Effect of Inhibition of GLUT1 Expression and Autophagy Modulation on the Growth and Migration of Laryngeal Carcinoma Stem Cells Under Hypoxic and Low-Glucose Conditions

Xiao-Hong Chen
Department of Otolaryngology, the Second Hospital of Jiaxing City(The Second Affiliated Hospital of Jiaxing University)

Jia Liu
Department of Otlaryngology,the First Affiliated Hospital, College of Medicine, Zhejiang University

Jiang-Tao Zhong
Department of Otolaryngology, the First Affiliated Hospital, College of Medicine,Zhejiang University

Shui-Hong Zhou (1190051@zju.edu.cn)
Zhejiang University, College of Medicine ,TheFirst Affiliated Hospital https://orcid.org/0000-0002-7163-2289

Jun Fan
State Key Laboratory for Diagnosis and reatment of Infectious Diseases, the First Affiliated Hospital, College of Medicine, Zhejiang University

Research article

Keywords: Laryngeal carcinoma, cancer stem cell, CD133+ cell, GLUT-1, autophagy

Posted Date: August 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-38920/v1

Abstract

Background: Enhanced glucose uptake and autophagy are means by which cells adapt to stressful microenvironments. We investigated the roles of glucose transporter-1 (GLUT-1) and autophagy in laryngeal carcinoma stem cells under hypoxic and low-glucose conditions.

Methods: $CD133^+$ Tu212 laryngeal carcinoma stem cells were purified by magnetic-activated cell sorting and subjected to hypoxic and/or low-glucose conditions. Proliferation was evaluated using a cell-counting kit and a clone-formation assay, and migration was evaluated through a Transwell assay. Autophagy was assessed via transmission electron microscopy. GLUT-1 and beclin-1 expression were silenced using an shRNA and autophagy was manipulated using rapamycin, 3-MA, or chloroquine. Gene expression levels were evaluated by quantitative reverse transcription-polymerase chain reaction and protein concentrations were assessing via Western blotting.

Results: Compared to $CD133^-$ stem cells, $CD133^+$ cells showed increased proliferation and migration, and reduced apoptosis, under hypoxic or low-glucose conditions. They also showed increased expression of GLUT-1 and autophagy markers. Finally, GLUT-1 knockdown or autophagy inhibition reduced their proliferation and migration.

Conclusions: Enhanced glucose uptake and autophagy maintain the functions of $CD133^+$ laryngeal carcinoma stem cells under hypoxic and low-glucose conditions.

Background

Laryngeal cancer is a common malignant tumor of the head and neck. The 5-year survival rate of laryngeal cancer has not improved in the past 40 years despite the near-continuous development of diagnostic and treatment methods [1]. The lack of understanding of the mechanisms underlying the functions of laryngeal cancer cells has hampered the development of effective therapeutic strategies.

Compared to ordinary tumor cells, cancer stem cells (CSCs) are typically resistant to apoptosis and therapeutic agents [2, 3]. CSCs are closely related to the occurrence, development, metastasis, recurrence, and chemoradiotherapy resistance of tumors [4]. Laryngeal CSCs reportedly express CD133 as a marker [5]. $CD133^+$ CSCs have higher tumorigenic and invasive abilities than $CD133^-$ cancer cells [5–7, 10]. However, whether $CD133^+$ CSCs have specific metabolic mechanisms is unclear.

Autophagy provides energy for cells from the degradation of proteins and organelles. It can restrain the growth of tumor cells. However, it is also an adaptive response to stressful tumor microenvironments, preventing apoptosis of tumor cells [11, 12]. A basal level of autophagy may promote the survival of cancer cells [11, 12]. However, prolonged and excessive activation of autophagy may induce self-degradation and death of tumor cells [13].

$CD133^+$ CSCs show greater proliferation and a lower frequency of apoptosis compared to $CD133^-$ cancer cells in multiple types of tumors, particularly under hypoxic or nutrient-deprived conditions [14, 15]. Interestingly, autophagy induction reportedly promotes the conversion of non-stem pancreatic cancer cells into $CD133^+$ stem-like cells under intermittent hypoxia [16]. The expression of glucose transporter-1 (GLUT-1) is reportedly associated with autophagy activation in CSCs under hypoxic or nutrient-deprived conditions [17–20]. Autophagy may also affect cellular glucose uptake [21–23]. Beclin-1, an autophagy marker, plays an important role in the initiation of autophagy [24]. It promotes the localization of other autophagy-related proteins to autophagosomes, thus promoting the formation and maturation of autophagosomes. High expression of beclin-1 is typically accompanied by enhanced autophagy, increased GLUT-1 expression, and increased glucose uptake in certain types of tumors, such as non-small-cell lung carcinoma and breast cancer [25–27]. However, in one study, beclin-1 and GLUT-1 expression were negatively correlated in 29 cases of head-and-neck squamous cell carcinoma [28], indicating that the association between autophagy and glucose metabolism may be cancer-type related. In this work, we investigated the regulation by GLUT-1 and autophagy of the functions of laryngeal carcinoma stem cells under hypoxic or low-glucose conditions, as well as the underlying mechanisms.

Methods

Cell culture and treatment

Tu212 cells were purchased from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). Tu212 cells were cultured in Roswell Park Memorial Institute-1640 medium (Gibco-BRL, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/mL penicillin, and 100 g/mL streptomycin at 37 °C in an atmosphere containing 5% CO_2.

Sorting and identification of Tu212 laryngeal carcinoma cells

Magnetic sorting. Briefly, 1×10^7 Tu212 cells were resuspended in phosphate-buffered saline (PBS). The resuspended cells were added to 100 μL FcR Blocking Reagent and 100 μL CD133 MicroBeads, mixed, and incubated at 4 °C for 30 min. Next, 2 mL PBS was added, and the cells were centrifuged at 300 × g for 10 min; the supernatant was discarded. Subsequently, the cell pellet was resuspended in 500 μL PBS. The magnetic separation (MS) column was clipped to the magnetic separator and 500 μL PBS was added to moisten the column. Next, the cell suspension was added to the MS sorting column. The MS separation column was washed three times with 500 μL PBS to remove unbound cells. The column was removed from the magnetic separator, 1 mL PBS was added, and the cells were expelled from the column using a push rod. After centrifugation at 300 × g for 10 min, the supernatant was discarded, and the cells were resuspended in 1 mL PBS and enumerated.

Determination of the purity of Tu212 CD133 + cells. Cells (2×10^5) were removed before and after separation, centrifuged, and the supernatant was discarded. Next, 80 μL PBS and 10 μL anti-human CD133-PE were added. The sample was gently mixed using a micropipette and incubated at 4 °C for 10 min. The cells were centrifuged at 1000 rpm for 5 min and the supernatant was discarded. Precooled PBS (1 mL) was added, and unbound excess antibody components were removed by two centrifugation and washing steps. After adding 4% paraformaldehyde and incubation at 4 °C for 20 min, the supernatant was centrifuged. The cells were transferred to a flow tube and stored at 4 °C protected from light. Flow cytometry was performed using the standard procedure (Beckman, Fullerton, CA).

Experimental groups

Relationship between the proliferation and migration of Tu212 CD133 + cells and the levels of GLUT-1 and autophagy under hypoxia and low-glucose conditions. Eight groups were used in this experiment: $CD133^+$ (20% O_2, 25 mM Glu); $CD133^-$ (20% O_2, 25 mM Glu); $CD133^+$+hypoxia (1% O_2, 25 mM Glu); $CD133^-$ +hypoxia (1% O_2, 25 mM Glu); $CD133^+$+low Glu (20% O_2, 2.5 mM Glu); $CD133^-$+low Glu (20% O_2, 2.5 mM Glu); $CD133^+$+hypoxia + low Glu (1% O_2, 2.5 mM Glu); and $CD133^-$+hypoxia + low Glu (1% O_2, 2.5 mM Glu).

Cell growth, *migration*, *and levels of GLUT-1 and autophagy-related proteins*. Fourteen groups were used in this experiment: $CD133^+$+hypoxia + low Glu (1% O_2, 2.5 mM Glu); $CD133^-$+hypoxia + low Glu (1% O_2, 2.5 mM Glu); $CD133^+$+hypoxia + low Glu (1% O_2, 2.5 mM Glu) + NC shRNA; $CD133^-$+hypoxia + low Glu (1% O_2, 2.5 mM Glu + NC shRNA); $CD133^+$+hypoxia + low Glu (1% O_2, 2.5 mM Glu + GLUT-1 shRNA); $CD133^-$+ hypoxia + low Glu (1% O_2, 2.5 mM Glu) + GLUT-1 shRNA); $CD133^+$+hypoxia + low Glu (1% O_2, 2.5 mM Glu + beclin-1 shRNA); $CD133^-$+hypoxia + low Glu (1% O_2, 2.5 mM Glu + beclin-1 shRNA); $CD133^+$+hypoxia + low Glu (1% O_2, 2.5 mM Glu + 3-MA); $CD133^-$+hypoxia + low Glu (1% O_2, 2.5 mM Glu + 3-MA); $CD133^+$+hypoxia + low Glu (1% O_2, 2.5 mM Glu + CQ); $CD133^-$+hypoxia + low Glu (1% O_2, 2.5 mM Glu + CQ); $CD133^+$+hypoxia + low Glu (1% O_2, 2.5 mM Glu) + rapamycin); and $CD133^-$+hypoxia + low Glu (1% O_2, 2.5 mM Glu) + rapamycin).

Clonogenic assay

The cell suspension was dispersed; the percentage of individual cells was greater than 95%. Next, cells were counted, and the cell density was adjusted to 250/mL by adding culture medium. The cell suspension was added to the wells of a six-well plate (2 mL per well), and the plate was gently shaken. The plate was placed in an incubator for 2 to 3 weeks, and the medium was replaced every 3 days. The culture was terminated when clones became visible. The medium was discarded, and the cells were gently washed twice with PBS, stained with 1% crystal violet at room temperature for 1 h, and photographed.

Cell-Counting Kit-8 assay

Cells were incubated at 37 °C in an atmosphere containing 5% CO_2 for 48 h. Next, 20 μL Cell Counting Kit-8 (CCK-8) solution was added, and the cells were incubated in the dark for 1 h. The absorption at 450 nm of the suspension was measured using a Spectra Plus Microplate Reader (Molecular Devices, Sunnyvale, CA).

Flow cytometry

Briefly, 10 × Binding Buffer was diluted 1:10 with deionized water. Cells were collected by centrifugation for 5 min, exposed to reagents, digested, and resuspended in 500 μL Annexin V binding buffer. Next, 5 μL fluorescein isothiocyanate and 10 μL propidium iodide (Sigma Aldrich Co., St. Louis, MO) were added for 10 min in darkness at RT. Finally, the proportions of non-apoptotic and apoptotic cells were determined in triplicate by flow cytometry with ModFit LT software (Becton Dickinson, Mountain View, CA)

Transwell assay

Cells were digested with trypsin and the culture medium was discarded. Next, the cells were washed once or twice with PBS and resuspended in serum-free medium (containing 0.2% bovine serum albumin) to a density of 1×10^6/mL. Cell suspension (200 μL) was added to the upper Transwell chamber and 600 μL FBS was added to the lower chamber. The cells were incubated in a 5% CO_2 atmosphere at 37 °C for 24 h. The Transwell chamber was removed, and the culture medium was discarded. Then the chamber was washed twice with calcium-free PBS, fixed in formaldehyde for 30 min, air-dried, and the cells were stained with 0.1% crystal violet for 20 min. Finally, the upper layer of unmigrated cells was gently removed using cotton swabs and washed three times with PBS.

Quantitative real-time polymerase chain reaction

The cells were collected, washed three times with precooled PBS, centrifuged at 1500 rpm for 3 min, and lysed on ice in the presence of TRIzol. Total RNA was extracted from the cells according to the manufacturer's instructions. Briefly, 1 μg RNA was reverse-transcribed using a First-Strand cDNA Synthesis Kit (K1622; Fermentas, Burlington, ON, Canada) and amplified by PCR using a SYBR Green qPCR Kit (Merck, Darmstadt, Germany). The PCR program was 37 °C for 60 min, 85 °C for 5 min, and 4 °C for 5 min. The amplification products were stored at − 20 °C. The primers for GLUT-1, Beclin-1, Atg7, Atg5, and LC3 were designed and synthesized by Sangon Biotech (Table 1). The $2^{\Delta\Delta Ct}$ method was used to calculate relative gene expression levels.

Table 1
The primers for GLUT-1, Beclin-1, Atg7, Atg5, and LC3

	forward	reverse
GLUT-1	5'-GTCAACACGGCCTTCACTG-3'	5'GGTCATGAGTATGGCACAACC-3'
Beclin-1	5'-CCATGCAGGTGAGCTTCGT-3'	5'-GAATCTGCGAGAGACACCATC-3'
Atg7	5'-CTGCCAGCTCGCTTAACATTG-3'	5'-CTTGTTGAGGAGTACAGGGTTTT-3'
Atg5	5'-TCAGCCACTGCAGAGGTGTTT-3'	5'-GGCTGCAGATGGACAGTTGCA-3'
LC3	5'-CATGAGCGAGTTGGTCAAGAT-3'	5'-TCGTCTTTCTCCTGCTCGTAG-3
GAPDH	5'-GAGCCCGCAGCCTCCCGCTT-3'	5'-CCCGCGGCCATCACGCCACAG-3'

Western blotting

Total proteins were extracted from cells and tumor tissues in radioimmunoprecipitation assay buffer. The cells were collected, washed three times with precooled PBS, and centrifuged at 1500 rpm for 3 min. An appropriate volume of cell lysate was added, and the cells were left on ice for 30 min. The supernatant was centrifuged at 1200 rpm at 4 °C for 30 min and stored at − 80 °C. After assaying the protein concentration, samples were added to 4· sodium dodecyl sulfate loading buffer, boiled for 5 to 10 min, and centrifuged at 12,000 · g for 1 min. Proteins (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis (SDSPAGE) and transferred to a polyvinylidene difluoride membrane (Millipore). Primary antibodies against GLUT-1 (Abcam), beclin-1, LC3 (Proteintech), Atg7, Atg5, and β-actin (Abcam) were added and incubated at 4 °C overnight; β-actin served as the internal control. After washing three times with Tris-buffered saline/Tween 20, the secondary antibodies were added for 1 h at room temperature. The signal was developed using an enhanced chemiluminescence assay kit (Beyotime Biotech) and analyzed semi-quantitatively using the ChemiDoc XRS + System (Bio-Rad).

Transmission electron microscopy

Cells were collected, washed in PBS, fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, and dehydrated in ethanol and acetone. After embedding in epoxy resin, sections were cut and stained with uranyl acetate and lead citrate. Autophagy was visualized by transmission electron microscopy (TEM; Thermo Fisher Scientific, Waltham, MA).

Results

The proliferation, migration and apoptosis of CD133 $^{+}$ laryngeal carcinoma stem cells under oxygen- or glucose-deprived conditions

To investigate the role of GLUT-1 in LCSCs, we first sorted $CD133^+$ cells from laryngeal carcinoma Tu212 cells by MACS. Cell purity was examined by flow cytometry. The results showed that the proportion of $CD133^+$ Tu212 cells was only ~ 8% before sorting, and reached more than 90% after sorting. Moreover, the isolated Tu212 $CD133^+$ cells showed good viability as demonstrated by SSC/FSC plots (Fig. 1a).

Next, we investigated whether the growth, proliferation, apoptosis, and migration of laryngeal carcinoma Tu212 $CD133^+$ cells under hypoxia and low glucose were significantly higher than those in $CD133^-$cells or $CD133^+$ under normal condition. Clonal formation test showed that the clonal numbers of Tu212 $CD133^+$cells were significantly higher than that of Tu212 $CD133^-$cells under hypoxia (1%O_2), low glucose (2.5 mM glucose) and hypoxia + low glucose conditions. However, the clonal-forming ability was similar between Tu212 $CD133^+$cells and Tu212 $CD133^-$cells under normal culture condition (Fig. 1b). CCK-8 assay also revealed that compared with $CD133^-$ cells, the proliferation of Tu212 $CD133^+$cells was significantly increased under hypoxia, low glucose and hypoxia + low glucose conditions (Fig. 1c). In addition, flow cytometry showed that the apoptotic rate of Tu212 $CD133^+$ cells was significantly decreased compared to Tu212 $CD133^-$ cells under hypoxia, low glucose and hypoxia + low glucose conditions (Fig. 1d). Transwell assay demonstrated that the migratory ability of Tu212 $CD133^+$ cells was also higher than that of Tu212 $CD133^-$ cells under the above three stress conditions (Fig. 1e). In contrast, the proliferation and apoptosis were comparable between Tu212 $CD133^+$ cells and Tu212 $CD133^-$cells under normal culture condition, with $CD133^+$ cells showing only a marginal increase in the migratory ability. Hence, $CD133^+$ laryngeal carcinoma stem cells showed increased proliferative and migratory capacity than $CD133^-$ cells under stressful conditions.

The expression of GLUT-1 and cell autophagy in CD133 + laryngeal carcinoma stem cells under oxygen- or glucose-deprived conditions

Previous studies showed that GLUT-1 overexpression promoted cancer cell proliferation, invasion and metastasis, especially in hypoxia and starvation conditions [6, 7]. Besides, activated autophagy promoted the functions of $CD133^+$ cells under hypoxia [15, 16], probably by enhancing cell glucose intake [21–23]. Thus, we explored whether the increased proliferation and migration of laryngeal carcinoma $CD133^+$ Tu212 cells were associated with high level of GLUT-1 expression and cell autophagy. To this end, we first investigated the expression of GLUT-1 and autophagy in Tu212 cells under hypoxia and low glucose conditions. qRT-PCR results showed that the expression of GLUT-1 mRNA in Tu212 $CD133^+$ cells was significantly higher than that in Tu212 $CD133^-$ cells under normal, hypoxia, low glucose, and hypoxia + low glucose conditions. The expression of GLUT-1 mRNA of Tu212 $CD133^+$ cells under hypoxia, low glucose, and hypoxia and low glucose was higher than that of Tu212 $CD133^+$cells under normal culture condition (Fig. 2a). Western blotting also showed that the protein level of GLUT-1 in Tu212 $CD133^+$ was significantly higher than that in Tu212 $CD133^-$ cells under the above four conditions (Fig. 2b, S1).

In terms of cell autophagy, we found that hypoxia or low glucose increased the expression of Beclin-1, Atg7, Atg5, and LC3 in Tu212 $CD133^+$ cells, but to a lesser extent in Tu212 $CD133^-$ cells. Compared with Tu212 $CD133^-$ cells, the levels of these autophagy markers were significantly higher in Tu212 $CD133^+$ cells under hypoxia, low glucose, and hypoxia + low glucose conditions (Fig. 2a, b, S1). Transmission Electron Microscopy (TEM) imaging also showed that the number of autophagosomes in Tu212 $CD133^+$ cells was significantly higher compared with that in Tu212 $CD133^-$ cells under the above three conditions, indicating the enhanced autophagy in stressed-laryngeal carcinoma stem cells (Fig. 2c). Thus, the increased survival and migration of laryngeal carcinoma stem cells under hypoxia and low glucose maybe associated with high GLUT-1 expression and cell autophagy.

The association between the expression of GLUT-1 and autophagy markers in CD133 + laryngeal carcinoma stem cells

To further study the association between the levels of GLUT-1 and autophagy marker genes. We adopted the following strategies: (1) silenced GLUT-1 expression by shRNA. (2) silenced Beclin-1 expression by shRNA. (3) inhibited autophagy by 3-MA and chloroquine. (4) activate autophagy by RAPA in Tu212 $CD133^+$ cells, then cell functions were evaluated. qRT-PCR and Western blotting results showed that the levels of GLUT-1, Beclin-1, Atg7, Atg5, and LC3II/LC3I ratio in Tu212 $CD133^+$ cells were higher than that in Tu212 $CD133^-$ cells under hypoxia + low glucose condition. After transfection with GLUT-1 shRNA, the levels of Beclin-1, Atg7, Atg5 mRNA and LC3II/LC3I ratio in Tu212 $CD133^+$ cells were significantly descended. After transfection with Beclin-1 shRNA, the LC3II/LC3I ratio in Tu212 $CD133^+$ cells were significantly descended, whereas the expression of GLUT-1, ATG7 and ATG5 did not obviously changed upon Beclin-1 silencing. In terms of autophagy inhibitors, 3-MA treatment significantly decreased the levels of Beclin-1, Atg7, Atg5 and LC3II/LC3I ratio in Tu212 $CD133^+$ cells, whereas GLUT-1 expression did not markedly change. In contrast, CQ treatment failed to obviously change the expression of GLUT-1, Beclin-1, Atg7, Atg5 and, LC3II/LC3I ratio in Tu212 $CD133^+$. On the other hand, RAPA treatment significantly increased the expression of Beclin-1 and LC3II/LC3I ratio in Tu212 $CD133^+$ cells (Fig. 3a, b, S2). The above results suggested that the expression of GLUT-1 and autophagy markers is closely associated in stressed laryngeal carcinoma stem cells. In addition, TEM imaging showed that silencing of GLUT-1 and Beclin-1, or inhibiton of autophagy significantly decreased the number of autophagosomes in Tu212 CD133 + cells under hypoxia + low glucose condition. In contrast, activation of autophagy by RAPA treatment significantly enhanced the number of autophagosomes in Tu212 $CD133^+$ cells (Fig. 3c).

GLUT-1 knockdown or autophagy inhibition reduced the proliferation and migration of CD133 + laryngeal carcinoma stem cells

In the functional analysis, we found that silencing of GLUT-1 markedly decreased the clonal-forming capacity, cell proliferation, and migration of Tu212 $CD133^+$ cells under oxygen- and glucose-deprived condition (Fig. 4a-c). In contrast, the apoptotic rate of Tu212 $CD133^+$ cells was significantly increased by the silencing of GLUT-1 (Fig. 4d). Similarly, Beclin-1 silencing or autophagy inhibitor (3-MA, CQ) treatment

also significantly decreased the above malignant behaviors of $CD133^+$ cells. Importantly, upon GLUT-1 silence or autophagy inhibition, the survival and migratory advantages of $CD133^+$ cells over $CD133^-$ counterparts were greatly compromised. To our surprise, autophagy activator Rapamycin also reduced the malignant behaviors of $CD133^+$ cells, suggesting that the excessive autophagy may be harmful for stem cells as well (Fig. 4a-d). Taken together, the enhanced glucose uptake and autophagy are responsible for maintaining the growth and migration of the stressed laryngeal carcinoma stem cells.

Discussion

Glucose is the main energy source for tumor cells and GLUT-1 is a key transporter of extracellular glucose [6, 7, 29]. GLUT-1 is overexpressed in many cancers, including laryngeal carcinoma, and is associated with their metastasis, drug resistance, and prognosis [6, 7, 30–36]. We investigated the role of GLUT-1 in Tu212 $CD133^+$ laryngeal carcinoma cells under hypoxic and low-glucose conditions.

A stressful extracellular microenvironment, such as hypoxia or low glucose, may upregulate GLUT-1 expression [37]. Indeed, hypoxia promotes the proliferation, migration, and chemoresistance of CSCs [4–7, 14, 15]. In this study, the growth and migration of $CD133^+$ Tu212 cells were greater than those of $CD133^-$ Tu212 cells under hypoxic and low-glucose conditions; this was associated with increased mRNA and protein levels of GLUT-1 in $CD133^+$ Tu212 cells. These findings are consistent with previous reports that high GLUT-1 expression facilitates glucose uptake to meet the energy demand of cancer cells. This adaptive response enables cancer cells to overcome external stresses, such as hypoxia or nutrient deprivation [6, 38], thereby suppressing apoptosis.

The levels of autophagy markers in Tu212 $CD133^+$ cells were increased by hypoxia and low glucose, whereas GLUT-1 silencing reduced the levels of these proteins. These results suggest mutual regulation of glucose uptake and autophagy in stressed laryngeal carcinoma stem cells. Autophagy modulates various metabolic pathways, including that centered on glucose [23]. Hypoxia and glucose deprivation may induce autophagy, promoting glucose uptake by upregulating GLUT-1 expression to increase the glycolytic flux and maintain nutrient uptake under stress conditions [21–23, 40, 41]. In airway progenitor cells, a lack of GLUT-1 impacts its recycling but not its expression, facilitating glucose uptake [40]. In mouse embryonic fibroblasts, however, autophagy enhances glucose uptake by increasing GLUT-1 expression and promoting GLUT-1 trafficking [21]. In this study, silencing of GLUT-1 decreased the levels of the autophagy markers beclin-1, Atg7, and Atg5, as well as the LC3II/LC3I ratio. However, inhibition or activation of autophagy by the beclin-1 shRNA/3-MA/CQ or rapamycin did not affect GLUT-1 expression. By contrast, rapamycin-induced autophagy activation increased the frequency of apoptosis of laryngeal CSCs, consistent with reports that excessive autophagy induces cell death.

This study had some limitations. First, we did not assay glucose metabolism, instead using GLUT-1 as a surrogate for glucose uptake. Second, we did not explore the *in vivo* implications of our findings using animal models. Third, the signaling mechanisms responsible for the enhanced GLUT-1 expression and

autophagy in laryngeal carcinoma stem cells under hypoxic and low-glucose conditions warrant further investigation.

Conclusions

In summary, hypoxia and low glucose increased the growth and migration of $CD133^+$ laryngeal carcinoma stem cells by enhancing the expression of GLUT-1 and activating autophagy.

Abbreviations

3-MA: 3-Methyladenine; GLUT-1: glucose transporter-1; CSCs: cancer stem cells; HNSCC: head and neck squamous cell carcinoma; ATG: autophagy-related proteins

Declarations

Ethics approval and consent to participate

This study was conducted under the guidelines and with the approval of Second Hospital of Jiaxin City, Jiaxin City, Zhejiang Province, 314000, China(No.jxey-201003).

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by Basic public welfare research plan of Zhejiang Province, China (No. LGF18H130001), Science and technology plan project of Medical and health of Zhejiang province, China (No. 2018KY801), and National Natural Science Foundation of China (No. 81372903). The funding LGF18H130001 has the role in the design of the study, the collection, analysis, and interpretation of data and in writing the manuscript. The funding 2018KY801 and 81372903 has the role in design of the study.

Authors' contributions

XH C designed the study and wrote the manuscript. JL, JT Z and SH Z reviewed the literatures and analyzed results.SH Z designed the study and revised the manuscript. J F conducted the experiments. All authors reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgemets

Not applicable.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin. 2020;70(1):7-30.
2. Zhu M, Yin F, Yang L, Chen S, Chen R, Zhou X, Jing W, Fan X, Jia R, Wang H, Zheng H, Zhao J, Guo Y. Contribution of TIP30 to chemoresistance in laryngeal carcinoma. Cell Death Dis. 2014;5:e1468.
3. Fu Q, Liu P, Sun X, Huang S, Han F, Zhang L, Xu Y, Liu T. Ribonucleic acid interference knockdown of IL-6 enhances the efficacy of cisplatin in laryngeal cancer stem cells by down-regulating the IL-6/STAT3/HIF1 pathway. Cancer Cell Int. 2017;17:79.
4. Wang C, Shao L, Pan C, Ye J, Ding Z, Wu J, Du Q, Ren Y, Zhu C. Elevated level of mitochondrial reactive oxygen species via fatty acid β-oxidation in cancer stem cells promotes cancer metastasis by inducing epithelial-mesenchymal ransition. Stem Cell Res Ther. 2019 13;10(1):175.
5. Garcia-Mayea Y, Mir C, Muñoz L, Benavente S, Castellvi J, Temprana J, Maggio V, Lorente J, Paciucci R, LLeonart ME. Autophagy inhibition as a promising therapeutic target for laryngeal cancer. Carcinogenesis. 2019; 40 (12), 1525-1534.
6. Zhong JT, Yu Q, Zhou SH, Yu E, Bao YY, Lu ZJ, Fan J. GLUT-1 siRNA Enhances Radiosensitization Of Laryngeal Cancer Stem Cells Via Enhanced DNA Damage, Cell Cycle Redistribution, And Promotion Of Apoptosis In Vitro And In Vivo. Onco Targets Ther. 2019;12:9129-9142..
7. Chen XH, Bao YY, Zhou SH, Wang QY, Wei Y, Fan J. Glucose transporter-1 expression in CD133+ laryngeal carcinoma Hep-2 cells. Mol Med Rep. 2013;8(6):1695-700.
8. Jang JW, Song Y, Kim SH, Kim JS, Kim KM, Choi EK, Kim J, Seo HR. CD133 confers cancer stem-like cell properties by stabilizing EGFR-AKT signaling in hepatocellular carcinoma. Cancer Lett. 2017;389:1-10.
9. Garg N, Bakhshinyan D, Venugopal C, Mahendram S, Rosa DA, Vijayakumar T, Manoranjan B, Hallett R, McFarlane N, Delaney KH, Kwiecien JM, Arpin CC, Lai PS, Gómez-Biagi RF, Ali AM, de Araujo ED, Ajani OA, Hassell JA, Gunning PT, Singh SK. CD133(+) brain tumor-initiating cells are dependent on STAT3 signaling to drive medulloblastoma recurrence. Oncogene. 2017;36(5):606-617.
10. Chen H, Zhou L, Dou T, Wan G, Tang H, Tian J. BMI1'S maintenance of the proliferative capacity of laryngeal cancer stem cells. Head Neck. 2011;33(8):1115-25.
11. Xu Z, Han X, Ou D, Liu T, Li Z, Jiang G, Liu J, Zhang J. Targeting PI3K/AKT/mTOR-mediated autophagy for tumor therapy. Appl Microbiol Biotechnol.2020;104(2):575-587.
12. Yang H, Ni HM, Ding WX. The double-edged sword of MTOR in autophagy deficiency induced-liver injury and tumorigenesis. Autophagy. 2019;15(9):1671-1673.
13. Pereira DL, Dos Santos Ferreira AC, de Faria GP, Kwee JK. Autophagy interplays with apoptosis and cell cycle regulation in the growth inhibiting effect of Trisenox in HEP-2, a laryngeal squamous cancer. Pathol Oncol Res. 2015;21(1):103-11

14. Song YJ, Zhang SS, Guo XL, Sun K, Han ZP, Li R, Zhao QD, Deng WJ, Xie XQ, Zhang JW, Wu MC, Wei LX. Autophagy contributes to the survival of CD133+ liver cancer stem cells in the hypoxic and nutrient-deprived tumor microenvironment. Cancer Lett. 2013;339(1):70-81.
15. Zhu H, Wang D, Zhang L, Xie X, Wu Y, Liu Y, Shao G, Su Z. Upregulation of autophagy by hypoxia-inducible factor-1α promotes EMT and metastatic ability of CD133+ pancreatic cancer stem-like cells during intermittent hypoxia. Oncol Rep. 2014;32(3):935-42
16. Zhu H, Wang D, Liu Y, Su Z, Zhang L, Chen F, Zhou Y, Wu Y, Yu M, Zhang Z, Shao G. Role of the Hypoxia-inducible factor-1 alpha induced autophagy in the conversion of non-stem pancreatic cancer cells into CD133+ pancreatic cancer stem-like cells. Cancer Cell Int. 2013;13(1):119.
17. Yuen CA, Asuthkar S, Guda MR, Tsung AJ, Velpula KK. Cancer stem cell molecular reprogramming of the Warburg effect in glioblastomas: a new target gleaned from an old concept. CNS Oncol. 2016;5(2):101-8.
18. Nazio F, Bordi M, Cianfanelli V, Locatelli F, Cecconi F. Autophagy and cancer stem cells: molecular mechanisms and therapeutic applications. Cell Death Differ. 2019;26(4):690-702.
19. Rothe K, Porter V, Jiang X. Current Outlook on Autophagy in Human Leukemia: Foe in Cancer Stem Cells and Drug Resistance, Friend in New Therapeutic Interventions. Int J Mol Sci. 2019;20(3). pii: E461. doi: 10.3390/ijms20030461. Review.
20. Li Q, Yin Y, Zheng Y, Chen F, Jin P. Inhibition of autophagy promoted high glucose/ROS-mediated apoptosis in ADSCs. Stem Cell Res Ther. 201;9(1):289.
21. Roy S, Leidal AM, Ye J, Ronen SM, Debnath J. Autophagy-Dependent Shuttling of TBC1D5 Controls Plasma Membrane Translocation of GLUT-1 and Glucose Uptake. Mol Cell. 2017;67(1):84-95.e5.
22. Zhu L, Wu G, Yang X, Jia X, Li J, Bai X, Li W, Zhao Y, Li Y, Cheng W, Liu S, Jin S. Low density lipoprotein mimics insulin action on autophagy and glucose uptake in endothelial cells. Sci Rep. 2019;9(1):3020.
23. Roy S, Debnath J. Autophagy enables retromer-dependent plasma membrane translocation of SLC2A1/GLUT-1 to enhance glucose uptake. Autophagy. 2017;13(11):2013-2014.
24. Wan B, Zang Y, Wang L. Overexpression of Beclin1 inhibits proliferation and promotes apoptosis of human laryngeal squamous carcinoma cell Hep-2. Onco Targets Ther. 2018 4;11:3827-3833.
25. Karpathiou G, Sivridis E, Koukourakis M, Mikroulis D, Bouros D, Froudarakis M, Bougioukas G, Maltezos E, Giatromanolaki A. Autophagy and Bcl-2/BNIP3 death regulatory pathway in non-small cell lung carcinomas. APMIS,2013,121(7):592-604.
26. Kim S,Kimdo H, Jung WH, Koo JS. Metabolic phenotypes in triple-negative breast cancer.Tumour Biol,2013,34(3):1699-712.
27. Choi J,Kimdo H, Jung WH, Koo JS. Metabolic interaction between cancer cells and stromal cells according to breast cancer molecular subtype.Breast Cancer Res,2013,15(5):R78.
28. Lin W, Yin CY, Yu Q, Zhou SH, Chai L,Fan J, Wang WD. Expression of glucose transporter-1, hypoxia inducible factor-1 alpha and beclin-1 in head and neck cancer and their implication. International Journal of Clinical and Experimental Pathology. 2018;11(7):3708-3717.

29. Coppock JD, Lee JH. mTOR, metabolism, and the immune response in HPV-positive head and neck squamous cell cancer. World J Otorhinolaryngol Head Neck Surg. 201620;2(2):76- 83.
30. Xi J, Wang Y, Liu H. GLUT-1 participates in the promotion of LncRNA CASC9 in proliferation and metastasis of laryngeal carcinoma cells. Gene. 2020 Feb 5;726:144194. doi: 10.1016/j.gene.2019.144194. Epub 2019 Oct 26. PubMed PMID: 31669650.
31. Lu ZJ, Yu Q, Zhou SH, Fan J, Shen LF, Bao YY, Wu TT, Zhou ML, Huang YP. Construction of a GLUT-1 and HIF-1α gene knockout cell model in HEp-2 cells using the CRISPR/Cas9 technique. Cancer Manag Res. 2019;11:2087-2096.
32. Luo XM, Xu B, Zhou ML, Bao YY, Zhou SH, Fan J, Lu ZJ. Co-Inhibition of GLUT-1 Expression and the PI3K/Akt Signaling Pathway to Enhance the Radiosensitivity of Laryngeal Carcinoma Xenografts In Vivo. PLoS One. 2015;10(11):e0143306.
33. Yan SX, Luo XM, Zhou SH, Bao YY, Fan J, Lu ZJ, Liao XB, Huang YP, Wu TT, Wang QY. Effect of antisense oligodeoxynucleotides glucose transporter-1 on enhancement of radiosensitivity of laryngeal carcinoma. Int J Med Sci. 2013;10(10):1375-86.
34. Luo XM, Zhou SH, Fan J. Glucose transporter-1 as a new therapeutic target in laryngeal carcinoma. J Int Med Res. 2010;38(6):1885-92.
35. Zhou SH, Fan J, Chen XM, Cheng KJ, Wang SQ. Inhibition of cell proliferation and glucose uptake in human laryngeal carcinoma cells by antisense oligonucleotides against glucose transporter-1. Head Neck. 2009;31(12):1624-33.
36. Li LF, Zhou SH, Zhao K, Wang SQ, Wu QL, Fan J, Cheng KJ, Ling L. Clinical significance of FDG single-photon emission computed tomography: Computed tomography in the diagnosis of head and neck cancers and study of its mechanism. Cancer Biother Radiopharm. 2008;23(6):701-14.
37. Meng Y, Xu X, Luan H, Li L, Dai W, Li Z, Bian J. The progress and development of GLUT-1 inhibitors targeting cancer energy metabolism. Future Med Chem. 2019;11(17):2333-2352.
38. Endo H, Owada S, Inagaki Y, Shida Y, Tatemichi M. Glucose starvation induces LKB1-AMPK-mediated MMP-9 expression in cancer cells. Sci Rep. 2018;8(1):10122.
39. Lin Z, Weinberg JM, Malhotra R, Merritt SE, Holzman LB, Brosius FC 3rd. GLUT-1 reduces hypoxia-induced apoptosis and JNK pathway activation. Am J Physiol Endocrinol Metab. 2000;278(5):E958-66.
40. Li K, Li M, Li W, Yu H, Sun X, Zhang Q, Li Y, Li X, Li Y, Abel ED, Wu Q, Chen H. Airway epithelial regeneration requires autophagy and glucose metabolism. Cell Death Dis. 2019 ;10(12):875.
41. Yan L, Raj P, Yao W, Ying H. Glucose Metabolism in Pancreatic Cancer. Cancers (Basel). 2019 Sep 29;11(10). pii: E1460. doi: 10.3390/cancers11101460

Figures

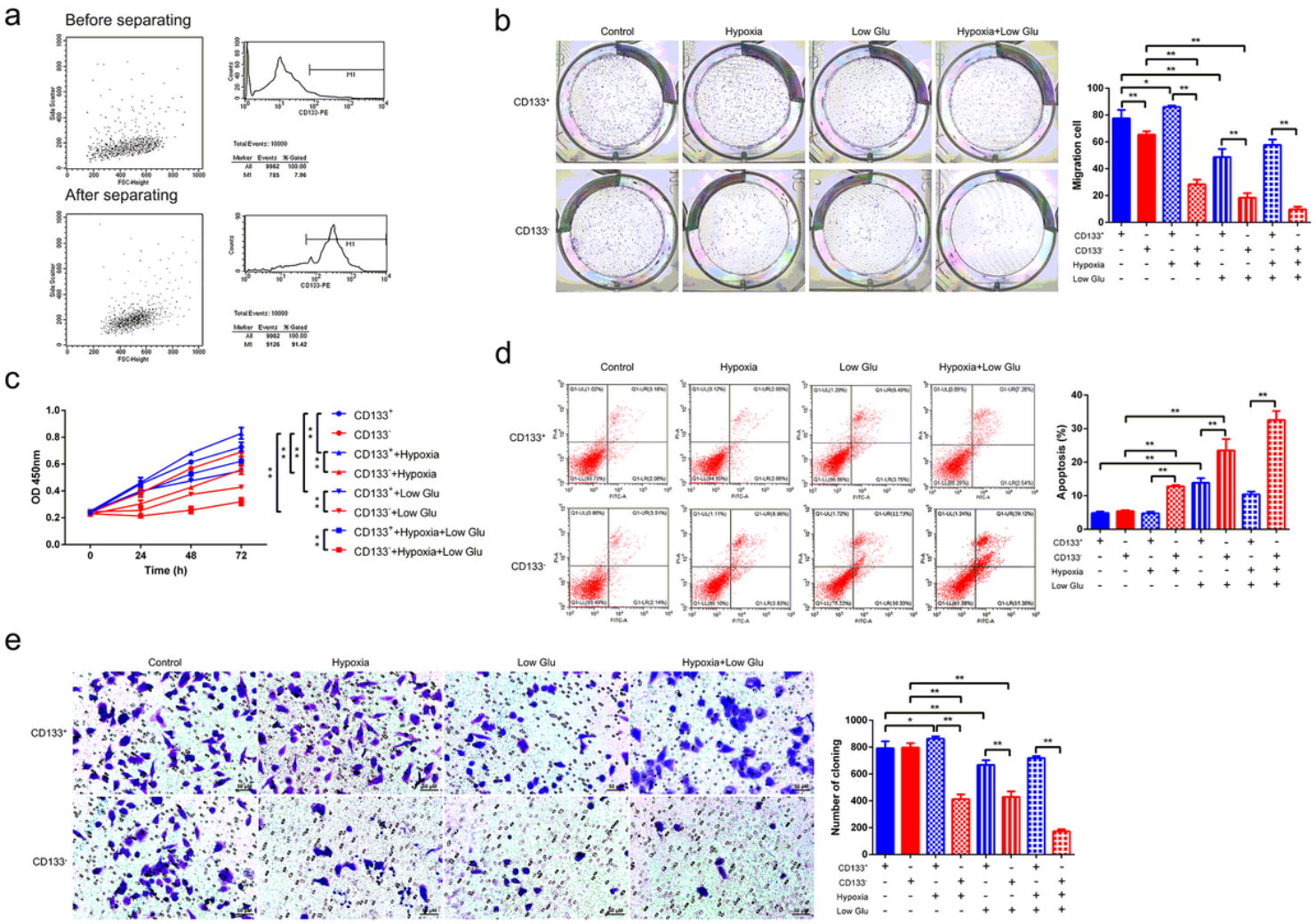

Figure 1

Determination of the proliferation, migration and apoptosis in stressed-CD133+ laryngeal carcinoma stem cells. (a) CD133+ population was isolated from Tu212 cells by MACS. The percentages of CD133+ cells before and after purification were determined by flow cytometry. (b-e) Indicated Tu212 cells were challenged with hypoxia or low glucose, either alone or in combination. Cell proliferation was evaluated by colony-formation assay (b) and CCK-8 assay (c). (d) Cell apoptosis was evaluated using AnnexinV-PI staining by flow cytometry. (e) Cell migration was determined by transwell assay. Data were mean ± SD and were representative of at least 3 independent experiments. *P<0.05; **P<0.01;

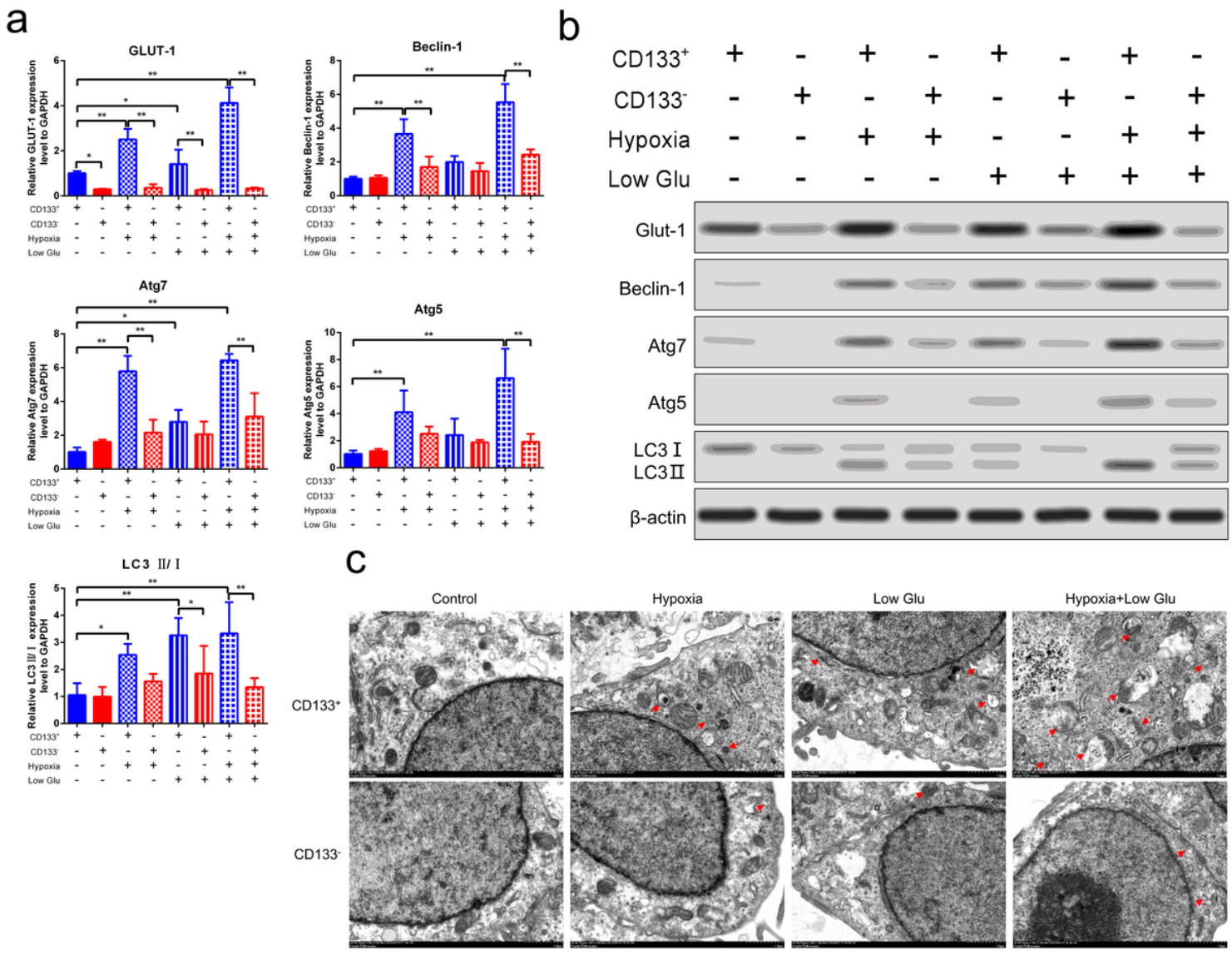

Figure 2

The expression of GLUT-1 and autophagy markers in stressed-CD133+ laryngeal carcinoma stem cells. Indicated Tu212 cells were challenged with hypoxia or low glucose, either alone or in combination. (a, b) The mRNA and protein levels of GLUT-1/Beclin-1/Atg7/Atg5/LC3 were evaluated by qRT-PCR (a) and Western blot (b), respectively. (c) Cell autophagy was examined by transmission electron microscopy. Data were mean ± SD and were representative of at least 3 independent experiments. *P<0.05; **P<0.01;

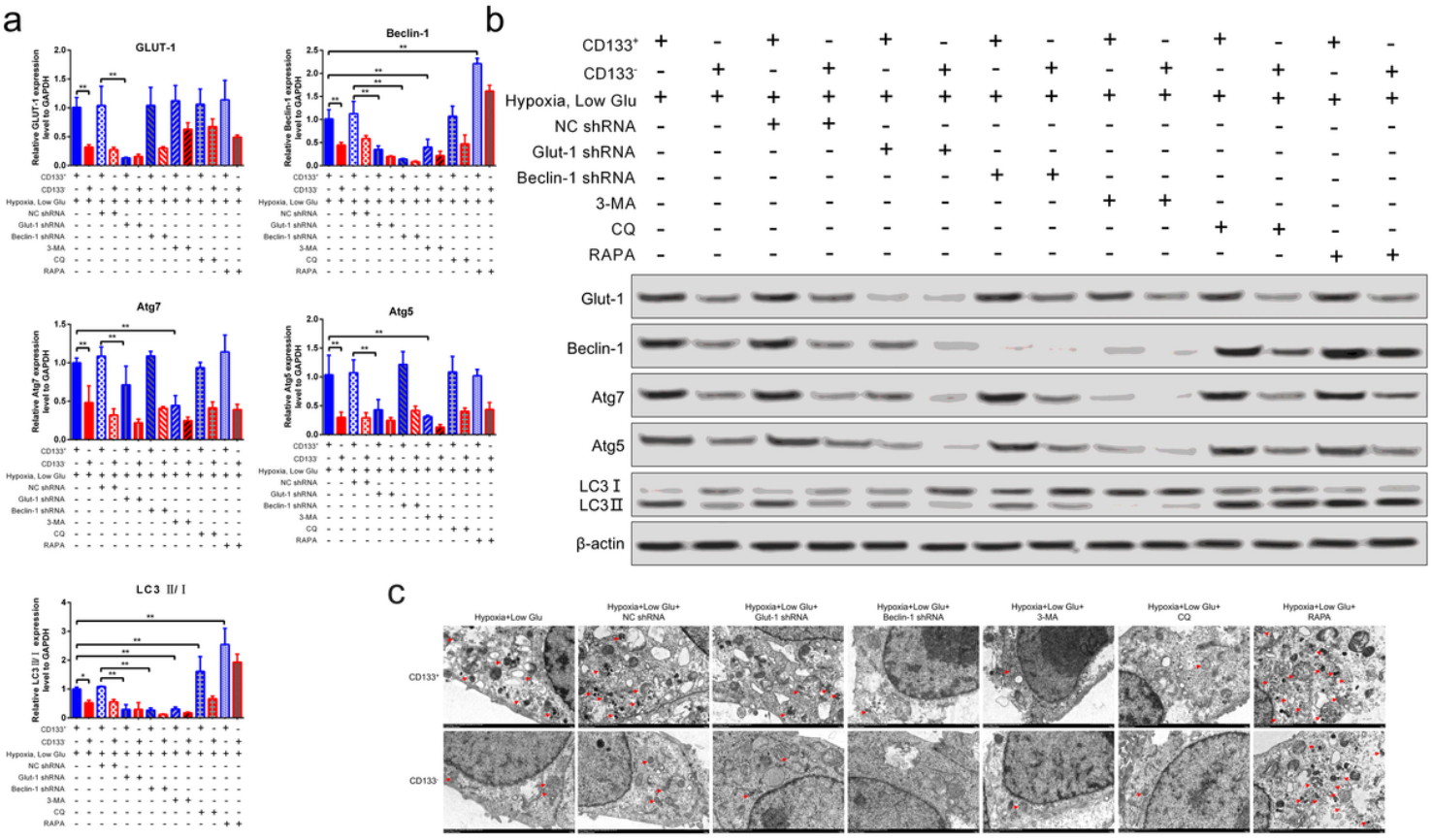

Figure 3

The relationships between GLUT-1 expression and cell autophagy in stressed-CD133+ laryngeal carcinoma stem cells. Indicated Tu212 cells were trasnfected with GLUT-1 and Beclin-1 siRNA, or treated with autophagy inhibitor (3-MA, CQ) or activator (Rapamycin). Then challenged with hypoxia or low glucose, either alone (a-c) or in combination (d). The mRNA and protein levels of GLUT-1/Beclin-1/Atg7/Atg5/LC3 were evaluated by qRT-PCR (a) and Western blot (b), respectively. (c) Cell autophagy was examined by transmission electron microscopy. Data were mean ± SD and were representative of at least 3 independent experiments. *P<0.05; **P<0.01;

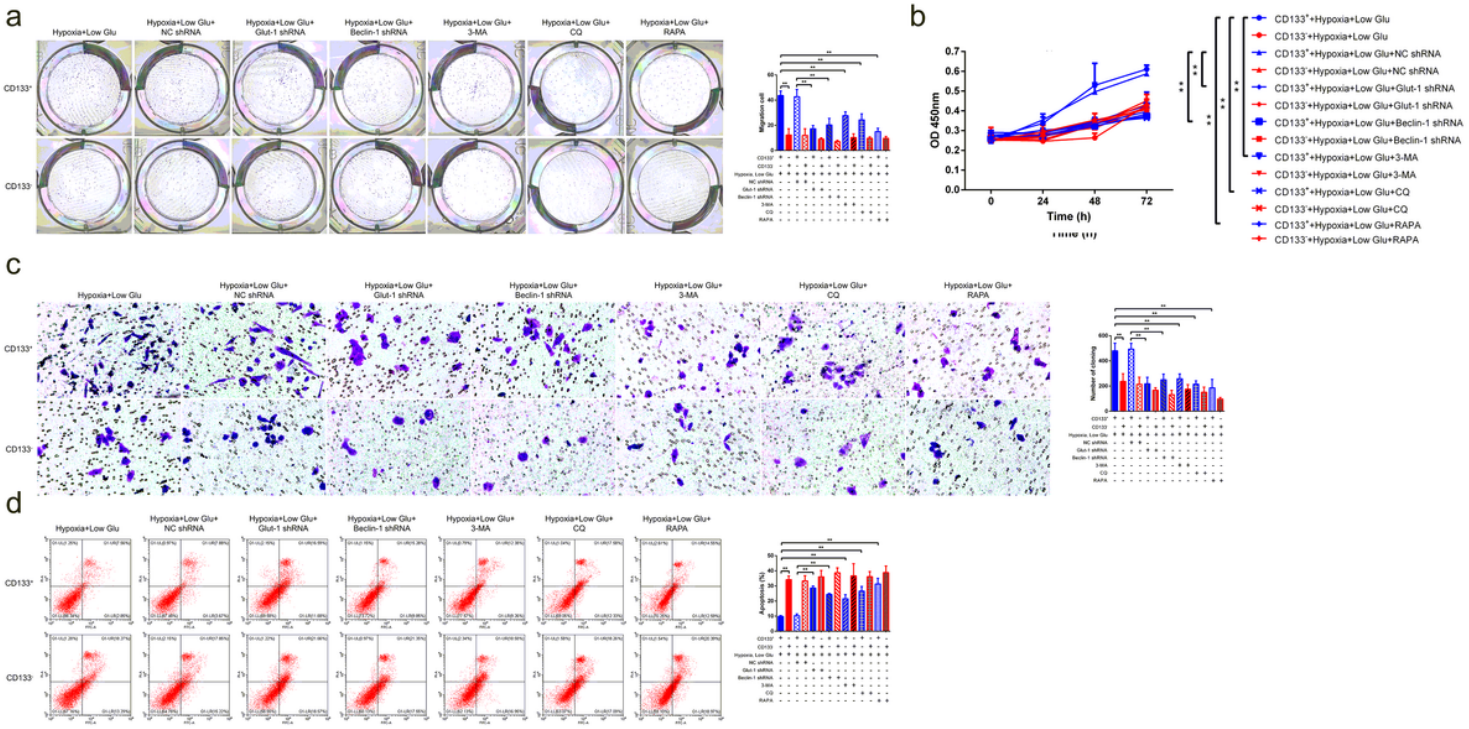

Figure 4

The impact of GLUT-1 knockdown or autophagy change on the malignant behaviors of stressed-CD133+ laryngeal carcinoma stem cells. Indicated Tu212 cells were trasnfected with GLUT-1 and Beclin-1 siRNA, or treated with autophagy inhibitor (3-MA, CQ) or activator (Rapamycin). Then challenged with hypoxia plus low glucose. (a, b) Cell proliferation was evaluated by colony-formation assay (a) and CCK-8 assay (b). (c) Cell apoptosis was evaluated using AnnexinV-PI staining by flow cytometry. (d) Cell migration was determined by transwell assay. Data were mean ± SD and were representative of at least 3 independent experiments. *P<0.05; **P<0.01;

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.tif
- s2.tif
- s1.tif