Inspiratory Hyperoxia Suppresses Lung Cancer Metastasis Through A MYC/SLC1A5-Dependent Metabolic Pathway

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Abstract

**Background** The lack of knowledge about the effect of inspiratory hyperoxia (IH) on the lung-specific tumor microenvironment and progression of lung cancer has attracted considerable attention.

**Methods** The effects of different oxygenation parameters on the proliferation, apoptosis, invasion and migration of lung cancer cells were systematically evaluated *in vitro* and *in vivo*, including CCK-8, transwell and metastasis models. Mechanistically, transcriptome, proteome and metabolome analysis were combined to reveal the effects of IH on the malignant phenotype of lung cancer cell. Luciferase reporter analysis, Western blot, RNA immunoprecipitation and immunohistochemical staining were performed to examine the detailed regulatory mechanism of IH regulating metabolic reprogramming of lung cancer cells.

**Results** Our results reveal that IH treatment (60% O$_2$, 6h/day) not only has no tumor progression-promoting effects, but also suppresses lung cancer metastasis and promotes long-term survival. MYC/SLC1A5-induced metabolic reprogramming and glutamine addiction serves as a new mechanism that drives lung cancer metastasis, which can be significantly suppressed by IH.

**Conclusion** MYC/SLC1A5-induced metabolic reprogramming and glutamine addiction is a new mechanism that drives lung cancer metastasis, which can be significantly suppressed by IH treatment. These findings are relevant to the debate on the perils, promises and antitumor effect of IH, especially for patients with lung cancer.

**Background**

The tissue of tumor origin is a crucial factor determining the intrinsic malignant phenotype of a tumor and the characteristics of external tumor microenvironment (TME)[1–3]. Tumors originating in the same organ usually have similar gene expression signatures since they retain the transcriptional features of the parental tissue. The tissue of origin can also create an organ-specific TME for cancer cells, including epigenetically regulated gene expression, cellular composition and tissue architecture[4, 5]. The TME with organ-specific characteristics will impose a variety of non-cell independent pressures on cancer cells and participate in the regulation of tumor initiation, progression, and metastasis. Notably, the TME of lung tumors is extremely unique due to distinct environment created by the gas exchange in which the oxygen concentration is constantly changing[6, 7]. However, although this must be an acute challenge to lung tumors, few studies have been conducted to explain the impact of this lung-specific TME on the progression of lung cancer.

The prognosis of patients with lung cancer remains dismal due to its aggressive malignancy and profound resistance to various forms of therapy. Thus, there is a strong impetus to identify new therapeutic targets for lung cancer. Inspiratory hyperoxia (IH) has been widely used as an adjuvant therapy in many clinical settings[8]. The effect of IH on cancer growth and metastasis remains controversial, especially in lung cancer. Existing studies have reported that, due to its ability to improve
the oxygenation status of tumors, IH can be used in conjunction with radiotherapy or chemotherapy, but this application also raises concerns that an increased oxygen pressure may stimulate tumor growth via reoxygenation of hypoxic tumor cells and increased neovascularization[9–11]. Recently, Hatfield et al.[12] suggested that supplemental oxygenation (60%) can weaken the hypoxia-driven and A2A or A2B adenosine receptors (A2AR/A2BR)-mediated (hypoxia-A2-adenosinergic) immunosuppression in the TME and serve as an immunological co-adjuvant in combination with current lung cancer immunotherapies. Notably, the effect of IH on the TME of lung tumors is comprehensive and subversive. Not only immune cells, but also mixed cells, including cancer cells, endothelial cells and stromal cells, are under stress from lung-specific TME. Therefore, whether IH has anti- or pro-tumorigenic effect on lung tumors needs to be further studied.

In recent decades, the advanced metabolic analysis of cancer cells has expanded the understanding of the consequences and mechanisms of cancer metabolism in different stages of tumorigenesis[13, 14]. The classical oncogenic drivers, such as MYC, KRAS, etc., can induce metabolic reprogramming in cancer cells, which allows them to actively assist tumor growth and dissemination in nutrient- and oxygen-limited environments[15–17]. Glucose and glutamine are major anabolic carbon sources supporting cancer cell survival and biosynthesis in most tumors. In some malignant tumors, aberrantly activated oncogenes drive cancer cells into a state of nutritional addiction or to use unconventional nutritional catabolism pathways, such as the Warburg effect and glutamine addiction[18]. Recent studies have shown that the metabolic phenotype of tumors is also affected by the organ of origin and TME. An excellent example is that MYC activates glutamine catabolism in lung tumors, but in liver tumors it activates glutamine synthesis [19].

Here, we hypothesized that the change in oxygen concentration is of unique significance to the TME and the malignant phenotype of lung tumor cells. To confirm this hypothesis, in this study the effects of different oxygenation parameters on the proliferation, apoptosis, invasion and migration of lung cancer cells were systematically evaluated in vitro and in vivo. Transcriptome, proteome, and metabolome analysis were combined to determine the effect of supplemental oxygen on the malignant phenotype of lung cancer and the detailed underlying mechanism. The findings of this study will provide an adequate theoretical basis to clarify the safety of oxygenation therapy for lung cancer patients and ascertain whether it has the potential to be used as an adjuvant in combination with current lung cancer treatment methods.

**Methods**

**Cell Lines**

The immortalized human lung epithelial cell line BEAS-2B, Lewis lung carcinoma (LLC) cells and the human lung cancer cell lines H1299 and A549 were obtained from FuHeng Biology (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 medium (Gibco/Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, and
1% penicillin/streptomycin solution, in an incubator at 37 °C, with a humidified atmosphere containing 5% CO₂.

**Mice**

Balb/c nude mice (4-6 weeks old) were purchased from Beijing Weitong Lihua Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were approved by the Animal Protection and Utilization Committee of Xuzhou Medical University (Xuzhou, China). No preference in mouse sex was given for any of the studies.

**Clinical samples**

A series of tissue samples from patients with non-small cell lung cancer (NSCLC) who did not receive radiotherapy or chemotherapy were obtained from the Department of Pathology of Shanghai Pulmonary Hospital. All specimens were obtained under the guidance of the U.S. Health Insurance Portability and Accountability Act (HIPAA) protocol and supervised by the ethics committee of the hospital, and were pathologically confirmed as NSCLC.

**Establishment of lung cancer model**

An orthotopic lung tumor model was established as previously reported[20]. Briefly, Balb/c nude mice were anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). After confirming that mice have been adequately anesthetized, LLC cells (1.0 × 10⁶) with 50% Matrigel matrix (Corning Inc., Corning, NY, USA) were injected orthotopically into the left lung. Mice were examined daily for infection, bleeding, weight loss, lethargy, and changes in food and/or water consumption.

For the lung metastasis model, luciferase-labelled LLC and H1299 cells×10⁶ were injected into mice through the tail vein. The fluorescence expression was detected after 1 months, and then the lungs were harvested.

**Hyperoxic exposure**

Mice were placed in an ATTENDOR animal gas control system (China Innovation Instrument Co., Ltd., Ningbo, China), and exposed to a well-controlled gas composition to mimic protocols of supplemental oxygen delivery to humans. This system provides an independent environment with a controllable oxygen concentration for animals. The gas monitoring module can effectively control carbon dioxide emissions and supply gas of excellent quality for the breathing of mice. The oxygen concentrations of 21, 30, 60, 90 and 98% were selected and supplied to mice for 3, 6 and 12 h per day for 30 days.

A Smartor-118-three gas incubator (China Innovation Instrument Co., Ltd.) was used to mimic protocols of supplemental oxygen delivery in vitro. BEAS, LLC, H1299 and A549 cells were exposed to hyperoxia (60% O₂ / 5%CO₂ / 35%N₂) for 4, 8, 12, 24, 48 and 72 h.
Tumor xenograft study

H1299 cells (5 × 10^6) were injected subcutaneously into the flanks of mice. Then, mice in the hyperoxia group were treated with IH (60% oxygen) for 21 days, and the mice in the control group were routinely fed. Tumor volume (V) was monitored every 4 days by measuring the long axis (L) and the short axis (W) of xenograft tumors and calculated using the following formula: \( V = \frac{(L \times W^2)}{2} \).

RNA sequencing analysis

Total RNA was isolated from the H1299 cells exposed to hyperoxia (24 h) and control cells, quantified and its integrity checked. The high-throughput RNA sequencing analysis was performed by Shanghai Aksomics Biotech Co. Ltd. (Shanghai, China). Briefly, the ribosomal RNA (rRNA) was removed from the total RNA using the NEBNext® rRNA Depletion Kit (New England Biolabs, Inc., Ipswich, MA, USA) following the manufacturer's instructions. RNA libraries were constructed using the NEBNext® Ultra™ II Directional RNA Library Prep Kit (New England Biolabs, Inc.) according to the manufacturer's instructions. Then, the HTSeq software (v0.9.1) was used to obtain the transcript level raw count as the expression profiling, and the edgeR software (v3.16.5) was used to perform normalization, and differentially expressed genes were identified by their p-value and expression fold change.

Liquid chromatography tandem Mass spectrometry (LC-MS/MS) for proteome analysis

H1299 cells (1.0 × 10^7) were lysed in 1,000 µL RIPA buffer at 4 °C. The total protein content of each sample was then determined using a bicinchoninic acid (BCA) protein assay. Then, after performing acetone precipitation and trypsin digestion, the samples were labeled with tandem mass tag (TMT). Subsequently, after removing the sodium deoxycholate (SDC) from the samples, the peptides were desalinated. After reverse-phase high-performance liquid chromatography (RP-HPLC), peptides were fractionated into 120 fractions using high pH RPRP-HPLC, and then combined into 8 fractions. For each fraction, ~2 µg peptide were separated and analyzed using a nano-UPLC (Easy-nLC 1200) coupled to Q-Exactive mass spectrometry (Thermo Fisher Scientific Inc.).

The original data obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) were searched and quantified using the Max Quant software (version 1.5.6.0; Max-Planck-Institute of Biochemistry, Martinsried, Germany). The protein sequence database (Uniprot_organism_2016_09) was downloaded from UNIPROT. This database and its reverse decoy were then searched using the Max Quant software. Both peptide and protein FDR should be less than 0.01. Only unmodified unique peptides were used for quantification. All the other parameters were kept as default.

Metabolomics

The 1260 infinity high-performance liquid chromatography (HPLC) system (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with Q-Exactive MS/MS (Thermo Fisher Scientific Inc.) was used for metabolomics analysis in this study. Cells were washed twice with ammonium carbonate (75 mM) at pH
7.4 and snap frozen in liquid nitrogen. Metabolites were extracted with MeOH: acetonitrile (ACN) (1:1, v/v). The samples were incubated for 1 h at -20 °C, followed by 15 min centrifugation at 20,000 g and 4 °C to precipitate proteins. After drying and reconstituting with ACN: H₂O (1:1, v/v), the extracts were centrifuged for 15 min at 20,000 rpm to remove insoluble debris. Samples were stored at −80°C until LC/MS analysis.

For LC/MS analysis, samples were separated on an amide column with a mobile phase A consisting of water mixed with ammonium acetate (25 mM) and ammonium hydroxide (25 mM) and a mobile phase B consisting of ACN. The MS/MS analysis coupled with HPLC was performed on a Q-Exactive MS/MS system (Thermo Fisher Scientific Inc.). The different data-dependent analysis (DDA) methods were performed as follows: full scan range: 60 to 900 (m/z); resolution for MS1 and ddMS2: 70,000 and 17,500, respectively; maximum injection time for MS1 and ddMS2: 100 and 45 ms, respectively; automatic gain control (AGC) for MS1 and ddMS2: 3e⁶ and 2e⁵, respectively; isolation window: 1.6 m/z; normalized collision energies (NCE): 10, 17, 25 or 30, 40, 50. The full scan method was performed as follows: the full scan range was 60–900 (m/z); the AGC was 3e⁶ ions; resolution was 140,000; and the maximum injection time was 100 ms.

**Preparation of plasmids, lentivirus, and stable cells**

Short hairpin RNAs (shRNAs) were designed and synthesized by GeneChem Co., Ltd. (Shanghai, China). The shRNA-control vector and shRNA-SLC1A5 or shRNA-MYC were transfected into H1299 and A549 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). Cells were infected with lentivirus for 48 h and then selected with 2 ng/ml puromycin for 2 weeks, refreshing the medium every 3 days.

**Cell proliferation**

Cell proliferation was measured by the cell counting kit-8 (CCK-8) and 5-ethynyl-2'-deoxyuridine (EdU) incorporation. Cells (5×10³) were seeded in 96-well plates, and the recommended volume of CCK-8 solution was added at 24, 48, and 72 h, respectively. The EdU incorporation assay was performed using an EdU Incorporation kit, following the instructions. Briefly, cells were inoculated in 96-well plates (3×10³), cultured in 60% oxygen for 24 h, and incubated in EdU at a concentration of 50 µM for 2 h. Cells were then fixed and stained with Apollo dye (which reacts with EdU to detect it). Ultimately, cell nuclei were stained with Hoechst33342.

**Cell invasion, migration, and wound healing assays**

For the migration assay, H1299, A549 and LLC cells (2~4×10⁴) were seeded in serum-free medium in the upper chamber and cultured in 60% oxygen for 24 h. For the invasion assay, the Transwell filter inserts were coated with Matrigel, and 1×10⁵ cells were seeded in serum-free medium in the upper chamber and in 60% oxygen for 24 h. Cancer cells that traversed the membrane were stained with crystal violet (0.04%) and counted.
Analysis of cell apoptosis

The Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (KeyGen Biotech Co., Ltd., Nanjing, China) was used for the detection of apoptotic cells with corresponding treatment.

RNA extraction, reverse transcription, and qRT-PCR analysis

RNA was extracted with TRIzol (Invitrogen) and cDNA was synthesized according to the method described in the HiScript First Strand cDNA synthesis kit (Vazyme Biotech Co., Ltd., Nanjing, China). The qRT-qPCR analysis was performed according to the procedure described in the UltraSYBR one-step RT-qPCR kit (CWBIO, Beijing, China). The primers used for qRT-PCR analysis are listed in Supplementary Table 1.

Western blot analysis

Cells were lysed using the Cell Total Protein Extraction Kit (Sangon Biotech, Shanghai, China) for whole cell lysate. Total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 μm polyvinylidene fluoride (PVDF) membranes (MilliporeSigma, Burlington, MA, USA). After blocking with 5% non-fat milk for 1 h, primary antibodies against SLC1A4, SLC1A5, c-MYC, TBX4, TBX15, BACH1, HK2, PFK2, MCT1, GPI, HIF1α, GAPDH and Tubulin were separately incubated with the membranes at 4 °C overnight. Subsequently, the membranes were incubated with the corresponding secondary antibody for 1 h at room temperature. Eventually, the immunoreacted protein bands were visualized. The antibody used in this study were listed in Supplementary Table 3.

Enzyme-linked immunosorbent assay (ELISA)

To detect intracellular glutamine and glutathione, cells were incubated overnight under normal conditions, followed by a change to fresh medium and continued incubation at 21% oxygen and 60% oxygen for 12, 24, and 48 h. The culture medium was removed and cells were rinsed with PBS before adding the appropriate amount of lysis buffer to obtain the cell lysate. Then, the cell lysate was centrifuged at 10,000-14,000g for 3-5 minutes and the supernatant was collected and used in subsequent measurements as described in the kit.

Extracellular flux measurements

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were determined using an Agilent Seahorse XFe96 extracellular flux analyzer (Agilent Technologies Inc.) as described in the manufacturer’s protocol. Cells were seeded in 96-well microplates the day before the experiment and incubated in a normal incubator or a 60% hyperoxia incubator for 12 h. The hydrated probe plates were placed in a CO₂-free incubator overnight. Glucose (10 mM), oligomycin (1 mM) and 2-Deoxy-d-glucose (2-DG; 50 mM) were added successively to conduct the glycolytic stress test. Oligomycin (1.5 mM), carbonyl
cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 1 mM) and rotenone and antimycin A (0.5 mM) were added successively to conduct the mitochondrial stress test.

**Immunohistochemical (IHC) staining**

The tissues microarrays were blocked with goat serum for 30 min before adding the primary antibody. Specimens were incubated with primary anti-SLC1A5 antibody for 12 h at 4 °C. Eventually, the microarrays were stained with 3, 3-diaminobenzidine solution and hematoxylin. The slides were photographed with an inverted microscope (Olympus Corporation, Tokyo, Japan).

Two pathologists separately assessed the specimens under blinded experimental conditions. The expression levels of SLC1A5 were evaluated by combining the percentage of cells with the staining intensity. The intensity of immunoreacted SLC1A5 was scored using a 0-3 scale (0, negative; 1, weak; 2, moderate; 3, strong); the percentage of immunoreactivity cells was graded as 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The staining index value (values, 0-12) was determined by multiplying the score for staining intensity by the score for the positive area. For statistical analysis, the level of SLC1A5 expression was categorized as low (0-4), middle (4-8) and high (8-12) expression.

**Chromatin immunoprecipitation (ChIP) analysis**

Cells were cultured under normal conditions for 24 h and chromatin immunoprecipitation ChIP Assay was performed using a ChiP assay kit (Beyond) using the procedure described by the manufacturer of the kit. Cells (1x10⁶) were fixed in 1% formaldehyde for 10 min. Nuclei extracts were sonicated to fragment DNA to 200-1000bp. The fragmented chromatin obtained was divided into two ChiP reactions (C-MYC or control IgG). DNA was enriched by immunoprecipitation using 4 mg of anti-C-MYC or normal rabbit IgG control antibody complexed with protein A agarose beads. Protein K treatment removed the protein and unenriched DNA samples were treated in the same way as control input. PCR was performed on the CHIP and input DNA using primers for the SLC1A5 promoter region containing the MYC binding site.

**Luciferase reporter assay**

The luciferase reporter assay was performed according to a previous report[21]. Briefly, the luciferase reporter vector was constructed using 0.5 μg of pGL3 plasmid containing the putative MYC binding site or response element or its mutant. The constructed luciferase reporter vector was transiently co-transfected in triplicate into 293T cells. The Dual Luciferase Kit (Promega Corporation, Madison, WI, USA) was used to determine the luciferase activity. Firefly luciferase activity was normalized to the Renilla luciferase control value and shown as the average of triplicate measurements.

**Quantification and statistical analysis**

The statistical analyses were performed using GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Values are presented as the mean ± SEM unless stated otherwise. Details of the specific statistical analysis are indicated in the figure legends.
Results

1. Inspiratory hyperoxia suppresses lung cancer metastasis.

To determine the effects of long-term IH on lung cancer cell, we established a lung cancer model in immunodeficient mice. Using a gas control delivery system, mice were exposed to various concentrations of oxygen (21, 30, 60, 90 and 98% oxygen) to mimic protocols of supplemental oxygen delivery to humans (Fig. 1A). Tumor-free mice exposed to 98% oxygen (6h/day) had a mortality rate higher than 50% within 30 days. Such mortality rate may be the result of oxygen toxicity, nonspecific inflammatory responses and extensive alveolar hemorrhage (Fig. S1A, 1C). Tumor-free mice exposed to 60% oxygen for less than 6 h did not affect their survival. Histological examination revealed that about 14% of the mice exposed to 60% oxygen had non-fatal emphysema or pulmonary bulla (Fig. S1B, 1D). In addition, mice exposed to 60% oxygen showed no significant difference in general physical signs (heart rate, body temperature, and respiration rate) except for weight loss (Fig. S1E). In general, the exposure to 60% oxygen (6 h/day) proved to be safe in long-term treatment.

Tumor-bearing mice exposed to 60% oxygen (6h/day) showed significantly improved survival and suppressed lung tumor progression (Fig. 1B, 1C). The incidence of lymph node metastasis and distant metastasis in the control group was 2.5-fold and 3-fold higher than that in the IH exposed mice, respectively (Fig. 1D). Immunohistochemical (IHC) staining showed that exposure to IH downregulated the expression of high mobility group AT-hook 2 (HMGA2) protein, which has been reported to be a marker of tumor metastasis (Fig. S1F)[22].

In Balb/c nude mice, IH also prevented LLC cells and H1299 cells from metastasizing to the lung and reduced the primary tumor burden and improved survival after intravenous (i.v.) injection (Fig. 1E-H). The results of a luciferase reporter assay using H1299 cells stably expressing firefly luciferase implanted into Balb/c nude mice by i.v. injection further confirmed that IH has antimetastatic effect (Fig. 1I).

2. Effects of Inspiratory hyperoxia on the proliferation, invasiveness and apoptosis of lung cancer cells.

To further evaluate the effects of IH on lung tissues and tumors, BEAS, H1299, A549, and LLC cells were cultured under high oxygen concentration (60% O₂, 5% CO₂, 35% N₂) to simulate IH in vitro. The measurement of the proliferation rate using the cell counting kit-8 (CCK-8) assay and 5-ethynyl-2'-deoxyuridine (EdU) incorporation showed that exposure to 60% oxygen for 24 h significantly reduced the proliferation rate of human normal lung epithelial cells (BEAS cell line), human lung cancer cells (H1299, A549, and LLC cell lines) (Fig. S2A-E). However, it did not alter the growth of primary tumors when H1299 cells wereinjected subcutaneously into the mice (Fig. S2F, 2G). Experiments using hypoxia reagent to label hypoxia area in primary tumors revealed that IH did not effectively reduce or reverse the hypoxic area in tumor xenografts (Fig. S2H). These data suggest that the inhibition of lung tumor growth by IH may be closely related to lung specific TEM.
In addition, exposure to hyperoxia for less than 24 h had no effect on apoptosis and necrosis in BEAS and lung cancer cell lines, but in LLC, H1299 and A549 cells exposed to 60% oxygen for more than 48 h caused significant apoptosis and necrosis. BEAS cells had a higher tolerance to hyperoxia-induced apoptosis and necrosis (Fig. S3). Cell migration, invasion and scratch assays showed that lung cancer cells exposed to 60% oxygen had lower invasive and migratory abilities (Fig. S4).

3. Transcriptomic and proteomic profiling of hyperoxia exposed H1299 cells.

RNA sequencing (RNA-seq, GEO: GSE192839) and tandem mass tag (TMT)-based quantitative proteomics analyses (iProX data license: IPX0003908000) on controls and hyperoxia exposed H1299 cells, and identified 59, 577 and 5,970 expressed RNAs and proteins, respectively (Fig. 2A, 2B). The expression levels of 5,460 identified proteins and their corresponding mRNAs showed a moderate correlation ($R^2 = 0.0312$, Fig. 2C, 2D). For mRNA and protein differential expression analyses, a false discovery rate (FDR) cut off of 5% and a 2-fold change threshold were used. When integrating these datasets, we identified 43 overlapping differentially expressed genes and proteins (Fig. 2E).

Gene ontology enrichment analysis revealed that these significantly differentially expressed genes and proteins were enriched in “carbohydrate metabolic process”, “trans-epithelial transport”, “ADP metabolic process” and cell motility and migration regulation process (Fig. 2F). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed a marked enrichment in cell metabolism as well as pathways associated with cancer development (Fig. 2G). To identify cellular pathways involved in lung cancer metastasis and metabolism, Gene Set Enrichment Analysis (GSEA) performed on the RNA-seq data determined that, compared with control cells, cells exposed to hyperoxia showed reduced enrichment of metastasis-related genes reported in the Human Cancer Metastasis Database (Fig. 2H)[23]. Furthermore, GSEA also revealed a significant decrease in metabolic pathways, including mitochondrial inner membrane, oxidative phosphorylation, glucose metabolism and glutamine metabolism genes, upon hyperoxia exposure (Fig. 2I, 2J, S5). In general, the multi-omics data suggested that exposure to IH may lead to significant metabolic reprogramming of lung cancer cells, which may explain its antitumor metastasis activity.


Our analysis of the cellular metabolome of normoxia and hyperoxia-exposed H1299 cells using untargeted metabolomics to establish a holistic overview of cancer metabolism mechanisms underlying hyperoxia treatment identified a total of 319 structurally named metabolite across both treatment groups. Compared with the normoxia group, in hyperoxia treated H1299 cells 13 metabolites, including 6-methylquinoline, hippuric acid and pyridoxal, were significantly increased, and 15 metabolites, including 4-hydroxybenzaldehyde, L-glutamine, asparagine and pyruvic acid, were significantly decreased (Fig. 3A-3G). Additionally, analysis of the KEGG metabolic library using the Metaboanalyst (3.0) software revealed that altered metabolites were enriched in “alanine, aspartate and glutamate metabolism”, “arginine biosynthesis”, “glyoxylate and dicarboxylate metabolism”, “TCA cycle” and “D-glutamine and D-glutamate
metabolism” processes (Fig. 3H). It is known that glucose and glutamine are the main anabolic carbon sources that support tumor cell proliferation and invasion. Therefore, we hypothesize that IH may cause changes in the metabolism of glutamine and glucose, thereby regulating the metastasis of lung cancer.

5. Inspiratory hyperoxia decreases the glutamine uptake of lung tumor.

The assessment of the effect of hyperoxia on glutamine metabolism in lung cancer cells through measuring the level of intracellular glutamine by enzyme-linked immunosorbent assay (ELISA) revealed that hyperoxia exposure caused a continuous decrease in glutamine in H1299 and A549 cells (Fig. 4A-4C). Heat map and gene enrichment analysis indicated that the metabolic pathway of glutamine was inhibited in lung cancer cells exposed to hyperoxia (Fig. 4D-4F). Quantitative real-time polymerase chain reaction (qRT-PCR) and immunoblotting analyses were used to confirm the regulatory effects of hyperoxia on the expression of genes largely involved in the transport, synthesis and catabolism of glutamine. The results indicated that solute carrier family 1 member 5 (SLC1A5), also known as ASCT2, a major glutamine transporter, which was highly expressed in normoxia H1299 and A549 cells, was downregulated by 60% oxygen treatment (Fig. 4G-4J).

In an in vivo lung metastasis mouse model, we found that SLC1A5 expression was significantly reduced in IH exposed lung tumors compared with that in the control group (Fig. 4K). However, 60% oxygen treatment had no effect on the expression of SLC1A5 in subcutaneously transplanted tumors in mice (Fig. 4L). We inferred that the IH-induced decrease in SLC1A5 expression in lung tumor led to reduced glutamine uptake and metabolic demands to regulate tumor metastasis. The measurement of glutamine uptake further confirmed that hyperoxia significantly decreased the intracellular transport of glutamine in lung cancer cells (Fig. 4M, 4N).

Furthermore, the level of glutathione was significantly increased in BEAS cells treated with 60% oxygen, suggesting that hyperoxia may play a role in the maintenance of cellular redox homeostasis. However, the opposite effect on intracellular glutathione was observed in H1299 and A549 cells (Fig. S6A-6C). We speculate that the hyperxia induced decrease of glutamine level may led to the obstruction of glutathione synthesis in lung cancer cells. Hyperoxia caused a more significant increase in reactive energy species (ROS) levels in lung cancer cells compared with BEAS cells (Fig. S6D-6E). Remarkably, the treatment with the antioxidant N-acetylcysteine (NAC) could prevent the apoptosis caused by exposure to hyperoxia for more than 48 h, but promoted cancer progression (Fig. S6G-6I).

6. The progression of lung cancer depends on glucose and glutamine catabolism.

Non-small cell lung cancer (NSCLC) cells have been reported to use glycolysis as the main pathway of glucose catabolism, even in an oxygen enriched environment, that is, the Warburg effect[24]. Previous metabolomic and transcriptomics studies have shown that exposure to hyperoxia leads to an increase in the heterogeneity of carbohydrate small molecule compounds and gene expression levels involved in glucose metabolism in lung cancer cells (Fig. S7A, 7B ). We analyzed the protein expression levels of glycolysis related enzymes and found lower levels of HK2, PFK2 and MCT1 in H1299 and A549 cells.
exposed to 60% oxygen (24 h) compared to controls (Fig. S7C). The measurement of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in BEAS, H1299 and A549 cells revealed that H1299 and A549 cells exposed to hyperoxia showed decreased basal and maximum ECAR and OCR compared to the control group (Fig. S7D-7I). The glycolytic capacity of lung cancer cells exposed to 60% oxygen for 12 h was 40–50% lower than that in controls. However, hyperoxia did not significantly affect the glycolytic capacity and mitochondrial respiratory capacity of BEAS cells (Fig. S7J-7O).

In addition, we also found that the proliferation and survival of both lung cancer cells and BEAS cells are heavily dependent on glucose. Compared with glucose deprivation, glutamine deprivation is more associated with reducing the aggressiveness of tumor cells, but less with inducing proliferation arrest and apoptosis of BEAS, H1299 and A549 cells (Fig. S8). These results suggest that antitumor strategies that target glutamine catabolism have greater safety and feasibility.

7. SLC1A5 mediates the hyperoxia-induced inhibition of glutamine catabolism.

Functional analysis of SLC1A5 showed that short hairpin RNA (shRNA)-mediated knockdown of SLC1A5 expression significantly decreased the proliferation, migration and invasion abilities of H1299 and A549 cells (Fig. 5A-5E, S9). In in vivo metastasis assays, SLC1A5 knockdown significantly inhibited metastasis of H1299 cells to the lung and reduced the primary tumor burden (Fig. 5F-5H). Further investigation of the role of SLC1A5 in the growth of lung tumor in a mouse xenograft model indicated that a low expression level of SLC1A5 could reduce tumor weight and volume (Fig. 5I-5L). Moreover, SLC1A5 overexpression increased the level of intracellular glutamine in lung cancer cells and partially reverse the hyperoxia-induced inhibition of invasion (Fig. S10).

8. MYC directly activates SLC1A5 transcription.

The possible mechanism of the down-regulation of SLC1A5 was further investigated by determining the expression of RNA-seq identified differentially expressed transcription factors by qRT-PCR and Western blotting analyses. The results showed that hyperoxia exposure significantly reduced the level of MYC in H1299 cells (Fig. 6A-6C). We proposed that the SLC1A5 gene may be a target of oncogenic MYC. The inactivation of C-MYC by JQ1 consistently reduced expression of SLC1A5 in H1299 and A549 cells. Similarly, MYC depletion led to the decrease of SLC1A5 expression in H1299 cells (Fig. 6D-6G). Among the 441 hyperoxia-induced differentially expressed genes, we identified a MYC-binding motif in the proximal promoters of 82 genes, including SLC1A5 (Fig. 6H, 6I). Analysis of a publicly available ChIP sequencing dataset (GSE80151) further suggested that SLC1A5 may be a MYC target gene. To determine whether the MYC-binding sequence participates in SLC1A5 transcriptional activation, we cloned the 400- to 500-bp promoter region containing the putative (wt) MYC binding sequence and a mutated (mut) version into the luciferase reporter vector (Fig. 6J). As expected, the luciferase activity from the SLC1A5 promoter was 2.5-fold higher in MYC-REwt than in RE-mut in 293T cells (Fig. 6K). The results of the ChIP-qPCR assay further confirmed the decreased MYC occupancy on the MYC promoter in H1299 and A549
cells treated with JQ1 or MYC knockdown (Fig. 6L-6O). Thus, the oncogene MYC activates transcription of SLC1A5, which raises the possibility that MYC regulates glutamine uptake.

9. SLC1A5 expression level is associated with tumor stage, lymph node metastases and poor survival in human NSCLC.

We performed microarray analysis using the Gene Expression Profiling Interactive Analysis (GEPIA) online platform and database to identify the expressions of SLC1A5 and MYC in NSCLC. The results indicated that the SLC1A5 level was higher in NSCLC than in the adjacent tissues. Notably, MYC was only significantly expressed in lung squamous cell carcinoma (LUSC), and did not appear to play a significant role in lung adenocarcinoma (LUAD) (Fig. 7A-7D). Similar findings were also observed in cervical squamous cell carcinoma, cholangiocarcinoma, pancreatic cancer and other malignant tumors (Fig. S11A, 11B). In addition, there was a significant positive correlation between MYC and SLC1A5 mRNA levels in NSCLC (Fig. 7E, S11C, 11D). Analysis of The Cancer Genome Atlas (TCGA) datasets also showed that the levels of SLC1A5 and MYC were upregulated in LUAD with lymph node or distant metastasis (Fig. 7F-7I, S11E-11L).

We also constructed a tissue microarray (TMA) of 219 human NSCLC specimens, and used it for immunohistochemical (IHC) analysis (Fig. 7J). We found that the level of SLC1A5 was significantly associated with clinical tumor stage and lymph node metastasis (Fig. 7K-7N, Supplementary Table 2). Additionally, Kaplan–Meier analysis indicated that NSCLC patients with high levels of SLC1A5 have poor survival prognosis (Fig. 7O).

Discussion

In this study, we hypothesized that the change in oxygen concentration has a unique significance for the lung-specific TME and the malignant phenotype of lung tumor cells. To confirm such hypothesis, the effects of different oxygenation parameters on the proliferation, apoptosis, and invasion of lung cancer cells were systematically evaluated in vitro and in vivo. In addition, transcriptome, proteome, and metabolome analysis were combined to determine the effect of supplemental oxygen on the malignant phenotype of lung cancer and the detailed mechanism mediating this effect. The findings of this study will provide a satisfactory theoretical basis to clarify the safety of oxygenation therapy for lung cancer patients and ascertain whether it has the potential to be used as an adjuvant in combination with current lung cancer treatment methods.

Effectively reducing tumor burden and metastasis risk is the main challenge in the clinical response and management of advanced lung cancer[25, 26]. This study demonstrated that MYC/SLC1A5-induced metabolic reprogramming and glutamine addiction serves as a new mechanism that drives lung cancer metastasis, which can be significantly suppressed by IH (60% oxygen). These findings are relevant to the debate on the perils, promises and antitumor effect of IH, especially for patients with lung cancer. The clinical protocol of respiratory hyperoxia is widely used in patients with oxygenation deficiency caused by various diseases, including lung cancer. Previous studies have shown that respiratory hyperoxia has a
confirmed stimulatory effect on the proliferation of endothelial and epithelial cells in wound healing[27–30]. However, the unknown impact of IH on local lung cancer progression has raised some concerns. Over the last decade, studies have strongly suggested that respiratory hyperoxia did not have a stimulatory effect on tumor growth, recurrence, and metastasis in most cancers[31]. Evidence from clinical and animal studies has shown that hyperbaric oxygen (HBO) can enhance the effects of chemotherapy and radiotherapy in cervical and breast cancer[32, 33]. A possible explanation is that HBO overcomes the hypoxia and insufficient vascular development inside the tumor, thereby increasing the transport of drug molecules and therapeutic sensitivity[34]. Recently, Kim et al.[9] intravenously injected mice with LLC cells and exposed them to a 24 h-normobaric (95% oxygen)/normoxia cycle for two weeks, and found that respiratory hyperoxia inhibited the progression of lung cancer by inducing apoptosis. Additionally, studies by Hatfield et al. also revealed the antitumor effects of respiratory hyperoxia by reversing the hypoxia-adenosinergic immunosuppression in the TME[12, 35, 36]. In this study, our data indicated that exposure to 60% oxygen (6h/day) inhibited lung cancer metastasis and increased the survival time of tumor-bearing mice. We emphasize that it is very important to take the proper time of application, duration, pressure and dose to achieve the overall benefit for mice with lung tumors. It is not recommended to use more than 60% oxygen and a duration of more than 12 h, to avoid serious complications, including pulmonary bullae, alveolar hemorrhage, oxygen toxicity, and even death.

Glutamine is an essential amino acid for NSCLC growth in vitro and in vivo. Recent studies have proposed that glutamine supports lung cancer growth through a KRAS-regulated metabolic pathway under hypoxia[37, 38]. Momcilovic et al.[39] also showed that LUSC could circumvent inhibition of mTOR and glycolysis by upregulation of glutamine metabolism. Another major finding of our untargeted metabolomics study is that IH had a profound effect on intracellular metabolism of lung cancer cells. We found that hyperoxia exposure significantly decreased the expression of SLC1A5 and the level of intracellular glutamine in lung cancer cells. Additional data confirmed that hyperoxia-induced suppression of the aggressiveness of lung cancer is mediated by inhibition of glutamine uptake, in a manner similar to glutamine deprivation. In vitro study, hyperoxia-induced suppression of glycolysis may be the main contributor to its inhibition of the proliferation of lung cancer cells and BEAS cells. However, hyperoxia did not significantly affect the growth of tumor xenografts in vivo. We believe that there may be two reasons for this inconsistency. First, oxygen supplementation is not enough to counteract the limitations of tumor volume increase and vascular development defects. Second, the inhibitory effect of IH on lung tumor growth may depend on lung-specific TEM. In fact, tumor cells cultured in vitro and early primary lung tumors do not experience significant hypoxia, which suggests that reversing hypoxia is not the main mechanism of hyperoxia-induced inhibition of the proliferation of lung cancer cells and reduction of the lung tumor burden.

The transcription factor MYC is one of the most common somatically mutated oncogenes in human cancer. High level of MYC is found in both high-grade premalignancy and invasive tumors and is associated with poor outcome in different human tumor types[40, 41]. Recent studies suggest that MYC is specifically necessary for the metastasis of tumor cells independent of its effects on proliferation and survival[42]. Our data indicated that exposure to 60% oxygen for 12 h significantly downregulated MYC
expression at both mRNA and protein levels. Additionally, we found that MYC directly activates SLC1A5 transcription, leading to the reprogramming of glutamine metabolism. In summary, we discovered a MYC/SLC1A5 signaling pathway regulated by hyperoxia, which is the basis for glutamine uptake and lung cancer metastasis.

Moreover, our preliminarily investigation of the role of IH-induced oxidative stress in the progression of lung cancer revealed that hyperoxia exposure caused a decrease in glutathione level and a significant increase in intracellular ROS in BEAS, H1299 and A549 cells. Recent studies provide compelling evidence that ROS have anti-tumorigenic roles with the potential to become a novel anticancer target[43–46]. Our study also confirmed that exogenous antioxidants promote the metastasis of lung cancer under IH, which is consistent with previous reports[16]. However, exposure to hyperoxia for more than 48 h could significantly increase intracellular ROS to toxic levels and induced severe apoptosis and necrosis in both lung cancer cells and BEAS cells. This reminds us that balancing the antitumor activity and toxic effect of ROS needs to be cautiously considered when using IH.

**Conclusion**

In conclusion, our study reveals that IH exposure not only has no tumor progression-inducing effects, but also suppresses lung cancer metastasis. We also propose that intracellular metabolic reprogramming driven by the oncogene MYC/SLC1A5 axis is the main mechanism by which IH inhibits cancer metastasis. These findings will have implications for future therapeutic approaches as IH can potentially synergize with therapies that target glutamine metabolism, glycolysis or ROS, such as chemotherapy, radiation and novel targeted drug.

**Abbreviations**

IH: inspiratory hyperoxia; NSCLC: non-small cell lung cancer; siRNAs: Short interfering RNAs; CCK-8: Cell Counting Kit-8; RT-qPCR: Quantitative real-time PCR; RIP: RNA immunoprecipitation; H&E: Hematoxylin and eosin; IHC: Immunohistochemical staining; TME: tumor microenvironment; TMT: tandem mass tag.

**Declarations**

**Author contributions**

H.Z and C.C conceived the project. X.C.L and H.Q performed all experiments on cell lines and mice. W.Z, S.J.F, C.G and Y.L helped establish lung cancer models. X.C.L, X.Y.Q, C.L.J and Z.X.C wrote the manuscript. All authors contributed to experimental design and data analysis. XCL composed the manuscript. All authors reviewed the manuscript and discussed the work.

**Competing interests**

The authors declare that they have no competing interests.
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Findings

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Availability of data and materials

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

Ethics approval and consent to participate

All participants provided written informed consent, and the study was approved by the ethics committee of Shanghai Pulmonary Hospital (KL281-02). All animal experiments complied with the Policy of Xuzhou Medical University on the Care and Use of Laboratory Animals.

Consent for publication

All authors agree to publication of the article.

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**Figures**
Figure 1

**Inspiratory hyperoxia suppress lung cancer metastasis.**

(A) Schematic of workflow for the establishment of lung cancer models and IH treatment.
(B) Effects of IH treatment (30% O$_2$, 60% O$_2$, 90% O$_2$) on the long-term survival in the orthotopic lung tumor models (n=15). Statistical significance was calculated using a log-rank test.

(C) Representative lung sections.

(D) Percentage of mice with lymph node metastasis ($P<0.01$) and distant metastasis ($P<0.05$) and representative photos, Chi-square test.

(E) Sixty percent oxygen treatment led to long-term survival in the lung metastasis models with LLC cells i.v. injection (n=15), log-rank test.

(F) Sixty percent oxygen treatment led to long-term survival in the lung metastasis models with H1299 cells i.v. injection (n=15), log-rank test.

(G) Representative lung photos and lung sections of tumor-bearing mice.

(H) IH decreased lung tumor burden in mice 3 weeks after i.v. injection of H1299 cells (n=10), green dot indicated mice with distant metastasis.

Left, bioluminescence imaging four weeks injection into mice via tail vein. Right, box plot (n=5), *$P<0.05$, ***$P<0.001$ vs the indicated group, two-tailed Student's t-test.
Figure 2

Multi-omics data of H1299 cells treated with hyperoxia.

(A) Schematic diagram of workflow for transcriptome, proteome and metabolome analysis.

(B) Dynamic range of protein expression.
(C) Scatter plot of the correlation between genes quantified in both transcriptomic and proteomic data sets. The red plot indicates genes that change consistently.

(D) Venn diagram shows the number of mRNAs and proteins quantified.

(E) Venn diagram showing 5460 genes jointly identified by RNA-seq and TMT, as well as significantly enriched mRNAs and proteins, and their overlap.

(F) GO enrichment analyses were used to classify the significant differentially expressed mRNA and proteins.

(G) KEGG enrichment analyses of the significant differentially expressed mRNA and proteins.

(H) Upregulation of HCMDB genes in 60% O₂ treated H1299 cells versus controls.

(I, J) Gene set enrichment analysis of significant differentially expressed mRNA with normalized enrichment score (NES) and false-discovery rate (FDR) Q value.
Figure 3

Metabolic profiles with metabolite compositions and metabolic pathways.

(A) Heat map analysis for metabolite compositions between 60% O₂ and normoxia groups.
(B-G) Quantitation of the levels of 6-methylquinoline, argininosuccinic acid, DL-4-Hydroxyphenyllactic acid, 4-hydroxybenzaldehyde, L-glutamine and pyruvic acid, two-tailed Student’s t-test.

(H) The metabolic pathways from MetaboAnalyst 3.0.

Figure 4
**Inspiratory hyperoxia decreases the glutamine uptake of lung tumor.**

(A) Schematic diagram of workflow for the measurement of intracellular glutamine levels.

(B, C) Hyperoxia treatment decreases the level of glutamine in H1299 and A549 cells (n=5), one-way ANOVA followed by the Tukey’s post hoc test.

(D-F) Effects of 60% O\textsubscript{2} treatment on the expressions of genes related to glutamine metabolism in H1299 cells.

(G, H) The levels of glutamine metabolism related genes were determined by qRT-PCR in H1299 and A549 cells (n=3).

(I, J) Western blot analysis for the expressions of SLC1A4 and SLC1A5 (n=3), one-way ANOVA followed by the Tukey's post hoc test.

(K) IH treatment reduce the expression of SLC1A5 in lung tumor in lung metastasis models, bar (left)=200 μm, bar (right)=50 μm.

(L) IHC staining for the expression of SLC1A5 in xenograft tumors, 50 μm.

(M, N) Effect of hyperoxia treatment on the relative glutamine uptake in H1299 and A549 cells (n=5).

* \( P \leq 0.05 \), ** \( P \leq 0.01 \), *** \( P \leq 0.001 \), NS, \( P > 0.05 \) vs the normoxia or indicated group, two-tailed Student's t-test.
Figure 5

SLC1A5 mediates the hyperoxia-induced suppression of glutamine catabolism.

(A, B) Knockdown of SLC1A5 was confirmed at the mRNA and protein level in H1299 and A549 cells (n=5).
(C) Determination of the relative glutamine uptake in H1299 and A549 cells with shSLC1A5 treatment (n=5).

(D, E) The migration and invasion of H1299 and A549 cells with shSLC1A5 treatment (n=5), bar=50 μm.

(F) Representative bioluminescence images of mice four weeks after i.v. injection.

(G, H) Lung tumor burden in mice four weeks after i.v. injection and representative lung section (n=5).

(I-K) Photographs of matrigel plugs excised from mice after 21 days of growth in vivo and quantitative analysis of the tumor volume and tumor weight (n=10).

(L) Detection of SLC1A5 expression in xenograft models by western blot (n=6).

*P<0.05, **P<0.01, ***P<0.001 vs the indicated group, two-tailed Student’s t-test.
Figure 6

MYC directly activates SLC1A5 transcription.

(A) qRT-PCR is used to detect the levels of transcription factors with significant differences identified by RNA-seq in H1299 cells (n=3).
(B, C) Western blots showing exposure to 60% oxygen for 12 h resulted in a decrease in MYC protein expression in H1299 cells (n=5).

(D-F) Analysis of MYC and SLC1A5 expressions in H1299 and A549 cells with JQ1 treatment.

(F) Effect of MYC knockdown on the expression level of SLC1A5 protein in H1299 cells (n=3).

(G) Venn diagram of RNA-seq data showing the number of genes differentially expressed in H1299 cell with 60% O₂ treatment versus the control cells (blue). Small circle show genes with MYC binding motifs in their proximal promoter.

(H) Predicted binding site MYC-binding sites of SLC1A5. Yellow arrows show primers used for ChIP-qPCR.

(I) Schematic of the MYC binding site, the MYC response elements (MYC REwt), and its mutants (REmut) within the promoter of SLC1A5.

(J) Luciferase activity of 293T cell with and without ectopically expressed MYC (n=3).

(L-O) Binding of MYC to SLC1A5 analyzed by ChIP assays in H1299 and A549 cells with and without JQ1 and MYC knockdown treatment (n=3).

*P<0.05, **P<0.01, ***P<0.001 vs the normoxia or indicated group, two-tailed Student’s t-test.
Figure 7

SLC1A5 is associated with the tumor stage, lymph node metastases and poor survival in human NSCLC.

(A-D) SLC1A5 and MYC expressions are analyzed in NSCLC tissues (n=969) and their adjacent noncancerous tissues in the GEPIA database.
(E) Correlation between mRNA levels of SLC1A5 versus MYC in NSCLC tissues according to TCGA database, \( r=0.430, P<0.001 \).

(F-I) Data of TCGA datasets showing SLC1A5 is closely related to lymph node metastasis of NSCLC, \( **P<0.01 \), NS, \( P>0.05 \) vs indicated group.

(J) Representative images from human NSCLC tumors stained with SLC1A5, bar=100 μm.

(K-N) Correlation of SLC1A5 expression with tumor grade, lymph node metastasis, distant metastasis and EGFR mutation in 219 NSCLC patients, Chi-square test.

(O) Kaplan–Meier survival curve analysis is performed to explore the effects of the genes on the survival rate in NSCLC (\( P<0.001 \)), log-rank test.

**Supplementary Files**

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