

ORIGINAL ARTICLE

Inhibition of glutathione metabolism attenuates esophageal cancer progression

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Esophageal squamous cell carcinoma (ESCC) is a deadly malignancy with regard to mortality and prognosis, and the 5-year survival rate for all patients diagnosed with ESCC remains poor. A better understanding of the biological mechanisms of ESCC tumorigenesis and progression is of great importance to improve treatment of this disease. In this study, we demonstrated that the glutathione metabolism pathway is highly enriched in ESCC cells compared with normal esophageal epithelial cells in an *in vivo* mouse model. In addition, treatment with L-buthionine-sulfoximine (BSO) to deplete glutathione decreased the ESCC tumor burden in mice, thus demonstrating the critical role of glutathione metabolism in ESCC progression. BSO treatment also led to decreased cell proliferation and activation of cell apoptosis in ESCC. Finally, BSO treatment blocked NF- κ B pathway activation in ESCC. Our study reveals a new pathway that regulates ESCC progression and suggests that inhibition of glutathione metabolism may be a potential strategy for ESCC treatment.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most prevalent cancers in China, with an incidence of 43 cases per 100 000 persons annually.¹ Current standard treatments for ESCC include surgery, chemotherapy and radiotherapy. However, the 5-year survival rate of ESCC patients remains relatively low at ~10%.² Hence, identification of effective therapeutic targets is essential for ESCC treatment.

Glutathione, the most prevalent cellular antioxidant in mammals, participates in two metabolic processes, including reducing glutathione to its oxidized product and a conjugation reaction mediated by glutathione S-transferase (GST).³ The liver is typically the site with the highest levels of GSTs. However, certain isozymes of GSTs are also present in high amounts in the esophagus.⁴ Proteins associated with glutathione metabolism have long been associated with ESCC. For example, GSTO1, GSTP1 and GST-pi have been recognized as tumor-associated antigens and act as indicators of prognosis in human ESCC.^{5–7} Moreover, previous findings

have demonstrated that a GSTM1 deletion polymorphism is associated with an increased ESCC risk.⁸ In contrast, another study has demonstrated that the GSTM1 non-null genotypes are highly represented in ESCC patients compared with control patients in China.⁹ However, the functional role of glutathione metabolism in ESCC remains largely uncharacterized. In skin SCC, the glutathione metabolism pathway is specifically enriched in the TGF- β -activating cancer stem cell population and is responsible for the chemoresistant property of these cells,¹⁰ thus suggesting that glutathione metabolism might be critical for ESCC tumor progression.

NF- κ B has a critical role in a various stages of cancer initiation and progression. A previous study has demonstrated that the levels of NF- κ B p65 in ESCC samples correlate with clinical staging, lymph node metastasis and tumor differentiation.¹¹ Furthermore, survivin and NF- κ B p65 have important roles in ESCC tumor progression.¹² Importantly, depletion of glutathione with diethyl maleate leads to inhibition of NF- κ B target gene expression induced by TNF α ,¹³ thus

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indicating that glutathione metabolism block ESCC tumor progression by functioning through the NF- κ B signaling pathway.

In this study, we used an ESCC mouse model to examine the unique gene profiles in ESCC. We found that glutathione metabolism was among the top enriched pathways in ESCC. To characterize the role of glutathione metabolism in ESCC progression, we treated mice with L-buthionine-sulfoximine (BSO), which depleted the glutathione levels in ESCC. BSO treatment led to a decreased tumor burden, inhibition of cell proliferation and activation of cell apoptosis. Finally, we demonstrated that depletion of glutathione caused inhibition of the NF- κ B pathway. Our *in vivo* findings provide evidence that the glutathione metabolism pathway contributes to ESCC progression via inhibition of the NF- κ B signaling pathway and provides a groundwork for further development of a glutathione metabolism inhibitor to treat ESCC.

MATERIALS AND METHODS

Mice and chemical administration

Six- to eight-week-old Balb/c mice were purchased from Guangdong Laboratory Animal Center, Guangzhou, China. All animal procedures were performed by following the Institutional Animal Care and Use Committee (IACUC) approved protocols. For ESCC induction, 4-nitroquinoline 1-oxide (Sigma, Shanghai, China) was dissolved in propylene glycol (Sigma) and diluted in the drinking water at a final concentration of 100 μ g ml⁻¹. For glutathione depletion in mice, BSO (Sigma) was dissolved in drinking water at a concentration of 20 mM. Control and BSO-treated esophagi were dissected from mice, flash frozen in liquid nitrogen and homogenized. Approximately 50 mg of tissue was collected for determination of GSH content, as previously described.¹⁴ GST and GPX activity were measured using a GST Activity Assay Kit (Abcam, Shanghai, China) and Glutathione Peroxidase Cellular Activity Assay Kit (Sigma), respectively.

Immunohistochemistry

Paraffin sections of ESCC tissue samples from mice were antigen retrieved, blocked and processed as previously described.^{15,16} Primary antibodies to mouse K14 (ab7800, 1:500), p63 (ab53039, 1:100), PCNA (ab29, 1:200) and cleaved Caspase3 (ab13847, 1:100) were purchased from Abcam.

RNA isolation and quantitative RT-PCR

ESCC samples were flash frozen in liquid nitrogen and homogenized with a mortar and pestle, as previously described.¹⁷⁻¹⁹ Total RNA was extracted using TRIzol (Invitrogen, China).²⁰ Quantitative RT-PCR was performed as previously described.¹⁹ The primers used for RT-PCR is listed below:

GAPDH F: 5'-TGGCCTCCGTGTTCTCTAC-3'
GAPDH R: 5'-GAGTTGCTGTGAAGTCGCA-3'
IL1A F: 5'-CGAAGACTACAGTTCTGCCATT-3'
IL1A R: 5'-GACGTTTCAGAGGTTCTCAGAG-3'
IL1B F: 5'-GCAACTGTTCTGAACTCAACT-3'
IL1B R: 5'-ATCTTTGGGGTCCGTCAACT-3'
IL6 F: 5'-TAGTCCTTCTACCCCAATTTCC-3'
IL6 R: 5'-TTGGTCCTTAGCCACTCCTTC-3'
MMP3 F: 5'-ACATGGAGACTTTGTCCCTTTTG-3'
MMP3 R: 5'-TTGGCTGAGTGGTAGAGTCCC-3'
MMP9 F: 5'-CTGGACAGCCAGACACTAAAG-3'

MMP9 R: 5'-CTCGCGGCAAGTCTTCAGAG-3'
CCL2 F: 5'-TTAAAAACCTGGATCGGAACCAA-3'
CCL2 R: 5'-GCATTAGCTTCAGATTTACGGGT-3'
CCL4 F: 5'-TTCCTGCTGTTTCTCTTACACCT-3'
CCL4 R: 5'-CTGTCTGCCTCTTTTGGTCAG-3'
BCL2 F: 5'-GTCGCTACCGTCGTGACTTC-3'
BCL2 R: 5'-CAGACATGCACCTACCCAGC-3'
BCL2A1A F: 5'-GGCTGAGCACTACCTCAGTA-3'
BCL2A1A R: 5'-TGGCGGTATCTATGGATTCCAC-3'
CCND2 F: 5'-GAGTGGAACTGGTAGTGTG-3'
CCND2 R: 5'-CGCACAGAGCGATGAAGGT-3'

RNA-Sequencing (RNA-Seq) library preparation and data analysis

For RNA-Seq library preparation, total RNA was first extracted using Trizol, as previously described,^{21,22} from control and ESCC tissues ($n=3$ each). mRNA was then isolated, fragmented and reverse-transcribed into the cDNA libraries with an mRNA-Seq preparation kit (Illumina, San Diego, CA, USA). Library quality was assessed on an Agilent Bioanalyzer 2100 (Agilent, Shanghai, China). The Illumina HiSeq2000 platform was used for single-end sequencing of cDNA libraries. Alignment of reads was performed using Tophat2 with the mm10 build of the mouse genome. Transcript assembly and differential expression genes were identified via Cufflinks software (Seattle, WA, USA).²³ RNA-Seq data were analyzed using the CummeRbund package in R.²⁴ Functional enrichment analysis of differential expression genes was performed using the DAVID Functional Annotation Clustering Tool. A heat map of differentially expressed genes involved in glutathione metabolism was generated using Heatmap Builder.²⁵

Western blot

Western blot analysis was performed as previously described.^{26,27} Primary antibodies included anti-phospho-p65 antibody (Cell signaling #3033, 1:1000), anti-p65 antibody (Cell Signaling #6956, 1:1000), anti-I κ B α antibody (Cell Signaling #3033, 1:1000), anti-I κ B α antibody (Cell Signaling #9242, 1:1000), anti-phospho-I κ B α antibody (Cell Signaling #6956, 1:2859) and anti- α -tubulin (Sigma-Aldrich #T-9026, 1:5000).

Statistics

Data are presented as the means \pm s.d. or s.e. The two-tailed Student's *t*-test and one-way analysis of variance were used to calculate statistical significance. A *P*-value of <0.05 was considered significant.

RESULTS

Mice developed ESCC after carcinogen treatment

To study ESCC *in vivo*, we modified a previously described carcinogen-induced mouse model.²⁸ Mice were administered drinking water containing 100 μ g ml⁻¹ carcinogen 4-nitroquinoline 1-oxide for 15 weeks and then were switched back to dH₂O. We analyzed the changes in the histopathology of the esophageal epithelia 15 weeks and 22 weeks after the initial carcinogen treatment. H&E staining results showed the esophageal epithelia from carcinogen-induced mice were noticeably thicker than those in the controls (Figure 1a). Immunohistochemical results revealed that the expression pattern of Keratin14 (K14) was expanded at both dysplasia and ESCC compared with that in normal controls (Figure 1a). K14 is typically specifically expressed in the proliferative basal

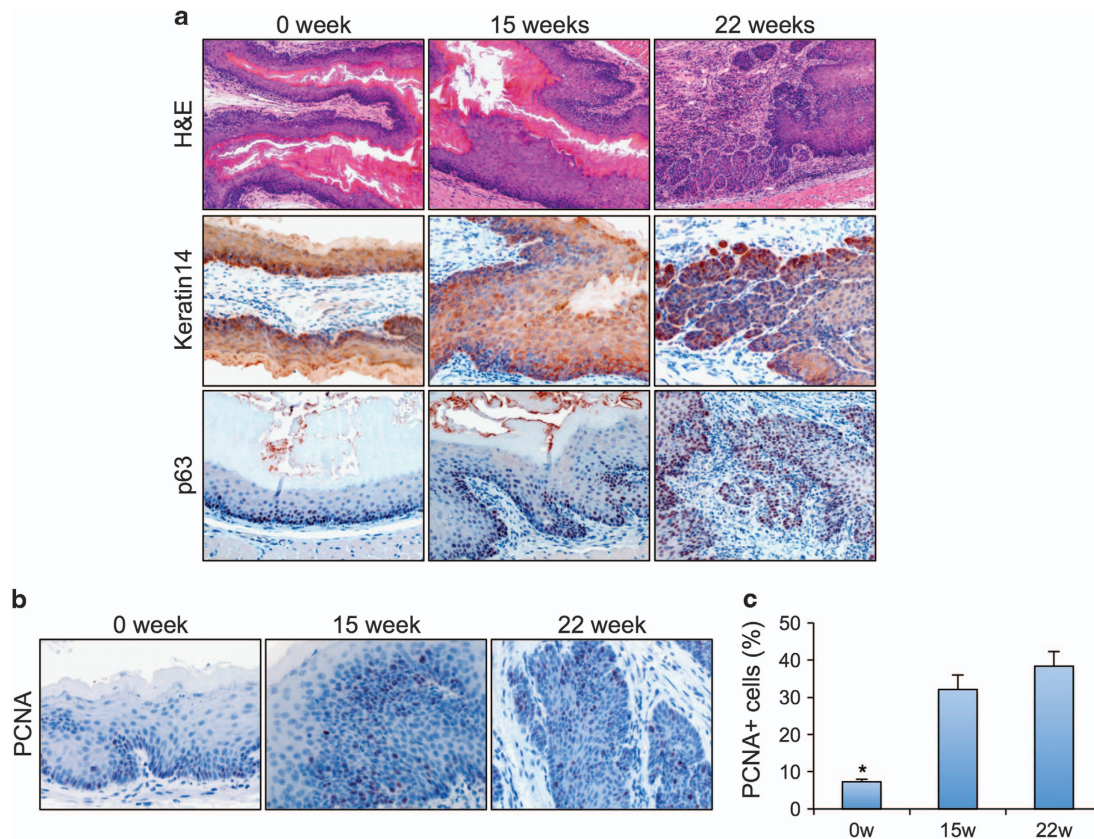


Figure 1 Mice treated with carcinogen developed dysplasia and ESCC. (a) H&E staining and K14 and p63 IHC staining of normal esophageal epithelium, dysplasia and SCC. (b) PCNA IHC for cell proliferation in normal esophageal epithelium, dysplasia and SCC. (c) Quantification of cell proliferation in normal esophageal epithelium, dysplasia and SCC. H&E, hematoxylin and eosin. IHC, immunohistochemical.

layer. However, we observed expression of K14 in the suprabasal layer of dysplasia and SCC. Furthermore, we examined the expression pattern of p63, which is elevated in human ESCCs.²⁹ In the control esophageal epithelia, we detected weak p63 protein expression levels in the basal cell layer. However, we observed upregulated p63 expression in both basal and suprabasal layers of dysplasia and ESCC samples (Figure 1a). Next, we quantified the proliferation index of the epithelial cells in the esophagus on the basis of the results from immunohistochemical, by using an antibody against PCNA (Figure 1b). We detected significant increases in cellular proliferation rates in both dysplasia ($32.1 \pm 3.9\%$, $P < 0.05$) and SCC ($38.4 \pm 3.8\%$, $P < 0.05$) compared with the normal control ($7.3 \pm 0.7\%$; Figure 1c).

Components of the glutathione metabolism pathway were enriched in ESCC

To probe the molecular events responsible for ESCC development, we extracted RNA from normal control and ESCC samples ($n = 3$ each) and performed RNA-Seq. To determine the differences between control and ESCC, we generated density plots of transcriptome-wide differential expression in both conditions (Figure 2a). Our data indicated that 797 transcripts were upregulated, and 1383 transcripts were

downregulated in ESCC compared with the control (Figure 2a and Supplementary Table 1). We performed KEGG pathway analysis to obtain a more comprehensive understanding of the transcripts that were enriched in the ESCC sample. Our results revealed that the specific upregulated pathways in ESCC included cell cycle, glutathione metabolism, DNA replication, p53 signaling pathway, focal adhesion, small cell lung cancer, ECM-receptor interaction, pyrimidine metabolism, progesterone-mediated oocyte and drug metabolism (Figure 2b). We generated a heat map of 16 differentially expressed transcripts involved in glutathione metabolism, including *Gsta1*, *Gclc*, *Gclm*, *Gsta3*, *Gsta4*, *Gpx2*, *Odc1*, *Rrm2*, *Rrm1*, *Mgst2*, *Gstp1*, *Gsr*, *Srm*, *Gsta2*, *Gss* and *Mgst1* (Figure 2c).

Inhibition of glutathione metabolism by BSO decreased the ESCC tumor burden

To investigate whether glutathione metabolism contributed to the progression of ESCC development, we treated mice bearing ESCC 20 weeks after initial carcinogen treatment with buthionine sulfoximine (BSO), a commonly used chemical inhibitor of γ -glutamylcysteine synthetase (γ -GCS) that decreases tissue glutathione concentrations (Figure 3a). Two weeks after BSO treatment, mice were killed for analysis. Mice treated

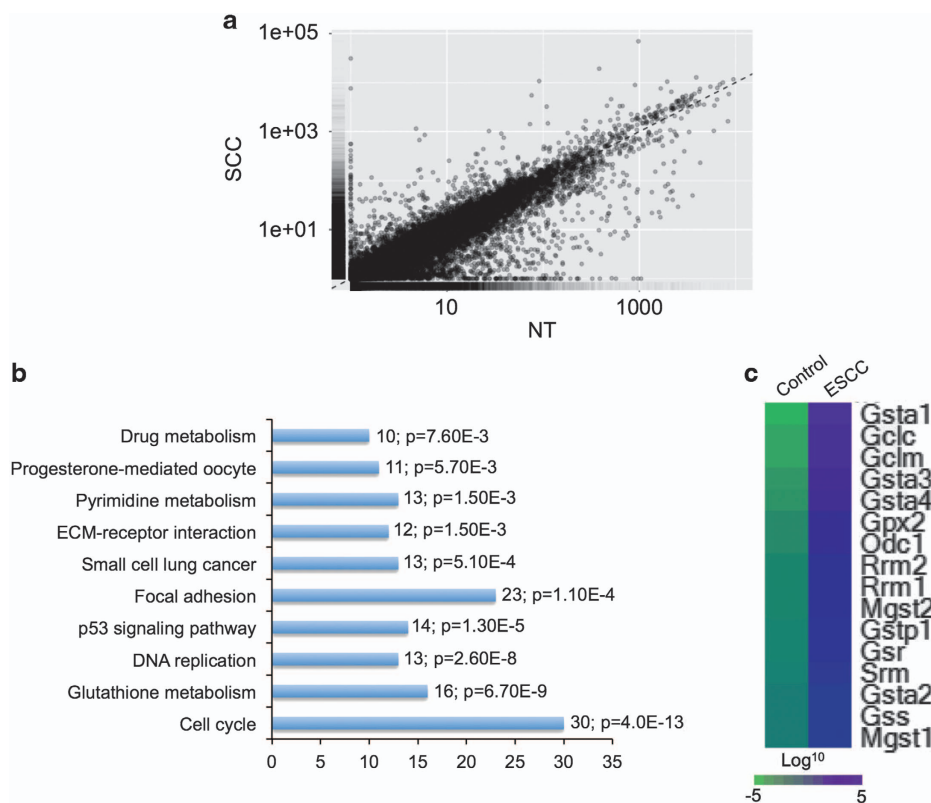


Figure 2 RNA-Seq revealed the upregulation of the glutathione metabolism pathway in ESCC. **(a)** Scatter plot generated from RNA-Seq data demonstrates that a large number of transcripts were altered in ESCC compared with control. **(b)** Selected enriched KEGG pathways in ESCC, ordered from top to bottom from highest to lowest *P*-value. Numbers of transcripts and *P*-value in each pathway are indicated in the text next to each pathway. **(c)** Heat map of 16 differentially expressed genes involved in glutathione metabolism.

with BSO, compared with control mice, exhibited a significant recovery of body weight (Figure 3b). In addition, the tumor lesion numbers per mouse were significantly decreased in mice treated with BSO (Figure 3c). Finally, we validated that glutathione concentrations, GSH transferase and GSH peroxidase activity were inhibited in the esophageal tissues treated with BSO (Figures 3d–f).

Next, we examined the cell proliferation rates of ESCC cells after BSO treatment by examining PCNA expression. Our immunohistochemical and quantification results demonstrated a clear inhibition of cell proliferation in ESCC treated with BSO (Figures 4a and b). Furthermore, the apoptotic cells in ESCC treated with BSO, compared with the control samples, exhibited a dramatic increase (Figures 4c and d).

Treatment of BSO inhibited the NF- κ B signaling pathway in ESCC

A previous report has demonstrated that the NF- κ B signaling pathway is constitutively activated in ESCC cell lines and is a potential target for ESCC treatment.³⁰ Moreover, depletion of glutathione leads to decreased expression of NF- κ B genes in hepatocytes. However, whether BSO treatment inhibits NF- κ B signaling transduction in ESCC *in vivo* remains unknown. To test this possibility, we examined the expression of total p65 and phosphorylated-p65 (p-p65). As expected, we detected

decreased levels of p-p65 in ESCC samples treated with BSO (Figure 5a). Furthermore, p-I κ B α was significantly repressed by BSO (Figure 5a), thus suggesting that BSO blocks NF- κ B signaling transduction. Moreover, mRNA expression levels of NF- κ B downstream targets, including *Il1a*, *Il1b*, *Il6*, *Mmp3*, *Mmp9*, *Ccl2*, *Ccl4*, *Bcl2*, *Bcl2a1a* and *Ccnd2*, were downregulated in ESCC by BSO (Figure 5b).

DISCUSSION

ESCC is often refractory to current therapeutic treatment and has poor outcomes. Therefore, effective approaches for treating esophageal cancer are urgently needed. To explore the cellular and molecular events that regulate ESCC development, we utilized a previously established mouse model combined with a high-throughput sequencing assay. Glutathione metabolism was among the top pathways enriched in ESCC. Our study provides the first report that glutathione metabolism is required for ESCC progression in an animal model, thus suggesting that this model is an excellent tool for testing glutathione metabolism inhibitors in the prevention and treatment of ESCC.

Numerous malignancies contain higher glutathione levels than those found in normal tissues. Glutathione content is involved in mutagenic mechanisms, DNA synthesis and drug resistance.¹⁰ Although it has long been proposed that

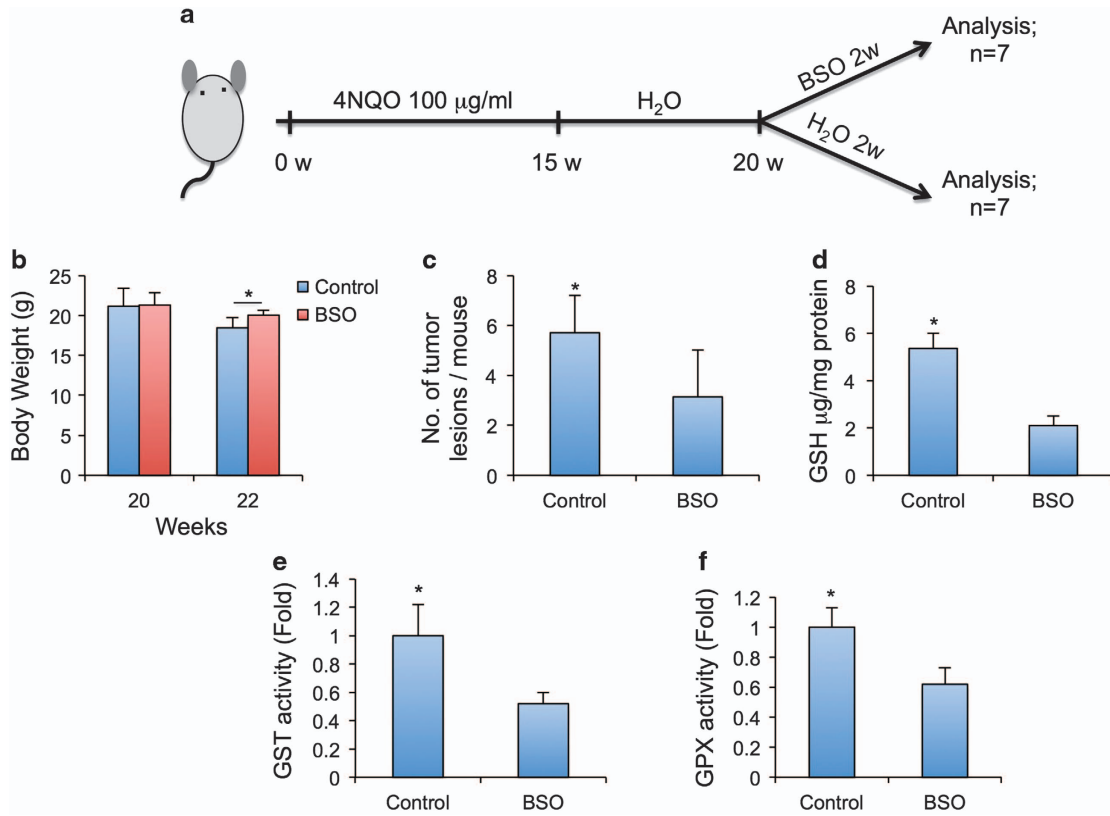


Figure 3 Depletion of glutathione by BSO reduced the ESCC tumor burden. (a) Experimental schematic representation. (b) Body weights of control and BSO-treated mice before and 2 weeks after BSO treatment. (c) Incidence of esophageal tumors after BSO treatment in ESCC-bearing mice. (d) Glutathione concentration in BSO-treated and control esophagi. (e) BSO treatment decreases cellular GSH activity in ESCC cells. (f) BSO treatment decreases cellular GPX activity in ESCC cells.

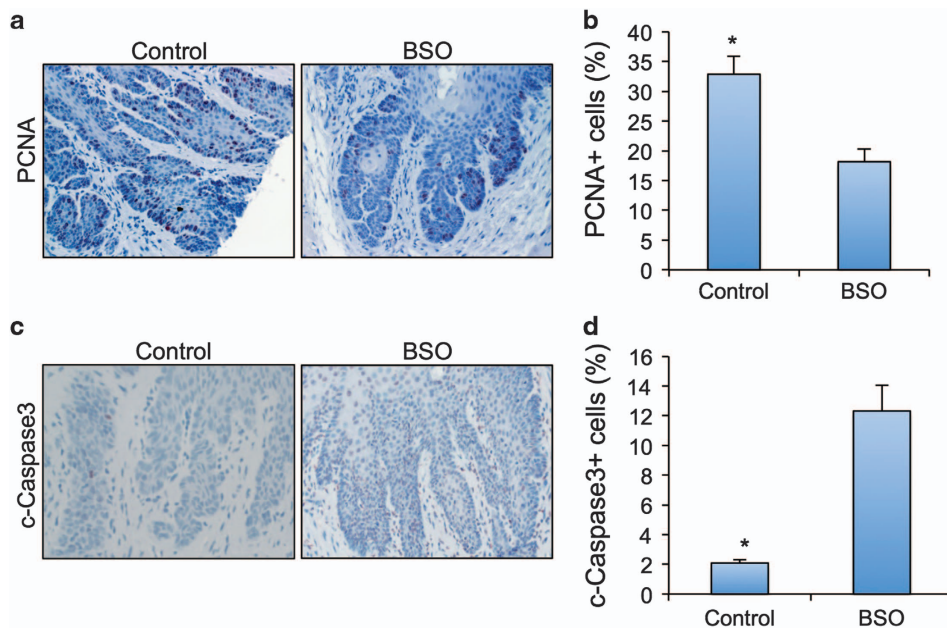


Figure 4 BSO decreased cell proliferation and induced cell apoptosis in ESCC. (a) PCNA IHC for cell proliferation in control and BSO-treated ESCC. (b) Quantification of cell proliferation in control and BSO-treated ESCC. (c) Cleaved caspase3 indicating cell apoptosis in control and BSO-treated ESCC. (d) Quantification of cell apoptosis in control and BSO-treated ESCC. IHC, immunohistochemical.

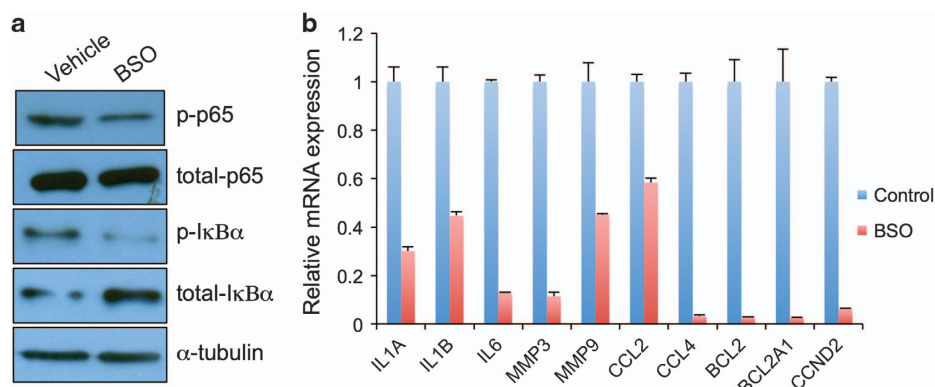


Figure 5 BSO downregulated the NF- κ B signaling pathway. (a) Western blot analysis of p-p65, total p65, p-I κ B α and total I κ B α proteins. Equal protein loading was controlled for with an anti- α -tubulin antibody. (b) mRNA expression levels of target genes of the NF- κ B signaling pathway.

application of selective GSH-depleting approaches will be beneficial in cancer therapy, the strategy remains elusive.³¹ In our study, we demonstrated inhibition of the glutathione metabolism pathway by BSO, which decreased the ESCC tumor burden in mice. However, the full toxicity of this treatment must be more rigorously tested in the future to prevent any major side effects.

The activation of key NF- κ B signaling pathway components was inhibited by BSO treatment, thus suggesting that NF- κ B might have an important role in ESCC progression. Consistently with these results, a recent report has established a positive correlation among the expression of NF- κ B p65 and clinical staging, lymph node metastasis and tumor differentiation of ESCC.¹¹ In addition, the NF- κ B pathway was constitutively activated in the ESCC cell lines. Silencing of p65 exhibits anti-proliferative effects and confers the chemodrug sensitivity of the ESCC cells.³⁰ NF- κ B is a positive regulator of GSTP1-1, thus suggesting the importance of NF- κ B in the GSH pathway.³² However, the detailed mechanism regarding how glutathione depletion leads to inhibition of the NF- κ B signaling pathway remains largely uncharacterized. A previous study using hepatocytes has shown that glutathione depletion represses TNF α -triggered NF- κ B activity through both I κ B kinase-dependent and -independent routes.³³ In addition, endothelial cells treated with GSH exhibit blockade of NF- κ B activity decreased expression of NF- κ B downstream target gene inter-cellular adhesion molecule-1 (ICAM-1).³⁴ Our data showed that BSO treatment indeed caused inhibition of the phosphorylation status of I κ B α in ESCC, suggesting that inhibition of the NF- κ B signaling pathway in ESCC by BSO depends on I κ B α . However, we could not exclude the possibility that other alternative pathways might be involved in this inhibition.

In summary, we combined a carcinogen-induced ESCC mouse model with RNA-Seq to investigate the molecular changes associated with the progression of ESCC. We demonstrated that BSO treatment significantly decreased the ESCC tumor burden in mice. Additionally, BSO treatment attenuated NF- κ B signaling transduction and repressed NF- κ B target gene

expression. Our model provides an excellent tool to determine the prognostic markers for ESCC and provides support for the development of potential drugs for prevention or treatment of ESCC.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contributions: LP and RL conceived the ideas, designed the experiments, collected the data and wrote the manuscript. DC, JY, XK, XW and YH participated in data analysis and manuscript editing. YH, YZJ and JY supervised the project.

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Supplementary Information accompanies the paper on Experimental & Molecular Medicine website (<http://www.nature.com/emm>)