

Original Article

Glyoxalase 1, regulated by LncRNA MALAT1, promotes malignant development of esophageal squamous cell carcinoma

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Abstract: In previous study we found that long noncoding RNA (lncRNA) MALAT1 promotes proliferation and metastasis of esophageal squamous cell carcinoma (ESCC), and that the following microarray chip screening of MALAT1 target genes showed that Glyoxalase I (GLO1) was a potential downstream effector of MALAT1. In this study, we further confirmed that GLO1 was regulated by MALAT1. GLO1 belongs to the glyoxalase system, which encodes a ubiquitous detoxification pathway being implicated in the progression of multiple malignancies. However, currently, the role of GLO1 in human ESCC remains unclear. To explore the clinical significance of GLO1 in ESCC, we first determined the expression of GLO1 in 40 paired ESCC tissues and adjacent normal tissues. We found that the expression level of GLO1 was higher in human ESCC tissues ($P=0.0040$). Knockdown of GLO1 by siRNA significantly inhibited the proliferation and migration of ESCC cells. In vivo assays showed that knockdown of GLO1 decreased tumor growth. Overall, GLO1 might be an essential effector of lncRNA MALAT1 which promotes ESCC progression and can be identified as a potential therapeutic target for ESCC in the future.

Keywords: ESCC, GLO1, oncogene, MALAT1

Introduction

Esophageal cancer is one of the most frequently diagnosed invasive malignancies and ranks as the sixth cancer-related mortality worldwide [1]. ESCC is the predominant histologic type of esophageal cancer in China [2]. LncRNAs are widely recognized to promote the initiation and progression of malignant carcinoma. However, there are still many unsolved mysteries about its mechanisms. In our previous study, it has been demonstrated that high expression of MALAT1 contributes to proliferation and metastasis of ESCC [3], but its detailed mechanisms was unclear. In this study, we performed gene chip screening to identify its downstream targets. The results showed that GLO1, a player in methylglyoxal metabolism, was significantly regulated by MALAT1, and bioinformatic analysis indicated that the metabolic pathway was most changed by knockdown of MALAT1. More

and more evidence has shown that metabolic dysregulation plays an important role in cancer initiation and progression [4]. It is known that many tumor cells exhibit high glycolysis rate and lactate production, as originally discussed by Warburg [5]. GLO1 belongs to the glyoxalase system, and the biochemical function of the glyoxalase system is to convert the dicarboxylic reactive metabolites glyoxal and methylglyoxal into low-toxicity products [6]. Glyoxal and methylglyoxal can react with nucleotides and protein to result in serious endogenous damage to the functional integrity of genome and proteome which can induce cell apoptosis or inhibit cell proliferation and growth [7, 8], while degradation of glyoxal and methylglyoxal by glyoxalase system may reduce the harmful effects to tumor cells, resulting in accelerated growth of tumor cells. Overexpression of GLO1 has been shown in various human tumors, such as gastric cancer [9], hepatocellular carcinoma [10],

GLO1 promotes ESCC proliferation

Table 1. Differentially expressed genes down-regulation by Microarray screening in ECA109

Gene Symbol	EC109-NC_ (HTA-2_0).rma-gene-full-Signal	EC109-SI1_ (HTA-2_0).rma-gene-full-Signal	Fold change (SI1/NC)
GLO1	9.326839	7.758554	0.337209012
CTH	7.116726	5.963578	0.449643027
SPINK13	9.560634	8.484088	0.474162673
CEACAM7	5.072638	4.091531	0.506590876
TAF13	6.408698	5.436994	0.509903448
HGD	5.563014	4.616415	0.518854165
USH1C	7.14222	6.247835	0.537976478
SLC16A6	9.958321	9.076439	0.542659069
ASAP2	8.652463	7.781674	0.546847702

EC109-NC_ (HTA-2_0).rma-gene-full-Signal, log2 as the base; EC109-SI1_ (HTA-2_0).rma-gene-full-Signal, log2 as the base; Fold change (SI1/NC), the ratio of the same probe signal value between groups.

Table 2. The expression of GLO1 in ESCC tissues and normal tissues

Tissue	Relative GLO1 expression level	P value
Normal tissue	1 ± 0.1354	
ESCC	2.182 ± 0.4004	0.0040

cutaneous neoplasms [11]. However, the functional significance of GLO1 in ESCC remains unknown.

In this study, GLO1 was found to be overexpressed in ESCC tumor tissues compared with paired normal esophageal tissue. In addition, ESCC cell lines (ECA109 and TE13) infected with GLO1-siRNA exhibited a reduced proliferation and lower invasion ability. The clinical parameters were positively correlated with the expression level of GLO1, indicating that GLO1 may be used as an ideal diagnostic and therapeutic target of ESCC.

Materials and methods

Microarray screening and bioinformatic analysis

Total RNA from the ECA109 cells with knock-down of MALAT1 and control cells was isolated and quantified. The expression profiles were determined using the Human Transcriptome Array 2.0 (Affymetrix, USA) by Qiming Bio-tech Company (Shanghai, China). Differentially expressed mRNAs (fold change >1.2) was picked

out for further bioinformatic analysis. GO analysis was applied to analyze the main function of the differentially expressed genes according to the Gene Ontology, which is the key functional classification of NCBI, that organize genes into hierarchical categories and uncover the gene regulatory network on the basis of biological process and molecular function. Pathway analysis was utilized to find out the significant pathway of the differential genes according to KEGG, Biocarta and Reatome.

Tissues specimens and cell lines

40 paired ESCC and normal tissues were collected in the Jinling Hospital Affiliated to Southern Medical University/Nanjing General Hospital of Nanjing Military Region from 2016 to 2017, and all patients received neither chemotherapy nor radiotherapy prior to surgery. Every sample was frozen and stored at -80°C. All pathological results were confirmed by two senior pathologists. Written informed consent for biological research was obtained from all participants. ECA109 and TE13 were donated by the Department of Thoracic Surgery, Cancer Institute of Jiangsu Province. All cells were cultured at 37°C with 5% CO₂ in RPMI-1640 medium (GIBCO) supplemented with 10% fetal calf serum (FBS; GIBCO) and 100 U/mL penicillin/streptomycin.

qRT-PCR

GLO1 mRNA expression was analyzed by qRT-PCR, total RNA was extracted from tissues and cell cultures with Trizol reagent (Takara, Japan). qRT-PCR analysis was performed using the SYBR Green Supermix kit (Takara, Tokyo, Japan). Relative GLO1 and MALAT1 expression levels in each specimen were normalized to the housekeeping gene β -actin. Primers used are presented in **Table 4**.

siRNA transfection

Small interfering RNA (siRNA) against GLO1/MALAT1 and one negative control (si-control) with no definite target were synthesized at Genepharma (shanghai, china), control group were only added in PBS. The sequences of siRNAs and negative control were: si-GLO1: 5'-GAAACCUGAUGAUGGUAATT-3'; 5'-UUUAC-

GLO1 promotes ESCC proliferation

Table 3. Correlation between GLO1 expression and clinical parameters in ESCC tissues

Clinical parameters	Number	GLO1 expression		P-value
		Low*	High*	
Gender				
Male	32	9 (22.5%)	23 (57.5%)	0.619
Femal	8	2 (5%)	6 (15%)	
Age (year)				
<65	18	4 (10%)	14 (35%)	0.723
≥65	22	7 (17.5%)	15 (37.5%)	
Clinical stage				
I	8	5 (12.5%)	3 (7.5%)	0.024*
II	19	5 (12.5%)	14 (35%)	
III	13	1 (2.5%)	12 (30%)	
Lymph node metastasis				
N	20	9 (22.5%)	11 (27.5%)	0.013*
Y	20	2 (5%)	18 (45%)	

2-ΔΔCt>1 means GLO1 mRNA high expression, 2-ΔΔCt≤1 means GLO1 mRNA low expression. *P<0.05.

Table 4. Primers were used for qRT-PCR

Gene	Primers
GLO1	Forward: 5'-CCGTTCTTGGGTCCCGTCG-3' Reward: 5'-TCTGGAGAGCGCCAGGCTAT-3'
β-actin	Forward: 5'-GGGACCTGACTGACTACCTC-3' Reward: 5'-TCATACTCCTGCTTGCTGAT-3'
MALAT1	Forward: 5'-TGCGAGTTGTCTCCGTCTAT-3' Reward: 5'-CTTATCTGCGTTTCTCAAG-3'

CAUCAUCAGGUUUCTT-3'. si-control: 5'-UUCU-CCGAACGUGUCACGUTT-3'; 5'-ACGUGACAGU-UCGGAGAATT-3. si-MALAT1: 5'-GAGGUGUAA-AGGGAUUUAUTT-3'. si-control: 5'-UUCUCCGA-ACGUGUCACGUTT-3'. ECA109 and TE13 were seeded in 6-well plates overnight to 60% confluence, then transfection reagent (invitrogen, USA) was incubated with 250 ul serum free media for 5 min and added in siRNA oligos resuspended in 250 ul of serum free media for 20 min at room temperature. The mixture continue to incubate at room temperature for 5 min. The interfering efficiency was determined by qRT-PCR 24 hr post transfection.

Western blot

Cellular proteins were lysed using protein extraction reagent RIPA (Beyotime Shanghai) 48 hr post transfection. Proteins were separated by 10% SDS-PAGE, and then transferred to polyvinylidenedifluoride membranes (Sigma, USA). The membranes were blocked with 5% skimmed milk in PBS-T, and incubated at

4°C overnight with antibodies to GLO1 and β-actin. After incubation (1:10000) with secondary HRP-conjugated rabbit anti-goat IgG (ABCAM, USA) for 1 hr. Signals were obtained with an ECL-chromogenic.

Cell proliferation assay

Cells seeded on 96-well plates (5000/well) were transfected with si-GLO1, si-control and negative control. The cell proliferation assays were conducted every 24 hr using CCK-8 (Sigma, USA) according to the manufacture's protocol, the optical density was measured at 450 nm using a microtiter plate reader.

Cell cycle analysis

Cells seeded on 6-well plates were harvested 48 h after transfection by trypsinization, washed twice in cold PBS, and fixed in 70% ethanol at 4°C overnight. After fixation, the cells were resuspended in cold PBS, and then stained with propidium iodide at 37°C for 30 min in the dark. Finally the DNA content was determined by flow cytometry (BD bioscience, USA). The percentage of cells in the G0/G1, S, and G2/M phases was determined using Cell Quest acquisition software (BD Bioscience, USA).

Transwell migration and invasion assay

Cell migration assays were performed using 6.5-mm transwell chambers with a pore size of 8.0 μm (Corning, USA). The invasion assay was performed similarly, but using matrigel pre-coated membranes. 3×10⁴ cells (ECA109 and TE13) from each group were suspended in serum free medium, and were seeded into the upper chamber 24 hr post siRNA transfection. The lower chamber contained medium mixed with 10% FBS. After incubation for 48 hr, cells in the upper chamber were removed by cotton swap, whereas the cells that had migrated or invaded through the lower chamber (below the surface) were fixed with methanol and stained with 0.1% crystal violet, imaged and counted under a microscope (400×) in five random fields.

Tumor xenografts in nude mice

To explore the effects of GLO1 on tumor growth in vivo, eight mice were divide into two groups,

GLO1 promotes ESCC proliferation

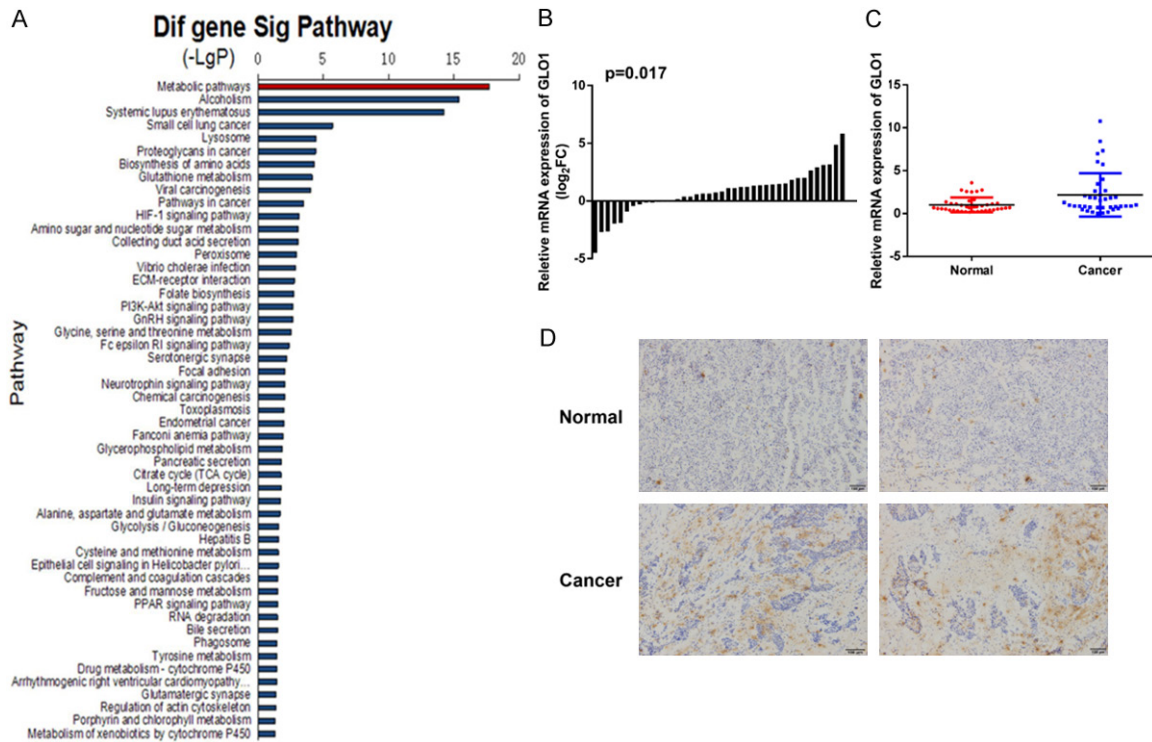


Figure 1. GLO1 is upregulated in ESCC tissues and correlates with metabolic pathway. A. Metabolic pathway is the most significantly up-regulated pathway upon MALAT1 knockdown on ECA109 cells by KEGG pathway analysis. B. Relative expression of GLO1 was measured by qRT-PCR in 40 ESCC tissues and matched adjacent normal tissues. The relative GLO1 expression was calculated by $2^{-\Delta\Delta Ct}$ method. C. Relative GLO1 expression was normalized against the expression level of β -actin. D. Immunohistochemistry experiments revealed that the GLO1 protein was expressed at high levels in tumor tissues. GLO1 is localized in the cytoplasm, the strong brown staining shown in ESCC tissues compared to normal tissues.

one of which received sh-GLO1 transfected cells according to the Lipofectamine 3000 reagent (Invitrogen, USA) protocol, while the other group was treated with control shRNA. A volume of 0.1 ml of suspended cells was subcutaneously injected into the right axilla of each mouse. Ten days later, the tumors became visible with an average size of 2 mm. The tumor volumes were calculated using $V = (\text{length} \times \text{width}^2)/2$. Once the tumor of any mouse has grown to 20 mm, all mice will be killed, and it happens at the 35th day. Tumor nodules were weighed and immunohistochemically stained for ki67 to evaluate cell proliferation.

Immunohistochemistry

The paraffin-embedded ESCC tissues were examined for the expression of GLO1. Sections were blocked with dual endogenous enzyme block (DAKO, Glostrup, Denmark), followed by incubation of goat anti-GLO1 (RD, USA) at 4°C overnight, next, sections were re-warmed at 37°C for 1 hr and washed with PBS three times

before incubation with secondary rabbit anti-goat antibody (ABCAM, USA). The brown color was visualized using 3,3-diaminobenzidine (DAB) staining.

Statistical analysis

Data were expressed as the mean $\pm$ SD. Statistical analysis was conducted using SPSS 18.0. Statistical significance was tested by Student's t-test. Correlation between GLO1 and MALAT1 expression was evaluated using liner correlation. Clinical parameters were evaluated using cross tabulation. The paired groups were analyzed by paired t test. $P < 0.05$ was considered as significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

Target mRNA screening and bioinformatic analysis

To illustrate the underlying mechanisms by which MALAT1 contribute to ESCC proliferation,

GLO1 promotes ESCC proliferation

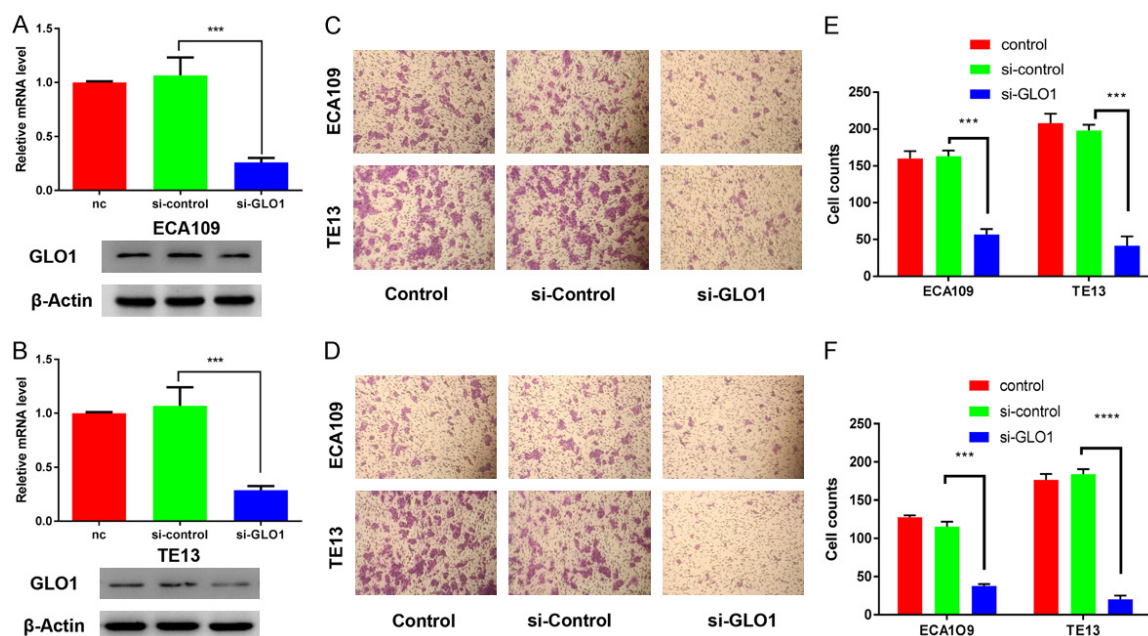


Figure 2. Down-regulation of GLO1 inhibited invasive and malignant capacity in ECA109B and TE13 cells. A and B. QRT-PCR and western blot assays verified reduced GLO1 expression by ECA109 and TE13 upon GLO1 siRNA transfection. C and D. Representative images of the transwell migration and invasion assays showed clearly decreased in the si-GLO1 group. E and F. A statistical plot of the average number of cells migrated and invaded in each groups (*** $P < 0.001$, **** $P < 0.0001$).

we performed target mRNAs screening using microarray chip, and the result showed that GLO1 is the most significantly changed mRNA upon MALAT1 knockdown in ECA109 (**Table 1**). More detailed result of the differentially expressed genes down-regulation by microarray screening in ECA109 (**Table S1**). Functional and pathway enrichment analyses indicated the role of MALAT1 in ESCC metabolism regulation and other cancer-related pathways, further supporting its involvement in carcinogenesis from molecular level (**Figure 1A**).

GLO1 is highly expressed in ESCC tissues and correlated with clinical parameters

Following we detected the GLO1 expression in 40 pairs of human ESCC tumor tissues and matched adjacent non-cancerous tissues by qRT-PCR. The expression of GLO1 was notably 2-fold higher than adjacent normal tissues ($P = 0.0040$, **Table 2**; **Figure 1B**). GLO1 was over-expressed in tumor tissues in 72.5% (29/40) of all ESCC patients (**Figure 1C**). In order to explore the association between GLO1 expression and clinical pathological characteristics. We divided the 40 patients into two groups (high-expression and low-expression). Association with TNM stage ($P = 0.024$) and

lymph nodes metastasis ($P = 0.031$, **Table 3**) were analyzed between the two groups. Immunohistochemistry staining showed strong expression in the ESCC tissues of GLO1 compared with normal tissues (**Figure 1D**).

Knockdown of GLO1 inhibits ESCC cells migration and invasion

To study the role of GLO1 in ESCC cell metastasis, we first silenced GLO1 expression in EC109 and TE13 by small interfering RNA. Efficacy of genetic antagonism of GLO1 expression by si-GLO1 was confirmed at the mRNA and protein level (**Figure 2A** and **2B**). It is shown that GLO1 disruption significantly inhibited the migration ($P < 0.001$ **Figure 2C** and **2E**) and invasion ($P < 0.001$ **Figure 2D** and **2F**) of ECA109 and TE13.

Knockdown of GLO1 inhibits ESCC cells proliferation and induces G0/G1 cell cycle arrest

CCK-8 assays revealed that cell growth was repressed in ECA109 and TE13 cells transfected with si-GLO1 compared with control group after 3 days (**Figure 3A** and **3B**). To investigate the effect of GLO1 knockdown on ESCC cell cycle distribution, transfected ECA109 and TE13 cells were analyzed by flow cytometry

GLO1 promotes ESCC proliferation

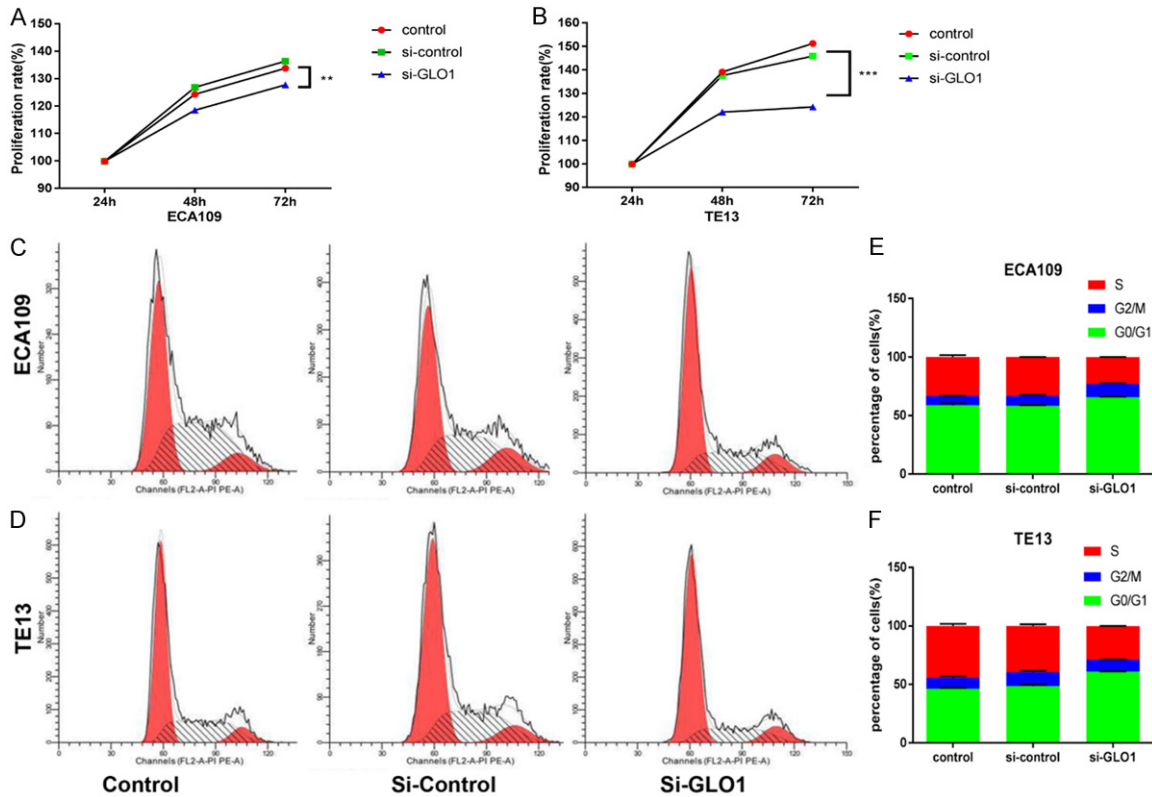


Figure 3. GLO1 increased proliferation in the ECA109 and TE13 cells. A and B. Cellular proliferation of ECA109 and TE13 cells were measured using a CCK-8 assay daily for 3 days. C and D. Untransfected or transfected ECA109 and TE13 cells were stained by propidium iodide analyzed by flow cytometry. E and F. The percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle were calculated. Results are expressed as mean $\pm$ SD from three independent experiments.

(Figure 3C and 3D). The result indicated that GLO1 knockdown increased the percentage of G0/G1 cells, indicating its role in cell cycle controlling ($P < 0.001$ Figure 3E and 3F).

Knockdown of GLO1 increases ESCC cells apoptosis

The percentage of apoptotic cells transfected with si-GLO1 or si-control were detected by flow cytometry analysis of Annexin V- PE staining. (Figure 4A and 4B). The result indicated apoptotic rate of cells transfected with si-GLO1 was notably elevated compared with si-control group ($P < 0.0001$ respectively) (Figure 4C and 4D).

Knockdown of GLO1 expression decreased tumor formation in vivo

We assessed the effect of GLO1 suppression on TE13 cells combined with nude mice. Mice with sh-GLO1 transfected TE13 cells had smaller tumors compared with those treated with

control shRNA since 5 weeks after cell inoculation (Figure 5A). The measure of tumors weight showed significant differences between sh-GLO1 group and sh-control group (Figure 5B). The images of tumors in nude mice formed by sh-GLO1 group and sh-control group transfected TE13 cells have a significant difference (Figure 5C). We also investigate the Ki-67 immunohistochemistry staining in nude mice carcinoma tissues (Figure 5D), the expression intensities of the proliferation marker Ki-67 were weaker in the sh-GLO1 group than in the sh-control group.

Confirmation of GLO1 expression correlated with lncRNA MALAT1 in ESCC tissues and cell lines

To further confirm GLO1 is regulated by MALAT1, we analyzed the association of expression level between expression of GLO1 and MALAT1 by qRT-PCR in 29 cases with high expression of GLO1 ESCC tissues (Figure 6A) and found significant correlation between MALAT1 and

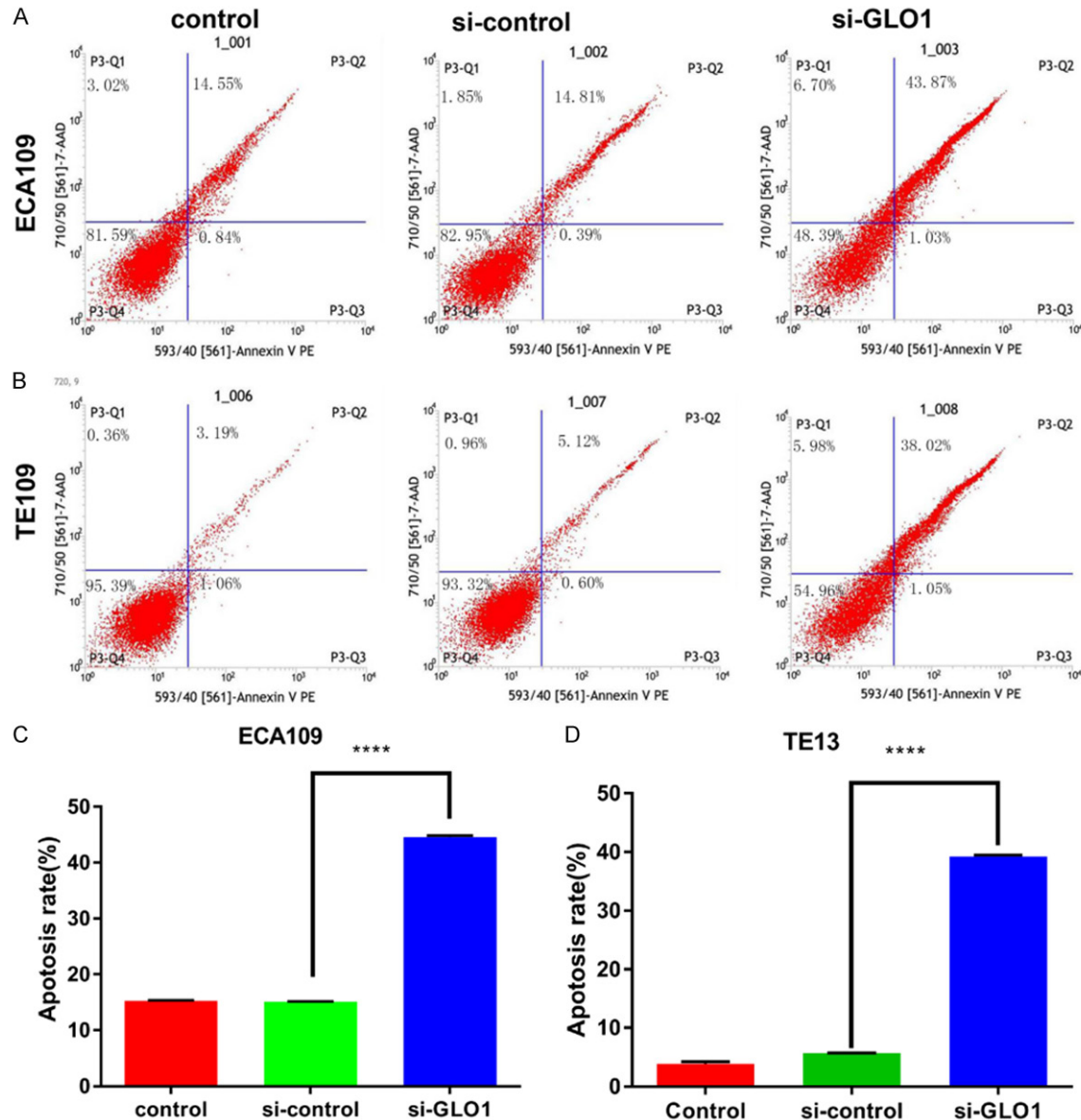


Figure 4. GLO1 increased apoptosis in the ECA109 and TE13. A and B. The percentage of apoptotic cells transfected with si-GLO1 or untransfected ECA109 and TE13 cells were detected by flow cytometry analysis of Annexin V/PE staining. C and D. Apoptosis rate was assessed by calculating the percentage of early apoptotic (P3-Q2) and late apoptotic (P3-Q3) cells (****P<0.0001).

GLO1. Furthermore, down-regulation of MALAT1 (Figure 6B) suppressed the expression of GLO1 by western-blot (Figure 6C). All the results confirmed that GLO1 was regulated by MALAT1 directly.

Discussion

lncRNAs are defined as non-protein coding transcripts longer than 200 nucleotides [12]. lncRNAs can control gene activities through

multiple mechanisms [13]. As a classic lncRNA, the role of MALAT1 in ESCC proliferation and metastasis have been characterized clearly by previous studies [14], but the exact mechanism of MALAT1 function in ESCC remains unclear. In our study, microarray screening and bioinformatic analysis suggested MALAT1 might participate in metabolic process of cancer, which has not been reported by any previous study. GLO1 was most significantly regulated by MALAT1, which was further verified in tissues

GLO1 promotes ESCC proliferation

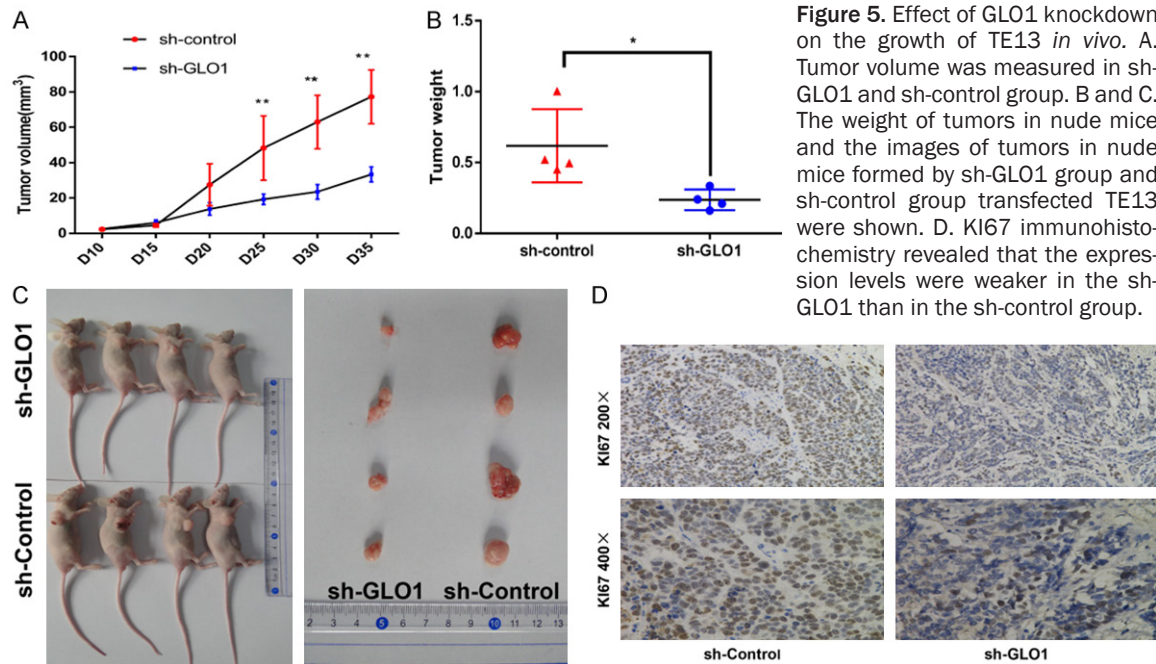


Figure 5. Effect of GLO1 knockdown on the growth of TE13 *in vivo*. A. Tumor volume was measured in sh-GLO1 and sh-control group. B and C. The weight of tumors in nude mice and the images of tumors in nude mice formed by sh-GLO1 group and sh-control group transfected TE13 were shown. D. Ki67 immunohistochemistry revealed that the expression levels were weaker in the sh-GLO1 than in the sh-control group.

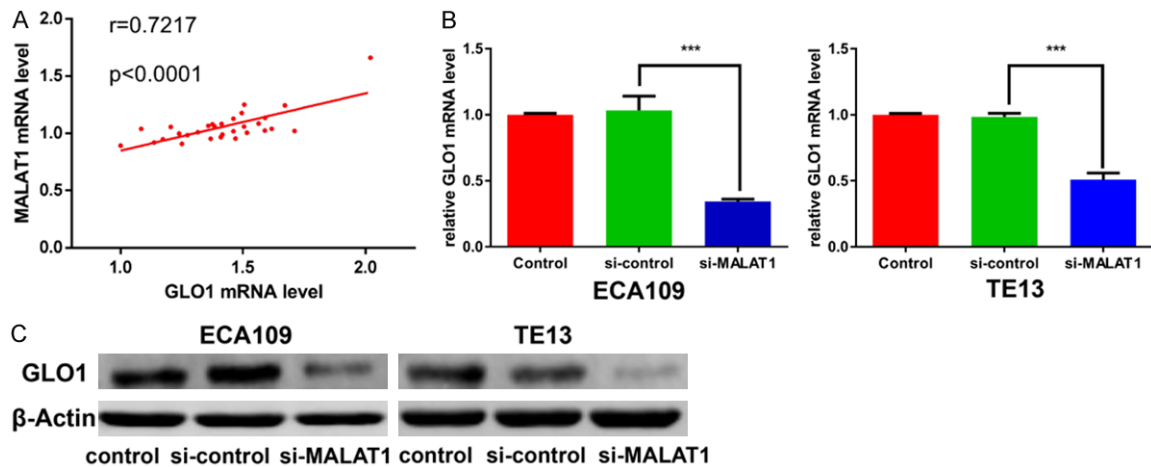


Figure 6. The positive correlation between GLO1 and MALAT1 in ESCC tissues. A. There was a significantly positive correlation between GLO1 and MALAT1 in the 29 high expression of GLO1 ESCC tissues ($R=0.7217$, $P<0.0001$). Each symbol indicated one patient and the line represented the regression liner. B. The MALAT1 mRNA expression level in ECA109 and TE13 cells transfected with si-MALAT1, si-control and control group were measured by qRT-PCR. C. MALAT1 down-regulation suppressed the protein expression of GLO1 after 48 h by Western blot.

and cell lines, indicating the involvement of MALAT1 in glyoxalase system.

Recently, mounts of studies rapidly came into the focus on tumor metabolism. Studies have shown that inhibition of GLO1 expression led to methylglyoxal accumulation while over expression of GLO1 promote the metabolism of methylglyoxal [15]. High expression of GLO1 was verified at protein levels in human pancreatic-melanoma tissues compared to adjacent nor-

mal tissues [16]. Positive correlation between GLO1 expression and high tumor grade was found in breast cancer by immunohistochemistry on tissue microarrays (TMA) [17]. Knockdown of GLO1 in the cancer cells significantly reduced tumor-associated properties [18]. However, GLO1 has not so far been characterized in ESCC. Our results demonstrated that GLO1 was highly expressed in ESCC tissues compared to adjacent normal tissues at mRNA and protein level, and knockdown of GLO1 sig-

nificantly inhibited cell proliferation, migration and invasion in two ESCC cell lines (TE13 and ECA109). Xenograft tumor model also showed that knockdown of GLO1 inhibited tumor growth and proliferation in vivo. Roles of GLO1 in glyoxalase system protects tumor cells against cellular damage and apoptosis caused by cytotoxic metabolites have been recognized by previous studies [19]. In vitro, GLO1 catalyzes the conversion of methylglyoxal (MG) to S-D-lactoylglutathione, which in turn is converted to D-lactate by glyoxalase-II [20]. If MG was not efficiently catalyzed by GLO1, it would accumulate to cytotoxic level and then induce cell death by apoptosis induction [21]. Accordingly this study has shown that down-regulation GLO1 resulted in a significant increase of apoptosis ratio of ESCC.

Glyoxal and methylglyoxal can react with nucleotides and protein and to form advanced glycation endproducts (AGEs), resulting in serious damage of genome [22]. Methylglyoxal has shown significant anti-proliferative properties that prevents cells from early-DNA synthesizing (stage G1) into the DNA synthesis phase (phase S) [23]. Flow cytometry analysis revealed that in the siRNA-treated ESCC cells, the cell cycle was arrested at G0/G1 phase in this study. Totally speaking, upregulation of GLO1 may promote ESCC proliferation by reducing the damaging chemicals to ESCC cells.

In summary, our study has shown the role of MALAT1 in the metabolism in ESCC for the first time, which explain the mechanisms that lncRNAs participate in carcinogenesis from a new angle. We found that GLO1 was over-expressed in ESCC tissues at mRNA and protein level, which may promote the ESCC associated properties and induce apoptosis resistance. Our study also suggested that GLO1 might be a promising target for esophageal cancer. However, the mechanism of how GLO1 be regulated by MALAT1 still need further research and discussion.

Acknowledgements

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Disclosure of conflict of interest

None.

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GLO1 promotes ESCC proliferation

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