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Regular paper

Efficacy of glucose transporter inhibitors for the treatment of ERRα-overexpressed colorectal cancer

Miao Zhao#, Cairong Xu#✉ and Hui Zhu

Department of General Surgery, Jiangyin Changjing Hospital, Yangzhou University Medical College Teaching Hospital, Wuxi 214411, Jiangsu, China

Background: Colorectal cancer is the most-incidence associated extremely high mortality rate worldwide. The overexpression of estrogen-related receptor α (ERRα) is contributing to a poor prognosis. Obtaining a better understanding of the mechanisms of ERRα in colorectal cancer is important for developing cancer therapies. **Methods:** Western blotting and qRT-PCR were used to determine the protein and mRNA levels of ERRα, OUTB1, and solute carrier family 7 member 11 (SLC7A11) in HCT-116 cells. Short hairpin RNA (shRNA) was used to knockdown ERRα in HCT-116 cells. The level of reactive oxygen species (ROS), the nicotinamide adenine dinucleotide phosphate NADP+/NADPH, and the oxidized glutathione (GSSG)/reduced glutathione (GSH) ratio were measured by HPLC-MS to determine the redox state in HCT-116 cells. Lastly, tumor xenograft experiments were carried out to determine the effect of glucose transporter (GLUT) inhibitor. **Results:** Knockdown of ERRα decreased the expression of OTUB1 and SLC7A11 in HCT-116 cells. SLC7A11 overexpression induced NADPH-dependent redox system collapse. Aberrant expression of ERRα significantly reduced NADPH level and resulted in collapse of the redox system under glucose deprivation. Furthermore, ERR overexpression of ERRα sensitized cancer cells to inhibition of GLUTs. Treatment with GLUT inhibitor significantly reduced tumor volume after 6 weeks of tumor xenograft experiment. Our study demonstrates that the over-expression of ERRα causes redox system collapses via regulating the expressions of OUTB1 and SLC7A11. **Conclusion:** Up-regulation of SLC7A11 mediates the disruption of cell metabolism and the balance of redox state in colorectal cancer. Additionally, the GLUT inhibitor significantly reduces colorectal tumor volume, suggesting that the GLUT inhibitor could serve as a potential therapy for colorectal treatment.

Keywords: ERRα; OTUB1; SLT7A11; GLUTs; colorectal cancer

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✉e-mail: xcr392768@163.com
#these authors contributed equally to this work

Abbreviations: ANOVA, analysis of variance; COAD, colorectal adenocarcinoma; ERRα, estrogen-related receptor α; GLUTs, glucose transporters; GSH, glutathione; GSSG, glutathione disulfide; OTUB1, Otubain-1; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; shRNA, short hairpin RNA; SLC7A11, solute carrier family 7 member 11; S.D., standard deviation; NADPH, nicotinamide adenine dinucleotide phosphate

INTRODUCTION

Estrogen-related receptor α (ERRα) has been reported to contribute to cell metabolism, tumorigenesis, cancer proliferation, and migration. It has been found that ERRα is overexpressed in tissues of colorectal cancer, and high levels of ERRα indicate a poor prognosis (Cavallini *et al.*, 2005; Liang *et al.*, 2018). Otubain-1 (OTUB1), a deubiquitinating enzyme, is a member of the ovarian tumor (OUT) family of cysteine proteases. It has been proven that ERRα regulates the expression of OTUB1 by binding to the promoter region of OTUB1 and promotes the migration of colorectal cancer cells through Vimentin (Zhou *et al.*, 2019).

Additionally, studies have found that OTUB1 can directly interact with the key component solute carrier family 7 member 11 (SLC7A11) of the cystine-glutamate antiporter Xc- (composed of the catalytic subunit SLC7A11 and the chaperone subunit SLC3A2) to stabilize the protein (Liu *et al.*, 2019). Glutathione is the most abundant antioxidant in the cell, and cysteine is the rate-limiting precursor of glutathione synthesis. Most cancer cells mainly rely on the cystine transporter system Xc- to obtain cysteine from the extracellular environment (Stipanuk *et al.*, 2006). The amino acid is then converted to cysteine in the cytoplasm by a reduction reaction that consumes nicotinamide adenine dinucleotide phosphate (NADPH), which is then used to synthesize glutathione (Combs & DeNicola, 2019). Therefore, cystine uptake mediated by SLC7A11 plays a key role in inhibiting oxidative reactions and maintaining cell survival under conditions of oxidative stress (Koppula *et al.*, 2020).

Overexpression of OTUB1 will increase the level of SLC7A11, thus inhibiting iron death and promoting tumor development, which is beneficial for tumor growth (Liu *et al.*, 2019). However, the establishment of a cysteine-derived antioxidant defense system has cost SLC7A11 high-expressing cancer cells, including glutamate export, cystine uptake, and NADPH supply to reduce cystine in the cell. These make cancer cells dependent on glucose and glutamine. In view of the glucose dependence of high-expressing cancer cells of SLC7A11, the growth of cancer cells can be inhibited by glucose transporter inhibitors, such as BAY-876 or KL-11743 (Liu *et al.*, 2020).

Therefore, we hypothesize that colorectal cancer tissues that highly expressERRα could increase the expression of OTUB1, which could up-regulate SLC7A11 in a strong glucose-dependent fashion. We hypothesize that glucose transporter inhibitors could

serve as a pharmacological approach to the treatment of colorectal cancer overexpressing ERRα.

METHODS

Cell culture and shRNA transfection

The human colorectal cancer cell line (HCT-116) was purchased from the Cell Bank of the Chinese Academy of Sciences. HCT-116 cells were cultured in DMEM / Ham F12 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37°C, 5% CO_2, in a humidified atmosphere.

1×10^5 cells HCT-116 cells were seeded per well in 6-well plates and incubated for 24 h. Subsequently, cells were transfected with 1 mg of ERRα specific shRNA expression vector and control shRNA-NC (GenScript, Nanjing, China), using jet PRIME transfection reagent (PolyPlus, Shanghai, China).

Quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 200 ng of RNA was used for cDNA synthesis using the PrimeScript TM RT reagent kit with gDNA Eraser (Takara, Dalian, China). mRNA levels were determined using TB GreenTM Premix Ex TaqTM II (Takara Bio Inc., Shiga, Japan) by quantitative real-time PCR (qRT-PCR) on the CFX ConnectTM Real-Time System (Bio-rad, Hercules, CA, USA). The mRNA levels of the target genes were normalized to *GAPDH*.

Western blot

Total protein was extracted using ice-cold radioimmunoprecipitation lysis buffer containing the protease inhibitor PMSF. Protein concentrations were measured using the BCA assay kit (Pierce, Waltham, MA, USA). The same amount of proteins was loaded into 10% sodium dodecyl sulfate polyacrylamide gels. The following antibodies were used against: ERRα (1:1000, Abcam, Cambridge, MA, USA), OTUB1 (1:1000, Abcam), SLC7A11 (1:1000, Abcam), β-actin (1:2000, Sigma, MO, USA). Blots were imaged using the SuperLumia ECL Plus HRP Substrate Kit (Abbkine, Wuhan, China).

Measurements of NADP+, NADPH

Cells were seeded in 6-well plates and incubated overnight. Reactive oxygen species (ROS) levels were measured using CM-H2DCFDA (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocol. Cells were lysed in 300 μL extraction buffer (20 mM nicotinamide, 20 mM $NaHCO_3$, 100 mM Na_2CO_3). The supernatants were saved for further analysis. Total NADP and NADPH were measured as described in the published work (Koppula *et al.*, 2020). Briefly, for the measurement of NADPH, the supernatant was first incubated at 60°C for 30 min to remove NADP+ without destroying NADPH. Then NADPH and total NADP were measured in NADP cycling buffer (100 mM Tris-HCl pH8.0, 0.5 mM thiazolyl blue, 2 mM phenazine ethosulfate, 5 mM EDTA) containing 0.75 U of the G6PD enzyme and 10 mM glucose 6-phosphate. The absorbance at 570 nm was measured every 1 min for 6 min at 30 degrees. Subtracting [NADPH] from [total NADP] was used to calculate the concentration of NADP+.

Cell death analysis

The cells were seeded in a 12-well plate and cultured for 24 h. After treatment, cells were collected in a 1.5 mL tube and washed with phosphate buffered saline (PBS). Cells were stained with 1 μg/mL propidium iodide (PI) in cold PBS. Cell death was analyzed using the BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the FlowJo 10 software.

Quantification of intracellular glutathione, glutathione disulfide by HPLC–MS Determination of intracellular glutathione

Intracellular levels of glutathione (GSH) and glutathione disulfide (GSSG) were extracted and quantified using methods published as described (Koppula *et al.*, 2020).

Glucose uptake assays

Cells were washed with PBS and cultured with glucose-free medium containing 0.1 μCi 2-[1-14C]-Deoxy-D-Glucose for 2 h at 37°C The cells were then washed twice with cold PBS and lysed with 0.1 mM NaOH. Radioactivity (DPM) was measured using the Tri-Carb Liquid Scintillation Analyzer.

Tumor xenograft experiments

4-6 week old nude mice were used for HCT-116 cell line xenotransplantation experiments. The HCT-116 and ERRα knockdown cells were resuspended in FBS-free DMEM medium, and the same number of cells was subcutaneously injected into the mice. The tumor growth of the mice was monitored by two-dimensional tumor measurement. Tumor volume is calculated according to

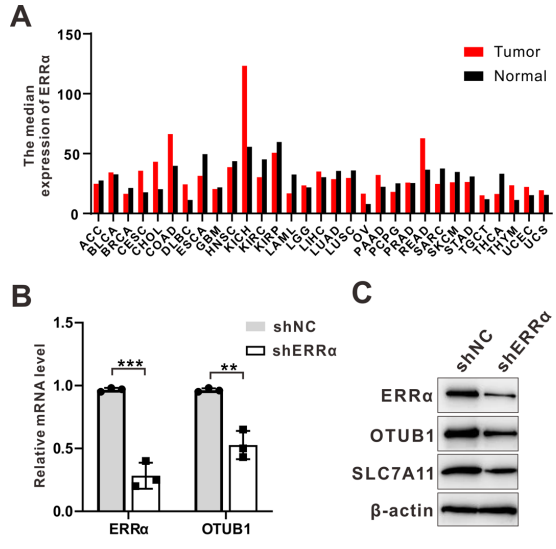

Figure 1. ERRα knockdown leads to decreased expression of OTUB1 and SLC7A11 in HCT-116 cells.
(**A**) The analysis of the TCGA database showed that the level of ERRα was increased in a broad spectrum of human cancers, including COAD. (**B**) HCT-116 cells were transfected with ERRα shRNA. The mRNA levels of ERRα and OTUB1 were determined by qRT-PCR. (**C**) The protein level of ERRα, OTUB1 and SLC7A11 was determined by Western blotting. Data are presented as means ±S.D.

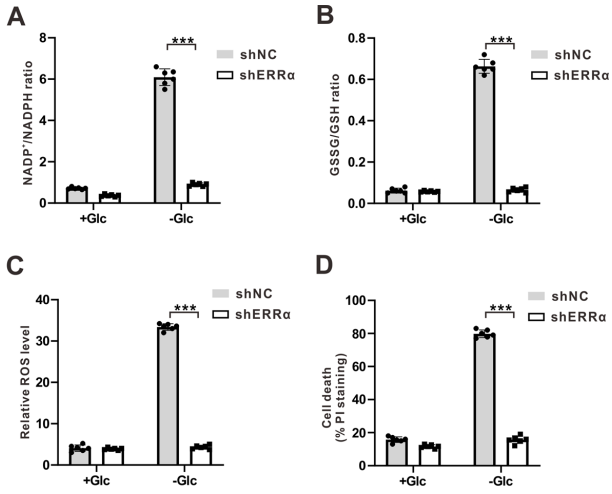

Figure 2. Overexpression of ERRα deplete NADPH and causes the collapse of the redox system under glucose deprivation.
(**A–D**) NADP+/NADPH ratios (**A**), GSSG/GSH ratios (**B**), ROS levels (**C**) and cell death (**D**) in ERRα shRNA or NC shRNA transfected HCT-116 cells cultured with or without glucose (Glc). Data are presented as means ± S.D. ***P*<0.01; ****P*<0.001.

the formula volume = length×width^2×1/2. When tumor volume increased to about 50-100 mm^3 mice were randomly divided into two groups and administered intraperitoneally with 100 mg/kg KL-11743 or vehicle every two days. The study was approved by Jiangyin Changjing Hospital.

Statistical analysis

Data were analyzed using GraphPad Prism V8.0 software (Graphpad, San Diego, CA, USA). Data were represented as means ± standard deviation (S.D.). Statistical significance was determined using the two-tailed unpaired *t*-test, one-way or two-way analysis of variance (ANOVA) with a post hoc test. $P<0.05$ was considered statistically significant.

RESULTS

Knockdown of ERRα reduced the expression of OTUB1 in HCT-116 cells)

The level of ERRα (has been found that the level of ERRα was increased in a broad spectrum of human cancers compared to normal tissues, including colorectal adenocarcinoma (COAD) (Fig. 1A). Next, to determine whether ERRα could regulate ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 (OTUB1) in the ovarian tumor domain, we transfected HCT-116 cells with ERRα shRNA to knockdown ERRα expression. ERR mRNA and protein levels of ERRα were significantly downregulated in HCT-116 cells compared to negative control ($P<0.001$, Fig. 1B–C). Consequently, the mRNA level of OTUB1 was significantly reduced ($P<0.01$, Fig. 1B–C). Additionally, we measure protein levels of solute carrier family 7 member 11 (SLC7A11), which is an amino acid transporter and plays a pivotal role in providing cysteine for the synthesis of GSH. We found that the level of SLC7A11 protein level was markedly reduced in shERRα transfected cells compared to the negative control (Fig. 1C). In general, these findings revealed the overexpression of ERRα in colorectal cancers and knockdown of ERRα could suppress the expressions of OTUB1 and SLC7A11 expressions in HCT-116 cells) were overexpressed.

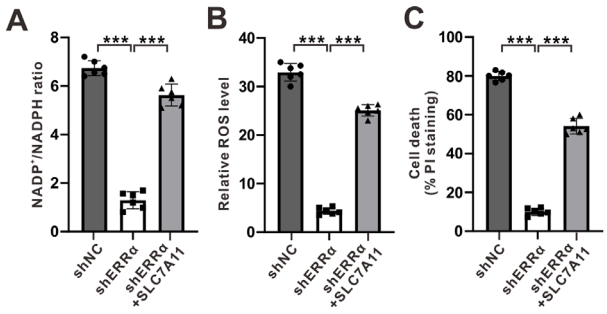

Figure 3. SLC7A11 mediates the effect of ERRα on the level of NADPH and the collapse of the redox system.
(**A–C**) NADP+/NADPH ratios (**A**), ROS levels (**B**) and cell death (**C**) in overexpressing SLC7A11 HCT-116 cells after knockdown of ERRα. Data are presented as means ± S.D. ****P*<0.001.

The aberrant expression of ERRα depleted NADPH and disturbed redox homeostasis under glucose deprivation

Furthermore, we investigated the role of ERRα in regulating NADPH level and redox homeostasis under glucose replete or starvation. We found that the NADP+/NADPH ratio increased markedly under glucose starvation, which was significantly reduced by the knockdown of ERR ($P<0.001$, Fig. 2A). In parallel, we observed that the GSSG/GSH ratio and ROS level were dramatically increased under glucose starvation in the control group, suggesting a collapse of the redox system under glucose starvation. In contrast, the knockdown of ERRα significantly reduced the GSSG/GSH ratio and ROS level in HCT-116 cells ($P<0.001$, $P<0.001$, respectively, Fig. 2B–C). As a result, collapse of the redox system dramatically increased cell death, which was significantly inhibited in the ERRα knockdown HCT-116 cells ($P<0.001$, Fig. 3D). However, in a glucose-replete state, no statistically significant differences were observed in terms of the NADP+/NADPH ratio, GSSG/GSH ratio, ROS levels and the number of cell death between control and ERRα knockdown HCT-116 cells (Fig. 2A–D).

SLC7A11 depleted the level of NADPH and led to the collapse of the redox system in ERRα knockdown HCT-116 cells

SLC7A11 has been shown to be involved in the regulation of redox homeostasis and ferroptosis (Lin *et al.*, 2020). It has been found that SLC7A11 is highly expressed in cancer cells and promotes resistance to chemotherapy, e.g., cisplatin treatment (Okuno *et al.*, 2003). In this study, the upregulation of SLC7A11 in ERRα knockdown HCT-116 cells significantly increased the NADP+/NADPH ratio ($P<0.001$) and ROS level ($P<0.001$) to that of the negative control (Fig. 3A–B). Ultimately, the percentage of cell death was significantly elevated when upregulated SLC7A11 in ERRα knockdown HCT-116 cells ($P<0.001$) compared with ERRα silent cells (Fig. 3C). These data indicate that overexpression ERR in colorectal cells causes redox system collapse through up-regulation of SLC7A11.

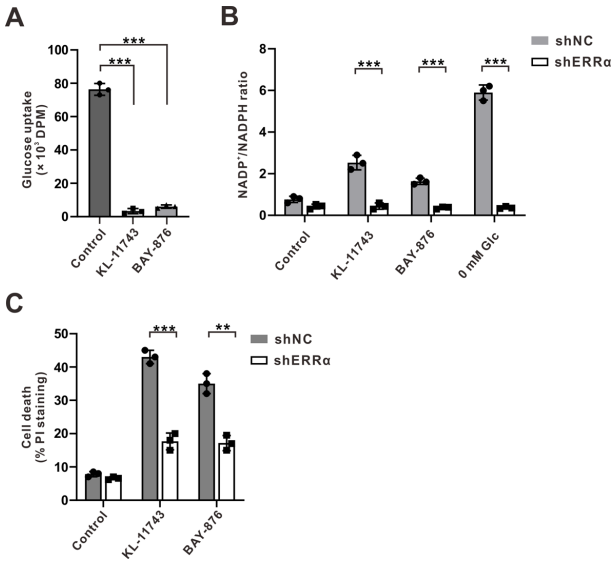

Figure 4. Overexpression of ERRα sensitizes cancer cells to GLUT inhibition.
(**A**) Glucose uptake in HCT-116 cells treated with 2 μM KL-11743 or BAY-876. DPM, disintegrations per min. (**B**) Measurement of the NADP+/NADPH ratios in ERRα shRNA or NC shRNA transfected-HCT-116 cells, treated with KL-11743, BAY-876, or cultured in glucose-free medium. (**C**) Cell death was measured by propidium iodide staining in ERRα shRNA or NC shRNA transfected-HCT-116 cells treated with 2 μM KL-11743 or BAY-876. Data are presented as means ± S.D. ***P*<0.01; ****P*<0.001.

Aberrant expression of ERRα increased sensitivity of HCT-116 cells to glucose transporters (GLUTs) inhibition

To further assess whether the aberrant expression of ERRα will affect sensitivity of HCT-116 cells to inhibition of GLUTs), we treated HCT-116 cells with a highly potent GLUT inhibitor KL-11743 (GLUT1 and GLUT3) or BAY-876 (GLUT1). Both KL-11743 and BAY-876 significantly inhibited glucose uptake in HCT-116 cells compared to control (*P*<0.001, *P*<0.001, respectively, Fig. 4A). Subsequently, we found that KL-11743 and BAY-876 treatment increased the NADP+/NADPH ratio in HCT-116 cells. On the contrary, the NADP+/NADPH ratio was significantly reduced when knockdown ERRα in HCT116 cells compared to the negative control (*P*<0.001, *P*<0.001, *P*<0.001, Fig. 4B). Furthermore, our results showed that KL-11743 and BAY-876 induced more cell death relative to the control, which was significantly reduced in ERRα knockdown cells (Fig. 4C). Taken together, ERRα highly expressed colorectal cancer cells are highly sensitive to GLUTs inhibition. Therefore, these findings highlighted that targeting ERRα in colorectal could be a potential approach for the treatment of colorectal cancer.

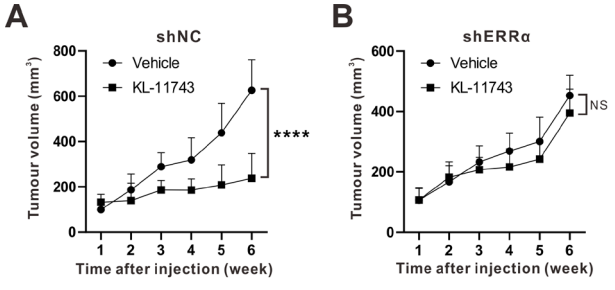

Figure 5. ERRα^{high} tumors are sensitive to the GLUTs inhibitor.
(**A–B**) Tumor volumes in control tumors (shNC) (**A**) and ERRα-knockdown (sh ERRα) (**B**) HCT-116 xenograft tumors at different times after injection with KL-11743 or vehicle. Data are presented as means ± S.D., n = 6 independent repeats. ****P*<0.0001.

ERR-high tumors were sensitive to GLUTs inhibition

Due to the fact that both GLUT1 and GLUT3 are highly expressed in tumors, we only treated with KL-11743 to evaluate the sensitivity of ERRα-high expression tumor to GLUTs inhibition. We found that KL-11743 significantly reduced the growth of ERR-high HCT-116 xenograft tumors (*P*<0.0001, Fig. 5A). Notably, knockdown of ERRα abolished the elevated sensitivity of tumors to GLUTs inhibition (Fig. 5B).

DISCUSSION

Colorectal cancer is one of the leading malignancies worldwide associated with a high mortality rate (Siegel *et al.*, 2021). It has been found that ERRα is highly expressed in colorectal tumor tissue, which contributes to a poor prognosis (Cavallini *et al.*, 2005; Liang *et al.*, 2018). In this study, we showed that ERRα played a critical role in maintaining redox hemostasis in colorectal cancer. OUTB1 is a cysteine protease that belongs to the ovarian tumor domain protease family and has been shown to be associated with the development, proliferation and metastasis of colorectal cancer (Zhou *et al.*, 2014). OTUB1 has been shown to increase significantly in colon cancer patients (Yuan *et al.*, 2017). Targeting OTUB1 directly by miR-542-3p treatment effectively inhibited colon cancer cell proliferation, migration, and invasion in human colon cancer cell lines (Yuan *et al.*, 2017). Furthermore, a study showed that OTUB1 persulfidation contributed to stabilizing xCT and further promoted colon cancer development (Chen *et al.*, 2021). Consistent with these results, we showed that OTUB1 mRNA and protein levels were highly expressed in HCT-116 cells, which were significantly reduced by knockdown of ERRα. These findings suggest that the upregulated OTUB1 in colorectal cancer could be reduced by ERRα silencing, which could further inhibit the expression of SLC7A11.

Redox hemostasis plays an important role in maintaining cell metabolism and cell survival (Serrano *et al.*, 2020). Targeting the redox state in tumor cells has been used to treat colon cancer (Sun & Rigas, 2008), such as oxidoreductase thioredoxin-1 has been reported to promote redox-mediated cell death in colon cancer (Sun & Rigas, 2008). The pentose phosphate pathway plays an important role in the regulation of glucose metabolism by supplying NADPH and ribose 5-phosphate (R5P). NADPH is an essential electron donor in maintaining redox balance during the biological process, such as biosynthesis of fatty acids and nucleotides (Ju *et al.*, 2020; Wamelink *et al.*, 2008). Additionally, the conversion of GSSG to GSH through glutathione reductase, which is produced from NADPH, is critical for cellular antioxidation (Ju *et al.*, 2020). NADPH is also a substrate for ROS production mediated by NADPH oxidases (Gianni *et al.*, 2010; Lambeth *et al.*, 2007). In this study, we demonstrated that the NADP+/NADPH and GSSG/GSH ratio and ROS level were dramatically increased in ERR aberrant expressed HCT-116 cells under glucose starvation. These observations were abolished when knockdown ERRα under glucose deprivation, suggesting that targeting glucose uptake could be a potential approach to collapse the redox system in colon cancer.

SLC7A11 (xCT), a member of the solute carrier family, mediates cystine uptake and promotes glutathione biosynthesis (Lin *et al.*, 2020). Consequently, SLC7A11 exerts effects on maintaining redox in cells and protecting cells against oxidative stress, thus protecting cells from iron-dependent ferroptosis (Wang *et al.*, 2020). Colorectal cancer cells have been found to have a high level of SLC711 (Xu *et al.*, 2020). Consistent with this result, our data showed that SLC7A11 is highly expressed in HCT-116 cells, which was significantly reduced by knockdown of ERRα. Interestingly, up-regulation of SLC7A11 significantly collapsed the redox state in HCT-116 cells, evidenced by elevated levels of the NADP+/NADPH ratio and ROS level compared to that of the negative control (shNC). These findings imply that the aberrant expression of ERRα induced collapse of the redox system is possibly mediated by SLC7A11.

It has been well documented that glucose uptake is markedly increased in cancer cells (Vander Heiden *et al.*, 2009). Targeting GLUTs has been considered a potential colorectal cancer therapy. The Warburg effect proposes that cancer cells are under hypoxic conditions and prefer ATP production through glycolysis (Jang *et al.*, 2013). Therefore, to maintain normal function, cancer cells require a large amount of glucose. As expected, GLUTs have been reported to overexpress in cancer cells, especially GLUT1 and GLUT3 (Brown & Wahl, 1993; Kunkel *et al.*, 2003). Shriwas *et al.* have shown that GLUT inhibitor effectively reduced cell proliferation and metabolism in human lung and cervical cancer cells (Shriwas *et al.*, 2021). A recent study found that overexpression of GLUT is related to 5-fluorouracil resistance, and inhibition of GLUT significantly improved the outcome of treatment in colorectal cancer (Chang *et al.*, 2021). SLT7A11 overexpression in colon cancer cells has been shown to be vulnerable to GLUT inhibitors, which could be induced by mutation of tumor suppressor genes, eg BAP1 or KEAP1 (Zhang *et al.*, 2019; Zhang *et al.*, 2018). Consistently, our results showed that the redox state was balanced under glucose deprivation. Therefore, we investigated the role of GLUTs in the management of colorectal cancer. We used the GLUT1 selective inhibitor BAY-876 and the GLUT1 and GLUT3 inhibitor KL-11743. We found that aberrant expression of ERRα in HCT-116 cells were significantly more sensitive to GLUTs inhibition than ERRα knockdown cells, resulting in the promotion of the death of colorectal cancer cells. To confirm this finding, we performed a tumor xenograft experiment. Tumor volumes were significantly reduced by GLUTs inhibitor treatment compared to that of the control (vehicle). This effect was abolished in ERRα silencing HCT-116 cells injection, indicating that overexpression of ERRα is responsible for increased sensitivity to inhibition of GLUT in colorectal cells.

CONCLUSIONS

In conclusion, our study for the first time demonstrated that aberrant expression of ERRα mediated the redox system through the regulation of OUTB1 and SLC7A11 expression, which, in turn, could further disrupt cell metabolism and balance the redox state in colorectal cancer. Furthermore, our results for the first time showed that a GLUT inhibitor has a good therapeutic effect on colorectal cancer overexpressing ERRα.

Declarations

Disclosure of potential conflicts of interest. The authors declare that they have no conflict of interest.

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