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Original Article

Glut-1 inhibits proliferation and invasion of laryngeal cancer cells through regulating AKT/mTOR

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Abstract: The goal of this study was to investigate the role of Glut-1 in patients with Larynx squamous carcinoma (LSCC). Real-time fluorescent quantitative PCR (RT-PCR) and Western blot were used to measure differential expression of Glut-1 in LSCC tumor tissues and adjacent tissues. Cultured LSCC cell line Hep-1 was randomly assigned into control group, Glu-1 negative control (NC) group and Glut-1 siRNA group. RT-PCR and Western blot measured Glut-1 expression across all groups of cells, whose proliferation was measured by MTT assay, and cell invasion was described using Transwell assay. Hep-2 cell apoptosis was measured by caspase-3 activity. Western blot measured the effect of Glut-1 on AKT/mTOR signal pathway. Glut-1 mRNA and protein showed up-regulation in LSCC tumor tissues ($P < 0.05$ comparing to adjacent tissues). Glut-1 siRNA transfection into Hep-2 cells suppressed Glut-1 expression and inhibited Hep-2 cell proliferation, invasion, or caspase-3 activity. Glut-1 siRNA also suppressed mTOR expression or AKT phosphorylation ($P < 0.05$ comparing to NC group). Glut-1 showed up-regulation in LSCC tumor tissues. Therefore, down-regulation of Glut-1 can modulate AKT/mTOR pathway, further mediating LSCC cell proliferation, apoptosis and invasion.

Keywords: Glut-1, larynx cancer, AKT/mTOR signal pathway, cell proliferation, cell invasion, apoptosis

Introduction

Laryngeal squamous cell carcinoma (LSCC), or larynx cancer, is one commonly occurred high malignant tumor in head-neck region, and is the second popular cancer in respiratory tract [1, 2]. Due to advancement of social-economy and life standards, plus rapid life style transition and diet habit, LSCC incidence is progressively increasing with high frequency in males and in middle to elder age groups [3, 4]. Complex pathogenic mechanism existed for LSCC, making it one polygenetic disease and under the modulation of various factors including viral infection, genetics, diet habit and environmental factors [5, 6]. Treatment approaches for LSCC are diversifying with advancement of medical diagnostic and treatment approaches, and individualized therapy has been developed covering surgical operation, chemo-/radio-therapy, immune therapy, and intervention therapy. However, the overall treatment efficiency of LSCC is still unfavorable and the prognosis has not been improved, resulting in lower survival rate plus more complications and higher metastatic rate and recurrent rate, all of which bring heavy burdens for global economy [7, 8]. In addition, due to the lack of biomarkers with higher sensitivity and specificity, a majority of LSCC patients are already at a terminal stage at the time of primary diagnosis. So far, the incidence and mortality of LSCC are still relatively high worldwide [9]. A prominent biological feature of LSCC is invasion and metastasis of tumor cells, which are also primary factors inducing patient death [10]. Therefore, investigation for effective molecular targets of LSCC can largely benefit disease treatment and can better inhibit tumor invasion and progression.

Biological activities and energy metabolism of cells are dependent on energy provided by glucose, which is critical for tumor proliferation and invasion [11]. Glucose transporter (Glu) can help the trafficking of cellular glucose, further providing energy for tumor cell proliferation and migration [12]. Glut-1 is the major carrier for cellular intake of glucose and can provide energy via glycolysis pathway [13]. Tumor cells can obtain energy via glycolysis pathway under

hypoxic environment, a process known as Warburg effect [14]. Up-regulation of Glut-1 is known to facilitate energy uptake of tumors [15]. However, the functional role or related mechanism of Glut-1 in LSCC has not been fully illustrated.

Materials and methods

General information

A total of 78 patients who were diagnosed as LSCC by pathophysiology examination in Jinan Third People's Hospital between January 2017 and January 2018 were recruited in this study. All patients have received surgical operation, with aging between 39 and 63 years (average age = 51.3 ± 5.5 years). Inclusive criteria [16, 17]: All patients were diagnosed as LSCC for the first time, and have not received surgery before. No patient has received other treatment such as radiotherapy or chemotherapy before surgery. All patients signed informed consent. Exclusive criteria: Patients having recurrent LSCC, received surgery before, or chemo-/radio-therapy previously. Patients were complicated with other disease such as infectious disease, malignant tumor or severe diabetes, or major organ failure, systemic immune disorder or cancer complications. Both tumor cells and adjacent tissues were collected during surgery for storage in liquid nitrogen.

This study has been approved by the Ethics Committee of Jinan Third People's Hospital (Shandong, China) and all the enrolled objects signed informed consent.

Major reagent and equipment

LSCC cell line Hep-2 (CCl-23™) was purchased from ATCC cell bank (US). DMEM culture medium, streptomycin-penicillin dual antibiotic were purchased from Hyclone (US). DMSO, fetal bovine serum (FBS) and MTT powder were purchased from Gibco (US). Trypsin-EDTA digestion buffer was purchased from Sigma (US). PVDF membrane was purchased from Pall Life Science. Western blot reagents were purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human Glut-1 monoclonal antibody, mTOR monoclonal antibody and phosphorylated AKT (pAKT), and rabbit anti-human AKT monoclonal antibody, goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG secondary antibody were purchased from Cell signaling (US). RNA extraction kit and reverse transcription kit were purchased from Axygen (US). Caspase-3 activity assay kit was purchased from R&D (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US). Glut-1 siRNA sequence and si-NC sequence were synthesized by Toyobo (China). XO1000D ultrasonic rupture was purchased from Xianou (China). ABI 7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (US). Ultrapure workstation was purchased from Suzhou Purification equipment (China). Transwell chamber was purchased from Corning (US). DNA amplifier was purchased from PE Gene Amp PCR System 2400 (US). CO_2 incubator was purchased from Thermo (US).

Hep-2 cell culture and grouping

Hep-2 cells kept in liquid nitrogen were resuscitated and passed for generations, and were incubated into culture dish at 1×10^7 cells per cm^2 density, using 90% high-glucose DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 ug/ml streptomycin. Cells were kept in a 37°C incubator with 5% CO_2, and were randomly assigned into three groups: control group, Glut-1 negative control (NC) group, and Glut-1 siRNA group.

Liposome transfection of Glut-1 siRNA

Separately transfected Glut-1 siRNA (5'-GCGGAGUGUGUGGAAUCA-3') and siRNA-NC (5'-UUGAGAGCUAGUGUGGU-3') were introduced into Hep-2 cells. In brief, cells were kept in 6-well plate until reaching 70-80% confluence. Glut-1 siRNA and siRNA-NC liposomes were added into 200 μl serum-free DMEM medium for mixture and room temperature incubation for 15 minutes. Lipo2000 was mixed with dilutions for 30 minutes at room temperature. Serum was removed from cell culture, and cells were gently rinsed by PBS. Then, 1.6 ml of serum-free DMEM medium was added into each system, and cells were incubated in a 37°C chamber with 5% CO_2 for 6 hours. Serum-containing DMEM medium was switched for 48 hours of continuous incubation in further studies.

Real-time PCR for measuring Glut-1 expression in LSCC cells and tissues

Trizol reagent was used for extract mRNA from LSCC cancer tissues and adjacent tissues, in

Table 1. Primer sequences

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGTAGTCACCTGTTGCTGG	TAATACGGAGACCTGTCTGGT
Glut-1	ATACTGATCCATGGATGG	TCAACCGTAGC CTCG GGTA

addition to Hep-2 tumor cells from all groups. Reverse transcription of DNA was performed following the manual instruction of test kit. Primers were designed using PrimerPremier6.0 software based on target gene sequence and were synthesized by Invitrogen (China) as in **Table 1**. Real-time PCR (RT-PCR) was performed on target gene expression using the following conditions: 55°C for 1 minute, following by 35 cycles each containing 92°C 30 seconds, 58-60°C 45 seconds and 72°C 35 seconds. Data were collected and CT values of all samples and standards were calculated using fluorescent quantification and PCR software. Using CT values of standard samples as the reference, a standard linear function was plotted for semi-quantitative analysis using $2^{-\Delta Ct}$ approach.

MTT assay for the effect on Hep-2 cell growth

Hep-2 cells at log-growth phase were digested, enumerated, and were seeded into 96-well plate at 3000 cells per well density. Cells were randomly assigned into three groups, all of which were treated for 48 hours in triplicated manners. Then, 20 μl MTT solution (5 g/L) was added into each well for 4 hours of continuous incubation. The supernatant was removed and 150 μl DMSO was added into each well. After 10 minutes vortex until the complete dissolution of violet crystals, absorbance (A) values at 570 nm wavelength were measured on a microplate reader for calculating cell proliferation rate.

Caspase 3 activity assay

Caspase 3 activity was measured from each group of cells following the manual instruction of test kit. In brief, cells were digested by trypsin, centrifuged at 600 × g for 5 min under 4°C. The supernatant was removed and cells were lysed by lysis buffer for 15 minutes incubation on ice. The lysate was centrifuged at 20000 × g for 5 minutes under 4°C. 2 mM Ac-DEVD-pNA was added for measuring optical density (OD) values at 405 nm wavelength to calculate caspase 3 activity.

Transwell chamber assay for measuring cell invasion

The assay for cell invasion followed manual instruction of test kit. In brief, serum-free DMEM medium was applied. Then 24 hours later, transwell chambers were coated with 1:5 diluted Matrigel solution (50 mg/L) on bottom and upper membrane, and were air-dried at 4°C. 500 μl DMEM medium containing 10% FBS, and 100 μl tumor cell suspensions were added into the interior and exterior side of the chamber, respectively. Three replicated wells were adopted in each group, and all chambers were placed in a 24-well plate. The control group utilized Transwell chamber without Matrigel. After 48 hour incubation, Transwell chamber was rinsed in PBS to remove cells on the membrane, followed by fixation on iced ethanol. After staining in crystal violet, cells at the lower phase of membrane were enumerated. The experimental was repeated for three times.

Western blot for measuring Glut-1 and AKT/mTOR signal pathway

Hep-2 cellular proteins were extracted from LSCC tumor tissues and adjacent tissues on ice. In brief, cells were lysed on ice for 15~30 minutes on ice, and were ruptured using 5 s × 4 times of ultrasound. Cell lysate was centrifuged at 10000 g for 15 minutes under 4°C, and the supernatant was saved to a new tube. After quantification by Bradford approach, proteins were kept at -20°C for Western blot. Proteins were separated using 10% SDS-PAGE, and were transferred onto PVDF membrane using semi-dry approach (100 mA, 1.5 hours). Non-specific background was removed by 5% skimmed milk powder at room temperature for 2 hour incubation. Then, 1:1000 diluted monoclonal primary antibody against phosphorylated pAKT or AKT, or 1:1000 diluted monoclonal antibody against mTOR and Glut-1 was added for 4°C overnight incubation. After rinsing in PBST, 1:2000 diluted goat anti-rabbit secondary antibody was added for dark incubation at room temperature for 30 minutes. With PBST rinsing, ECL reagent was added for 1 min development, followed by X-ray exposure. Protein imaging processing software and Quantity One system was used to scan the film for measuring

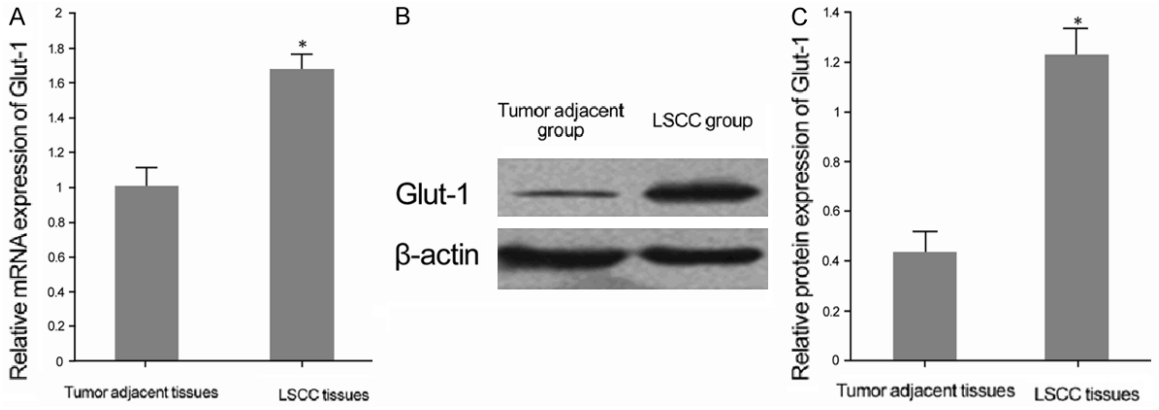

Figure 1. Expression of Glut-1 in LSCC. A. Glut-1 mRNA in LSCC tissues. B. Western blot for Glut-1 protein expression in LSCC tumor tissues. C. Glut-1 protein expression in cancer tissues. $^{*}P < 0.05$ comparing to tumor adjacent tissues.

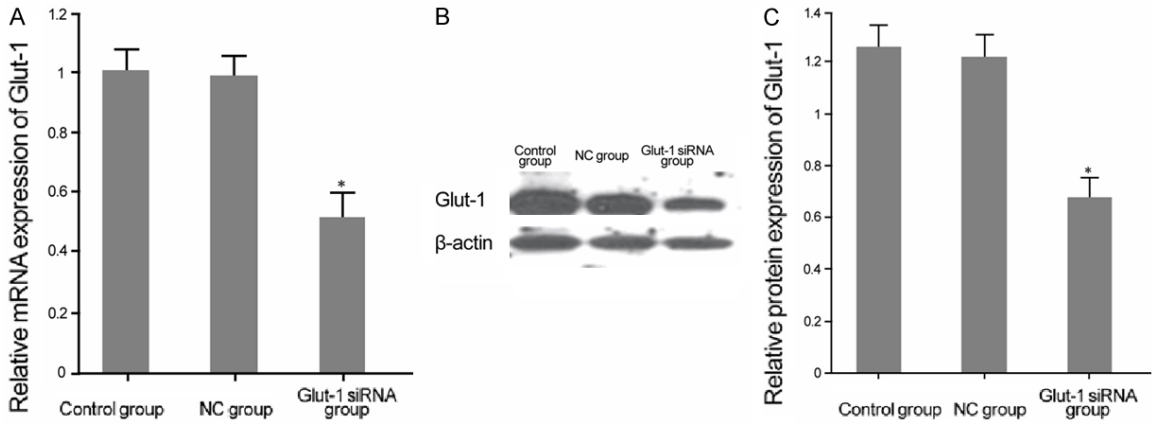

Figure 2. Effect of siRNA transfection on Glut-1 expression in Hep-2 cells. A. Glut-1 mRNA expression in Hep-2 cells. B. Western blot for Glut-1 protein in Hep-2 cells. C. Analysis for Glut-1 protein expression in Hep-2 cells. $^{*}P < 0.05$.

band density. Each experiment was repeated for four times (n = 4) for statistical analysis.

Statistical analysis

SPSS 11.5 software was sued for analysis of all data, which were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used for measuring between-group difference. Student t-test was employed for comparing means between two groups. A statistical significance was identified when $P < 0.05$.

Results

Expression of Glut-1 in LSCC

Real-time PCR and Western blot were used to measure Glut-1 mRNA and protein expression in LSCC. Results showed that comparing to adjacent tissues, Glut-1 mRNA and protein showed significantly elevated expression in cancer tissues ($P < 0.05$, **Figure 1**).

Effects of siRNA transfection on Glut-1 expression in LSCC cell line Hep-2

After transfecting Glut-1 siRNA into Hep-2 cells, expression of Glut-1 was measured. Results showed that comparing between control group and siRNA-NC group, Glut-1 mRNA and protein showed decreased expression in Hep-2 cells ($P < 0.05$, **Figure 2**).

Effect of Glut-1 knockdown on Hep-2 cell proliferation

After using Glut-1 siRNA to knockdown Glut-1 expression in Hep-2 cells, MTT assay was used to analyze the effect on Hep-2 cell proliferation.

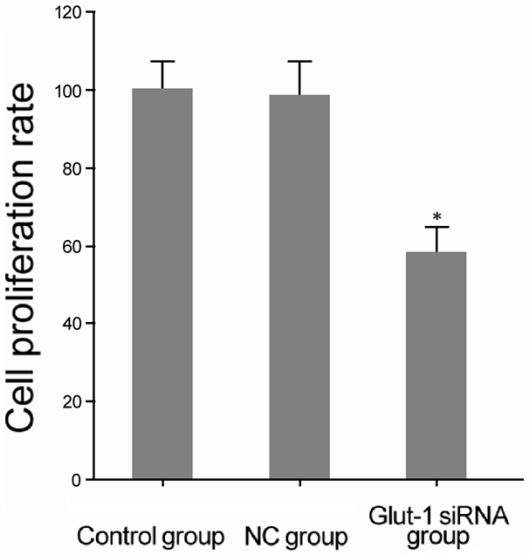

Figure 3. Effects of Glut-1 knockdown on Hep-2 cell proliferation. *P < 0.05 comparing to the control group.

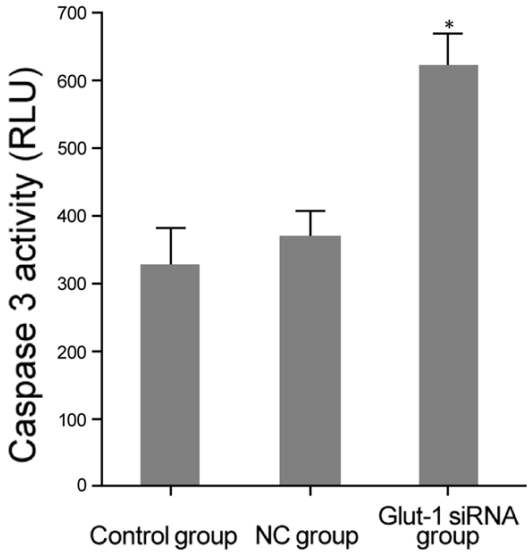

Figure 4. Effects of Glut-1 expression modulation on Caspase 3 activity of Hep-2 cells. *P < 0.05 comparing to the control group.

Results showed that transfection of Glut-1 siRNA for 48 h remarkably inhibited Hep-2 cell proliferation (P < 0.05 comparing to control group, **Figure 3**). These results suggested that the modulation of Glut-1 had significant inhibition on Hep-2 cell proliferation.

Effect of Glut-1 expression modulation on Caspase 3 activity of Hep-2 cells

By activity assay, we measured the effect of Glut-1 expression on Caspase 3 activity in Hep-22 cells. Results showed that transfection of Glut-1 siRNA into Hep-2 cells remarkably increased Caspase 3 activity in tumor cells (P < 0.05 comparing to control group, **Figure 4**).

Effects of Glut-1 expression modulation on invasion of Hep-2 cells

Transwell chambers were used to measure the effect of Glut-1 expression on Hep-2 cell invasion. The transfection of Glut-1 siRNA into Hep-2 cells remarkably suppressed Hep-2 cell invasion (P < 0.05 comparing to control group, **Figure 5**).

Effect of Glut-1 modulation on AKT/mTOR in Hep-2 cells

Western blot was used to measure the effect of Glut-1 on AKT/mTOR signaling pathway protein expression in Hep-2 cells. The transfection of Glut-1 siRNA into Hep-2 cells facilitated mTOR inhibition and down-regulation of phosphorylated AKT (P < 0.05 comparing to control group, **Figure 6**).

Discussion

Early identification, diagnosis and timely treatment largely improve prognosis of larynx cancer, which frequently has misdiagnosis due to atypical early symptoms. Therefore, the investigation for LSCC pathogenic mechanism and precise molecular target can benefit optimization of treatment strategy [18]. Glut-1 has a wide distribution and is mainly localized in transmembrane glycoprotein on cell membrane, and can induce transmembrane trafficking of glucose [19]. Under sufficient oxygen supply, tumor cells can obtain energy by the means of glycolysis. Glut-1 has relatively higher affinity with glucose molecules, and can still assist the transportation of glucose molecule even under low-glucose condition, thus providing energy for tumor cells and making it the critical factor for rapid proliferation and invasion of tumor cells [20]. Glut-1 is further found to be closely related with tumor differentiation stage. Previous study believed that Glut-1 up-regulation in tumors was positively related with tumor malignancy, metastasis and invasion, indicating unfavorable prognosis, and is thus one important index for tumor prognosis [21]. This study demonstrated up-regulation of Glut-1 mRNA and protein in LSCC tumor tissues, and

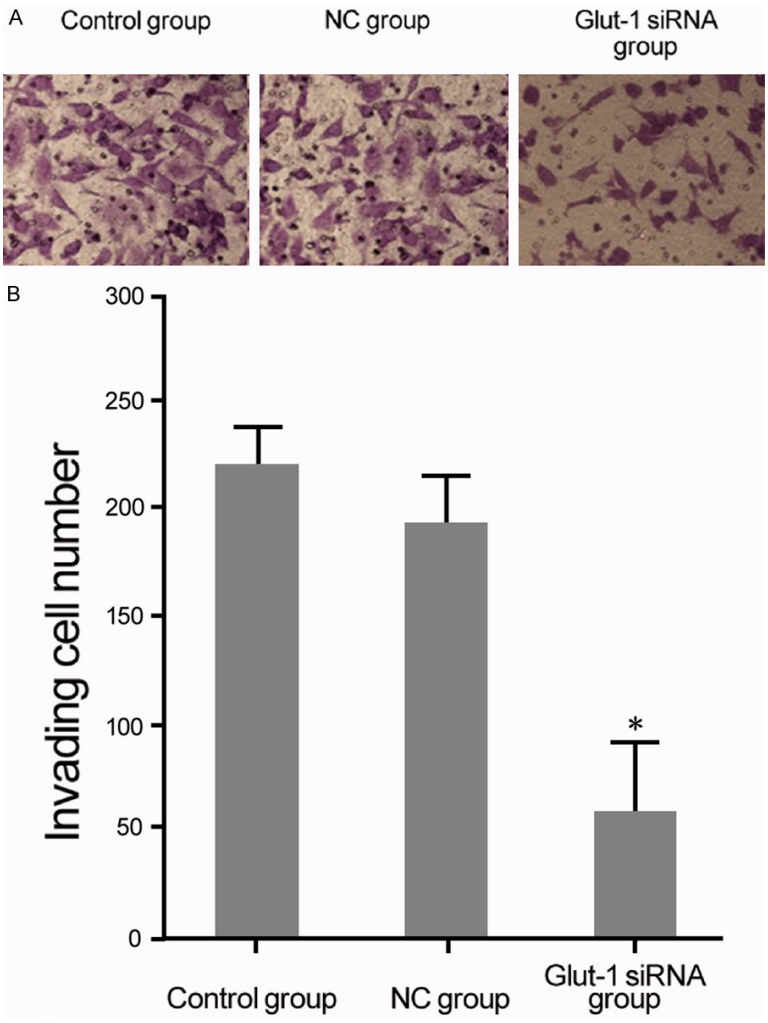

Figure 5. Effect of Glut-1 expression modulations on Hep-2 cell invasion. A. Transwell chamber assay for the effect of Glut-1 expression on Hep-2 cell invasion. B. Effect of Glut-1 expression on invasion of Hep-2 cells. $^{*}P < 0.05$ comparing to the control group.

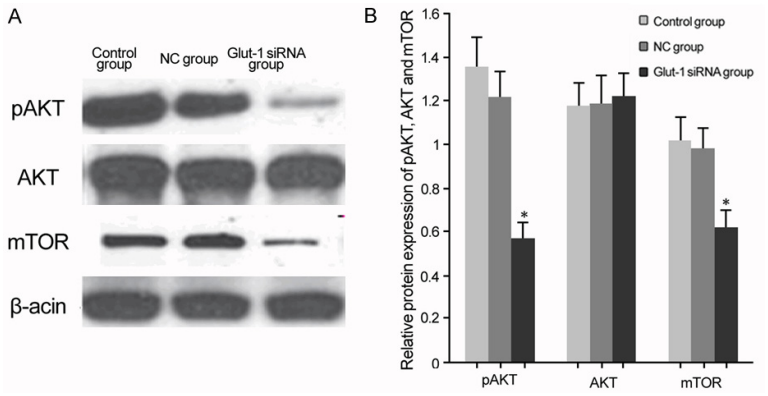

Figure 6. Regulation of Glut-1 expression on AKT/mTOR signal pathway proteins in Hep-2 cells. A. Western blot for measuring the effect of Glut-1 on AKT/mTOR signal pathway proteins in Hep-2 cells. B. Analysis for the effect of Glut-1 on AKT/mTOR signal pathway protein expression in Hep2 cells. $^{*}P < 0.05$ comparing to the control group.

further siRNA approach to interfere Glut-1 expression in LSCC cell line Hep-2 for its down-regulation could inhibit tumor cell proliferation or invasion, plus enhancement of apoptosis, indicating the role of Glut-1 in LSCC onset and progression.

Mammalian target of rapamycin (mTOR)/protein kinase B (AKT) signaling pathway plays crucial roles in tumor cell growth, proliferation and invasion [11]. Previous study showed the close correlation between AKT/mTOR signaling pathway and onset and progression of LSCC [22]. Therefore the regulation of AKT/mTOR signaling pathway has become one focus for targeted therapy of LSCC. AKT/mTOR pathway is known to be closely related with onset, progression and prognosis of LSCC [23]. As one important downstream factor of AKT, mTOR participates in regulating tumor cell proliferation, growth, survival and angiogenesis. AKT/mTOR signaling pathway frequently facilitates tumor survival via inhibiting apoptotic signal and activating anti-apoptotic factors [24, 25]. This study confirmed that down-regulation of Glut-1 expression can lead to down-regulation of AKT phosphorylation (pAKT), whose activation is thus suppressed for further down-regulation of target protein mTOR, indicating the close correlation between the regulatory effect on LSCC and the AKT/mTOR pathway upon changes in Glut-1 expression. However, this study only covers cellular levels, and further investigations are thus required to illustrate the role of Glut-1 on LSCC by *in vivo* assay in addition to mechanistic study.

Conclusion

Glut-1 is up-regulated in LSCC tumor tissues. The suppression of Glut-1 expression exerts functions for LSCC cell proliferation, apoptosis and invasion via modulating AKT/mTOR pathway.

Disclosure of conflict of interest

None.

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