Long non-coding RNA SLC2A1-AS1 induced by GLI3 promotes aerobic glycolysis and progression in esophageal squamous cell carcinoma by sponging miR-378a-3p to enhance Glut1 expression

Hongtao Liu 1*, Qing Zhang 1,†, Yinsen Song 2, Yibin Hao 2, Yunxia Cui 1, Qin Zhang 1, Xueying Zhang 1, Yue Qin 1, Guangzhao Zhu 1, Feng Wang 3,4, Jinghan Dang 5, Shanshan Ma 1, Yanting Zhang 1, Wenna Guo 1, Shenglei Li 6*, Fangxia Guan 1* and Tianli Fan 7*

Abstract

Background: Emerging evidence demonstrates that IncRNAs play pivotal roles in tumor energy metabolism; however, the detailed mechanisms of IncRNAs in the regulation of tumor glycolysis remain largely unknown.

Methods: The expression of SLC2A1-AS1 was investigated by TCGA, GEO dataset and qRT-PCR. The binding of GLI3 to SLC2A1-AS1 promoter was detected by Luciferase Reporter Assay System and Ago2-RIP assay. FISH was performed to determine the localization of SLC2A1-AS1 in ESCC cells. Double Luciferase Reporter assay was used to investigate the interaction of miR-378a-3p with SLC2A1-AS1 and Glut1. Gain-of-function and Loss-of-function assay were performed to dissect the function of SLC2A1-AS1/miR-378a-3p/Glut1 axis in ESCC progression in vitro and in vivo.

Results: We identified a novel IncRNA SLC2A1-AS1 in ESCC. SLC2A1-AS1 was frequently overexpressed in ESCC tissues and cells, and its overexpression was associated with TNM stage, lymph node metastasis and poor prognosis of ESCC patients. Importantly, GLI3 and SLC2A1-AS1 formed a regulatory feedback loop in ESCC cells. SLC2A1-AS1 promoted cell growth in vitro and in vivo, migration and invasion, and suppressed apoptosis, leading to EMT progression and increased glycolysis in ESCC cells. SLC2A1-AS1 functioned as ceRNA for sponging miR-378a-3p, resulting in Glut1 overexpression in ESCC cells. MiR-378a-3p inhibited cell proliferation and invasion as well as induced apoptosis, resulting in reduced glycolysis, which was partly reversed by SLC2A1-AS1 or Glut1 overexpression in ESCC cells.

© The Author(s). 2021, corrected publication 2021. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
SLC2A1-AS1 plays important roles in ESCC development and progression by regulating glycolysis, and SLC2A1-AS1/miR-378a-3p/Glut1 regulatory axis may be a novel therapeutic target in terms of metabolic remodeling of ESCC patients.

Keywords: Esophageal squamous cell carcinoma, SLC2A1-AS1, miR-378a-3p, Glucose transporter 1, Glycolysis.

Background

Esophageal cancer (ESCA) is the seventh leading cause of cancer-related death in the world, which consists of two histological types, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) [1, 2]. In China, ESCC accounts for 90% of all ESCA types, and mainly distributes in Lin County and An Yang area, Henan province [3]. Although tremendous advances in diagnosis and treatment of ESCC patients, the 5-year survival rate for ESCC patients is quite poor [4, 5], which may be mainly due to diagnosis at an advanced stage, high recurrence and metastasis [6, 7]. Therefore, it is extremely urgent for us to seek for novel prognostic markers and therapeutic targets for ESCC patients.

Tumor cells mainly obtain energy via a high rate of glycolysis for tumor survival, and this metabolic feature is now considered as one of tumor hallmarks [9]. Otto Warburg has verified that tumor cells preferentially take glucose as main energy source to produce lactate, even in the presence of plentiful oxygen, to support the increased metabolic rate [9], which is well known as “Warburg effect” or “aerobic glycolysis” [10]. The metabolic characteristics implicated in a series of the alterations of metabolic enzymes, such as glucose transporter 1 (Glut1, also named SLC2A1), hexokinase 2 (HK2), pyruvate kinase type M2 (PKM2) and lactate dehydrogenase A (LDHA) [11-16]. Notably, many transcriptional factors including c-myc, hypoxia inducible factor-1α (HIF-1α), nuclear factor kappa-B (NF-κB) promote tumor lactogenesis by regulating the expressions of metabolic enzymes [17-19]. Recent studies have demonstrated that targeting glycolysis-related pathways may be a novel therapeutic target for a variety of tumors [20-22], which will link many metabolic enzymes involved in glycolysis. Despite tremendous advances in tumor glycolysis, its regulatory mechanisms implicated its metabolic enzymes and transcriptional factors remain to be further identified.

Long non-coding RNAs (lncRNAs) as key regulatory elements of cell biological processes are longer than 200 nucleotides, without protein-coding potential, which is transcribed by RNA polymerase II [23]. lncRNAs are considered to be involved in many biological processes, including gene expression regulation, decoy for transcription factors, competing endogenous RNAs (ceRNAs) and scaffolding, etc. [24, 25]. Increasing reports have demonstrated that lncRNAs widely participate in cell growth, survival, differentiation, apoptosis, invasion and metastasis [26-29]. Recently lncRNAs have been reported to be tightly associated with tumor glycolysis [30-32], and thus targeting these lncRNAs may be novel therapeutic strategies for a variety of tumors. Recently, SLC2A1-AS1 as a novel lncRNA has been verified to promote tumor proliferation by absorbing miR-508-5p in lung adenocarcinoma (LUAD) [33]. Besides, SLC2A1-AS1 triggered the suppression of Glut1 expression, and thus resulting in the inhibition of glycolysis of hepatocellular carcinoma (HCC) by competitively binding to transketolase and signal transducer and activator of transcription 3 (STAT3) [34]. Our previous report revealed differential expression of lncRNAs in ESCA [35], and SLC2A1-AS1 was given our exclusive attention due to the same human chromosome 1 with Glut1, suggesting that it may be implicated in the regulation of glycolysis in ESCA. However, its roles and regulatory mechanisms involved in glycolysis remain elusive.

In the present study, we reported a novel lncRNA SLC2A1-AS1 in ESCC. We found that SLC2A1-AS1 was frequently overexpressed in ESCC tissues and cells, which might be due to the high expression of transcription factor GLI3, and its depletion suppressed ESCC cell growth in vitro and in vivo, migration and invasion as well as glycolysis, and induced cell apoptosis in ESCC cells. Mechanistically, SLC2A1-AS1 sponged miR-378a-3p to enhance the Glut1 expression, which further triggered the increased glycolysis in ESCC. Taken altogether, our current data presented herein suggest that SLC2A1-AS1/miR-378a-3p/Glut1 regulatory axis plays an essential role in ESCC development and progression, and thus targeting the signal axis may be a novel therapeutic target for ESCC patients.

Materials and methods

Tissue samples

Sixty cases of ESCC samples and paired normal esophageal epithelial tissues were obtained during surgical resection at the First Affiliated Hospital of Zhengzhou University from 2010 to 2016. Tissue samples confirmed using H&E staining by experienced pathologists were immediately frozen in liquid nitrogen after resection, which was further investigated by quantitative real-time PCR (qRT-PCR), Western blot and immunohistochemistry (IHC). Informed consent of all tissue samples was obtained from each patient and the utilization of tissue...
samples was approved by the Research and Ethics Committee of Zhengzhou University in this study. Meanwhile, the study methodologies completely conformed to the standards set by the Declaration of Helsinki.

IHC assay
IHC assay was performed according to previous reports [36]. In brief, tissue slides were fixed using formalin, embedded in paraffin and cut continuously for 4-6 μm. After deparaffinization, rehydration and antigen retrieval using microwave heating in citrate buffer (pH 6.0). Primary antibodies against Glut1 (Abcam, Cambridge, MA, USA) were incubated with tissue sections. After rinsing, the corresponding second antibody was added to tissue slides. Staining signals were developed using DAB reagent. The staining results were evaluated by two excellent pathologists.

Public database assay
Gene expression profiles from ESCA patients were downloaded from The Cancer Genome Atlas (TCGA) database using the GDC data portal (https://portal.gdc.cancer.gov/repository), which was analyzed according to our previous publication [35]. GEO DataSet (GSE111011) was applied to examine the SLC2A1-AS1, GLI3 and Glut1 expression in 7 cases of ESCC patients and 7 cases of paired normal samples, and GSE43732 was utilized to detect the miR-378a-3p expression in 119 cases of ESCC tissues and paired normal tissues. TCGA database from StarBase (ENCORI) (The Encyclopedia of RNA Interactomes) was used to investigate the SLC2A1-AS1, GLI3, miR-378a-3p and Glut1 expression in 162 cases of ESCA patients and 11 cases of normal samples. GSE111011 was performed to analyze the expression of glycolysis-related proteins in 7 cases of ESCC tissues and corresponding normal tissues.

Cell lines, cell culture and transfection
Human ESCC cell lines including EC9706, TE1, Eca109, KYSE70, KYSE30, KYSE180, KYSE450, KYSE510 and KYSE90 as well as normal esophageal epithelial cell Het-1A were obtained from the Chinese Academy of Sciences Cell Bank, which was maintained in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (Gibco, Invitrogen, USA) in a humidified incubator harboring 5% CO₂. GLI3 siRNA #1, 2 and 3, control siRNA (con-siRNA), SLC2A1-AS1 siRNA #1 and 2, negative control (NC) (Supplementary Table 1), pcDNA3.1, pcDNA3.1-SLC2A1-AS1, pcDNA3.1-GLI3, pcDNA3.1-Glut1 (Supplementary Table 2), NC-mimic, miR-378a-3p mimic, NC-inhibitor, miR-378a-3p inhibitor (GenePharma Company, Shanghai, China), control siRNA and Glut1 siRNA (Santa Cruz company, USA) were transfected into EC9706, TE1 and KYSE180 cells by Lipofectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer’s instruction.

Cell counting Kit-8 (CCK-8) assay for cell proliferation
CCK-8 kit was employed to evaluate cell proliferation in ESCC cells. Briefly, various treatment ESCC cells (EC9706, TE1 and KYSE180 cells) at a density of 2 × 10⁴ cells per well were seeded into 96-well plate. At the indicated time points, CCK-8 (Beintime Biotech, Haimen, China) was added to corresponding cells according to manufacturer’s protocol, and absorbance value at 450 nm was determined in a microplate reader (Thermo Scientific, Waltham, MA).

Colony formation
Colony formation was performed to access the cell proliferation ability. Briefly, ESCC cells (1 × 10⁶) with different transfections were added to 6-well plate for 72 h. Then, fresh medium was used to replace the old medium to culture ESCC cells for continuous 10 days. Finally, ESCC cells were fixed using 4% of paraformaldehyde for 1 h and stained with 0.1% crystal violet for 30 min.

EdU staining assay
EdU staining assay was performed according to manufacturer’s protocol. EC9706, TE1 and KYSE180 cells (6 × 10³ cells/well) were seeded into 96-well plate, and then were transfected with NC, SLC2A1-AS1 siRNA, pcDNA3.1 and pcDNA3.1-SLC2A1-AS1 according to manufacturer’s protocol. Cells were labeled with EdU reagent in a final concentration of 50 μM for 2 h. Cells were rinsed using PBS buffer for 5 min. Subsequently, cells was fixed in PBS buffer containing 4% polyformaldehyde for 30 min, and glycine (2 mg/ml) in a volume of 50 μl was added to cells for 5 min. Finally, TritonX-100 in a volume of 100 μl was used for decolorization for 10 min. Regarding Apollo staining, a total of 100 μl of 1 × Apollo staining liquid was applied to each well, and incubated for 30 min. PBS buffer containing 0.5% TritonX-100 in a volume of 100 μl was used for decolorization for 10 min. Finally, DNA staining was performed using 1 × Hoechst33342 according to manufacturer’s instruction. The photo was taken using florescent microscope.

Migration and invasion assay by Transwell chamber
Cell migration and invasion were investigated by Transwell chamber without or with Matrigel (BD Biosciences, San Diego, CA, USA) according to previous report [37]. Briefly, EC9706, TE1 and KYSE180 cells (1 × 10⁵) were placed in the upper layer of chamber, whereas 20% FBS was added to underlayer of chamber. Subsequently, migratory and invasive cells were fixed using methanol, followed by staining with crystal violet 48 h after
transfection. Finally, the number of invasive cells was investigated under the field of 200× magnification.

**Glucose uptake and lactate production assays**

Glucose and lactate assay kits (Nanjing Jiancheng Biotechnology Institute, Nanjing, China) were used to determine the glucose consumption and lactate production according to manufacturer’s instructions. All data obtained were normalized to protein quantitative values.

**Subcellular fractionation**

Cell nucleus and cytoplasm RNA isolation kit (Beibei, Biotech, Co. Ltd., China) was used to extract the nuclear RNA and cytoplasmic RNA, respectively, according to manufacturer’s instruction, and then were investigated using qRT-PCR (Supplementary Table 3).

**Fluorescence in situ hybridization (FISH)**

SLC2A1-AS1 probe (SAAAGCAAGGCTTGGCTCAACA-3') was synthesized and labeled using Cy3 by GenePharma Company, Shanghai, China. For FISH assay, EC9706, TE1 and KYSE180 cells were grown in 24-well plates with glass cover slips for 24 h. After immobilization and permeabilization, EC9706, TE1 and KYSE180 cells were hybridized with 20 μM Cy3-labeled SLC2A1-AS1 probe, and 6-diamidino-2-phenylindole (DAPI) was used to stain cell nuclei of ESCC cells. The images were observed with a florescent microscope.

**qRT-PCR**

Total RNA was isolated by Trizol reagent (Invitrogen) according to the manufacturer’s instructions. For mRNA analysis, qRT-PCR was performed using Power SYBR green PCR master mix (Applied Biosystems) on an ABI 7500 series PCR machine (Applied Biosystems) according to manufacturer’s instructions. For mRNA analysis, qRT-PCR was performed using Power SYBR green PCR master mix (Applied Biosystems) on an ABI 7500 series PCR machine. Amplification for miR-378a-3p expression was determined using qRT-PCR (Supplementary Table 3). Experiments were normalized to U6.

**Western blot**

Total proteins were extracted from ESCC cells using RIPA lysis (Solarbio, Beijing, China) and the concentration of the proteins was measured by Bradford method. The proteins were separated by SDS-PAGE, and then transferred to PVDF membranes (Millipore Corporation, Bedford, MA, USA). The primary antibodies against E-cadherin, N-cadherin, Vimentin, Glut1, HK2, PKM, LDHA and β-actin (1: 200 dilution, Abcam, Cambridge, MA, USA) were incubated with PVDF membrane (Roche, Switzerland) overnight at room temperature after blocking with skimmed milk. Subsequently, the secondary antibody (ZSGB-BIO, Guangzhou, China) was added to PVDF membrane. Finally, enhanced chemiluminescence (ECL) reagent (Beyotime, Haimen, China) was utilized to develop the protein signal.

**The detection of luciferase activity**

The dual luciferase reporter assay system was conducted to determine the direct interaction of SLC2A1-AS1 and miR-378a-3p as well as miR-378a-3p and Glut1. Recombinant vector pmirGLO-SLC2A1-AS1-wild type (WT) and pmirGLO-SLC2A1-AS1-mutation (MUT) as well as pmirGLO-Glut1-WT and pmirGLO-Glut1-MUT (TSINGKE Biological Technology, Beijing, China) were used in experiments. miR-378a-3p mimic and NC-mimic were transfected into EC9706, TE1 and KYSE180 cells, respectively. For luciferase assay, pGL3-basic with SLC2A1-AS1 promoter MUT and WT sequences (Supplementary Table 5) were combined with pcDNA3.1 or pcDNA3.1-GLI3 were transfected into EC9706, TE1 and KYSE180 cells. The Luciferase activity was determined using the Luciferase Reporter Assay System (Promega, USA) 48 h after transfection according to manufacturer’s instruction.

**RNA immunoprecipitation (RIP)**

RIP assay was performed in EC9706, TE1 and KYSE180 cells by using RNA-binding protein immunoprecipitation kit (Millipore company, Billerica, MA, USA) as described previously [38, 39]. Briefly, RIP lysates were prepared from EC9706, TE1 and KYSE180 cells transfected with miR-378a-3p mimic or NC-mimic, and then were subjected to immunoprecipitation using 5 μl of either a normal mouse IgG or 5 μl of Anti-Ago2 antibody and the Mana RIP™ RNA-binding Protein Immunoprecipitation Kit. The mRNA levels of SLC2A1-AS1 and miR-378a-3p enriched on beads was determined by qRT-PCR (Supplementary Table 3).

**In vivo nude mouse model**

Four- to six-week old female BALB/c nude mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. were utilized for the animal experiment. The protocols of animal experiments were approved by the Animal Experiment Administration Committee of Zhengzhou University. For tumorigenesis assay, EC9706 cells (1 x 10⁶ cells/mouse) were subcutaneously injected into the right flank of nude mice at their back, with 5 mice per group. When tumor volume reached around 100mm³, NC, SLC2A1-AS1 siRNA, pcDNA3.1 and pcDNA3.1-SLC2A1-AS1 were used to inject intratumorally into the nude mice (NC and SLC2A1-AS1 siRNA modified by methylation and cholesterol, 2 nM for each mouse; pcDNA3.1 and pcDNA3.1-SLC2A1-AS1 for each mouse with 2.5 μg) twice every week for a total of 4 weeks.
Tumor volume was measured twice every week, and tumor growth curve was made using the formula “length×width² ×3.14/6”. The mice were euthanized when the experiment was terminated. The tumor tissues were obtained for the isolation of total RNAs and proteins, qRT-PCR was used to investigate the SLC2A1-AS1 and miR-378a-3p expression, and Western blot was performed to determine the expressions of Glut1, HK2, PFKM, PKM and LDHA proteins.

**Statistical analysis**

All experimental data from at least three independent repeats were investigated using GraphPad Prism 6.0 software. Data were presented as mean with standard deviation (SD). The IHC results were examined using chi-square, and survival assay were performed using Log-rank test. For the matched samples, the data was analyzed using Wilcoxon signed rank, and for non-matched samples, the data were compared by Mann-Whitney test. The comparison between two groups was determined using Student’s t-test, and the comparison of > 3 groups was determined using one-way ANOVA, and then Bonferroni test was selected for further statistical assay when datasets contain > 3 groups. A P value less than 0.05 was regarded to be statistical significance.

**Results**

SLC2A1-AS1 is upregulated in ESCC and correlated with TNM stage, lymph node metastasis and patient prognosis

In this study, we investigated SLC2A1-AS1 expression in ESCA tissues by TCGA database and GEO dataset, the data from TCGA database revealed that SLC2A1-AS1 expression in 162 cases of ESCA samples was markedly higher than that of 111 cases of normal samples (Fig. 1A), with no difference between EAC samples and normal samples (P > 0.05) (Fig. 1B), but significance difference exhibited in ESCC samples and normal samples (P < 0.0001) (Fig. 1C), suggesting SLC2A1-AS1 may be a novel biomarker for discriminating EAC and ESCC historical types. Further GEO dataset assay demonstrated that SLC2A1-AS1 level in 7 cases of ESCC tissues was significantly higher than that in paired normal esophageal tissues (Fig. 1D). To validate these data, we detected SLC2A1-AS1 expression in 60 cases of ESCC tissues and corresponding normal tissues by qRT-PCR using SLC2A1-AS1 specific primers. The results showed that ESCC tissues displayed higher SLC2A1-AS1 expression than normal tissues (Fig. 1E). Afterwards, FISH assay demonstrated that SLC2A1-AS1 expression in ESCC tissues was dramatically higher than that in normal tissues (Fig. 1F). Meanwhile, we also found the high expression of SLC2A1-AS1 in 4 ESCC cell lines, including EC9706, TE1, KYSE180 and KYSE450 (Fig. 1G). These findings suggest that SLC2A1-AS1 may participate in ESCC development.

To further dissect the role of SLC2A1-AS1 in progression and metastasis of ESCC, we investigated the expression of SLC2A1-AS1 in ESCC patients with diverse clinicopathological features. We found that SLC2A1-AS1 expression was not related to the patients’ gender, age, smoking status, drinking, differentiation degree and tumor size (P > 0.05), however, its expression was tightly associated with TNM stage and lymph node metastasis (Fig. 1H). Notably, the survival rate of ESCC patients with high SLC2A1-AS1 level was extremely lower than that of ESCC patients with low SLC2A1-AS1 level (P < 0.05) (Fig. 1I). These data indicate that SLC2A1-AS1 may be a novel predictor for metastasis and prognosis for ESCC patients.

Transcriptional factor GLI3 binds to SLC2A1-AS1 promoter and induces its expression in ESCC

To explore possible molecular events implicated in SLC2A1-AS1 high expression in ESCC, UCSC Genome Browser (http://genome.ucsc.edu/) was used to obtain the promoter sequence of SLC2A1-AS1, hTFTarget and JASPAR online software was used to predict the binding site of transcription factors, we found that transcriptional factor GLI3 bound to three potential sites in SLC2A1-AS1 promoter region (Fig. 2A). pGL3-basic with SLC2A1-AS1-promoter-WT or –MUT vectors along with pcDNA3.1 or pcDNA3.1-GLI3 were co-transfected into ESCC cells, the results demonstrated that co-transfection of pcDNA3.1-GLI3 and pGL3-SLC2A1-AS1-promoter-WT extremely enhanced the activity of luciferase in EC9706, TE1 and KYSE180 cells, whereas the other groups displayed no difference (Fig. 2B). To verify the precise interaction site of GLI3 in SLC2A1-AS1 promoter region, three mutation vectors harboring different SLC2A1-AS1 promoter mutation region were constructed, these vectors combined with pcDNA3.1 or pcDNA3.1-GLI3 were co-transfected to ESCC cells, we found that the mutation of site 2 significantly reduced the activity of luciferase in EC9706, TE1 and KYSE180 cells (Fig. 2C), suggesting GLI3 mainly binds to the site2 region of SLC2A1-AS1 promoter.

To further elucidate the expression of GLI3 in ESCC, the result of TCGA database revealed that there was no difference in GLI3 expression between ESCA samples and normal samples (Fig. 2D), in which there was no difference between EAC samples and normal samples (Supplementary Fig. 1), however, significant difference was found in ESCC samples and normal samples from TCGA database (Fig. 2E), which was further confirmed by GEO dataset in 7 cases of ESCC tissues and paired normal tissues (Fig. 2F). Further investigation showed that GLI3 expression and SLC2A1-AS1 expression
Fig. 1 (See legend on next page.)
exhibited evidently positive correlation (P < 0.0001) (Fig. 2G). To further verify whether GLI3 expression affected the SLC2A1-AS1 expression in ESCC cells, three GLI3 siRNAs designed were transfected to ESCC cells, we found GLI3 siRNA#1, 2 and 3 significantly downregulated the GLI3 expression in EC9706, TE1 and KYSE180 cells (Fig. 2H), whereas pcDNA3.1-GLI3 markedly upregulated the GLI3 expression in EC9706, TE1 and KYSE180 cells (Fig. 2I). Stepwise investigation showed that GLI3 downregulation triggered the decrease of SLC2A1-AS1 expression (Fig. 2I), whereas GLI3 upregulation evoked the increase of SLC2A1-AS1 expression in EC9706, TE1 and KYSE180 cells (Fig. 2K). These findings suggest that GLI3 can bind to the promoter region of SLC2A1-AS1 and manipulate its expression in ESCC cells.

Whether SLC2A1-AS1 affected GLI3 expression in ESCC cells, to this end, we firstly analyzed the possible regulatory correlation of SLC2A1-AS1, by LncACTdb2.0 online software, we found that SLC2A1-AS1 might manipulate the GLI3, VEGFA, RUNXI, etc. (Supplementary Fig. 2A and B), further investigation showed that SLC2A1-AS1 was tightly associated with self-sufficiency in growth signals, evading apoptosis, tissue invasion and metastasis as well as reprogramming energy metabolism (Supplementary Fig. 2C). These findings suggest that SLC2A1-AS1 is widely implicated in tumor progression, metastasis and metabolism, therefore, we further detected several key signaling pathways, which was confirmed to be closely involved in tumor development and progression. We found that SLC2A1-AS1 mainly affected Shh and Wnt signaling pathways. SLC2A1-AS1 downregulation suppressed the expressions of GLI1, GLI3, PTCH1, CD44 and Wnt10A (Supplementary Fig. 2D), whereas SLC1A1-AS1 overexpression enhanced GLI1 and GLI3 expressions in EC9706, TE1 and KYSE180 cells (Supplementary Fig. 2E), in which GLI3 displayed the most significant alteration among all genes, suggesting that SLC2A1-AS1 manipulates GLI3 expression in feedback regulatory manner.

SLC2A1-AS1 depletion suppresses cell proliferation and induces cell apoptosis in ESCC cells
Sustaining proliferation ability and resisting cell death are two representative tumor hallmarks, which have been verified to be involved in tumor development and progression. Hence, we further investigated the roles of the alteration of SLC2A1-AS1 expression in cell proliferation and cell apoptosis in ESCC cells. Two siRNAs against SLC2A1-AS1 or NC was transfected into EC9706, TE1 and KYSE180 cells, qRT-PCR was used to verify the SLC2A1-AS1 expression. We found that two SLC2A1-AS1 siRNAs were both markedly downregulated in the SLC2A1-AS1 expression, in which SLC2A1-AS1 siRNA #2 had the better interference effect (Fig. 3A), whereas pcDNA3.1-SLC2A1-AS1 significantly upregulated the SLC2A1-AS1 expression in EC9706, TE1 and KYSE180 cells (Supplementary Fig. 3A). Subsequently, CCK-8, colony formation and EdU staining was used to assess the proliferation ability of ESCC cells, the results demonstrated that SLC2A1-AS1 downregulation significantly suppressed cell proliferation, reduced colony formation and EdU positive staining cell numbers (Fig. 3B-F), whereas the opposite data was presented after SLC2A1-AS1 overexpression (Supplementary Fig. 3B-F). Further Flow cytometry assay revealed that SLC2A1-AS1 depletion contributed to cell apoptosis (Fig. 3G and H). In contrast, SLC2A1-AS1 overexpression suppressed cell apoptosis (Supplementary Fig. 3G and H). Overall, these data imply that SLC2A1-AS1 functions as an oncogenic lncRNA in ESCC cells.

SLC2A1-AS1 downregulation inhibits cell migration and invasion accompanying reduced glycolysis in ESCC cells
Metabolic reprogramming has been verified to be involved in tumor progression and metastasis through affecting the expressions of glycolysis-related proteins. Here, we found that SLC2A1-AS1 depletion suppressed cell migration and invasion in ESCC cells (Fig. 4A, B and C), coupled with increased E-cadherin protein expression and reduced N-cadherin and Vimentin protein expressions (Fig. 4D and E), whereas SLC2A1-AS1 overexpression promoted cell migration and invasion in ESCC cells (Supplementary Fig. 4 A, B and C), accompanying reduced E-cadherin protein expression and
increased N-cadherin and Vimentin protein expressions (Supplementary Fig. 4 D and E). Further investigation revealed that SLC2A1-AS1 depletion markedly downregulated the expression of Glut1 protein, and suppressed glucose consumption and lactate production (Fig. 4F-I), whereas SLC2A1-AS1 overexpression showed opposite effects (Supplementary Fig. 4 F-I). To further elucidate the underlying mechanisms of glucose and lactate alterations mediated by SLC2A1-AS1, GSE111011 was used to investigate the expressions of glycolysis-related proteins in 7 cases of ESCC tissues and paired normal tissues, we found that Glut1, HK2, PFKM, ALDOA, PKM,
GPI, LDHA and PGK1 displayed high expression in ESCC tissues, compared to normal tissues (Fig. 4J), which was also confirmed in ESCC samples, EAC samples and normal samples from TCGA database (Supplementary Fig. 5) as well as in 60 cases of ESCC samples and paired normal samples (Supplementary Fig. 6). To further dissect whether SLC2A1-AS1 affected the above altered glycolysis-related proteins, we selected 4 key proteins (HK2, PFKM, PKM and LDHA) as rate-limiting enzymes of glycolysis. We found that SLC2A1-AS1 downregulation dramatically suppressed the expressions of HK2, PFKM, PKM and LDHA proteins in EC9706, TE1 and KYSE180 cells (Fig. 4K and L), whereas SLC2A1-AS1 overexpression evidently promoted the expressions of HK2, PFKM, PKM and LDHA in EC9706, TE1 and KYSE180 cells (Supplementary Fig. 4J and K). These findings suggest that SLC2A1-AS1 depletion suppresses cell migration and invasion by inhibiting EMT progression and glycolysis in ESCC cells.

SLC2A1-AS1 acts as a ceRNA and competitively absorbs miR-378a-3p in ESCC cells

To investigate how SLC2A1-AS1 exerts its function in ESCC cells, qRT-PCR assay of SLC2A1-AS1 level in the nucleus and cytoplasm demonstrated that SLC2A1-AS1 was mainly localized in the cytoplasm of ESCC cells (Fig. 5A), which was further confirmed by FISH assay (Fig. 5B). Based on the localization of SLC2A1-AS1, we hypothesized that SLC2A1-AS1 functioned as a ceRNA in ESCC cells, and thus DIANA LncBase Predicted v.2 was utilized to predict the possible binding miRNAs of SLC2A1-AS1. We found that SLC2A1-AS1 harbored the binding sites of miR-378a-3p (Fig. 5C). SLC2A1-AS1 expression displayed the negative correlation with miR-378a-3p in ESCA tissues (Fig. 5D). To test the interaction of SLC2A1-AS1 and miR-378a-3p in ESCC cells, Double Luciferase Report experiment was used to verify their interaction. The results demonstrated that the luciferase intensity was significantly reduced by co-transfecting miR-378a-3p mimic and SLC2A1-AS1 WT, but not in the

SLC2A1-AS1 depletion contributes to the suppression of cell proliferation and the induction of cell apoptosis in ESCC cells. A. qRT-PCR was used to determine SLC2A1-AS1 expression following transfection with SLC2A1-AS1 siRNA in ESCC cells. B. CCK-8 assay for cell proliferation at 24h, 48h, 72h and 96h after transfection with SLC2A1-AS1 siRNA in ESCC cells. C. SLC2A1-AS1 downregulation markedly reduced colony formation in ESCC cells. D. Statistical assay for the number of colony formation in ESCC cells treated with NC or SLC2A1-AS1 siRNA. E. EdU staining assay for EdU-positive cell numbers in ESCC cells treated with NC or SLC2A1-AS1 siRNA. F. Quantitative assay of the ratio of EdU-positive cell numbers in ESCC cells treated with NC or SLC2A1-AS1 siRNA. G. Flow cytometry detection for cell apoptosis in different treatment ESCC cells. H. Quantitative analysis for apoptotic cell numbers in a variety of ESCC cells. Compared to NC group, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001, indicating statistical significance.
mutant vector without miR-378a-3p binding site in EC9706, TE1 and KYSE180 cells (Fig. 5E). As a core component of the RNA-induced silencing complex (RISC), Ago2 is involved in miRNA-mediating mRNA destabilization or translational repression, therefore, we further performed RIP assay by anti-Ago2 antibody, we found that SLC2A1-AS1 and miR-378a-3p levels precipitated by anti-Ago2 antibody were dramatically increased compared to IgG group (Fig. 5Fa and G). Besides, Ago2-RIP assay showed that SLC2A1-AS1 enrichment in miR-378a-3p mimic group was markedly higher than that in NC group (Fig. 5H). Subsequently, we found that SLC2A1-AS1 silencing obviously enhanced miR-378a-3p level (Fig. 5I), whereas SLC2A1-AS1 overexpression dramatically reduced miR-378a-3p level in EC9706, TE1 and KYSE180 cells (Fig. 5J). These data suggest that SLC2A1-AS1 directly regulates miR-378a-3p level in ESCC cells.
Fig. 5  SLC2A1-AS1 competitively absorbs miR-378a-3p by ceRNA mechanism in ESCC cells. A. qRT-PCR assay of subcellular SLC2A1-AS1 level in the nucleus and cytoplasm of EC9706, TE1 and KYSE180 cells. GAPDH and U6 are employed as endogenous controls. B. Subcellular localization of SLC2A1-AS1 in EC9706, TE1 and KYSE180 cells is investigated by FISH experiment, SLC2A1-AS1 is labeled by Cy3 (red) and nuclei are stained with DAPI (blue). C. The miR-378a-3p binding sites in SLC2A1-AS1 transcript are predicted by DIANA LncBase Predicted v.2. D. The correlation of SLC2A1-AS1 with miR-378a-3p is examined in ESCA samples using the bioinformatics tool StarBase. E. Double luciferase report experiment is performed to verify the interaction of SLC2A1-AS1 with miR-378a-3p by co-transfecting pmirGLO-SLC2A1-AS1-WT or pmirGLO-SLC2A1-AS1-MUT along with NC-mimic or miR-378a-3p mimic into ESCC cells, compared to NC group, **P < 0.01 and ****P < 0.0001, indicating statistical significance. F. Fold enrichment of SLC2A1-AS1 by Ago2 antibody or IgG in EC9706, TE1 and KYSE180 cells. G. Fold enrichment of miR-378a-3p by Ago2 antibody or IgG in EC9706, TE1 and KYSE180 cells. H. Enrichment of SLC2A1-AS1 in EC9706, TE1 and KYSE180 cells transfected with NC mimic or miR-378a-3p mimic, (F, G and H, compared to IgG group, ****P < 0.0001, indicating statistical significance). I. SLC2A1-AS1 depletion promotes the expression of miR-378a-3p in EC9706, TE1 and KYSE180 cells compared to NC group, ****P < 0.0001, indicating statistical significance. J. SLC2A1-AS1 overexpression suppresses the expression of miR-378a-3p in EC9706, TE1 and KYSE180 cells, compared to pcDNA3.1, ****P < 0.0001, indicating statistical significance.
The associations of miR-378a-3p and Glut1 expressions with clinicopathological features and Glut1 is a target of miR-378a-3p in ESCC cells

To explore the possible roles of miR-378a-3p and Glut1 in ESCC development and progression, we detected their expressions and investigated their correlations with clinicopathological features. TCGA assay revealed that there was no difference in the miR-378a-3p or miR-378a expressions between ESCC and normal samples or EAC and normal samples (Fig. 6A, Supplementary Fig. 7 A and B), but GEO assay exhibited significant difference in miR-378a-3p expression level between ESCC tissues and normal tissues (Fig. 6B), which was further validated by qRT-PCR in 60 cases of ESCC tissues and paired normal tissues (Fig. 6C) as well as ESCC cells and normal esophageal epithelial cell Het-1A (Fig. 6D). Besides, TCGA and GEO assay demonstrated that Glut1 expression in ESCC and EAC tissues was significantly higher than that in normal tissues (Fig. 6E and F, Supplementary Fig. 8 A and B), which was also confirmed in ESCC cells and normal esophageal epithelial cell Het-1A by Western blot (Fig. 6G and H) as well as in 60 cases of ESCC tissues and paired normal tissues by IHC assay (Fig. 6I, Supplementary Table 6). These findings suggest that low miR-378a-3p or high Glut1 expression may participate in ESCC development and progression.

To further investigate the associations of miR-378a-3p and Glut1 with clinicopathological features of ESCC patients, GraphPad Prism 8.0 software was used to analyze the their correlations. We found that miR-378a-3p level was not related to ESCC patients’ gender, age, smoking, drinking and differentiation degree (P > 0.05), but was closely related with tumor size, TNM stage and lymph node metastasis (P < 0.05) (Supplementary Table 7). Besides, Glut1 expression was not related to ESCC patients’ gender, age, smoking, drinking and tumor size (P > 0.05), but was tightly correlated with differentiation degree, TNM stage and lymph node metastasis (P < 0.05, Supplementary Table 8). These findings suggest that miR-378a-3p and Glut1 may be novel predictors for TNM stage and metastasis of ESCC patients.

To further explore the regulatory role of miR-378a-3p on Glut1 in ESCC cells, miRDB online software was used to predict the target genes of miR-378a-3p. We found that Glut1 had the potential binding sites of miR-378a-3p (Fig. 6J). Further Double Luciferase Report assay revealed that the luciferase intensity was significantly reduced by co-transfecting miR-378a-3p mimic and Glut1 WT, but not in the mutant vector without miR-378a-3p binding site in EC9706, TE1 and KYSE180 cells (Fig. 6K). Subsequently, we detected the miR-378a-3p expression in ESCC cells by transfecting miR-378a-3p mimic or inhibitor, we found that miR-378a-3p mimic significantly upregulated the level of miR-378a-3p, whereas miR-378a-3p inhibitor markedly downregulated the level of miR-378a-3p in EC9706, TE1 and KYSE180 cells (Fig. 6L). Western blot assay showed that miR-378a-3p mimic dramatically downregulated the expression of Glut1 protein (Fig. 6M and N), whereas miR-378a-3p inhibitor extremely upregulated the expression of Glut1 protein in EC9706, TE1 and KYSE180 cells (Fig. 6O and P). These findings suggest that Glut1 is a direct target of miR-378a-3p in ESCC cells.

The alterations of proliferation, apoptosis and invasion triggered by miR-378a-3p are reversed by SLC2A1-AS1 and Glut1 overexpression in ESCC cells

To verify whether the biological roles of miR-378a-3p exerted in a SLC2A1-AS1- or Glut1-dependent manner, we transfected ESCC cells using NC mimic, miR-378a-3p mimic plus pcDNA3.1-SLC2A1-AS1 and miR-378a-3p mimic plus pcDNA3.1-Glut1 as well as NC inhibitor, miR-378a-3p inhibitor, miR-378a-3p inhibitor plus SLC2A1-AS1 siRNA and miR-378a-3p inhibitor plus Glut1 siRNA. We found miR-378a-3p mimic suppressed cell proliferation and invasion and increased apoptotic cell numbers (Fig. 7A-E), whereas SLC2A1-AS1 and Glut1 overexpressions recovered in part the effects in ESCC cells (Fig. 7A-E). Conversely, miR-378a-3p inhibitor promoted cell proliferation and invasion and decreased apoptotic cell numbers (Supplementary Fig. 9A-E), whereas SLC2A1-AS1 and Glut1 silencing reversed in part the effects in ESCC cells (Supplementary Fig. 9A-E). These findings revealed that miR-378a-3p functions as a tumor suppressor in a SLC2A1-AS1- or Glut1-dependent manner.

Given the links of SLC2A1-AS1 with miR-378a-3p, we put forward to whether miR-378a-3p triggered the alterations of glycolysis in ESCC and whether these changes were also reversed by SLC2A1-AS1 or Glut1. Thus, we investigated the changes of glucose consumption and lactate production as well as glycolysis-related gene expressions. We found that miR-378a-3p mimic reduced glucose consumption, lactate production and the expressions of HK2, PFKM, PKM and LDHA proteins (Fig. 7F-I), whereas SLC2A1-AS1 and Glut1 overexpressions recovered in part the effects (Fig. 7F-I). Conversely, miR-378a-3p inhibitor promoted glucose consumption, lactate production and the expressions of HK2, PFKM, PKM and LDHA proteins (Supplementary Fig. 9F-I), whereas SLC2A1-AS1 and Glut1 silencing exhibited the opposite effects (Supplementary Fig. 9F-I). These findings suggest that SLC2A1-AS1 functions as an oncogenic lncRNA that promoted cell proliferation, migration and invasion as well as glycolysis via SLC2A1-AS1/miR-378a-3p/Glut1 signal axis in ESCC cells (Fig. 7I).
Fig. 6 (See legend on next page.)
SLC2A1-AS1 promotes tumorigenesis and glycolysis in vivo in ESCC xenografted nude mice

To verify the roles of SLC2A1-AS1 in ESCC cells xenografted nude mice, chemically modified siRNA and overexpression vector of SLC2A1-AS1 were used to treat the tumor. The tumor growth curve demonstrated that SLC2A1-AS1 knockdown dramatically suppressed tumor growth (Fig. 8A and B), but not affected the body weight of nude mice (Fig. 8C), coupled with SLC2A1-AS1 downregulation and miR-378a-3p upregulation in EC9706 cells xenografted tumor tissues (Fig. 8D and E). Further Western blot assay showed that SLC2A1-AS1 depletion markedly reduced the expressions of Glut1, HK2, PFKM, PKM and LDHA in EC9706 cells xenografted tumor tissues (Fig. 8F and G); whereas SLC2A1-AS1 overexpression displayed the opposite effects on tumor growth (Fig. 8H and I), mice weight (Fig. 8C), the expressions of SLC2A1-AS1 and miR-378a-3p (Fig. 8K and L) as well as the expressions of glycolysis-related proteins (Glut1, HK2, PFKM, PKM and LDHA) (Fig. 8M and N) in EC9706 cells xenografted tumor tissues. These data indicate that SLC2A1-AS1 contributes to ESCC tumorigenesis and glycolysis in vivo.

Discussion

Currently, a great number of lncRNAs implicated in tumor progression and glycolysis are rapidly expanding [30, 40]. In this study, we reported a novel lncRNA SLC2A1-AS1 involved in ESCC glycolysis. SLC2A1-AS1 was frequently overexpressed in ESCC tissues and cells, and its overexpression was tightly correlated with TNM stage, lymph node metastasis and poor prognosis of ESCC patients. GLI3 as a transcriptional factor drove the SLC2A1-AS1 expression in ESCC cells, whereas SLC2A1-AS also manipulated the GLI3 expression, which formed an important regulatory feedback loop in ESCC. SLC2A1-AS1 silencing suppressed cell growth in vitro and in vivo, migration and invasion ability, and induced cell apoptosis, coupled with alterations of EMT-related molecules and glycolysis-related proteins. Importantly, SLC2A1-AS1 sponged miR-378a-3p to increase the Glut1 expression, further promoted the expressions of glycolysis-related proteins, which led to ESCC progression and increased glycolysis, and thus targeting SLC2A1-AS1/miR-378a-3p/Glut1 signal axis may be a novel therapeutic target for ESCC patients.

Genome-wide analysis has identified a large number of differential lncRNAs in a number of tumor types. Our previous report has unveiled many differential lncRNAs in ESCC [35]. Based on this study, we focused on SLC2A1-AS1 as a novel lncRNA molecule in ESCA. We found SLC2A1-AS1 was highly expressed in ESCC tissues and cells, and its overexpression was tightly correlated with TNM stage, lymph node metastasis and poor prognosis of ESCC patients, which was similar to the study reported in LUAD [33], but was inconsistent with the data previously reported in HCC [34]. These different expression of SLC2A1-AS1 in different tumor types suggest that SLC2A1-AS1 expression may be tumor dependent. Many studies have revealed that transcriptional factors play important regulatory roles in the expressions of many genes. Wang C, et al. found that c-myc bound to the promoter region of PVT1 to enhance the expression of PVT1 in cervical cancer [41]. HIF-1 was identified to directly bind to the promoter region of PDA13P1, further resulting in its transcription activation in glioma [42]. Notably, the transcription factor activating enhancer binding protein 2 α (TFAP2A) regulated the transcription of SLC2A1-AS1 by directly binding to its promoter region in LUAD cells [33]. To explore the possible regulatory factors of SLC2A1-AS1 in ESCC cells, hTFTarget and JASPAR online software were used to predict the binding sites of transcription factors in the promoter region of SLC2A1-AS1, we found that GLI3
Fig. 7 (See legend on next page.)
ceRNA to control gene expression [50], implying that body markedly enriched the SLC2A1-AS1 and miR-378a-3p from Ago2-RIP assay demonstrated that Anti-Ago2 antibody markedly enriched the SLC2A1-AS1 and miR-378a-3p in ESCC cells by Double Luciferase Report Assay. Stepwise investigation SLC2A1-AS1 sponged miR-378a-3p in ESCC cells by mechanism. Our data supported the hypothesis that esized that SLC2A1-AS1 exerted its function via ceRNA plasm of ESCC cells by qRT-PCR and FISH. We hypoth-
ized that SLC2A1-AS1 may function as oncogene in tumor cells, we firstly examined subcellular localization of SLC2A1-AS1 in ESCC cells. We found that SLC2A1-AS1 mainly localized in cyto-
plasm of ESCC cells by qRT-PCR and FISH. We hypothesized that SLC2A1-AS1 exerted its function via ceRNA mechanism. Our data supported the hypothesis that SLC2A1-AS1 sponged miR-378a-3p in ESCC cells by Double Luciferase Report Assay. Stepwise investigation from Ago2-RIP assay demonstrated that Anti-Ago2 anti-
body markedly enriched the SLC2A1-AS1 and miR-378a-3p in ESCC cells, not only, when miR-378a-3p was overexpressed, the expression of SLC2A1-AS1 was dra-
matically enhanced, suggesting that SLC2A1-AS1 and miR-378a-3p appeared in the same RISC in ESCC cells. Meanwhile, SLC2A1-AS1 depletion significantly upregulated the miR-378a-3p level, whereas SLC2A1-AS1 over-
expression extremely downregulated the miR-378a-3p level in ESCC cells. These findings indicate that SLC2A1-AS1 functions as the sponge of miR-378a-3p to regulate miR-378a-3p expression in ESCC cells.

There is growing evidence that miRNAs are widely deregulated in a number of tumors, highlighting its key regulatory role in onset, growth and metastasis of tumors [51]. Emerging data suggest that miRNAs play essential regulatory roles in different physiological and pathological processes by targeting a majority of mRNAs [52]. At present, miR-378a-3p has been reported to be involved in tumor development, progression and drug resistance by manipulating a series of targeted genes. For example, miR-378 functions as an enhancer of cell growth, cell survival and angiogenesis by targeting SuFu and Fus-1 [53]. MiR-378a-3p has been confirmed to sensitize tumor cells to cisplatin in ovarian cancer by targeting MAPK2/GRB2 [54]. To further unveil the functions of miR-378a-3p in ESCC, we firstly examined the expression of miR-378a-3p in ESCC. We found that miR-378a-3p was frequently downregulated in ESCC tissues and cells by TCGA database, GEO dataset and qRT-PCR, and its expression was tightly associated with tumor size, TNM stage and lymph node metastasis in ESCC patients, suggesting its implication in ESCC develop-
ment and progression. To uncover its underlying target genes, miRDB database was used to predict the possible downstream target genes. We found that Glut1 was a potential target of miR-378a-3p. To test the prediction, Double Luciferase Report experiment was used to validate it. We confirmed that miR-378a-3p bound to 3′-UTR region of Glut1 to manipulate its expression in ESCC cells. MiR-378a-3p overexpression markedly suppressed the Glut1 expression, whereas miR-378a-3p inhibitor dramatically promoted the Glut1 expression in a panel of ESCC cells. These findings highlight the link of miR-378a-3p with Glut1 in ESCC cells.
Recent investigation revealed that miR-378a-3p was recognized as the pivotal regulator of energy and glucose homeostasis [55], which will enable us to firmly believe that SLC2A1-AS1/miR-378a-3p axis participates in tumor metabolism. Wang Y, et al. verified that Lnc-p23145 bound to the promoter of miR-378a-3p and suppressed the expression of miR-378a-3p, further released the Glut1 expression in oral squamous cell carcinoma.

**Fig. 8** The downregulation of SLC2A1-AS1 suppresses tumor growth and glycolysis in vivo in EC9706 cells xenografted nude mice. A. Representative image of mice from each group after treatment with chemically modified NC or SLC2A1-AS1 siRNA. B. Tumor volumes are measured twice a week for assessment of therapeutic efficacy of SLC2A1-AS1 siRNA in EC9706 cells xenografted nude mice. C. The body weight of mice is determined twice a week in EC9706 cells xenografted nude mice. D. The expression of SLC2A1-AS1 is detected after the end of therapy by qRT-PCR in tumor tissues of nude mice. E. The miR-378a-3p level is examined at the end of therapy by qRT-PCR in nude mouse tumor tissues. F. Western blot assay for the expressions of Glut1, HK2, PFKM, PKM and LDHA proteins in NC group and SLC2A1-AS1 siRNA group, and β-actin is used as loading control. G. Quantification of the relative levels of Glut1, HK2, PFKM, PKM and LDHA proteins in NC group and SLC2A1-AS1 siRNA group. H. Representative image of mice from each group after treatment with 2.5 μg pcDNA3.1 or pcDNA3.1-SLC2A1-AS1. I. Tumor volumes are measured twice a week for assessment of therapeutic efficacy of pcDNA3.1-SLC2A1-AS1 in EC9706 cells xenografted nude mice. J. The body weight of mice is determined twice a week in EC9706 cells xenografted nude mice. K. The expression of SLC2A1-AS1 is detected after the end of therapy by qRT-PCR in tumor tissues of nude mice. L. The miR-378a-3p level is examined at the end of therapy by qRT-PCR in nude mouse tumor tissues. M. Western blot assay for the expressions of Glut1, HK2, PFKM, PKM and LDHA proteins in pcDNA3.1 group and pcDNA3.1-SLC2A1-AS1 group, and β-actin is utilized as loading control. N. Quantification of the relative levels of Glut1, HK2, PFKM, PKM and LDHA proteins in pcDNA3.1 group and pcDNA3.1-SLC2A1-AS1 group. Compared to NC group or pcDNA3.1 group, P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 were regarded as statistical significance. Note: 1: NC; 2: SLC2A1-AS1 siRNA; 3: pcDNA3.1; 4: pcDNA3.1-SLC2A1-AS1.
and thus resulting in the acceleration of tumor glycolysis and tumor progression [56]. MiR-378a-3p evoked the metabolic shift by regulating PGC-1β/ERRγ pathway in breast carcinoma [57]. Here, we found miR-378a-3p mimic suppressed cell proliferation and invasion, promoted cell apoptosis, and triggered the decreases of glucose consumption, lactate production as well as glycolysis-related proteins (HK2, PFKM, PKM and LDHA), which was reversed in part by SLC2A1-AS1 and Glut1 overexpression, whereas miR-378a-3p inhibitor exhibited the opposite effects, which was also recovered partly by SLC2A1-AS1 and Glut1 knockdown. Overall, these findings indicate that SLC2A1-AS1/miR-378a-3p/Glut1 may be a key player in ESCC progression and glycolysis.

Conclusions
In conclusion, SLC2A1-AS1 functions as oncogene in ESCC, and its expression is tightly associated with TNM stage, lymph node metastasis and poor prognosis. Functional and mechanistic assay suggests that SLC2A1-AS1 silencing suppresses cell growth in vitro and in vivo, migration and invasion, and expressions of glycolysis-related proteins by acting as a ceRNA that sponges miR-378a-3p, which will lead to increased Glut1 expression and enhanced glycolysis-related protein expressions. Our current results indicate that SLC2A1-AS1 may play an essential role in driving tumorigenesis, progression and glycolysis, and SLC2A1-AS1/miR-378a-3p/Glut1 signal axis may be a novel therapeutic target for ESCC patients.

Abbreviations
LncRNAs: Long non-coding RNAs; ESCC: Esophageal squamous cell carcinoma; ESCA: Esophageal cancer; EAC: Esophageal adenocarcinoma; HIF-1α: Hypoxia inducible factor-1α; NF-κB: Nuclear factor kappa-B; Glut1: Glucose transporter 1; HK2: Hexokinase 2; PFKM2: Pyruvate kinase type M2; LDHA: Lactate dehydrogenase A; ERR: Competing endogenous RNAs; qRT-PCR: Quantitative real-time PCR; ENCORI: The Encyclopedia of RNA Interactomes; IgG: Immune globulin; KIT-8: FISH: Fluorescence in situ hybridization; ESCC: Esophageal squamous cell carcinoma; HCC: Hepatocellular carcinoma; TFAP2A: Transcription factor activating enhancer binding protein 2 a

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13046-021-02081-x.

Additional file 1. Supplementary Tables 1-8.
Additional file 2. Supplementary Figs. 1-9.

Acknowledgements
None.

Authors’ contributions
Hongtao Liu, Tianli Fan, Fangxia Guan, and Shenglei Li supervised the whole project. Hongtao Liu, Fangxia Guan and Qing Zhang designed the study. Hongtao Liu, Qing Zhang and Tianli Fan performed the majority of the experiments; Yinsen Song, Yibin Hao, Yunxia Cui and Xin Zhang contributed to animal experiments; Xueying Zhang, Yue Qin and Guangzhao Zhu performed the glucose consumption and lactate production assay; Feng Wang, Jinhang Dang, Shanshan Ma, Yanting Zhang and Wenna Guo participated in the design and interpretation of some experiments; Shenglei Li critically reviewed the paper; Fangxia Guan and Tianli Fan interpreted all the results and Hongtao Liu wrote the manuscript. The author(s) read and approved the final manuscript.

Funding
This study was supported by the National Natural Science Foundation of China (82073084, 81372677 and 81272691), the Key R&D Promotion Projects in Henan Province (No. 182108410414), the Natural Science Foundation of Henan Province (No. 172300410010), Key Research Projects of Henan High Education Institutions (No.17A180016), and the Key Discipline Construction Projects Prevention and Treatment of Esophageal Cancer in Zhengzhou University (XKZDJC202001), the Central Plains Thousand People Plan of Henan Province (204200510013), the Discipline Innovation fund of Henan Province (202001).

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
This study was reviewed and approved by the Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). The study was conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects. All subjects provided informed consent to participate in the study.

Consent for publication
Not applicable.

Competing interests
All authors declare no competing interests.

Author details
1School of Life Sciences, Zhengzhou University, Zhengzhou 450001, Henan, China. 2Translational Medicine Research Center, Zhengzhou People’s Hospital, Zhengzhou 450003, Henan, China. 3Institute of Genomic Medicine, College of Pharmacy, Jinan University, Guangzhou 510632, Guangdong, China. 4International Cooperative Laboratory of Traditional Chinese Medicine Modernization and Innovative Drug Development of Chinese Ministry of Education (MOE), College of pharmacy, Jinan University, Guangzhou 510632, Guangdong, China. 5Department of Clinical Medicine, Zhengzhou University, Zhengzhou 450052, Henan, China. 6Department of Pathology, the First Affiliated Hospital of Zhengzhou University, 40 Dauxe Road, Zhengzhou 450052, Henan, China. 7Department of Pharmacology, School of Basic Medicine, Zhengzhou University, 100 Kexue Road, Zhengzhou 450001, Henan, China.

Received: 13 July 2021 Accepted: 20 August 2021
Published online: 13 September 2021

References
1. Roshandel G, Nourouzi A, Pourshams A, Semnani S, Mosat S, Khoshnia M. Endoscopic screening for esophageal squamous cell carcinoma. Arch Iran Med. 2013;16(6):351–7.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424.
3. Wang C, Wang J, Chen Z, Gao Y, He J. Immunohistochemical prognostic markers of esophageal squamous cell carcinoma: a systematic review. Chin J Cancer. 2017;36(1).https://doi.org/10.1186/s12952-017-0232-5.
4. Fakhrian K, Ordu AD, Lordick F, Theisen J, Haller B, Omrcen T, et al. Long-term outcomes of trimodality treatment for squamous cell carcinoma of the esophagus with cisplatin and/or 5-FU: more than 20 years’ experience at a
single-cell determination. Strahlenther Onkol. 2014;190(12):1133–40. https://doi.org/10.1007/s00061-014-0711-4.

5. Soquist KM, Burmeister BH, Smithers BM, Zalicke JR, Simes RJ, Barbour A, et al. Australasian gastrointestinal trials: G survivor after neoadjuvant chemotherapy or chemoradiation therapy for resectable oesophageal carcinoma: an updated meta-analysis. Lancet Oncol. 2011;12(7):881–92. https://doi.org/10.1016/S1470-2045(11)70142-6.

6. Shapiro J, van Lanschot JJB, Hulshof M, van Hagen P, van Berge Henegouwen MJ, Wijnhoven BPL, et al. Neoadjuvant chemoradiotherapy plus surgery versus surgery alone for oesophageal or junctional cancer (CROSS): long-term results of a randomised controlled trial. Lancet Oncol. 2015;16(9):990–8. https://doi.org/10.1016/S1470-2045(15)00080-6.

7. Oppedijk V, van der Gaast A, van Lanschot JJ, van Hagen P, van Ot R, van Rij CJ, et al. Patterns of recurrence after surgery alone versus neoadjuvant chemoradiotherapy and surgery in the CROSS trials. J Clin Oncol. 2014;32(5):385–91. https://doi.org/10.1200/JCO.2013.15.2186.

8. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74. https://doi.org/10.1016/j.cell.2011.02.013.

9. Warburg O, Wind F, Negelein E. The metabolism of tumours in the body. J Gen Physiol. 1932;6(3):553–60. https://doi.org/10.1085/jgp.8.6.559.

10. Racker E. Bioenergetics and the problem of tumor growth. Am Sci. 1972;60(1):56–63.

11. Mayer A, Schmidt M, Seeger A, Serras AF, Vaupel P, Schmidberger H. GLUT-1 glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem. 1994;269(25):13037–43. https://doi.org/10.1074/jbc.269.25.13037.

12. Prakash G, Singh RK, Iqbal MA, Saini SK, Tiku AB, Bansal MN. Pyruvate kinase M2 knockdown-induced signaling via P-M-activated protein kinase promotes mitochondrial biogenesis, autophagy, and cancer cell survival. J Biol Chem. 2017;292(37):2561–76. https://doi.org/10.1074/jbc.M117.791343.

13. Wong N, Ojo D, Yan J, Tang D. PKM2 contributes to tumor metabolism. Cancer Lett. 2015;356(2):184–91. https://doi.org/10.1016/j.canlet.2015.01.031.

14. Fasinr VR, St-Pierre J, Leder P. Attenuation of tumor repression uncovers a link between glycolysis, mitochondrial physiology and tumor maintenance. Cancer Cell. 2006;10(6):425–34. https://doi.org/10.1016/j.ccr.2006.04.023.

15. Johnson RF, Perkins ND. Metabolic stress: regulation of cellular metabolism and the Warburg effect. Trends Biochem Sci. 2012;37(8):317–23. https://doi.org/10.1016/j.tibs.2012.04.002.

16. Shim H, Dolde C, Guo W, Wu CQ, Bao G, Jungmann RA, et al. c-Myc transcriptional upregulation of mTORC1 hijacks the mTORC1 signaling axis to promote aerobic glycolysis. Cell. 2018;173(4):1058–70. https://doi.org/10.1016/j.cell.2018.03.036.

17. Ren X, Zhou Y, Liu Y, Liu S, Hu Y et al. HIF-1alpha-activated miR-486-3p regulates HIF-1alpha-stabilizing lncRNA from tumour-associated macrophages regulates aerobic glycolysis of breast cancer cells. Nat Cell Biol. 2019;21(4):498–509. https://doi.org/10.1038/s41556-019-0299-0.

18. Zhao Y, Wang N, Zhang X, Liu H, Yang S. LncRNA ZEB1-AS1 down regulates tumor suppressor miR510. J Biochem. 2020;167(6):565–73. https://doi.org/10.1007/s12554-019-01157-x.

19. Shang R, Wang M, Dai B, Ju D, Wang J, Liu Z, et al. Long non-coding RNA CRHBP3 regulates cell apoptosis and cell growth in prostate cancer cells. Oncotarget. 2017;8(29):47905–17. https://doi.org/10.18632/oncotarget.17205.

20. Liu H, Zhang Q, Lou Q, Zhang X, Cui Y, Wang P, et al. Differential analysis of IncRNA, miRNA and mRNA expression profiles and the prognostic value of IncRNA in esophageal Cancer. Pathol Oncol Res. 2020;26(2):1029–39. https://doi.org/10.1007/s12053-020-0855-3.

21. Patino C, Guadés R, Cebolla D, et al. HOTAIR modulates cell survival and growth in glioma. Cell Death Dis. 2020;11(3):168. https://doi.org/10.1038/s41419-020-0064-4.

22. Tsai MC, Manor O, Wan Y, Morrisamparan N, Wang JK, Lan F, et al. Long non-coding RNA as modular scaffold of histone modification complexes. Science. 2010;329(5992):689–93. https://doi.org/10.1126/science.1192002.

23. Lin C, Wang Y, Wang Y, Zhang S, Guo C, et al. Transcriptional and posttranscriptional regulation of HOTAIR by IncRNA HOTTIP facilitates tumorigenesis and metastasis in esophageal squamous cell carcinomas. Oncogene. 2017;36(38):5392–406. https://doi.org/10.1038/onc.2017.133.

24. Chen F, Chen J, Yang L, Liu J, Zhang X, Zhang Y, et al. Extracellular vesicle-packaged HIF-1alpha-stabilizing IncRNA from tumour-associated macrophages regulates aerobic glycolysis of breast cancer cells. Nat Cell Biol. 2019;21(4):498–510. https://doi.org/10.1038/s41556-019-0299-0.

25. Wang C, Zou H, Chen A, Yang H, Yu X, Yu X, et al. C-Myc-activated long non-coding RNA PT1 enhances the proliferation of cervical cancer cells by sponging miR-486-3p. J Biochem. 2020;167(6):565–75. https://doi.org/10.1093/jb/mva005.

26. Wang S, Qi Y, Gao X, Qiu W, Liu Q, Guo X, et al. Hypoxia-induced IncRNA PDAI3P1 promotes mesenchymal transition via sponging of miR-234-3p in glioma. Cell Death Dis. 2020;11(3):168. https://doi.org/10.1038/s41419-02-0110-x.

27. Rodríguez M, Miguel L, De Andrade NP, Heguedusch D, Rodini CO, Moyses RA, et al. GLI3 knockdown decreases stemness, cell proliferation and invasion in oral squamous cell carcinoma. Int J Oncol. 2018;53(6):2485–72. https://doi.org/10.3892/ijo.2018.4572.
44. Zhu H, Xia L, Shen Q, Zhao M, Gu X, Bouamar H, et al. Differential effects of GLI2 and GLI3 in regulating cervical cancer malignancy in vitro and in vivo. Lab Investig. 2018;98(11):1384–96. https://doi.org/10.1038/s41374-018-0089-5.

45. Song J, Su ZZ, Shen QM. Long non-coding RNA MALAT1 regulates proliferation, apoptosis, migration and invasion via miR-374b-3p/SRSF7 axis in non-small cell lung cancer. Eur Rev Med Pharmacol Sci. 2020;24(4):1853–62. https://doi.org/10.26355/eurev_202002_20363.

46. Li WZ, Zou Y, Song ZY, Wei ZW, Chen G, Cai QL, et al. Long non-coding RNA SNHG5 affects the invasion and apoptosis of renal cell carcinoma by regulating the miR-363-3p/Twist1 interaction. Am J Transl Res. 2020;12(2):697–707.

47. Jin SJ, Jin MZ, Xia BR, Jin WL. Long non-coding RNA DANC6 as an emerging therapeutic target in human cancers. Front Oncol. 2019;9:1225. https://doi.org/10.3389/fonc.2019.01225.

48. Chaumeil J, Le Baccon P, Wutz A, Heard E. A novel role for Xist RNA in the formation of a repressive nuclear compartment into which genes are recruited when silenced. Genes Dev. 2006;20(16):2223–37. https://doi.org/10.1101/gad.380906.

49. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell. 2007;129(7):1311–23. https://doi.org/10.1016/j.cell.2007.05.022.

50. Cesana M, Cacchiarelli D, Legini L, Santini T, Sthandier O, Chinappi M, et al. A long non-coding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell. 2011;147(2):358–69. https://doi.org/10.1016/j.cell.2011.09.028.

51. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435(7043):834–8. https://doi.org/10.1038/nature03702.

52. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92–105. https://doi.org/10.1101/gr.082701.108.

53. Lee DY, Deng Z, Wang CH, Yang BB. MicroRNA-378a promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. Proc Natl Acad Sci U S A. 2007;104(11):20330–5. https://doi.org/10.1073/pnas.0706901104.

54. Xu ZH, Yao TQ, Liu W. miR-378a sensitizes ovarian cancer cells to cisplatin through targeting MAPK1. Cell Mol Pharmacother. 2018;70(4):1410–7. https://doi.org/10.1111/cmp.12132.

55. Machado IF, Teodoro JS, Palmeira CM, Rolo AP. miR-378a: a new emerging microRNA in metabolism. Cell Mol Life Sci. 2020;77(10):1947–58. https://doi.org/10.1007/s00018-019-03375-z.

56. Wang Y, Zhang X, Wang Z, Hu Q, Wu J, Li Y, et al. LncRNA-p23154 promotes the invasion-metastasis potential of oral squamous cell carcinoma by regulating Glut1-mediated glycolysis. Cancer Lett. 2018;434:172–83. https://doi.org/10.1016/j.canlet.2018.07.016.

57. Eichner JC, Perry MC, Dufour CR, Bertsos N, Park M, St-Pierre J, et al. miR-378(*) mediates metabolic shift in breast cancer cells via the PGC-1beta ERGgamma transcriptional pathway. Cell Metab. 2010;12(4):352–61. https://doi.org/10.1016/j.cmet.2010.09.002.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions