HIF-1-dependent heme synthesis promotes gemcitabine resistance in human non-small cell lung cancers via enhanced ABCB6 expression

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Abstract
Gemcitabine is commonly used to treat various cancer types, including human non-small cell lung cancer (NSCLC). However, even cases that initially respond rapidly commonly develop acquired resistance, limiting our ability to effectively treat advanced NSCLC. To gain insight for developing a strategy to overcome gemcitabine resistance, the present study investigated the mechanism of gemcitabine resistance in NSCLC according to the involvement of ATP-binding cassette subfamily B member 6 (ABCB6) and heme biosynthesis. First, an analysis of ABCB6 expression in human NSCLCs was found to be associated with poor prognosis and gemcitabine resistance in a hypoxia-inducible factor (HIF)-1-dependent manner. Further experiments showed that activation of HIF-1α/ABCB6 signaling led to intracellular heme metabolic reprogramming and a corresponding increase in heme biosynthesis to enhance the activation and accumulation of catalase. Increased catalase levels diminished the effective levels of reactive oxygen species, thereby promoting gemcitabine-based resistance. In a mouse NSCLC model, inhibition of HIF-1α or ABCB6, in combination with gemcitabine, strongly restrained tumor proliferation, increased tumor cell apoptosis, and prolonged animal survival. These results suggest that, in combination with gemcitabine-based chemotherapy, targeting HIF-1α/ABCB6 signaling could result in enhanced tumor chemosensitivity and, thus, may improve outcomes in NSCLC patients.

Keywords ABCB6 · Hypoxia-inducible factor-1 · Transcriptional activation · Non-small cell lung cancer · Gemcitabine resistance

Background
Lung cancer is one of the most common forms of malignancy and the leading cause of cancer-related deaths in both men and women worldwide [1]. An estimated 1.8 million people are diagnosed with lung cancer annually, and an estimated 1.6 million people die from this disease every year [2]. In China, approximately 733,300 new cases were diagnosed and 610,200 patients died of lung cancer in 2015 [3]. Non-small cell lung cancer (NSCLC), which is divided into three subtypes (large-cell carcinoma, squamous cell carcinoma, and adenocarcinoma), accounts for more than

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85% of all lung cancer cases [4]. Since most cases are diagnosed at advanced stage with advanced local invasion or distant metastasis, the 5-year survival rate for lung cancer patients is only 4–17% [5]. Despite advancements in surgical treatments and targeted therapies, systemic chemotherapy remains the mainstay of treatment for advanced lung cancer. Gemcitabine, a deoxycytidine analog, exerts an anticancer effect by catalyzing the complex intracellular conversion to gemcitabine diphosphate and triphosphate, compounds that are important for terminating DNA synthesis [6]. Clinically, gemcitabine has been used in one of the standard regimens for NSCLC [7]. However, gemcitabine-based chemotherapy resistance has continued to limit our ability to effectively treat advanced NSCLC. In addition to inherent resistance to gemcitabine, even cases that initially respond to gemcitabine treatment frequently develop acquired resistance. Hence, it is critically important to determine the mechanism of gemcitabine-based resistance in NSCLC and to urgently discover an effective approach to overcome chemotherapy resistance.

The adenosine triphosphate (ATP)-binding cassette (ABC) transporter family consists of 11 members that facilitate translocation of heterogeneous substrates, including saccharides, amino acids, peptides and proteins, lipids and sterols, metabolic products, inorganic and organic ions, metals and drugs, across the cell membrane [8]. Some of the ABC transporters have been shown to play crucial roles in the development of drug resistance via the efflux of anticancer agents outside of cancer cells [9]. However, this activity cannot fully explain the underlying mechanism of acquired chemotherapy resistance. ABC transporter subfamily B member 6 (ABCB6) encodes an 842-amino-acid protein [10], and as an energy-dependent transporter, ABCB6 is capable of binding with heme and is involved in heme biosynthesis [11]. To investigate the contribution of ABCB6 to gemcitabine resistance in NSCLC cells, in the present study, we established a gemcitabine-resistant (GR) human lung squamous cancer (LUSC) cell line and a GR lung adenocarcinoma (LUAD) cell line and then examined and analyzed ABCB6 expression as well as heme synthesis in these cells. We found that gemcitabine treatment alters intracellular heme biosynthesis by enhancing the levels of ABCB6, whereas inhibition of ABCB6 restrains intracellular heme accumulation. Interestingly, we also found this interaction is regulated by gemcitabine-induced hypoxia-inducible factor-1α (HIF-1α). HIF-1α, which is stably expressed in response to low oxygen conditions (hypoxia), is a transcriptional activator that regulates the expression of thousands of target genes [12]. In addition to hypoxia, stimuli such as chemotherapeutic agents and radiation increase the stabilization and accumulation of HIF-1α [13, 14]. We observed stable expression of HIF-1α in the GR lung cancer cell lines even under normoxic conditions. In addition, we demonstrate that ABCB6 is a direct HIF-1α target gene, and further identify relevant hypoxia response elements (HREs) in the ABCB6 gene.

Chemotherapeutic drug-induced cell apoptosis is associated with enhanced levels of reactive oxygen species (ROS), primarily superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), that can cause functional and structural damage to cell components [15]. Cancer cells, thus, protect themselves from chemotherapy by activating a variety of defense mechanisms to detoxify any adverse effects caused by an accumulation of chemotherapy-induced ROS. Catalase, an enzyme that degrades H$_2$O$_2$ by converting it into H$_2$O and O$_2$, is generally recognized as an antioxidant enzyme that confers cellular protection [16]. Heme metabolism is an important cellular mechanism that influences catalase activities. As a heme-containing enzyme, the regulation of H$_2$O$_2$ decomposition and peroxidase activities by catalase are heme-dependent [17]. To determine whether reprogramming of HIF-1α/ABCB6-regulated heme metabolism influences catalase activity, we analyzed catalase protein expression and activity in GR NSCLC cell lines and observed a dramatic induction of both catalase protein expression and activity, which further facilitates ROS scavenging to directly contribute to promoting gemcitabine-based resistance.

Materials and methods

Statistical analysis of microarray data and survival

Gene expression data from the LUSC and LUAD datasets of The Cancer Genome Atlas (TCGA) were obtained from http://ualcan.path.uab.edu/index.html or http://xena.ucsc.edu. Pearson’s correlation analysis was used to determine P values for co-expression. The prognostic significance of ABCB6 expression in lung cancer was analyzed using Kmplot (www.kmplot.com) [18]. Briefly, the gene was entered into the online database to obtain a Kaplan–Meier survival plot. The log-rank P values were calculated and displayed on the webpage. The survival rates were investigated according to a median cutoff (low represents patients with indicated gene mRNA levels less than the median, and high represents patients with indicated gene mRNA levels greater than the median).

Patients and clinical specimens

Patients diagnosed with advanced NSCLC were enrolled in the cancer center of the First Affiliated Hospital (Southwest Hospital) of Army Medical University (Chongqing, China) from January 2010 to December 2014. All patients received routine gemcitabine-platinum chemotherapy (completed 2–4 cycles). Forty-one specimens were obtained from patients with chemotherapy failure...
(including progressive disease, PD) after gemcitabine-based chemotherapy, and constituted the GR group. Forty specimens were obtained from patients who had a positive response after gemcitabine therapy including a partial response (PR), and these samples constituted the gemcitabine-sensitive group. Adjacent normal specimens were collected from 10 NSCLC cancer patients who underwent surgery in Southwest Hospital. The histomorphology of all specimens had been confirmed by the Department of Pathology. This clinical study complied with the ethical standards codified in the 1964 Declaration of Helsinki. The protocol for immunohistochemistry (IHC) examination of patient tissues was approved by the Ethics Committee of the First Affiliated Hospital (Southwest Hospital), Army Medical University, and all patients or family members involved provided written informed consent. All slides were prepared from stored pretreated, paraffin-embedded tissue blocks from lung cancer patients who underwent surgery at Southwest Hospital. All specimens were confirmed by pathological examination, and tissue microarrays were prepared as described previously [19].

Cell culture and drug treatment

All human NSCLC cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle Medium (DMEM, A549 cells) or RPMI 1640 medium (NCI-H1703 cells) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (Beyotime Institute of Biotechnology, China). Cells were cultured in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂. The GR cell lines (A549-GR and H1703-GR) were established as follows: A549 or H1703 parental cells were exposed to gradually increasing concentrations of gemcitabine (Sigma-Aldrich, St. Louis, MO, USA) from 10 nM initially up to 10 μM over a 7-month period. For hypoxia exposure, cells were placed in a modular incubator chamber that was flushed with a 1% O₂/5% CO₂/94% N₂ gas mixture and sealed. Dimethylxalylglycine (DMOG, Sigma-Aldrich) was initially dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in the treatment appropriate culture medium to the indicated concentrations. For the vehicle control group, a final concentration of 1% (v/v) aqueous DMSO was used throughout. Tomatidine was purchased from MedChemExpress (purity: >98.0% Cat No. HY-N2149, Shanghai, China), and fresh solutions were prepared by further dilution of a stock solution (100 mmol/l in DMSO). Tomatidine was added to cell culture wells to reach the final various working concentrations (0, 2, 6, 8, and 10 μmol/l). Cells were incubated for 24 h before subsequent experiments.

Lentivirus transduction

The pLKO.1-puro lentiviral vectors encoding short hairpin (sh)RNA targeting HIF-1α (sh1α-1, Clone ID:NM_001530.x-2671s1c1; sh1α-2, Clone ID:NM_001530.x-1048s1c1), ABCB6 (shABCB6-1, Clone ID:NM_005689.1-1581s1c1; shABCB6-2, Clone ID:NM_005689.1-2814s21c1), and non-targeting control (NTC) were purchased from Sigma-Aldrich. Lentiviruses were packaged, and cells were transduced and subjected to puromycin selection as described previously [20].

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

Total cellular RNA was extracted using TRIzol (Invitrogen), precipitated with isopropanol, treated with DNase I (Ambion), and reverse transcribed with the iScript cDNA Synthesis kit (Bio-Rad). qPCR analysis was performed using SYBR Green (Bio-Rad) with the iCycler Real-time PCR Detection System (Bio-Rad). The 2^ΔΔCt method was used to calculate relative gene expression levels as described previously [21]. The primer sequences used in this study are listed in Table S1.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as previously described [19]. Briefly, A549 and H1703 cells were cross-linked in 3.7% formaldehyde for 10 min and lysed with sodium dodecyl sulfate (SDS) lysis buffer. Chromatin was sheared by sonication, and lysates were pre-cleared with salmon sperm DNA/protein A-agarose slurry (Millipore) and incubated with IgG (Novus Biologicals) or antibodies against the following proteins: anti-HIF-1α (Santa Cruz Biotechnology), anti-HIF-2α (Cell Signaling Technology), anti-HIF-1β (Novus Biologicals), anti-H3K27ac (GeneTex) and anti-H3K4me3 (GeneTex). Salmon sperm DNA/protein A-agarose slurry was added, and the agarose beads were washed sequentially with low- and high-salt immune complex wash buffers; LiCl immune complex wash buffer; and twice with Tris–EDTA (TE) buffer (10 mM Tris–HCl/l mM EDTA). DNA was eluted in 1% SDS with 0.1 M NaHCO₃, and crosslinks were reversed by addition of 0.2 M NaCl. DNA was purified by phenol–chloroform extraction and ethanol precipitation and then suspended in 50-μl TE buffer. A 2-μl aliquot was used for qPCR with the primer sequences listed in Table S1.

Western blot assay

Immunoblot assays were performed as previously described [20]. Aliquots of whole-cell lysates were prepared in radioimmunoprecipitation assay (RIPA) lysis
buffer, and proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose membranes, and further probed with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare). The chemiluminescent signal was developed using ECL Plus (GE Healthcare). Antibodies used in immunoblot assays included: anti-HIF-1α (BD Transduction Laboratory); anti-ABCB6 (Novus Biologicals); anti-catalase (Novus Biologicals); and anti-β-actin (Santa Cruz Biotechnology).

Heme detection

The heme content in whole cells was determined by the fluorometric method [22]. Briefly, cells were washed and homogenized in phosphate-buffered saline (PBS) buffer, and the protein content was determined using the Bio-Rad protein assay system (Bio-Rad, Munich, Germany). For heme detection, 10-μg protein samples were incubated with 500 μl of 2 M oxalic acid (Sigma-Aldrich) at 100 °C for 30 min. Samples were subsequently centrifuged at 18,000 g for 10 min. The amount of heme in the supernatant was determined by fluorometric detection using a F4500 fluorescence spectrophotometer (Hitachi) with the excitation and emission wavelengths were set at 405 and 662 nm, respectively. The background was evaluated by measuring fluorescence in non-boiled samples.

Catalase activity

For the detection of catalase activity, cells were seeded into 6-well plates at a density of 10^5 cells/well and then later harvested and homogenized in 50 ml of 0.1 M PBS buffer. The mixture was centrifuged at 800 g for 5 min at 4 °C, and the supernatant was separated and assayed for protein content (Bradford assay, Bio-Rad). Catalase activity was determined calorimetrically in 10 μg protein samples using the Catalase Assay Kit (No. CAT-100, Sigma-Aldrich) according to the manufacturer’s instructions by spectrophotometry. Briefly, all working solutions were prepared from the reagents provided in the kit. The sample (10 μl), mixed with 750 μl of 1X assay buffer and 25 μl colorimetric assay substrate solution, was incubated for 5 min. The reaction was stopped using 900 μl of stop solution, and the tubes were kept inverted. Within 15 min after the enzymatic reaction, 10 μl aliquots of the reaction mixture were transferred to 1 ml of the color reagent. After incubation for 15 min, the absorbance of the reaction mixture was measured at 520 nm. Specific catalase activity is reported as μmol H_2O_2 consumed min^{-1} μg protein^{-1}.

Colony formation assay

For detection of the clonogenic ability of the cells after drug treatment, cells were trypsinized and seeded into 6-well plates at a density of 1000 cells per well. The cells were then cultured in medium containing gemcitabine at the indicated concentrations, which was refreshed every 3 days. After 15 days, cells were fixed with 4% paraformaldehyde for 10 min and then stained with 0.1% crystal violet in ddH_2O for 10 min.

Cell viability assay

To evaluate cell viability, 1 × 10^4 cells/well were seeded into 96-well plates and cultured at 37 °C overnight. Cells were then treated with various concentrations (0.001, 0.01, 0.1, 1, 10, or 100 μM) of gemcitabine for 72 h. Cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; Promega, USA) following the manufacturer’s instructions. All experiments were done in triplicate and repeated more than three times.

Soft agar assay

Cells were suspended in 1 ml top agar made of 0.35% agarose in medium supplemented with 10% FBS, and the mixture was seeded in 6-well plates containing a basal layer of 0.6% agarose at 5000 cells/well. After 3 weeks, colonies were stained with 0.05% crystal violet for 1 h, and the colony size and colony number were measured by pictomicrography. Viable colonies larger than 0.1 mm in diameter were counted.

TUNEL assay

To evaluate cell apoptosis in tumor tissues, we performed terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) on formalin-fixed tumor samples in paraffin blocks using the one-step TUNEL apoptosis assay kit (Beyotime Institute of Biotechnology). Tissue sections (4–5 μm) mounted on glass slides were deparaffinized, rehydrated with a graded series of alcohol solutions, treated with 20 μg/ml proteinase K (37 °C, 20 min), and then washed in PBS buffer. The TUNEL assay was performed according to the manufacturer’s instructions. Images of stained tissue sections were acquired using a confocal microscope (Leica TSC-SP5, Germany). Ten fields were randomly selected on each tumor slide.
ROS detection

Total intracellular ROS was determined by staining cells with dichlorofluorescin diacetate (DCFH-DA, Beyotime Institute of Biotechnology) following the manufacturer’s instruction. Briefly, cells were washed with PBS and incubated in darkness with 10 μM DCFH-DA at 37 °C for 30 min. As a positive control, cells were treated with Rosup (agent provided in ROS assay kit). Cells were then washed twice with PBS, and ROS levels were determined by fluorospectrophotometry (excitation wavelength 488 nm and emission wavelength 535 nm).

IHC staining

Tumors and adjacent normal tissue were fixed in 10% formalin and paraffin embedded. Tissue microarrays were dewaxed in xylene and hydrated with graded ethanol solutions, followed by antigen retrieval using citrate buffer (pH 6.1). The instantaneous SP supersensitive kit (SP-9000, Beijing Zhongshan Jinqiao Biotechnology) was used with antibodies against ABCB6 (Novus Biologicals). Sections were counterstained with Mayer’s hematoxylin (Sigma). For quantitative measurement of ABCB6-positive staining in clinical samples (adjacent normal lung tissue, GR tumors and gemcitabine-sensitive tumors), IHC staining of ABCB6 was quantified using ImageJ software (NIH) as described previously [23]. Specifically, five random fields on each sample (magnification, 200 ×) were selected for scoring, and a mean score for each slide was calculated in the final analysis. The percentage of stained cells was scored as follows: 0 (no positive cells), 1 (<10% positive cells), 2 (10–40% positive cells), 3 (40–70% positive cells), and 4 (>70% positive cells). The intensity of positive staining was scored as follows: 0 (negative staining), 1 (weak staining exhibited as light yellow), 2 (moderate staining exhibited as yellow brown), and 3 (strong staining exhibited as brown). The proportion and intensity scores were added to obtain a total score (range 0, 2–7). Samples were divided into two categories depending on the IHC score: low if < 4 and high if ≥4. Slides were examined and scored independently using the Gynecologic Oncology Group (GOG) criteria [24] by two histopathologists (F Wu and Y Ling) who were blinded to other pathological information.

Animal studies

The animal experiments were approved by the Institutional Animal Care and Use Committee of the Army Medical University (Chongqing, China) and were performed in accordance with the guidelines for animal experiments of the university, which meet the ethical guidelines for experimental animals in China. Six-to-eight-week-old male severe combined immunodeficiency (SCID) mice were purchased from the Institute of Experimental Animal of the Army Medical University. For transfer into the mice, A549 and H1703 GR and gemcitabine-sensitive subclones were harvested by trypsinization and resuspended at 10^7 cells/ml in a 50:50 mix of PBS:Matrigel (BD Biosciences) for subcutaneous injection of 2 × 10^6 cells into the inguinal area of mice (16 mice per group). The following groups were prepared: NTC-transfected A549 or H1703 wild-type (WT) group (treated with saline), NTC-transfected A549 or H1703 GR group (treated with saline), NTC-transfected A549 or H1703 GR group (treated with gemcitabine), shHIF-1α expression vector-transfected A549 or H1703 GR group (treated with gemcitabine), shABCB6 expression vector-transfected A549 or H1703 GR group (treated with gemcitabine), and shHIF-1α/ABCB6 expression vector-transfected A549 or H1703 GR group (treated with gemcitabine). Tumor volume (mm3) was calculated as 0.52 × L × W × T (L: Length, W: width, T: thickness). Tumor volumes and body weights were monitored twice weekly. After palpable tumors had formed (8 days after tumor implantation), mice received intraperitoneal injections of saline (250 μl) or gemcitabine (20 mg/kg) twice per week. After 42 days, tumors were excised and weighed (n = 6). Xenografts were harvested for analysis by TUNEL assay. To assess survival, the remaining animals (n = 10) were monitored for 100 days until being euthanized. The distribution of survival percentages over time was estimated using the Kaplan–Meier method. The log-rank test was used to determine P values. For the WT A549 animal experiment, 2 × 10^6 cells of A549 subclones (NTC, shHIF-1α, or shABCB6) were injected subcutaneously into the inguinal area of mice (n = 5 mice per group). Tumor volumes were monitored twice weekly. After palpable tumors had formed (26 days after tumor implantation), mice received intraperitoneal injection of gemcitabine (20 mg/kg) or saline (250 μl) twice per week until day 53.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Differences between two groups were analyzed using an unpaired two-tailed Student’s t test, and differences among multiple groups were detected by analysis of variance (ANOVA) followed by Bonferroni post-test. Survival data were analyzed using Kaplan–Meier survival plots, and P values were calculated using the Wilcoxon rank sum test. A P value of < 0.05 was considered statistically significant.
Results

**ABCB6 expression is increased after chemotherapy in GR lung cancer tissues and is associated with poor prognosis in human NSCLC**

To investigate whether ABCB6 expression has clinical significance in human NSCLCs, we compared ABCB6 gene expression in LUSC and LUAD tissues to that in normal lung tissue using data from TCGA database. This analysis revealed that ABCB6 mRNA levels were significantly greater in human NSCLC tissues (LUSC and LUAD) than in the normal lung tissues (Fig. 1A, B). Moreover, analysis of ABCB6 mRNA expression in 1926 human lung cancer specimens [19, 25] revealed that levels above the median were associated with a significant decrease in patient survival. (Fig. 1A, B). Moreover, analysis of ABCB6 mRNA expression in 1926 human lung cancer specimens [19, 25] revealed that levels above the median were associated with a significant decrease in patient survival.

**Fig. 1** High ABCB6 expression is associated with gemcitabine resistance and poor prognosis in human NSCLCs. Relative levels of ABCB6 mRNA expression from microarray analysis of primary NSCLC tumor samples compared with adjacent normal lung tissue from NSCLC patients (TCGA database) are shown. **A** Red, samples from lung squamous cell carcinoma (LUSC, n = 503); blue, samples from adjacent normal lung tissue (n = 52), P < 0.0001. **B** Red, samples from lung adenocarcinoma (LUAD, n = 515); blue, samples from adjacent normal lung tissue (n = 59), P < 0.0001. Mann–Whitney U test or ANOVA followed by Bonferroni post-test for multiple comparisons was used to determine P values. **C** Kaplan–Meier curves were constructed to analyze the association between ABCB6 mRNA levels in lung cancer tissues and the probability of overall survival (n = 1926, P = 0.006) using the KM plotter database. Low = ABCB6 mRNA levels less than the median. High = ABCB6 mRNA levels greater than the median. Statistical analysis was performed using log-rank tests. **D** Representative images of ABCB6 expression in tissue microarrays containing gemcitabine-resistant tumor tissues from 41 cases, gemcitabine-sensitive tumor tissues from 40 cases, and adjacent normal lung tissues from 6 cases are shown. The bottom right corner panel shows higher magnification of ABCB6 staining (brown, scale bar = 100 μm). **E** Image analysis was performed to determine the staining density of the ABCB6-positive area per field under x200 magnification based on 5 random fields per section. One-way ANOVA (mean ± SEM) was used to determine the P value, ***P < 0.0001.

![Figure 1](https://example.com/fig1.jpg)
survival (Fig. 1C). To evaluate whether the expression of ABCB6 in human NSCLC tissue differed according to varying gemcitabine-based chemotherapy sensitivity, we performed ICH staining to examine the expression of ABCB6 in the GR tumor tissues of 41 patients, gemcitabine-sensitive tumor tissues of 40 patients, and adjacent normal lung tissues of 6 individuals. Representative images of ABCB6 expression in GR tumor tissues, gemcitabine-sensitive tumor tissues, and adjacent normal lung tissues are shown in Fig. 1D. Quantification of the staining density of ABCB6 in multiple samples indicated that ABCB6 expression was increased by more than two-fold in GR tumors compared with gemcitabine-sensitive tumors (Fig. 1E). However, no positive or weak staining was seen in the adjacent normal lung tissues (Fig. 1D, E). The demographic and clinical characteristics of the patients are summarized in Table 1. We found no correlations between ABCB6 expression and patient’s age, gender, histology, differentiation, or clinical stage (all \( P > 0.05 \); Table 1). Interestingly, a high level of ABCB6 staining was significantly correlated with a negative response to gemcitabine treatment, including progressive disease (PD; \( P < 0.001 \)) as well as with distant metastasis (\( P < 0.001 \); Table 1). Moreover, the level of ABCB6 staining was positively correlated with gemcitabine resistance in NSCLCs (\( P < 0.001 \); Table 2).

| Variables                         | Total | Number of patients | Chi-square test |
|-----------------------------------|-------|--------------------|-----------------|
|                                   | Low ABCB6 expression | High ABCB6 expression | \( P \) value |
| Age (year)                        |       |                    |                 |
| < 60                              | 43    | 26                 | 17              | n.s             |
| ≥ 60                              | 38    | 14                 | 24              |                 |
| Gender                            |       |                    |                 |
| Male                              | 59    | 30                 | 29              | n.s             |
| Female                            | 22    | 10                 | 12              |                 |
| Grade                             |       |                    |                 |
| Well and moderately differentiated | 49    | 22                 | 27              | n.s             |
| Poorly and non-differentiated     | 32    | 18                 | 14              |                 |
| Cell type                         |       |                    |                 |
| Squamous                          | 66    | 34                 | 32              | n.s             |
| Adenocarcinoma                    | 15    | 6                  | 9               |                 |
| Clinical stage                    |       |                    |                 |
| Early stage (II)                  | 33    | 22                 | 11              | n.s             |
| Advanced stage (III–IV)           | 48    | 18                 | 30              |                 |
| cT stage                          |       |                    |                 |
| cT1–2                             | 40    | 24                 | 16              | n.s             |
| cT3–4                             | 41    | 16                 | 25              |                 |
| cN stage                          |       |                    |                 |
| cN0                               | 31    | 18                 | 13              | n.s             |
| cN +                              | 50    | 22                 | 28              |                 |
| cM stage                          |       |                    |                 |
| cM0                               | 45    | 31                 | 14              | ***             |
| cM1                               | 36    | 9                  | 27              |                 |
| Gemcitabine response              |       |                    |                 |
| PR                                | 34    | 25                 | 9               | ***             |
| SD                                | 6     | 4                  | 2               |                 |
| PD                                | 41    | 11                 | 30              |                 |

Median values were used as cutoff points for definition of age subgroups.

\( PD \) progressive disease, \( SD \) stable disease, \( PR \) partial response, \( n.s \) not significant

a, Pearson Chi-square test; b, Fisher’s exact test

Pearson Chi-square test was used to calculate \( P \) values, ***\( P < 0.001 \)
Establishment of GR NSCLC cell lines

To address the mechanism of gemcitabine resistance, we generated lung cancer cell line (A549 and NCI-H1703) models with acquired gemcitabine resistance. For this, the WT A549 and NCI-H1703 lung cancer cell lines were cultured with increasing concentrations of gemcitabine over a period of approximately 7 months. To determine the differences in resistant phenotypes between gemcitabine-treated cells and WT cells, we first measured the viability of each line after exposure to different concentrations of gemcitabine (0.001–100 μM). Results from the MTS colorimetric assay demonstrated that A549-GR cells were more resistant than A549-WT cells to increasing gemcitabine concentrations (Fig. S1A). Similar results for H1703 cells were obtained from cell viability assays (Fig. S1B). We also observed an increase in the clonogenic ability of the A549-GR cells (Fig. S1C–S1D) and H1703-GR cells (Fig. S1E–S1F) under control and gemcitabine treatment as well as increased colony number and size in soft agar assays. These data indicate that A549-GR and H1703-GR cells had an increased capability for chemoresistance to gemcitabine treatment.

ABCB6 expression is induced in GR lung cancer cells in a HIF-1-dependent manner

We next investigated ABCB6 gene and protein expression in GR lung cancer cell lines by performing RT-qPCR and immunoblot analyses, respectively. We observed that A549-GR and H1703-GR cells both expressed significantly elevated levels of ABCB6 mRNA (Fig. 2A, B) and protein (Fig. 2C) compared with the respective WT cells, indicating that ABCB6 may participate in gemcitabine-induced chemotherapy resistance in lung cancers. We also performed qPCR assays to detect altered expression of other genes in the ABC family in the GR cells. As shown in Supplemental Fig. 1, the mRNA expression levels of ABCA1, ABCB1, and ABCG2 also were increased in GR cells as compared to gemcitabine-sensitive cells (Fig. S2A, S2C and S2I). Interestingly, ABCA1, ABCB1, and ABCG2 are all regulated by hypoxia and HIFs [26–29]. It is possible that ABCB6 is also regulated by HIF-1α. We tested this hypothesis by qPCR analysis and found that similar to ABCA1, ABCB1, and ABCG2 expression, ABCB6 mRNA expression was increased remarkably in hypoxic cells compared to normoxic cells (Fig. S2A, S2C, S2F, S2I). HIF-1α is a transcription factor that serves as a master regulator of cellular responses to hypoxia and is always associated with aggressive phenotypes, including resistance to radiation and chemotherapy, metastasis, and poor patient prognosis [30]. Interestingly, chemotherapy induces HIF-1α expression even under normoxic conditions [31]. We observed that HIF-1α expression was induced in A549-GR and H1703-GR cells under normoxia (Fig. 2C), indicating that HIF-1α is participate in acquired gemcitabine resistance.

We next hypothesized that gemcitabine-induced ABCB6 expression is regulated by the transcription factor HIF-1α in GR lung cancer cells. To test this hypothesis, the gene expression data from 1267 human LUSC specimens and 706 LUAD specimens in TCGA database were analyzed to compare the expression levels of ABCB6 mRNA with a HIF metagene signature based on the expression of LDHA, PLOGD1, PLOGD2, P4HA1, P4HA2, SLC16A3, SLC2A1, ANGPTL4, PDK1, L1CAM, CA9, BNIP3, IGFBP1, PKM2, and MET mRNA, which are all HIF-regulated genes. Statistical analysis of the expression levels of individual genes in the HIF signature revealed that ABCB6 expression was significantly correlated with the expression levels of 12 of the 15 HIF target genes in the LUSC and LUAD databases, respectively (Tables 3, 4). These findings indicate that ABCB6 mRNA expression may be HIF-regulated in human NSCLCs. To confirm this possibility, we measured ABCB6 expression in A549 and H1703 (including WT and GR cells) subclones, which were stably transfected with an expression vector encoding shRNA targeting HIF-1α (sh1α-1 and sh1α-2) or a NTC shRNA. We observed that the induction of ABCB6 protein expression also was abrogated in A549 (Fig. 2F) and H1703 subclones (Fig. 2E) when HIF-1α was silenced. Induction of ABCB6 protein expression also was abrogated in A549 (Fig. 2F) and H1703 subclones (Fig. 2G) transfected with two different shRNAs (sh1α-1 and sh1α-2) targeting HIF-1α. It is well accepted that HIF-1α accumulates and is activated under hypoxia. In addition, DMOG, a small molecule inhibitor of the HIF prolyl hydroxylases, can also be used to mimic hypoxia in vitro. We next evaluated HIF-1α and ABCB6 protein expression in WT A549 or H1703 cells exposed to hypoxic conditions (1% O2 for 24 h or 48 h) or treated with varying concentrations
Fig. 2. ABCB6 expression is induced in GR cells in a HIF-1α-dependent manner. A, B, RT-qPCR was performed to quantify ABCB6 mRNA levels in A549 (A) and H1703 (B) gemcitabine-resistant (GR) and wild-type (WT) cell lines. For each sample, the expression of ABCB6 mRNA was quantified relative to 18S rRNA and then normalized to the result obtained from WT cells. Statistical analysis was performed before normalization. Data are shown as mean ± SEM; n = 3. ***P < 0.001 vs. WT cells (Student’s t test).

C. Immunoblot analysis was performed to analyze ABCB6 and HIF-1α protein expression in A549 and H1703 GR and WT cell lines.

D, E. ABCB6 mRNA expression was analyzed by RT-qPCR in A549 (D) and H1703 (E) GR and WT subclones, which were stably transfected with non-targeting control (NTC) or vector encoding HIF-1α shRNA (sh1α-1 or sh1α-2) (mean ± SEM; n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared to WT-NTC cells; ###P < 0.001 compared to GR-NTC cells (ANOVA with Bonferroni post-test).

F, G. Immunoblot analysis was performed using lysates prepared from A549 (F) and H1703 (G) subclones transfected with NTC or HIF-1α shRNAs.

H, I. Immunoblot analysis of HIF-1α and ABCB6 protein expression levels in A549-WT (H) and H1703-WT (I) exposed to hypoxia (1% O2) for 24 h or 48 h and DMOG (0 µM, DMSO vehicle, 100 µM, 500 µM, 1 mM) for 24 h in normoxia.
(0 µM, DMSO vehicle, 100 µM, 500 µM, 1 mM) of DMOG under normoxia for 24 h. As shown in Fig. 3H, I hypoxia or DMOG treatment stabilized HIF-1α expression in a dose- and time-dependent manner. The ABCB6 protein levels in A549 (Fig. 2H) and H1703 (Fig. 2I) cells were upregulated with the increase in HIF-1α expression under hypoxia and DMOG treatment. Taken together, the data presented in Figs. 1 and 2 indicate that ABCB6 expression is induced in GR lung cancer cells in a HIF-1α-dependent manner.

### Identification of HREs in the human ABCB6 gene

HIF proteins bind with co-factors to HREs in the promoter regions of their target genes to coordinate a wide-ranging transcriptional program in response to hypoxia [32, 33] as well as to other stimuli such as radiation and chemotherapy [34]. We hypothesized that ABCB6 is a direct HIF target gene. To test this hypothesis, we analyzed the ABCB6 locus for the presence of matches to the consensus HIF-binding site sequence (5′-RCGTG-3′) located in the DNase I-hyper-sensitive chromatin domains using the UCSC Genome Bioinformatics database. This analysis identified two candidate sites that met these criteria (Fig. S3). For validation, we performed ChIP-qPCR assays in A549-WT and A549-GR cells, which demonstrated binding of HIF-1α and HIF-1β, but not HIF-2α, to the two candidate sites (− 155 bp site and + 87 bp site) in A549-GR cells (Fig. 3A, H), which was in contrast to the findings in A549-WT cells (Fig. 3B, I). Notably, enrichment of IgG at these HREs was not significantly induced, verifying the specificity of the assay (Fig. 3B, I). Since HREs are primarily confirmed under hypoxic conditions, which promote HIF-1α protein stability, we confirmed our results by performing ChIP-qPCR assays in A549-WT cells exposed to normoxia (20% O2) or hypoxia (1% O2) for 16 h. The candidate binding sites (site 1 and site 2) showed significant hypoxia-induced binding of HIF-1α and HIF-1β but not HIF-2α (Fig. 3C, J).

In general, the transcription start sites of actively transcribed genes are marked by trimethylated acetylated H3K27 (H3K27ac) and H3K4 (H3K4me3) [35]. Thus, we next investigated the enrichment of these histone modifications on specific HREs. We performed ChIP assays with antibodies against H3K27ac and H3K4me3, followed by qPCR with primers flanking the HIF-1-binding sites (site 1 and site 2) of ABCB6 in A549-WT and A549-GR cells. Obvious enrichment of these histone marks of transcriptional activation (H3K27ac and H3K4me3) at HRE site 1 (Fig. 3D, E) and site 2 (Fig. 3K, L) was observed in GR cells compared to WT cells. We further exposed A549-WT cells to normoxia (20% O2) or hypoxia (1% O2) for 16 h and performed ChIP with antibodies against H3K27ac and H3K4me3. Again, hypoxic treatment significantly increased H3K27ac (Fig. 3F, M) and H3K4me3 (Fig. 3G, N) marks at the HIF-1-binding sites of ABCB6. Similar results were obtained by performing ChIP-qPCR assays in H1703-GR cells as compared to H1703-WT cells (Fig. S4C and S4E) and further confirmed in H1703 cells exposed to hypoxia compared to normoxia (Fig. S4D and S4F). Taken together, the results of these

### Table 3

Pearson’s correlation test of microarray data from The Cancer Genome Atlas (TCGA) dataset of 1267 lung squamous carcinomas

| Category | GENE | ABCB6 | CA9 | LDHA | PDK1 |
|----------|------|-------|-----|------|------|
| HIF targets | P4HA1 | *** | *** | *** | *** |
|           | LDHA  | *** | -  | -   | -    |
|           | PLOD2 | *** | *** | *** | *** |
|           | SLC16A3 | *** | *** | *** | n.s  |
|           | SLC2A1 | *** | *** | *** | n.s  |
|           | ANGPTL4 | n.s | *** | *** | n.s  |
|           | PDK1  | *** | *** | -   | -    |
|           | L1CAM | n.s | *** | n.s | ***  |
|           | CA9   | *** | -  | -   | -    |
|           | BNIP3 | *** | *** | *** | *** |
|           | IGFBP1 | *** | n.s | *** | n.s  |
|           | PKM2  | *** | *** | *** | *** |
|           | MET   | *** | *** | *** | *** |
|           | PLOD1 | *** | n.s | *** | *** |
|           | P4HA2 | *** | *** | n.s | ***  |
|           | ABCB6 | -   | *** | *** | *** |

Positive correlation *P<0.05, **P<0.01, ***P<0.001, n.s, not significant

### Table 4

Pearson’s correlation test of microarray data from The Cancer Genome Atlas (TCGA) dataset of 706 lung adenocarcinomas

| Category | GENE | ABCB6 | CA9 | LDHA | PDK1 |
|----------|------|-------|-----|------|------|
| HIF targets | P4HA1 | *** | *** | *** | *** |
|           | LDHA  | *** | -  | -   | -    |
|           | PLOD2 | *** | *** | *** | *** |
|           | SLC16A3 | *** | *** | *** | n.s  |
|           | SLC2A1 | *** | *** | *** | n.s  |
|           | ANGPTL4 | n.s | *** | *** | n.s  |
|           | PDK1  | *** | *** | -   | -    |
|           | L1CAM | n.s | *** | n.s | ***  |
|           | CA9   | *** | -  | -   | -    |
|           | BNIP3 | *** | n.s | *** | *** |
|           | IGFBP1 | *** | n.s | *** | *** |
|           | PKM2  | *** | *** | *** | *** |
|           | MET   | *** | *** | *** | *** |
|           | PLOD1 | *** | n.s | *** | *** |
|           | P4HA2 | *** | *** | n.s | ***  |
|           | ABCB6 | -   | *** | *** | *** |

Positive correlation *P<0.05, **P<0.01, ***P<0.001, negative correlation nP<0.01, n***P<0.001, n.s, not significant
HIF-1-dependent heme synthesis promotes gemcitabine resistance in human non-small cell lung…

ABCB6 is an ATP-binding cassette half-transporter and a specific heme-binding protein [36]. Heme plays a vital role in many biological processes such as oxidative metabolism and ATP generation, detoxification, oxygen transport, circadian rhythm, and regulation of transcription and translation [37, 38]. To investigate the mechanism by which the HIF-1α/ABCB6 axis regulates acquired gemcitabine resistance in NSCLC cells, we first explored the function of ABCB6 in heme synthesis. First, A549 and H1703 subclones were stably transfected with expression vectors encoding a shRNA targeting ABCB6 (shABCB6) or shRNAs targeting both HIF-1α and ABCB6 (shHIF-1α/ABCB6). Since HIF-1α protein is mostly degraded in WT cells under normoxia, we verified the efficiency of HIF-1α and ABCB6 double knockdown in A549-GR and H1703-GR subclones (Fig. 5A, B). HIF-1α/ABCB6 knockdown cells and to a greater extent in HIF-1α/ABCB6 double-knockdown cells (Fig. 4A, B). Knockdown of ABCB6 led to significantly decreased heme accumulation in both the GR and WT subclones (Fig. 4A, B). Gemcitabine-induced heme accumulation was also decreased clearly in HIF-1α knockdown cells and to a greater extent in HIF-1α/ABCB6 double-knockdown cells (Fig. 4A, B). These data suggest that gemcitabine-induced HIF-1α/ABCB6 activity promotes intracellular heme synthesis.

As a co-factor, heme has been demonstrated to be critically important in regulating the stability and activity of catalase, a key cellular and tissue antioxidant enzyme [11, 39]. We further confirmed that the protein expression and activity of catalase were highly induced in A549-GR and H1703-GR cells compared to WT cells (Fig. 4C–E). Again, catalase protein expression (Fig. 4C) and activity (Fig. 4D, E) were largely decreased in ABCB6 or HIF-1α knockdown subclones, particularly in the HIF-1α/ABCB6 double-knockdown groups, which indicates the vital role of the HIF-1α/ABCB6 axis in regulating the heme-dependent antioxidant defense enzyme catalase.

We then analyzed intracellular ROS-mediated oxidative stress and observed significantly decreased levels of intracellular ROS in A549-GR (Fig. 4F) and H1703-GR (Fig. 4G). As a co-factor, heme has been demonstrated to be critically important in regulating the stability and activity of catalase, a key cellular and tissue antioxidant enzyme [11, 39]. We further confirmed that the protein expression and activity of catalase were highly induced in A549-GR and H1703-GR cells compared to WT cells (Fig. 4C–E). Again, catalase protein expression (Fig. 4C) and activity (Fig. 4D, E) were largely decreased in ABCB6 or HIF-1α knockdown subclones, particularly in the HIF-1α/ABCB6 double-knockdown groups, which indicates the vital role of the HIF-1α/ABCB6 axis in regulating the heme-dependent antioxidant defense enzyme catalase.

Inhibition of the HIF-1α/ABCB6 axis sensitizes NSCLCs to gemcitabine therapy in animal models

Mouse xenograft models were established using A549 or H1703 subclones treated with or without gemcitabine to determine whether knockdown of ABCB6 and/or HIF-1α confers a sensitive phenotype to lung cancers previously resistant to gemcitabine therapy in vivo. The grouping and treatments of animals are outlined in Fig. 6. All animals received intraperitoneal injection of gemcitabine (20 mg/kg) or saline (250 µl) after palpable tumors had formed. Tumor growth was monitored twice per week. We observed similar results in both A549 (Fig. 6A) and H1703 (Fig. 6C) groups. The growth of GR tumors in mice transfected with shHIF-1α or shABCB6 was significantly decreased compared to that of GR tumors in mice transfected with NTC upon treatment with gemcitabine (Fig. 6A, C). The GR tumors in mice transfected with shHIF-1α/ABCB6 demonstrated a further decrease in the tumor volume compared with those in the
Fig. 3 The ABCB6 gene is a HIF-1α target gene. A Nucleotide sequence of the hypoxia response element-1 (HRE site 1; 5′-ACCGTG-3′) HIF-1-binding site is shown in red) in the 5′-flanking region of the ABCB6 gene, located ~0.1 kb from the transcription start site. ABCB6 exons and HRE are indicated by black bars and arrow, respectively. B Chromatin immunoprecipitation (ChIP) assays were performed using IgG or antibodies against HIF-1α, HIF-1β and HIF-2α in A549-GR and A549-WT cell lines. Primers flanking HRE site 1 were used for qPCR, and results were normalized to lane 1 (mean ± SEM; n = 3). ***P < 0.001 compared to WT (ANOVA with Bonferroni post-test). C ChIP assays were performed using IgG or antibodies against HIF-1α, HIF-1β and HIF-2α in A549-GR WT cells exposed to normoxia (20% O2) or hypoxia (1% O2) for 16 h. ***P < 0.001 compared to normoxia (ANOVA with Bonferroni post-test). D, E ChIP assays were performed using IgG or antibodies against H3K27ac (D) and H3K4me3 (E), followed by qPCR with primers flanking HIF-1-binding site 1 in the ABCB6 gene in A549-GR and A549-WT cells. **P < 0.01, ***P < 0.001 compared to WT (ANOVA with Bonferroni post-test). F, G ChIP assays were performed using antibodies against H3K27ac (F) and H3K4me3 (G), followed by qPCR with primers flanking HRE site 1 in A549 cells exposed to normoxia (20% O2) or hypoxia (1% O2) for 16 h (mean ± SEM; n = 3). ***P < 0.001 compared to normoxia (ANOVA with Bonferroni post-test). H, Nucleotide sequence of HRE site 2 (5′-CGGCGTG-3′ HIF-1-binding site and a direct repeat 5′-GCGTG-3′ sequence separated by 8 bp are shown in red) within exons 1 of the ABCB6 gene, located 487 bp from the transcription start site. Exons and intron are not drawn to scale. I, J ChIP assays were performed using IgG or antibodies against HIF-1α, HIF-1β and HIF-2α in A549 WT and A549-GR cells (I), and in A549-GR cells under normoxic or hypoxic conditions (J). Primers flanking the HRE site 2 were used for qPCR, and results were normalized to lane 1 (mean ± SEM; n = 3). I ***P < 0.001 compared to WT; J **P < 0.01, ***P < 0.001 compared to normoxia (ANOVA with Bonferroni post-test). K, L ChIP assays were performed using IgG or antibodies against H3K27ac (K) and H3K4me3 (L), followed by qPCR with primers flanking HIF-1-binding site 2 in the ABCB6 gene in A549-GR and A549-WT cells. ***P < 0.001 compared to WT (ANOVA with Bonferroni post-test). M, N ChIP assays were performed using antibodies against H3K27ac (M) and H3K4me3 (N), followed by qPCR with primers flanking HRE site 2 in A549-GR cells exposed to normoxia (20% O2) or hypoxia (1% O2) for 16 h (mean ± SEM; n = 3). ***P < 0.001 compared to normoxia (ANOVA with Bonferroni post-test).

Discussion

Although gemcitabine is approved as the first-line chemotherapeutic drug for pancreatic adenocarcinoma treatment, it is also critically and widely used to treat breast, bladder, and non-small cell lung cancers [44]. Unfortunately, patients commonly develop acquired resistance shortly after beginning gemcitabine treatment, which ultimately leads to treatment failure [45]. Cancer cells are known to employ a variety of mechanisms to acquire resistance against gemcitabine-based treatment. Most previous studies have focused on acquired resistance due to changes in the rate of drug influx or efflux. For instance, ABC transporters such as ABCB1, ABCC1 and ABCG2 have crucial roles in developing resistance via the efflux of anticancer drugs outside of cancer cells [46]. In general, ABC transporters use ATP to facilitate not only the transmembrane flux of drugs, but also drug metabolites...
Fig. 4 Effect of HIF-1α/ABCB6 signaling on heme-mediated ROS scavenging in GR cells. A, B, Heme content in A549 (A) and H1703 (B) GR and WT subclones, which were stably transfected with non-targeting control (NTC), shRNA targeting HIF-1α (shHIF-1α), shRNA targeting ABCB6 (shABCB6), or shRNAs targeting both HIF-1α and ABCB6 (shHIF-1α/ABCB6) (mean ± SEM; n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared to WT-NTC cells; ##P < 0.01, ###P < 0.001 compared to GR-NTC cells (ANOVA with Bonferroni post-test).

Catalase protein expression levels in A549 and H1703, GR and WT subclones (N: NTC; 1α: shHIF-1α; A6: shABCB6; DKD, double knockdown: shHIF-1α/ABCB6).

D, E Catalase activity in A549 (D) and H1703 (E), GR and WT subclones (NTC, shHIF-1α, shABCB6, and shHIF-1α/ABCB6) (mean ± SEM; n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared to WT-NTC cells; #P < 0.05, **P < 0.01 compared to GR-NTC cells (ANOVA with Bonferroni post-test).

F, G ROS generation in A549 (F) and H1703 (G), GR and WT subclones (NTC, shHIF-1α, shABCB6, and shHIF-1α/ABCB6). Intracellular hydrogen peroxide was detected by DCF-based measurement (mean ± SEM; n = 3). *P < 0.05, ***P < 0.001 compared to WT-NTC cells; #P < 0.05, ***P < 0.001 compared to GR-NTC cells (ANOVA with Bonferroni post-test).

H, I Effect of gemcitabine on the growth of A549 (H) and H1703 (I), GR and WT subclones (WT, NTC, shHIF-1α, shABCB6, and shHIF-1α/ABCB6). Cells were cultured in medium with or without gemcitabine (Gem, 1 μM) before measurement of cell viability. Relative survival is plotted as percent of WT control cells cultured in normal medium (mean ± SEM; n = 3). ***P < 0.001 compared to WT or GR-NTC cells with Gem; #P < 0.05, ##P < 0.01 compared to GR-NTC cells without Gem (ANOVA with Bonferroni post-test).
including saccharides, amino acids, peptides and proteins, and lipids and sterols, and they also participate in a variety of biological processes, such as antigen presentation, insulin secretion, and intracellular metabolic reprogramming [11]. However, the critical functions of ABC transporters as regulators or co-factors to activate intracellular signal pathways or metabolic reprogramming remain relatively unexplored. The present study demonstrates a mechanism of gemcitabine chemoresistance by which cancer cells increase intracellular ABCB6 expression in a HIF-1α-dependent manner that can, in turn, render antioxidant enzyme catalase effective by promoting heme biosynthesis. The results of the present study provide strong evidence for the inhibition of the HIF-α/ABCB6 axis in targeting lung cancer cell heme metabolism to disturb mitochondrial redox homeostasis, increase oxidant levels, and induce cell apoptosis, thereby abrogating gemcitabine resistance in NSCLCs (Fig. 7).

Fig. 5 ABCB6 inhibition by tomatidine reverses gemcitabine resistance in NSCLC cells. A, B Representative immunoblots from A549 (A) and H1703 (B) cells treated with tomatidine at indicated concentrations (0, 2, 6, 8, 10 μmol/l) for 24 h. C, D Heme content in A549 (C) and H1703 (D) GR and WT cells treated with DMSO or 10 μmol/l tomatidine for 24 h. E, F, Catalase activity in A549 (E) and H1703 (F) GR and WT cells treated with DMSO or 10 μmol/l tomatidine for 24 h. G, H Cell survival of A549 (G) and H1703 (H) GR and WT cells incubated with vehicle or 10 μmol/l tomatidine for 24 h, with or without the treatment with 1 μM gemcitabine (mean ± SEM; n=3). *P<0.05, **P<0.01, ***P<0.001 compared to WT cells; ###P<0.001 compared to GR cells (ANOVA with Bonferroni post-test).
Previous studies have established that intratumoral hypoxia increases the stabilization and accumulation of HIF-1α, a critical transcriptional factor that responds to low oxygen condition, and that HIF-1α expression is associated with the development of resistance to chemotherapy and radiotherapy in multiple human cancer types [47]. However, further research has revealed that in addition to hypoxia, chemotherapeutic agents and radiation also
HIF-1-dependent heme synthesis promotes gemcitabine resistance in human non-small cell lung cancer cells.

In Fig. 6 Inhibition of the HIF-1α/ABCB6 axis sensitizes NSCLCs to gemcitabine therapy in animal models. A549 and H1703, GR and WT subclones (2 × 10⁶ cells) were injected into the groin of 6- to 8-week-old male SCID mice. After palpable tumors had formed (8 days after tumor implantation), mice received intraperitoneal injection of gemcitabine (20 mg/kg) or saline (250 μl) twice per week. A, C Volume of primary tumor formed by A549 (A) or H1703 (C) subclones, as determined twice weekly. ***P < 0.001 vs. NTC by two-way ANOVA with Bonferroni post-test (mean ± SEM; n = 6). B, D Kaplan–Meier survival curves for the 6 treatment groups of A549 (B) and H1703 (D) cell-derived tumors as mentioned above. The log-rank test was used to demonstrate the statistical difference (n = 10; NTC, non-targeting control; GR, gemcitabine resistant; Gem, gemcitabine). E, F After 42 days, primary tumors were harvested, and apoptosis evaluated by TUNEL assay in A549 (E) and H1703 (F) cell-derived tumors (N: NTC; S: saline; G: gemcitabine; sh1α: shHIF-1α). Scale bar = 50 μm. G, H Percentages of TUNEL-positive cells in A549 (G) and H1703 (H) cell-derived tumors. Cells were counted in 10 randomly selected fields in 6 tumor samples from each group (mean ± SEM). ***P < 0.001

can increase the levels of HIF-1α, which regulates multiple downstream target genes associated with many key steps of cancer progression, particularly acquired chemotherapy resistance [48]. HIFs are heterodimers composed of HIF-1α or HIF-2α subunits and a constitutively expressed HIF-1β subunit. Under hypoxic conditions, the O2-regulated prolyl hydroxylation-induced proteasomal degradation of HIF-1α is inhibited, leading to rapid accumulation of HIF-1α, dimerization with HIF-1β, binding to the consensus DNA sequence 5′-RCGTG-3′ within HREs located in target genes, and transcriptional activation [19]. Conversely, in normoxic conditions, HIF-1α is subject to ubiquitination and degradation. Intriguingly, we observed obvious stabilization and accumulation of HIF-1α even under normoxic conditions in GR lung cancer cell lines that had been exposed to gemcitabine for months. Our results demonstrate that gemcitabine-induced HIF-1α increased ABCB6 transcriptional activities by binding with two HREs (−155 bp site and +87 bp site) in the ABCB6 gene sequence. Moreover, we observed induction of H3K27ac and H3K4me3 modifications at specific HREs of the ABCB6 gene that promotes chromatin accessibility and HIF-1 binding to these regions in response to chemotherapy or hypoxia. However, the activation mechanism of HIF-1α expression under normoxia in GR cells remains unclear. It is possible that another protein functions as a modulator or coregulator in coordination with HIF-1α to facilitate its stability under normoxia. A study by Chaika et al. demonstrated that type I transmembrane protein physically interacts with HIF-1α and p300 and stabilizes HIF-1α at the protein level [49]. Shukla et al. also reported upregulation of HIF-1α protein in normoxic GR pancreatic cancer cells [31].

Multiple molecular mechanisms contributed to the acquired resistance of cancer cells to chemotherapeutics, and metabolic rewiring of tumor cells has been identified as a critical step in acquired drug resistance [50]. Cancer cells alter their metabolism by monitoring nutrient uptake (such as glucose and glutamine) and metabolite secretion, which leads to the modulation of certain metabolic pathways in response. As a critical transcriptional factor, HIF-1α has been shown to modulate multiple genes related to cancer metabolic reprogramming, including glycolysis [51], branched-chain amino acid metabolism [52], nucleotide synthesis [53], and lipid synthesis [54]. In addition, our previous studies and results from other groups consistently showed the involvement of HIF-1α in the regulation of other important metabolic pathways of cancer cells, such as glutamine metabolism and glutathione synthesis [13, 19, 55]. In the present study, we focused on heme metabolism. Heme is a central metabolic and signaling molecule that regulates diverse molecular and cellular processes related to oxidative metabolism and ATP generation. A recent study by Sohoni et al. showed that heme synthesis and uptake are enhanced in NSCLC cells [38]. Here, we show that the levels of heme biosynthesis are upregulated in GR NSCLC cells relative to gemcitabine-sensitive NSCLC cells. Our results further demonstrated that this increase is regulated by the HIF-α/ABCB6 axis. Heme binds to and directly regulates the activities of many proteins controlling processes ranging from tyrosine kinase signaling to redox homeostasis [11, 56, 57]. The present study suggests that increased heme biosynthesis in GR cells causes the elevation of ROS scavenger catalase activity, which in turn leads to redox homeostasis destruction and tumor survival.

Gemcitabine combined with platinum is a standard therapy for NSCLC. However, platinum-based chemotherapy has been shown to produce limited clinical benefits for cancer patients due to various adverse effects, and acquired tumor resistance remains a common problem [58, 59]. Accordingly, increasing evidence has suggested that the combination of chemotherapy with another form of anticancer therapy such as a tyrosine kinase inhibitor (TKI) [60], angiogenesis inhibitor [61], or immune checkpoint inhibitor [62] can produce better therapeutic outcomes. Given that hypoxia and HIF-1α activation are prominent features of various solid tumors that are, at least in part, responsible for chemotherapy resistance in these tumors, reversal of hypoxia or inhibition of HIF-1α signaling can improve the effectiveness of cancer therapies [63–65]. Our results suggest that a combination therapy consisting of a HIF-1α inhibitor and gemcitabine offered improved anti-tumor efficacy in the NSCLC tumor model. This combination therapy inhibited tumor growth and prolonged animal survival through increased chemotherapy sensitivity, leading to suppression of tumor cell proliferation as well as increased tumor cell apoptosis. Therefore, gemcitabine/HIF-1α-trap combination therapy might represent a more effective alternative for human lung cancer. The results of
the present study provide rationale for human clinical studies to investigate the benefits of such combination therapy in lung cancer.

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Author contributions LX, BS and GX designed the study and wrote the paper. LX, GX, JL and FN performed the experiments. LX, SW, YG and HD analyzed the data. BS and GX advised on experimental procedures and revision of the paper. YW revised the paper. All the authors contributed to this manuscript. All the authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this article.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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