]. The ribosomal protein S6 kinase (S6K) is an important downstream target of mTOR pathway. The activation of S6K enhances translation of mRNAs that encode components of the translational machinery. S6K1 (p70S6K) is one of the best studied S6Ks, existing evidences show that mTOR and its downstream targets have emerged as novel targets for cancer therapeutics [5-7].

HRP-3 is the member of hepatoma-derived growth factor (HDGF) related proteins (HRPs) family that comprises six members including (HDGF, HRP1-4 and lens epithelium derived growth factor) [8]. All family members share a conserved HATH domain in NH_2-terminal while have no sequence homology among other regions [8]. HDGF, the first identified and most extensively studied member, was shown to play a significant part in progression and carcinogenesis of many types of human cancers [9-11] and associated with poor prognosis and high recurrence rate with HCC [12]. Compared with HDGF, the studies for the function of HRP-3 were limited. Initial studies on HRP-3 have defined its main functions only on modulating the development of neurons and brain [13, 14]. Our previous research has demonstrated that HRP-3 is overexpressed in human HCC tissues, promotes anchorage-independent growth of HCC cells in vitro and xenograft tumor growth in vivo through MAPK/ERK signaling pathway and enhances apoptosis of HCC cells induced by treatment with multiple chemotherapeutic drugs [15]. However, the role of HRP-3 in HCC during the temporary nutrient poor microenvironment has not been illustrated.

In this study, we focus on the adjustment that the carcinoma cells group makes to overcome the extracellular energy pressure during the HCC carcinogenesis. Our results show that HRP-3 expression in HCC cells is increased as prolonging the glucose deprivation condition. Both the intuitional morphology observation and fluorescence activating cell sorter (FACS) analysis reveal that knockdown of HRP-3 promotes HCC cells to apoptosis during glucose deprivation. Furthermore, we prove that HRP-3 function, at least in part, by inhibiting the glucose deprivation-induced de-phosphorylation of S6K1, which is the energy pressure effect gene involved in mTOR signaling pathway.

Materials and methods

Cell lines and cell culture

The human hepatocellular carcinoma cell line SK-Hep1 and human kidney epithelial 293T were obtained from ATCC. Human hepatocellular carcinoma cell line SMMC-7721 was preserved in our institution. All these cells were cultured in high-glucose DMEM (*Gibco*) supplemented with 10% (vol/vol) fetal bovine serum (FBS, *Hyclone*), at 37°C in a humidified incubator (*Heraeus*) with 5% CO_2. For energy pressure studies, we switch high-glucose DMEM (*Gibco*) to glucose-free DMEM (*Gibco*) supplemented with 10% FBS (vol/vol).

Isolation and culture of primary rat hepatocytes

Preparation of primary rat hepatocytes was documented two-step collagenase perfusion method [16]. Briefly, 500 ml of HEPES buffer (20 mM) was injected into hepatic portal vein to remove the residual blood and weaken the intercellular junctions. Followed by the step of collagenase (50 mg/100 ml) in perfusion solution injected for about 20 min, and hepatocytes were released. The isolated hepatocytes should culture in high-glucose DMEM (*Gibco*) with 10% FBS (*Hyclone*) and additional penicillin-streptomycin (*Hyclone*). Moreover, refresh the culture medium every other day.

Stable knockdown of HRP-3 in HCC cell lines

The siRNA and shRNA were mentioned in our previous study [15]. The efficient siRNA oligo sequences for HRP-3 are siRNA i1: 5'-GCCUCCAGCAAACAAGUAUdTdT-3', siRNA i3: 5'-AGGUGAUAGAGUAGAAGAAdTdT-3'. The sequence of negative control (NC) is 5'-ACAGACUUCGGAGUACCUGdTdT-3'. And the lentivirus-based small hairpin RNA (shRNA) expression vectors for siRNA i1 and siRNA i3 were constructed to generate the stable cell lines. Long-term knockdown of HRP-3 in SMMC-7721 and SK-Hep1 cell lines was mediated by infection with lentivirus harboring a HRP-3-specific siRNA sequence. Independent colonies were picked and the efficiency of knockdown was determined by Western Blot. Details of stable cell line construction were shown in previous research [15].

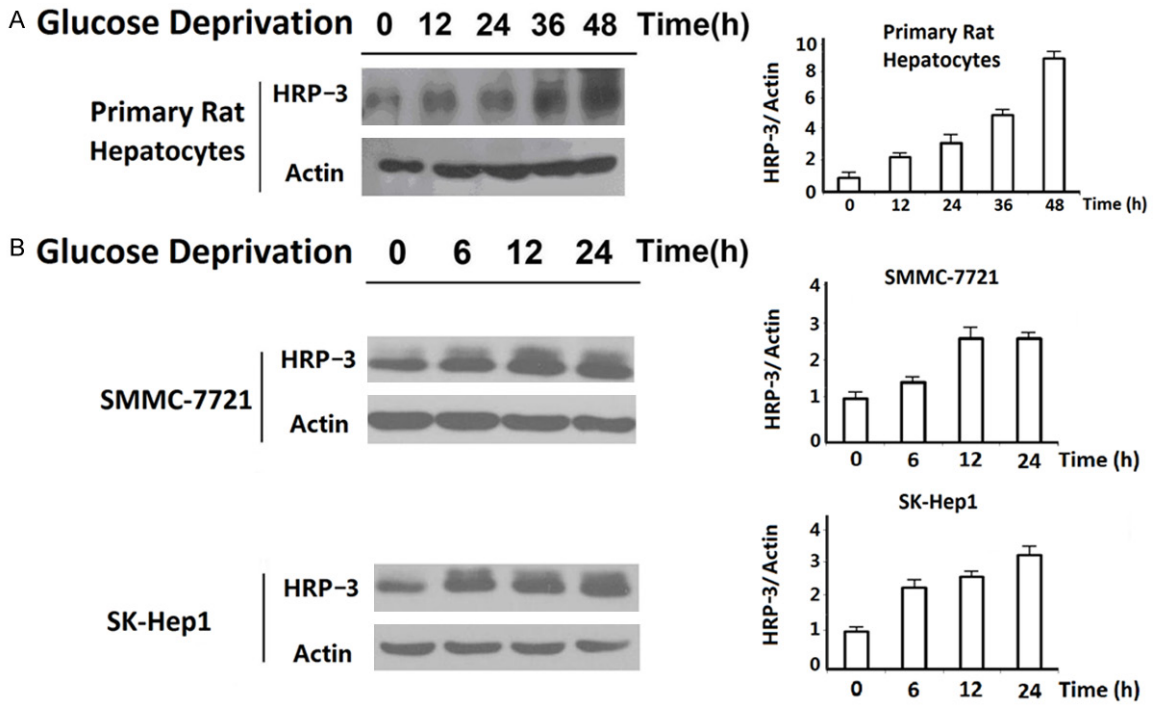

Figure 1. HRP-3 was up-regulated under glucose deprivation culturing. A. HRP-3 protein expression levels in primary rat hepatocytes with the extending time of glucose deprivation culturing, as detected by SDS-PAGE. B. The western blot results of HRP-3 expression in HCC cell lines at gradient time of glucose deprivation culturing. The quantification of each immunoblots was shown in the right-side panel, HRP-3 expression was normalized to actin.

Transient transfection and selection of stable cell lines for overexpression of HRP-3

Transfection was performed with Lipo2000 according to the manufacturer's instructions. For transient transfection, cells were transfected with HRP-3-specific siRNA (i1 and i3) and control siRNA (NC). The cells were harvested at 48 h after transfection.

Flow cytometry analysis

Cells were harvested by trypsin, suspended with ice-cold 70% filtered ethanol, followed by fixing at -20°C for at least 2 h. After twice centrifuging and washing, cells were stained with propidium iodide (50 μg/mL) and RNase (100 μg/mL) in filtered PBS for 30 min at room temperature in dark, then submitted to FACS Calibur (*BD Biosciences*) Apoptotic cells were calculated as cells in the area corresponding to sub-G1 relative to total cells.

Western blot

Protein samples were subjected to SDS-PAGE of appropriate concentration, and then electrotransferred to nitrocellulose membranes (*Millipore*, MA). The membranes were blocked with 5% skimmed milk, then incubated with primary antibodies according the interest proteins at 4°C overnight, followed by incubation with peroxidase-conjugated secondary antibody. Antibodies used were: anti-actin (1:5000, *Sigma*), anti-HRP-3 (1:1000, *Abnova*), anti-T-S6K1 (1:1000, *Abcam*), and anti-p-S6K1 (1:500, *Abcam*).

Statistical and image analysis

Protein expression was quantified by densitometric analysis with Quantity One (*Bio-Rad*). The HRP-3/Actin and p-S6K1/T-S6K1 were showed in histogram. Morphology of HCC Cells culturing in glucose-free medium were photographed under microscopy (*Leica*), and the spreading areas of cells were processed with Image J. Results were represented in histogram. These data were expressed as means and standard errors. Statistical analysis was performed with *Student t-test*. And a threshold of $P<0.05$ was defined as statistically significant.

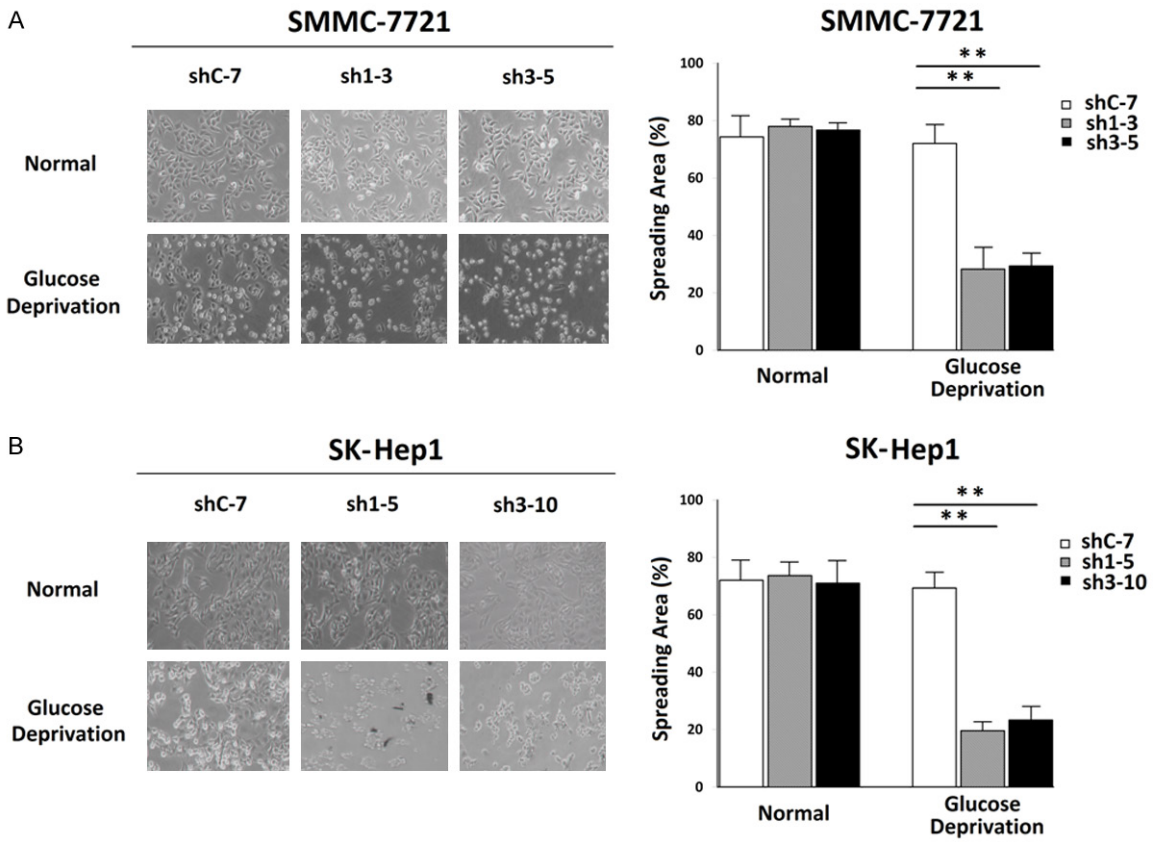

Figure 2. Stable Knockdown of HRP-3 in HCC cells sensitized cell to become shrunk morphology of apoptosis under the energy pressure. A. Effect of HRP-3 Knockdown on SMMC-7721 cell survival ability under glucose deprivation, as analyzed by morphology observation. B. Effect of HRP-3 Knockdown on SK-Hep1 cell survival ability under glucose deprivation, as analyzed by morphology observation. The statistical quantification of shrunk cells is shown in the right-side panel. (n=5 means 5 random areas; *stands for P<0.05, **stands for P<0.01).

Results

HRP-3 is up-regulated in respond to glucose deprivation in primary rat hepatocytes and HCC cell lines

To investigate the function of HRP-3 during energy pressure, we firstly evaluated the expression level of HRP-3 faced to changes of extracellular glucose concentration. We prepared primary rat hepatocytes and replaced the high-glucose DMEM by glucose-free DMEM, and obtained the cell lysates at gradient time. As showed in **Figure 1A**, the HRP-3 expression in primary rat hepatocytes significantly up-regulated with extending time of glucose deprivation condition. Parallelly, the expressions of HRP-3 in HCC cell lines were also dramatically increased when applying of glucose-free medium (**Figure 1B**). Furthermore, we find that the over-expression of HRP-3 of HCC cell lines was much faster than that of primary rat hepatocytes.

HRP-3 plays essential roles in protecting cells from glucose deprivation-induced apoptosis

In the following experiment, we used a loss-of-function approach to assess the role of HRP-3 in cell apoptosis induced by energy pressure. To estimate the long-term and stable effect of HRP-3 knockdown on HCC cells, we generated lentivirus-based small hairpin RNA (shRNA) expression vectors (Sh1 and Sh3) for two effective HRP-3-siRNA sequences (i1 and i3) and ShC as control vector for NS-siRNA. HRP-3 stable knockdown clones and control clones of SMMC-7721 (Sh1-3, Sh3-5 and ShC-7) and SK-Hep1 (Sh1-5, Sh3-10 and ShC-7), which infected with lentivirus haboring i1, i3 and NS siRNA respectively, were constructed for further analysis. The efficiency of knockdown was detected by Western Blot (**Figure 4**).

The adhesion and morphology of culture adherent cells could give an intuitional index to the cell viability and apoptosis. So we firstly con-

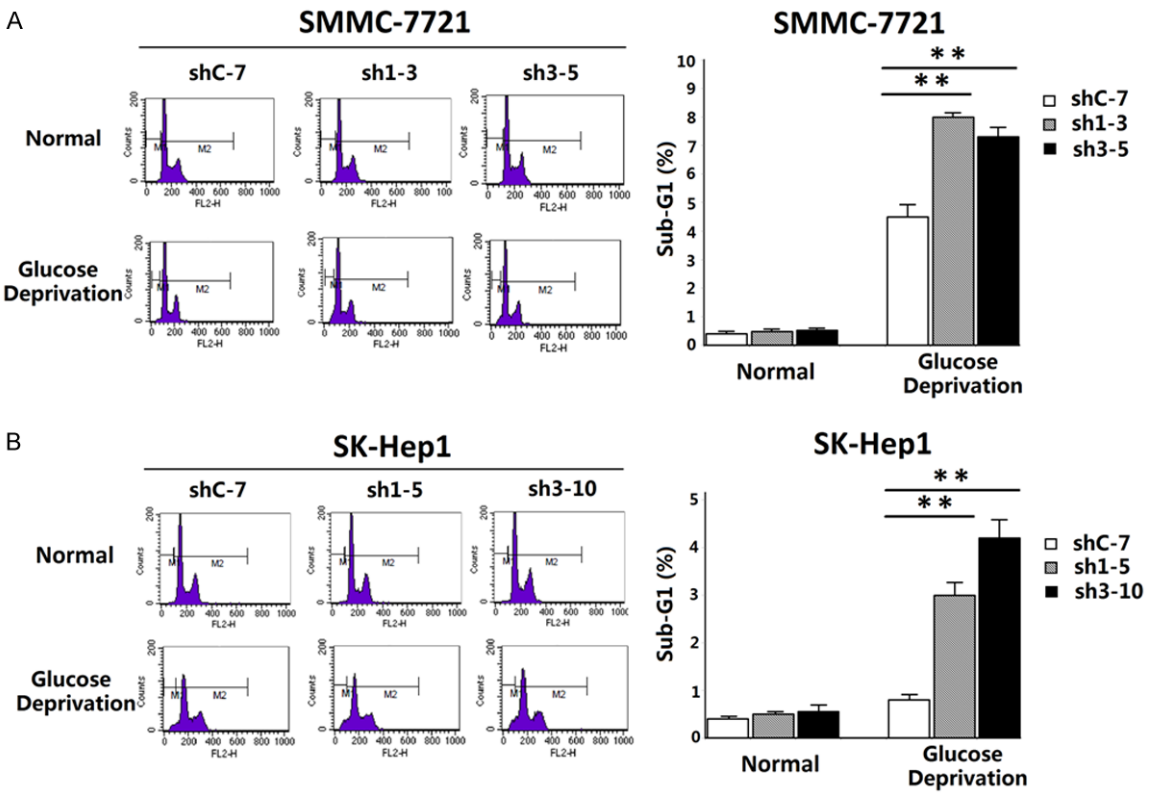

Figure 3. HRP-3 inhibits energy deprivation-triggered apoptosis of HCC cells. A. Sub-G1 analysis of stable knockdown clones (Sh1-3 and Sh3-5) and a corresponding control clone (ShC-7) of SMMC-7721 under glucose deprivation. B. Sub-G1 analysis of stable knockdown clones (Sh1-5 and Sh3-10) and a corresponding control clone (ShC-7) of SK-Hep1 under glucose deprivation. (n=3; *stands for P<0.05, **stands for P<0.01).

ducted the cell morphology analysis. All clones, counted for the same number and divided into two portions, were plated in 6 well plates of same diameter with normal high-glucose DMEM overnight. One portion of each clones were changed for glucose-free DMEM the next day and the other portion was used as control group maintained in high-glucose DMEM. After 9 hours, stable HRP-3 knockdown clones of SMMC-7721 (Sh1-5 and Sh3-10) became shrinking, round, even suspended compared with the shC-7 control clone which had no obviously injury morphology. Similarly results were observed between the stable HRP-3 knockdown clones of SK-Hep1 (Sh1-3 and Sh3-5) and the control clone of normal adhesion (ShC-7) after 7 hours cultured in glucose-free medium. The morphology of all clones was captured by microscope for stochastic observation for 3 areas and the pictures were analysis by software Image J for calculating spreading areas. (*stands for P<0.05, **stands for P< 0.01) (**Figure 2A**, **2B**).

Then all of these clone cells were digested and submitted to flow cytometry analysis. Consistent with the morphology results, during the glucose deprivation, a progressive aggregation in sub-G1 phase appeared in cells knocking down HRP-3 compared with in control cells (Sh1-3 and Sh3-5 Vs. ShC-7 of SMMC-7721; Sh1-5 and Sh3-10 Vs. ShC-7 of SK-Hep1), which indicating the influence of HRP-3 on cell apoptosis. (*stands for P<0.05, **stands for P<0.01) (**Figure 3A**, **3B**).

HRP-3 regulated de-phosphorylation of S6K1 in response to energy pressure

To investigate the molecular mechanism underlying HRP-3 mediated apoptosis of HCC cells under energy pressure, we determined the effect of HRP-3 on the activation of S6K1, which was the target gene of mTOR pathway and identified as an important effect gene response to energy pressure. As showed in **Figure 4** (*stands for P<0.05, **stands for P<0.01), phosphorylation of S6K1 in both

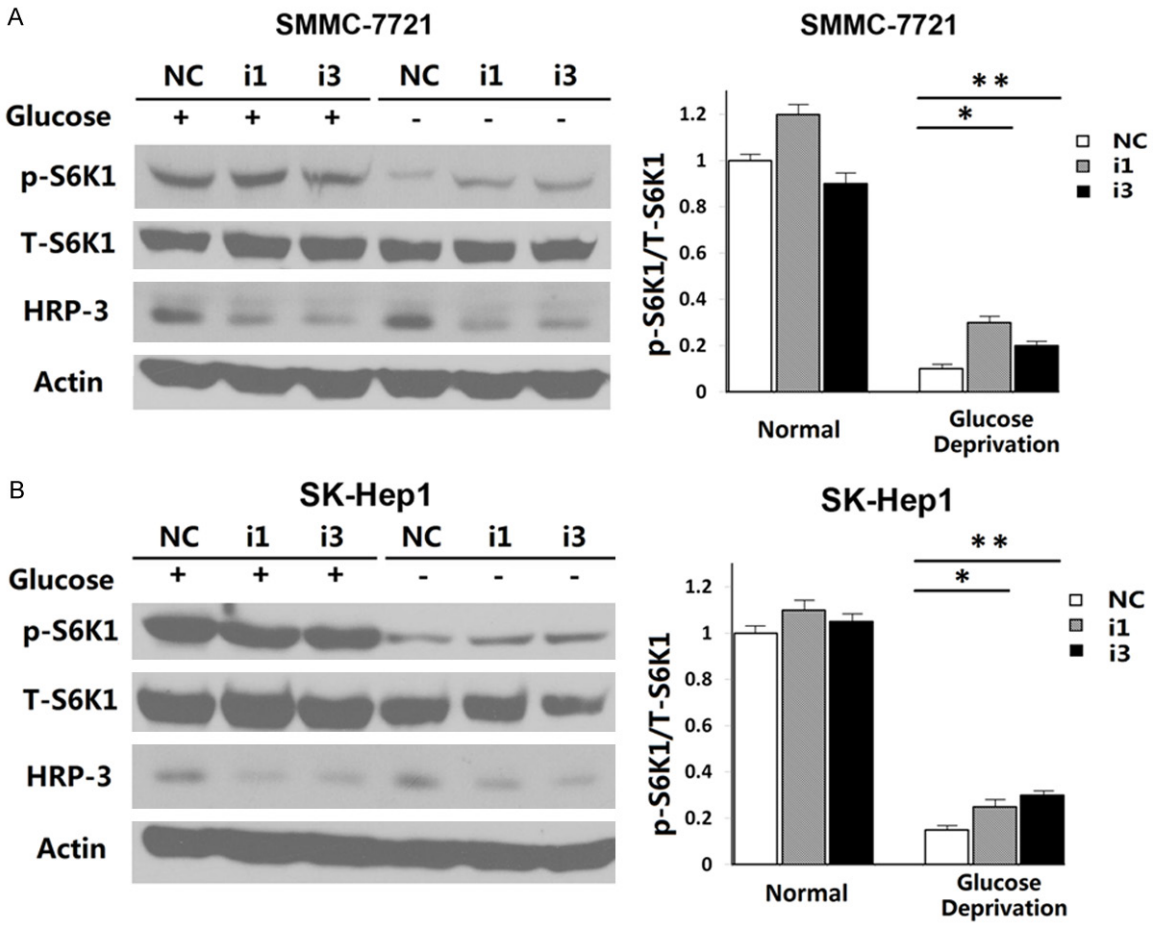

Figure 4. Silence of HRP-3 inhibits the de-phosphorylation of S6K1 induced by glucose deprivation. A. SMMC-7721 cells were transient transfected with HRP-3-specific siRNA (i1 and i3) and control siRNA (NC), then after 48 h, treated with glucose-free DMEM for 2 h and harvested to western blot analysis. Equal amounts of cell lysates were immunoblotted with the indicated antibodies. The corresponding densitometric histograms were shown in right-side panel. B. The western blot results for SK-Hep1 experiencing the same process as A.

SMMC-7721 and Sk-Hep1 cell lines was significantly decreased when treating with the glucose-free DMEM, while the decrease degree of phosphorylated S6K1 in transient transfected HRP-3-siRNA cells was significantly lower than that in the control cells. These results indicated that HRP-3 enhanced the tolerance of HCC cell against adverse energy microenvironment by, at least in part, regulating mTOR-mediated dephosphorylated S6K1 induced by glucose deprivation.

Discussion

Glucose deprivation is a major tumor microenvironment that subsequently induces cell apoptosis. One of the important characteristics of tumor cells is the resistance to cell apoptosis caused by this energy pressure. Our present results suggest that HRP-3, a glucose-sensitive protein, was up-regulated under the deprivation of glucose and protect the HCC cells against glucose deprivation-induced cell apoptosis via mTOR/S6K pathway.

Our previously research revealed that HRP-3 was overexpressed in human HCC tissues and promoted the anchorage-independent growth of HCC cells via ERK1/2 pathway. However, hardly is known about the cause for the up-regulation of HRP-3 in HCC. In present study, we fill this blank and find that extracellular glucose deprivation induced the expression of HRP-3 both in primary rat hepatocytes and human HCC cell lines. It is worthy to note that up-regulation of HRP-3 in HCC cell lines were much faster than that in primary rat hepatocytes (6 hours vs. 12 hours after glucose deprivation).

This result is make sense due to the well-known recognition that tumor cells have faster response to micro-environmental changes [17] and stronger ability to overcome the adverse tumor microenvironment than normal tissue cells [2].

Reduced extracellular glucose concentration can change the direction of cell fate towards apoptosis. Changes of several key intracellular proteins could respond to glucose availability, which resulted in an increased life span of cells in energy pressure. For example, Wang R and Liang H et al. reported that USF-1 could be induced by oxygen and glucose deprivation, which promoted apoptosis in HCC HepG2 cells [18]. Nishimoto A et al. reported that the well-known HIF-1α plays a central part in the acquisition of anti-apoptosis in human colon cancer cell under glucose deprivation [19]. We constructed stable HRP-3 knockdown clones of SMMC-7721 and SK-Hep1 to analyze the stable and long-term relationship between HRP-3 and energy tolerance. The adhesion and morphology of culture adherent cells, could give an intuitional index to the cell viability and apoptosis [20-22]. Our results demonstrated that 9 hours incubation of SMMC-7721 stable clones and 7 h incubation of SK-Hep1 stable clones with glucose-free DMEM initiated cell shrinking, round, even suspended only in HRP-3 knockdown clones but not in control clones (**Figure 2**). Further investigation for the sub-G1 percentage of these clones was performed and the results were agreed well with the morphology observations (**Figure 3**). These consistent results confirmed that HRP-3 in HCC cells will be induced to resist to apoptosis caused by glucose deprivation. These results were according with the clinical records and medicine researches that carcinoma cells group have tougher microenvironment range of tolerance than normal tissue cells [2, 23-26].

Our previous study showed that shRNA-mediated knockdown of HRP-3 failed to affect anchorage-dependent growth of HCC cells but markedly reduced the number and size of colonies when cells were cultured in suspension (in soft agar) [11]. In present study, we find that shRNA-mediated knockdown of HRP-3 has no effect on the morphology of HCC cells culturing in normal condition but obviously promotes cell to shrinking and apoptosis when treated with glucose-free medium. These observations demonstrate that HRP-3 functions only in adverse condition, which may help the HCC cell overcome the adverse microenvironment.

The mammalian target of rapamycin (mTOR) pathway has been documented as an important regulator of cell metabolism and survival in response to environmental cues in the past decade [27-30]. Phosphorylation of S6K, one of the downstream substrates of mTOR pathway, is characterized to increase cell survival by up-regulating further downstream molecular targets such as protein kinase C and ribosomal protein S6 [31]. Through mTOR pathway, exercise stimuli evoke synthesis by employing nutrients, including carbohydrates and amino acids [32-34]. Under energy pressure, the phosphorylation level of S6K1 decreased in order to reduce the use of nutrients to increase the tolerance [32, 35]. That brings us to the logical part, namely, to determine the molecular relationship between HRP-3 expression and S6K1 phosphorylation under glucose deprivation. We find that silence of HRP-3 slightly, but significantly inhibit the de-phosphorylation of S6K1 induced by glucose deprivation. Thus, HRP-3 may inhibit the glucose deprivation-induced apoptosis by, at least in part, mTOR/S6K pathway. Further study need to do to find the complementary mechanism under the HRP-3's role in HCC tolerance against energy pressure.

In summary, these results suggested that glucose deprivation-dependent HRP-3 induction involves a positive feedback mechanism to enhance HCC cells survival under glucose deprivation, one of the important adverse tumor microenvironment, which provide an important viewpoint on interaction between HRPs family and extracellular glucose levels.

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Disclosure of conflict of interest

None.

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