HIF-1-Dependent Heme Synthesis Promotes Gemcitabine Resistance In Human Non-Small Cell Lung Cancers Via Enhanced ABCB6 Expression

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Research

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Abstract

Background: Gemcitabine, a deoxycytidine analogue, is commonly used in the treatment of various types of tumors, including human non-small cell lung cancer (NSCLC). However, even the initial responders rapidly develop acquired resistance which limits our ability to effectively treat advanced NSCLC. The present study reports a widely prevalent mechanism of resistance to gemcitabine in NSCLC, whereby induced expression of ABCB6 is associated with poor prognosis and gemcitabine resistance in human NSCLCs which is in a HIF-1-dependent manner.

Methods: We established gemcitabine resistant LUSC and LUAD cells and examined ABCB6 expression as well as heme synthesis in these cells. Additionally, we analyzed catalase protein expression and activity in gemcitabine resistant NSCLC cell lines. To validate *ABCB6* gene as an HRE, we performed ChIP-qPCR assay. We also investigated the effect of inhibition of HIF- α /ABCB6 axis in *vitro* and in *vivo*.

Results: The activation of HIF-1 α /ABCB6 signaling leading to intracellular heme metabolic reprogramming and a corresponding increase in heme biosynthesis to enhance the activation and accumulation of catalase. Increased levels of catalase diminish the effective levels of reactive oxygen species (ROS) promoting gemcitabine-based resistance. Targeting HIF-1 α or ABCB6, in combination with gemcitabine, strongly restrains tumor proliferation, increases tumor cell apoptosis and prolongs animal survival.

Conclusion: In combination with gemcitabine-based chemotherapy, targeting HIF-1 α /ABCB6 signaling could result in enhanced tumor sensitivity to chemotherapeutic agent and may improve outcome in NSCLCs.

Background

Lung cancer is one of the most common form of [malignant tumor](#) and is the most frequent cause of cancer-related deaths in men and in women worldwide [1]. It is estimated that 1.8 million people will be diagnosed with lung cancer, and 1.6 million people will die from this disease every year [2]. In China, it was estimated that there were approximately 733,300 new cases diagnosed and 610,200 patients died of the disease in 2015 [3]. Non-small cell lung cancer (NSCLC), which consists of three subtypes (large-cell carcinoma, squamous-cell carcinoma and adenocarcinoma), accounts for more than 85% of all lung cancer cases [4]. Due to most patients are diagnosed at advanced stage with advanced local invasion or [distant metastasis](#), the 5-year survival of lung cancer is only 4-17% [5]. Despite the development in surgery and targeted therapy, systemic chemotherapy remains the current mainstay of treatment for advanced lung cancer. Gemcitabine, a deoxycytidine analogue, exerts an anticancer effect by catalyzing the complex intracellular conversion to gemcitabine diphosphate and triphosphate that are important for terminating DNA synthesis [6]. Clinically, gemcitabine has been used as one of the standard regimens for NSCLC [7]. However, gemcitabine-based chemotherapy resistance always constrains our ability to effectively treat advanced NSCLC. Besides the inherent resistance to gemcitabine, even the initial

responders frequently develop acquired resistance. Hence, it is critically important to determine the mechanism of gemcitabine-based resistance, moreover, the approach of overcoming chemotherapy resistance is urgently needed.

Adenosine triphosphate (ATP)-binding cassette (ABC) transporter family members consists of 11 members which 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 transporters have been found play crucial roles in development of drug resistance by the efflux of anticancer agents outside of cancer cells [9]. However, this could not fully explain the underline mechanism of acquired chemotherapy resistance. ATP binding cassette subfamily B member 6 (ABCB6) is one of the ABC transporter family members that encodes an 842 amino acid protein [10]. As an energy-dependent transporter, ABCB6 is capable of bind with heme and involved in heme biosynthesis [11]. To know attribution of ABCB6 to gemcitabine resistance in NSCLC cell, we established gemcitabine-resistant human lung squamous cancer cell line and lung adenocarcinoma cell line respectively, 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. Inhibition of ABCB6 restrain intracellular heme accumulation. Interestingly, we also found this interaction is regulated by gemcitabine-induced hypoxia-inducible factor-1 α (HIF-1 α). HIF-1 α , stably expressed response to low oxygen condition (hypoxia), is a transcriptional activator that regulate the expression of thousands of target genes [12]. In addition to hypoxia, stimuli such as chemotherapeutic agents or radiation increase the stabilization and accumulation of HIF-1 α [13, 14]. We observed a stable expression of HIF-1 α even under normoxic condition. Additionally, we demonstrated in our current paper that ABCB6 is a direct HIF-1 α target gene, and further identified hypoxia response elements (HREs) in *ABCB6* gene.

Chemotherapeutic drug-induced cell apoptosis was linked with enhanced levels of reactive oxygen species (ROS) which mainly contains superoxide (O_2^-) and hydrogen peroxide (H_2O_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 chemo-drugs induced ROS. Catalase, an enzyme degrading hydrogen peroxide by converting H_2O_2 to H_2O and O_2 , is generally recognized as antioxidant enzyme that confers cellular protection [16]. The heme metabolism is one of the important cellular mechanisms that influence catalase activities. As a heme containing enzyme, the regulations of H_2O_2 decomposition and peroxidase activities by catalase are heme dependent [17]. To determine whether HIF-1 α /ABCB6 regulated heme metabolism reprogramming influence catalase, we analyzed catalase protein expression and activity in gemcitabine resistant NSCLC cell lines and observed a dramatic induction of both catalase protein expression and activity, which further facilitates to ROS scavenging that directly contribute to promoting gemcitabine-based resistance.

Methods

Statistical analysis of microarray data and survival

Gene expression data from the lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) dataset of The Cancer Genome Atlas (TCGA) were obtained from <http://ualcan.path.uab.edu/index.html> or <http://xena.ucsc.edu>. Pearson's correlation coefficient was used to determine *P* values for co-expression. Prognostic significance of ABCB6 expression in lung cancer was analyzed using the Kmplot (www.kmplot.com) [18]. Briefly, gene was entered into the online database to obtain Kaplan–Meier survival plot. The log rank *P* were calculated and displayed on the webpage. The survival rates were investigated with median cut-off (Low represents patients with indicated gene mRNA levels less than the median. High represents patients with indicated gene mRNA levels greater than the median.)

Clinical and specimens

Patients diagnosed with advanced NSCLC were enrolled in the Cancer Centre of the First Affiliated Hospital (Southwest Hospital) of Army Medical University (Chongqing, China) from January 2010 to December 2014. The clinical and pathologic characteristics of the patients are summarized in Table 1. All patients received routine gemcitabine-platinum chemotherapy (completed 2–4 cycles). Forty-one of specimens were selected from patients with chemotherapy failure (including progressive disease, PD) after gemcitabine-based chemotherapy, and constituted the gemcitabine-resistant group. Forty of specimens were selected from patients who had positive response after gemcitabine therapy including partial response (PR), which 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. Clinical investigation complied with the ethical standards codified in the 1964 Declaration of Helsinki. The protocol of immunohistochemistry for 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 pretreatment paraffin-embedded tissue blocks from lung cancer patients who underwent surgery at Southwest Hospital. All specimens were confirmed by pathological examination and were made into tissue microarrays as described previously [19].

Cell culture and drug treatment

All human NSCLC cell lines were purchased from the ATCC (Manassas, VA, USA) and cultured in DMEM (A549) or RPMI 1640 medium (NCI-H1703) supplemented with 10% fetal bovine serum (GIBCO), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (Beyotime Institute of Biotechnology, China). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The gemcitabine-resistant cell lines (A549-GR and H1703-GR) was established as follow: A549 or H1703 parental cells were exposed to gradually increasing concentrations of gemcitabine (Sigma-Aldrich) from 10 nM initially up to 10 μM over a 7-month period. For hypoxic exposure, cells were placed in a modular incubator chamber that was flushed with a 1% O₂/5% CO₂/94% N₂ gas mixture and sealed.

Lentiviruses transduction

The pLKO.1-puro lentiviral vectors encoding shRNA targeting HIF-1 α (sh1 α -1, Clone ID: NM_001530.x-2671s1c1; sh1 α -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 NTC were purchased from Sigma. Lentiviruses were packaged, and cells were transduced and subjected to puromycin selection as described [20].

RT-qPCR

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 (BioRad). The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative gene expression as described [21]. The nucleotide sequence of the primers is as follows: 18S rRNA forward primer: 5'-, GAGGATGAGGTGGAACGTGT-3', reverse primer: 5'-AGAAGTGACGCAGCCCTCTA-3'; ABCB6 forward primer: 5'-TGCTGTTTCGCTTCTACG-3', reverse primer: 5'-CCTCCACCTCATCATTCC-3'.

ChIP assay

ChIP assays were performed as previously described [19]. A549 and H1703 cells were cross-linked in 3.7% formaldehyde for 10 min and lysed with 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: HIF-1 α (Santa Cruz), HIF-2 α (Cell signaling), and HIF-1 β (Novus Biologicals). 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 TE buffer (10 mM Tris-HCl/1 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, suspended in 50 μ l TE buffer, and a 2- μ l aliquot was used for qPCR. Primer sequences are listed as follows: ABCB6-HRE-site 1 forward primer: 5'-CTGGTCGTCAGATTTCCCG-3', reverse primer: 5'-CCATTGGCCAAATGTCTCCG-3'; ABCB6-HRE-site 2 forward primer: 5'-CAGTTGGCAGGAGGGTCC-3', reverse primer: 5'-ACGTGGACCAGGCCTCA-3'.

Western blot assay

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

Heme detection

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 by using the Bio-Rad protein assay system (Bio-Rad, Munchen, Germany). Ten mg of protein samples were incubated with 500 μ l of 2 M Oxalic Acid (Sigma-Aldrich) at 100°C for 30 min. Samples were subsequently centrifuged at 14000 rpm for 10 min. The amount of heme in the supernatant was determined by fluorometric detection using a F4500 fluorescence spectrophotometer (Hitachi). 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

Cells were seeded into 6-well plates at a density of 10^5 cells/well. Cells were harvested and homogenized in 50 ml of 0.1 M PBS buffer. The mixture was centrifuged at 3000 rpm for 5 minutes at 4°C. The supernatant was separated and assayed for protein content (Bradford assay, Bio-Rad). Catalase activity was determined calorimetrically in 10 μ g protein 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 μ l), mixed with 750 μ l of 1X assay buffer and 25 μ l colorimetric assay substrate solution, was incubated for 5 minutes. The reaction was stopped using 900 μ l of stop solution and the tubes were kept inverted. Within 15 minutes, after the enzymatic reaction, 10 μ l aliquot of the reaction mixture was transferred to 1 ml of the color reagent. After the incubation of 15 minutes, the absorbance of the reaction mixture was measured at 520 nm. Specific catalase activity is reported as μ mol H_2O_2 consumed min^{-1} μ g protein $^{-1}$.

Colony formation assay

For detection of clonogenic ability of the cells with drug treatment, cells were trypsinized and seeded into 6-well plates at a density of 1000 cells per well. The culture medium, which containing gemcitabine (concentrations indicated) for 72 hours and was refreshed every 3 days. After 15 days, cells were fixed with 4% paraformaldehyde for 10 min and then stained by using 0.1% crystal violet in ddH₂O for 10 min and imaged using a digital camera to record the results.

Cell viability assay

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 μ M, 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M, 100 μ M) 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 manufacturer's instructions. All experiments were done in triplicates and was 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 the cell apoptosis in tumor tissue, we performed terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) technique, to formalin-fixed tumor samples in paraffin blocks, using the one-step TUNEL apoptosis assay kit (Beyotime Institute of Biotechnology, China). The sections (4–5 μ m) mounted on glass slides were deparaffinized, rehydrated through graded alcohols to water, treated with 20 μ g/ml proteinase K (37 °C, 20 min) and then washed in PBS buffer. TUNEL assay was then performed according to the manufacturer's instructions. The images were acquired using a confocal microscope (Leica TSC-SP5, Germany). Ten fields were randomly selected in each tumor slide.

ROS detection

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

Immunohistochemistry

Tumors and adjacent normal tissue were fixed in 10% formalin and paraffin embedded. Tissue microarrays were dewaxed in xylene, hydrated with graded ethanol, followed by antigen retrieval using citrate buffer (pH 6.1). The instantaneous SP supersensitive kit (SP-9000, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd) was used with antibodies against ABCB6 (Novus Biologicals). Sections were counterstained with Mayer's hematoxylin (Sigma). For quantitative measurement of ABCB6 positive staining on clinical samples (adjacent normal lung tissue, Gemcitabine resistant tumors and Gemcitabine sensitive tumors), immunohistochemical staining of ABCB6 was quantified by Image J software (NIH) as described previously [23]. For detail, we used average optical density (AOD) to evaluate the intensity of the IHC reaction in order to compare expression levels between tumor and peritumoral fields of different markers [24]. AOD scores were calculated from the optical density of 5 spots selected randomly on each sample under the microscope ($\times 200$).

Criteria for assessing IHC results

For each sample, five random fields were selected for scoring and a mean score of each slide was calculated in 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 are added to obtain a total score (TS range: 0, 2-7). Samples were divided into two categories depending on the IHC score: Category low corresponded to IHC score less than 4; Category high corresponded to IHC score greater than or equal to 4. Slides were examined and scored independently using GOG criteria [25] by two histopathologists (F Wu and Y Ling) blinded to other pathological information.

Animal studies

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Army Medical University (Chongqing, China). Six to eight-week-old male SCID mice were purchased from the Institute of Experimental Animal of the Army Medical University. A549 and H1703 subclones were harvested by trypsinization, resuspended at 10^7 cells/ml in a 50: 50 mix of PBS: Matrigel (BD Biosciences), and 2×10^6 cells were injected subcutaneously into the inguinal area of mice (12 mice per group). Tumor volume (mm^3) was calculated as $0.52 \times L \times W \times T$ (L: Length, W: width, T: thickness). Tumor volumes and body weights were monitored twice weekly. After palpable tumors formed (8 days after tumor implantation), mice received intraperitoneal injection of gemcitabine (20 mg/kg) or saline (250 μl), which was administrated twice per week. After 42 days, tumors were excised and weighed ($n = 6$). Xenografts were harvested to be subjected to 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 the p value.

Statistical Analysis

All data were expressed as mean $\pm$ SEM and were analyzed using an unpaired two-tailed Student's t -test for two groups or ANOVA followed by Bonferroni posttest for multiple groups. 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 expressions are increased in lung cancer tissues of gemcitabine resistant patients after chemotherapy, and are associated with poor prognosis in human NSCLCs

To investigate whether ABCB6 expression has clinical significance in human NSCLCs, we compared *ABCB6* gene expression in lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) and normal lung tissue using the Cancer Genome Atlas (TCGA) database. Analysis of database revealed that ABCB6 mRNA levels were significantly greater in human NSCLCs tissue (LUSC and LUAD) than in the normal lung tissues (Figure 1A and 1B). Analysis of ABCB6 mRNA expression in 1926 human lung cancer specimens [19, 26] revealed that levels above the median were associated with a significant decrease in patient survival (Figure 1C). To evaluate whether the expressions of ABCB6 of human NSCLCs are different in patients with varying gemcitabine-based chemotherapy sensitivity, we used immunohistochemical staining to examine the expressions of ABCB6 in the 41 cases of gemcitabine-resistant tumor tissues, 40 cases of gemcitabine-sensitive tumor tissues and 6 cases of adjacent normal lung tissues. The positive expressions of ABCB6 in tumor tissues of gemcitabine-resistant patient were much higher than those of chemo-sensitive patients, but no positive or weak staining had been seen in the adjacent normal lung tissues (Figure 1D). Quantification of staining density of ABCB6 in multiple samples indicated that ABCB6 expression was increased by more than two-fold in these gemcitabine-resistant tumors compared with gemcitabine-sensitive tumors (Figure 1E). The patient characteristics were described in Table 1. We found that the expressions of ABCB6 were not correlated to the patient's age, gender, histology, differentiation, and Clinical stage ($p \geq 0.05$) (Table 1). Interestingly, the high levels of ABCB6 staining were significantly correlated with negative response to gemcitabine treatment including progressive disease (PD) ($p < 0.001$), as well as patient with distant metastasis (Table 1). The results of immunohistochemical expressions of ABCB6 in human non-small cell lung cancer tissues (41 cases of gemcitabine-resistant patients and 40 cases of gemcitabine-sensitive patients) were summarized in Table 1. The levels of ABCB6 staining were positively correlated with gemcitabine resistance of NSCLCs ($p < 0.001$) (Table 2).

Establishment of gemcitabine-resistant non-small cell lung cancer 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, wild-type (WT) lung cancer cell line of A549 and NCI-H1703 were cultured with increasing concentrations of gemcitabine over a period of approximately 7 months, respectively. To determine the differences in resistant phenotypes between gemcitabine-resistant (GR) cells and WT cells, we first measured the cell viability of each line after exposure to different concentrations of gemcitabine (0.001–100 μ M). The MTS colorimetric assay demonstrated that A549-GR cells were more resistant than A549-P cells to increasing gemcitabine concentrations (Figure 2A). Similar results were obtained by performing cell viability assays in H1703 cells (Figure 2B). We also observed an increase in clonogenic ability of the A549 Gem-R cells (Figures 2C–2D) and H1703 Gem-R cells (Figures 2E–2F) under control and gemcitabine treatment and increased colony number and size in soft agar assays. These data indicate that A549 and H1703 Gem-R cells have greater chemoresistance capability to gemcitabine treatment.

ABCB6 expression is induced in gemcitabine-resistant lung cancer cells in a HIF-1-Dependent manner

We next determined if ABCB6 expression increased in gemcitabine resistant lung cancer cell lines by performing RT and realtime qPCR and immunoblot. We observed that A549-GR and H1703-GR cells both express significantly higher levels of ABCB6 mRNA (Figure 3A, 3B) and protein (Figure 3C), indicating ABCB6 may participate in gemcitabine induced chemotherapy resistance in lung cancers. HIF-1 α is a transcription factors that serve as master regulators of cellular responses to hypoxia, and it is always associated with aggressive phenotypes, including resistance to radiation and chemotherapy, metastasis, and poor patient prognosis [27]. Interestingly, chemotherapy induce HIF-1 α expression even under normoxic conditions [28]. We observed that HIF-1 α expression was induced in A549-GR and H1703-GR cells under normoxia (Figure 3C), indicating HIF-1 α is participate in acquired gemcitabine resistance. We hypothesized that gemcitabine induced ABCB6 expression is regulated by transcription factor HIF-1 α in gemcitabine-resistant lung cancer cells. To determine our hypothesis, gene expression data from 1267 human lung squamous carcinoma specimens and 706 lung adenocarcinoma specimens in the TCGA database were analyzed to compare levels of ABCB6 mRNA with a HIF metagene signature based on the expression of LDHA, PLOD1, PLOD2, P4HA1, P4HA2, SLC16A3, SLC2A1, ANGPTL4, PDK1, L1CAM, CA9, BNIP3, IGFBP1, PKM2 and MET mRNA, which are all HIF-regulated genes. Statistical analysis of individual genes in the HIF signature revealed that ABCB6 expression was significantly correlated with 12 out of 15 HIF target genes in LUSC and LUAD databases respectively (Table 3 and 4), which indicated that ABCB6 mRNA expression may be HIF-regulated in human NSCLCs. To confirmed this, we measured ABCB6 expression in A549 and H1703 (including WT and GR cells) subclones, which were stably transfected with an expression vector encoding short hairpin RNA (shRNA) targeting HIF-1 α (sh1 α -1 and sh1 α -2) or a non-targeting control shRNA (NTC). We observed that the induction of ABCB6 mRNA expression was significantly decreased both in A549 (Figure 3D) and H1703 subclones (Figure 3E) when HIF-1 α was silenced. Induction of ABCB6 protein expression was also abrogated in A549 (Figure 3F) and H1703 subclones (Figure 3G) by two different shRNAs (sh1 α -1 and sh1 α -2) targeting HIF-1 α . Taken together, the data presented in Figures 1, 2 and 3 indicate that ABCB6 expression is induced in gemcitabine resistant lung cancer cells in a HIF-1 α dependent manner.

Identification of HIF-responsive elements (HREs) in the human ABCB6 gene

HIF- α protein binds with its cofactors to HIF-responsive elements (HREs) in the promoters of its target genes to coordinate a wide-ranging transcriptional programme in response to hypoxic environment [29, 30], as well as the stimulus from radiation and chemotherapy [31]. We hypothesized that *ABCB6* is a direct HIF-target gene. To this end, we analyzed the *ABCB6* locus for the presence of matches to the consensus HIF binding site sequence (5'-RCGTG-3') located in DNase I-hypersensitive chromatin domains using the UCSC Genome Bioinformatics database (Fig. 4C and 4D), by which two candidate sites that met these criteria were identified (Fig. S1). To validate, we performed ChIP-qPCR assay in A549 WT and A549 GR cells. The ChIP assays demonstrated that binding of HIF-1 α , HIF-1 β , but not HIF-2 α to the two candidate sites (-155 bp site and +87 bp site) was induced in A549 WT and A549 GR cells (Figure 4B and 4E). Conversely, the enrichment of IgG at these HREs were not observed an significant induction, suggesting the specificity of the assay (Figure 4B and 4E). We further confirmed our results by performing

ChIP-qPCR assays in H1703 WT and H1703 GR cells (Figure 4C and 4F). Taken together, the data presented in above demonstrate that HIF-1, but not HIF-2, bind directly to HRE sites adjacent to the transcription initiation site of the ABCB6 gene in gemcitabine resistant lung cancer cells.

HIF-1 α /ABCB6 axis regulate heme synthesis to acquire chemoresistance of gemcitabine treatments

ABCB6 is an ATP-binding cassette half-transporter and is a specific heme-binding protein [32]. Heme has a vital role in many biological processes such as oxidative metabolism and ATP generation, detoxification, oxygen transport, circadian rhythm, regulation of transcription and translation [33, 34]. To investigate the mechanism of HIF-1 α /ABCB6 axis regulate acquired gemcitabine resistance, we first identified the influence of ABCB6 in heme synthesis. To this end, A549 and H1703 subclones were stably transfected with expression vectors encoding shRNA targeting ABCB6 (shABCB6), or shRNAs targeting both HIF-1 α and ABCB6 (shHIF-1 α /ABCB6) (Figure S2A and S2B), and then, heme biosynthesis was detected in each subclones. Clearly, heme biosynthesis was increased in gemcitabine resistant A549 and H1703 non-targeting control (NTC) cells compared to that in WT groups (Figure 5A and 5B). Knockdown of ABCB6 led to significantly decreased heme accumulation in both GR and WT subclones (Figure 5A and 5B). Gemcitabine-induced heme accumulation was also decreased clearly in HIF-1 α knockdown cells, and to a greater extent in HIF-1 α /ABCB6 double knockdown cells (Figure 5A and 5B). These data suggests that gemcitabine-induced HIF-1 α /ABCB6 promote intracellular heme synthesis. As a co-factor, heme has been demonstrated critically in regulating the stability and activity of catalase, which is a key cellular and tissue antioxidant enzyme defenses against oxidative stress [11, 35]. We further confirmed that the protein expression and activity of catalase were both highly induced in A549 and H1703 GR cells compared to WT cells (Figure 5C, 5D and 5E). Again, catalase protein expression (Figure 5C) and activity (Figure 5D and 5E) were largely decreased in *ABCB6* or *HIF-1 α* knockdown subclones, particularly in *HIF-1 α /GLS1* double knockdown groups, which indicates the vital role of HIF- α /ABCB6 axis in regulating heme-dependent antioxidant defense enzyme catalase. We then analyzed intracellular reactive oxygen species (ROS)–mediated oxidative stress and observed a significantly decreased levels of intracellular ROS in A549 (Figure 5F) and H1703 (Figure 5G) GR cells but not in WT cells. knockdown of ABCB6, HIF-1 α or HIF-1 α /ABCB6 all elevated intracellular ROS accumulation (Figure 5F and 5G). We further observed an increased sensitivity to gemcitabine treatment in A549 (Figure 5H) and H1703 (Figure 5I) GR cells transfected with shHIF-1 α or shABCB6 expression vectors. Notably, there was a significant inhibition of cell survival in A549 and H1703 GR cells transfected with both shHIF-1 α and shABCB6 expression vectors when treated with gemcitabine (Figure 5H and 5I). Taken together, the data presented in Figure 5 demonstrate that gemcitabine resistant cells have increased HIF-1 α /ABCB6 axis-induced heme biosynthesis and catalase activity, and that their poor responsiveness to gemcitabine can be reversed by targeting HIF-1 α /ABCB6 axis.

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

Mouse xenograft models were established to determine whether knocking down of ABCB6 confers a sensitive phenotype of chemoresistant lung cancer to gemcitabine treatment *in vivo*. The groups consisted of the following (6 mice for each group): non-targeting control (NTC) transfected A549 or H1703 wide type group (treated with saline), NTC transfected A549 or H1703 gemcitabine (Gem) resistant group (treated with saline), NTC transfected A549 or H1703 Gem-resistant group (treated with gemcitabine), shHIF-1 α expression vector transfected A549 or H1703 Gem-resistant group (treated with gemcitabine), shABCB6 expression vector transfected A549 or H1703 Gem-resistant group (treated with gemcitabine), and shHIF-1 α /ABCB6 expression vector transfected A549 or H1703 Gem-resistant group (treated with gemcitabine). All animals received saline (250 μ l, *i.p.* administration, twice per week) or gemcitabine (20 mg/kg, *i.p.* administration, twice per week) after palpable tumors formed. Tumor growth was monitored twice per week. We observed similar results in both A549 (Figure 6A) and H1703 (Figure 6B) groups. The growth of Gem-resistant tumors in mice transfected with shHIF-1 α or shABCB6 were significantly decreased compared to Gem-resistant tumors in mice transfected NTC when treated with gemcitabine (Figure 6A and 6B). The Gem-resistant tumors in mice transfected with shHIF-1 α /ABCB6 demonstrated a further decrease in the tumor volume compared with the NTC group after gemcitabine administration (Figure 6A and 6B). Kaplan-Meier curves were plotted to evaluate the survival of mice in each group mentioned above (Figure 6C and 6D). The log-rank test demonstrated a statistical difference between the Gem-resistant NTC A549 tumors plus gemcitabine treatment groups and A549 Gem-resistant shABCB6 plus gemcitabine groups, or A549 Gem-resistant shHIF-1 α plus gemcitabine groups ($P = 0.0046$ and 0.0004 , respectively) (Figure 6C). The combination of HIF-1 α and ABCB6 inhibition (shHIF-1 α /ABCB6) with gemcitabine treatment also significantly increased survival of the mice compared with that of the gemcitabine alone treatment groups ($p = 0.0004$) (Figure 6C). Again, similar results were obtained in H1703 tumor-bearing mice groups as shown in figure 6D (GR NTC + Gem vs. GR shABCB6 + Gem, $p = 0.0008$; GR NTC + Gem vs. GR shHIF-1 α + Gem, $p = 0.0002$; GR NTC + Gem vs. GR shHIF-1 α /ABCB6 + Gem, $p = 0.0003$). However, A549 and H1703 Gem-resistant shHIF-1 α /ABCB6 plus gemcitabine groups did not prolong the survival significantly as compared with the Gem-resistant shABCB6 plus gemcitabine groups, or Gem-resistant shHIF-1 α plus gemcitabine groups (Figure 6C and 6D). At the end of the experiment, the animals were sacrificed and the xenografts were removed. A TUNEL assay was also performed to evaluate apoptosis. As expected, gemcitabine treatment caused marked apoptosis of Gem-resistant A549 tumor cells in shHIF-1 α , shABCB6, and particularly in shHIF-1 α /ABCB6 transfected groups (Figure 6D and 6E). We confirmed our data again in H1703 groups and observed similar results (Figure 6F and 6G). Taken together, the data presented in Figure 6 demonstrate that block HIF- α and ABCB6 dramatically delay tumor growth, promote apoptosis, and increased survival of mice in gemcitabine resistant tumor under chemotherapy, which indicate that inhibition of HIF- α /ABCB6 axis sensitizes NSCLCs to gemcitabine therapy.

Discussion

Although [gemcitabine](#) is the first-line chemotherapeutic drug for [pancreatic adenocarcinoma](#) treatment, it is critically and widely used to treat breast, bladder, and [non-small cell lung cancers](#) [36]. Unfortunately, it is routine for patients to develop acquired resistance shortly after beginning [gemcitabine](#) treatment and ultimately lead to anticancer failure [37]. Cancer cells employ a variety of mechanisms to acquire resistance against gemcitabine-based treatment. However, most previous studies have mainly addressed acquired resistance due to changes in the rate of drug influx or efflux. For instance, it is well demonstrated that ATP-binding cassette (ABC) transporters such as ABCB1, ABCC1 and ABCG2 have crucial roles in developing resistance by the efflux of anticancer drugs outside of cancer cells [38]. Virtually, ABC transporter families use ATP to facilitate not only the transmembrane flux of drugs, but also metabolites including saccharides, amino acids, peptides and proteins, lipids and sterols, as well as participating 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 regulator or co-factor to activate intracellular signal pathway or metabolic reprogramming is rarely explored. Our current study presents a mechanism of chemoresistance by which cancer cells increase intracellular ABCB6 expression in a HIF- α dependent manner that can in turn render antioxidant enzyme catalase effective by promoting heme biosynthesis. Here, we presents strong evidence for the inhibition of HIF- α /ABCB6 axis targeting lung cancer cell heme metabolism that disturbed mitochondrial redox homeostasis, escalated oxidant levels, and induced cell apoptosis by which abrogating gemcitabine resistance in [non-small cell lung cancers](#).

Previous studies have well demonstrated intratumoral hypoxia which increases the stabilization and accumulation of HIF-1 α , a critical transcriptional factor response to low oxygen condition, has been associated with the development of resistance to chemotherapy and radiotherapy in multiple types of human cancer [39]. However, increasing studies revealed that in addition to hypoxia, chemotherapeutic agents and radiation increase the levels of HIF-1 α , which regulates multiple downstream target genes associated with many key steps of cancer progression, particularly acquired chemotherapy resistance [40]. HIFs are heterodimers composed of HIF-1 α or HIF-2 α subunit and a constitutively expressed HIF-1 β subunit. Under hypoxic condition, the O₂-regulated prolyl hydroxylation-induced proteasomal degradation of HIF-1 α is inhibited, leading to rapid accumulation of HIF-1 α , dimerization with HIF-1 β , binding to the consensus DNA sequence 5'-RCGTG-3' within hypoxia response elements (HREs) located in target genes, and transcriptional activation [19]. Whereas HIF-1 α is subject to ubiquitination and degradation under normoxic condition. Intriguingly, we observe an obvious stabilization and accumulation of HIF-1 α even under normoxic conditions in gemcitabine resistant lung cancer cell lines which subjected to regularly treatment of gemcitabine for months. We demonstrate that gemcitabine-induced HIF-1 α increase ABCB6 transcriptional activities by binding with two HREs (-155 bp site and +87 bp site) in *ABCB6* gene sequence. However, the activation mechanism of HIF-1 α expression under normoxia in Gem-resistant cells is still not clear. It is possible due to a sort of protein function as modulator or coregulator which could interplay with HIF-1 α and facilitate to its stability under normoxia. A study by [Chaika et al.](#) demonstrates that type I transmembrane protein physically interacts with HIF-1 α and p300 and stabilizes

HIF-1 α at the protein level [41]. Shukla et al. also indicates the upregulation of HIF-1 α protein in normoxic gemcitabine resistant pancreatic cancer cells [28].

Multiple molecular mechanisms have been involved in cancer cells to acquire resistance against chemotherapeutics. In which, metabolic rewiring of tumors has been identified as a critical step in acquired drug resistance [42]. Cancer cells alter their metabolism by monitoring nutrient uptake (such as glucose and glutamine) and metabolites secretion, which results in modulating certain metabolic pathways in response. As a critical transcriptional factor, HIF-1 α has well been demonstrated in modulating multiple genes relating to cancer metabolic reprogramming, including glycolysis [43], branched-chain amino acids metabolism [44], nucleotide [45] and lipid synthesis [46]. Additionally, our previous studies and results from other groups all revealed that HIF-1 α is implicated in the regulation of other important metabolic pathways of cancer cells such as glutamine metabolism and glutathione synthesis pathway [13, 19, 47]. In our present study, we focused on heme metabolism. Heme is a central metabolic and signaling molecule that regulates diverse molecular and cellular processes relating to oxidative metabolism and ATP generation. A recent study by Sohoni et al. shows that levels of heme synthesis and uptake are enhanced in NSCLC cells [34]. Here, we show that the levels of heme biosynthesis are upregulated in gemcitabine-resistant NSCLC cells relative to gemcitabine-sensitive NSCLC cells. This elevation is regulated by HIF- α /ABCB6 axis as we present in the current paper. Heme binds to and directly regulates the activities of many proteins controlling processes ranging from tyrosine kinase signaling to redox homeostasis [11, 48, 49]. We further demonstrate that increased heme biosynthesis in Gem-resistant cells causes the elevation of ROS scavenger catalase activities in turn lead to redox homeostasis destruction and tumor survival.

Thus far, gemcitabine has been commonly prescribed in the treatment of patients with non-small-cell lung cancer (NSCLC) [50, 51], unfortunately, its benefits are limited due to frequent development of acquired tumor resistance. Gemcitabine combinations with platinum is one of the standard of care in non-small cell lung cancer patients. However, platinum-based chemotherapy has been shown to produce limited clinical benefits for cancer patients due to various adverse effects. Therefore, increasing evidence has suggested that the combination of chemotherapy with other anticancer therapy such as tyrosine kinase inhibitor (TKI) [52], angiogenesis inhibitors [53] and immune checkpoint inhibitors [54] produced better therapeutic outcomes. Giving that hypoxia and HIF-1 α activation represent a prominent feature of various solid tumors, being, at least in part, responsible for chemotherapy resistance in these tumors. Reversion of hypoxia or inhibition of HIF-1 α signaling can bring about greater benefit for cancer patients [55-57]. Our results suggested that the combination therapy of HIF-1 α inhibition and gemcitabine resulted in improved anti-tumor efficacy in the NSCLC tumor model. The combination therapy inhibited tumor growth and prolonged animal survival through increased chemotherapy sensitivity, leading to suppressed tumor cell proliferation, as well as increased tumor cell apoptosis. Gemcitabine/HIF-1 α -Trap combination therapy might present a more effective alternative for human lung cancer and may form the basis of a rationale for human clinical studies to investigate the benefits of these combination therapy in lung cancer.

Conclusions

The present study reports a widely prevalent mechanism of resistance to gemcitabine in NSCLC, whereby induced expression of ATP binding cassette subfamily B member 6 (ABCB6) is associated with poor prognosis and gemcitabine resistance in human NSCLCs which is in a hypoxia-inducible factor (HIF)-1–dependent manner. The activation of HIF-1 α /ABCB6 signaling leading to intracellular heme metabolic reprogramming and a corresponding increase in heme biosynthesis to enhance the activation and accumulation of catalase. Increased levels of catalase diminish the effective levels of reactive oxygen species (ROS) promoting gemcitabine-based resistance. Targeting HIF-1 α or ABCB6, in combination with gemcitabine, strongly restrains tumor proliferation, increases tumor cell apoptosis and prolongs animal survival. These results suggest that, in combination with gemcitabine-based chemotherapy, targeting HIF-1 α /ABCB6 signaling could result in enhanced tumor sensitivity to chemotherapeutic agent and may improve outcome in NSCLCs.

Abbreviations

ABCB6: ATP binding cassette subfamily B member 6

AOD: Average optical density

ChIP: Chromatin immunoprecipitation assay

DCFH-DA: Dichlorofluorescein diacetate

GR: gemcitabine-resistant

HIF-1: Hypoxia-inducible factor 1

HREs: Hypoxia response elements

IF: Immunofluorescence

IHC: Immunohistochemistry

LUAD: Lung adenocarcinoma

LUSC: Lung squamous cell carcinoma

NSCLC: Non-small cell lung cancer

PD: Progressive disease

PR: Partial response

ROS: Reactive oxygen species

TCGA: The cancer genome atlas

TS: Total score

TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling

WT: Wild type

Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the 1964 Declaration of Helsinki. The protocol of immunohistochemistry for patient tissues was approved by the Ethics Committee of the First Affiliated Hospital (Southwest Hospital), the Third Military Medical University (Army Medical University), and all patients or family members involved provided written informed consent.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and material

All data generated or analysed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

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

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Tables

Due to technical limitations, table 1 to 4 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Figure 1

ABCB6 high expression is associated with gemcitabine resistance and poor prognosis in human NSCLCs. Relative levels of ABCB6 mRNA from microarray analysis of primary NSCLC tumor samples relative to adjacent normal lung tissue from non-small cell lung cancer 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 of ABCB6 mRNA levels in the lung cancer with the probability of overall survival (n = 1926, $P = 0.006$) using the KM plotter database. Low = patients with ABCB6 mRNA levels less than the median. High = patients with 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 41 cases of gemcitabine-resistant tumor tissues, 40 cases of gemcitabine-sensitive tumor tissues and 6 cases of adjacent normal lung tissues are shown. The bottom right corner panel shows higher magnification of ABCB6 staining (brown, scale bar = 100 μ m). E, Image analysis was performed to determine the staining density of ABCB6 positive area per field under $\times 200$ magnification based on 5 random fields per section. For bar graph, one-way ANOVA (mean $\pm$ SEM) was used to determine the p value, ***p < 0.0001.

Figure 2

Figure 2

Establishment of gemcitabine-resistant NSCLC cell lines. Acquired gemcitabine resistant (GR) A549 lung adenocarcinoma cell line and H1703 lung squamous cell carcinoma cell line was generated by exposing the corresponding wild-types (WT) to an increasing concentration of gemcitabine over 7 months. The resistance status was confirmed by performing cell viability assays and soft agar assays at each step. A- B, NSCLC cells were treated with gemcitabine for 72 h and cell viability was analyzed using MTS. The viability of A549-WT (A) and H1703-WT (B) cells was respectively suppressed compared to A549-GR (A) and H1703-GR (B) by treatment with gemcitabine in a dose-dependent manner. Images of the colonies formed from A549-GR (A) and H1703-GR (B) cells as compared to relevant WT cells treated with 0.5 μ M or 1 μ M gemcitabine (as indicated) for 72 hours and plated for 15 days. C-D, Average colony number (C)

and colony size (D) in A549-GR compared to A549-WT as determined by soft agar assays. E-F, Average colony number (E) and colony size (F) in H1703-GR compared to H1703-WT was also determined. Colony number and size was determined by pictomicrography, and plotted for WT and GR. The results shown are the average of three independent experiments. Data are represented as mean $\pm$ SEM. For bar graph, Student's t-test was used to determine the p value, ***p < 0.0001.

Figure 3

Figure 3

ABCB6 expression is induced in gemcitabine resistant cells in a HIF-1α dependent manner. A-B, Reverse transcription and quantitative real-time PCR (RT-qPCR) was performed to quantify ABCB6 mRNA levels in A549 (A) and H1703 (B) gemcitabine resistant (GR) and wide 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 $\pm$ SEM; n = 3. ***P < 0.001 vs. WT cells (Student's t test). C, Immunoblot assays were performed to analyze ABCB6 and HIF-1 α protein expression in A549 and H1703 gemcitabine resistant (GR) and wide type (WT) cell lines. D-E, Expressions of ABCB6 mRNA were analyzed by RT-qPCR in A549 (D) and H1703 (E) gemcitabine resistant (GR) and wide type (WT) subclones, which were stably transfected with non-targeting control (NTC) or vector encoding HIF-1 α shRNA (sh1 α -1 or sh1 α -2) (mean $\pm$ SEM; n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. WT-NTC cells; ###P < 0.001 vs. GR-NTC cells (ANOVA with Bonferroni post-test). F-G, Immunoblot assays were performed using lysates prepared from A549 (F) and H1703 (G) subclones transfected with NTC or HIF-1 α shRNAs.

Figure 4

Figure 4

The ABCB6 gene is a HIF-1α target gene. A, The nucleotide sequence of HIF-responsive element-1 (HRE site 1; 5'-ACGTG-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, is shown. ABCB6 exons and HRE are indicated by black bars and arrow. B-C, chromatin immunoprecipitation (ChIP) assays were performed using IgG or antibodies against HIF-1α, HIF-1β and HIF-2α in A549 (B) and H1703 (C) gemcitabine resistant (GR) and wide type (WT) cell

lines. Primers flanking the HRE site 1 were used for qPCR and results were normalized to lane 1 (mean $\pm$ SEM; n = 3). ***P < 0.001 vs. WT (ANOVA with Bonferroni post-test). D, The nucleotide sequence of HRE site 2 (5'-GCGTG-3' HIF-1 binding site and an direct repeat 5'-GCGTG-3' sequence separated by 8 bp are shown in red) within exons 1 of the ABCB6 gene, located +87 bp from the transcription start site, is shown. Exons and intron are not drawn to scale. E-F, ChIP assays were performed using IgG or antibodies against HIF-1 α , HIF-1 β and HIF-2 α in A549 (E) and H1703 (F) gemcitabine resistant (GR) and wide type (WT) cell lines. Primers flanking the HRE site 2 were used for qPCR and results were normalized to lane 1 (mean $\pm$ SEM; n = 3). ***P < 0.001 vs. WT (ANOVA with Bonferroni post-test).

Figure 5

Figure 5

Effect of HIF-1α/ABCB6 signaling on the heme-mediated ROS scavenging of gemcitabine resistant cells. A-B, Heme content in A549 (A) and H1703 (B) gemcitabine resistant (GR) and wide type (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 vs. WT-NTC cells; ###P < 0.01, ###P < 0.001 vs.

GR-NTC cells (ANOVA with Bonferroni post-test). C, The protein levels of catalase in A549 and H1703, GR and WT subclones (N: NTC; 1α: shHIF-1α; A6: shABCB6; DKD, double knockdown: shHIF-1α/ABCB6). D-E, The catalase activity of 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 < 0.001 vs. WT-NTC cells; ##P < 0.01, ###P < 0.001 vs. GR-NTC cells (ANOVA with Bonferroni post-test). F-G, The generation of ROS 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 measurements. Data were presented as mean values with SEM (n = 3). *P < 0.05, ***P < 0.001 vs. WT-NTC cells; ###P < 0.001 vs. 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) followed by cell viability assays. Relative survival is plotted as percent of WT controls cultured in normal medium. Data were presented as mean values with SEM (n = 3). ***P < 0.001 vs. WT or GR-NTC cells with Gem; #P < 0.05, ##P < 0.01 vs. GR-NTC cells without Gem (ANOVA with Bonferroni post-test).

Figure 6

Figure 6

Block HIF-1 α /ABCB6 axis sensitizes NSCLCs to gemcitabine therapy in animal models. A549 and H1703, GR and WT subclones (2×10^6 cells) were implanted into the groin of 6-8 wk-old male SCID mice. After palpable tumors formed (8 days after tumor implantation), mice received intraperitoneal injection of gemcitabine (20 mg/kg) or saline (250 μ l), which was administrated twice per week. A-B, The volume of primary tumor formed by A549 (A) or H1703 (B) subclones were determined twice weekly. *** $P < 0.001$

vs. NTC by two-way ANOVA with Bonferroni post-test (mean $\pm$ SEM; n = 6). C-D, Kaplan–Meier survival curves were calculated for the 6 treatment groups of A549 (C) and H1703 (D) tumors as mentioned above. The log-rank test was used to demonstrate the statistical difference (N = 10; NTC, non-targeting control; GR, gemcitabine resistance; Gem, gemcitabine). E-F, After 42 days, the primary tumor was harvested. TUNEL assays were conducted with fluorescent microscopy for the measurement of cellular apoptosis in A549 (E) and H1703 (F) tumors (N: NTC; S: saline; G: gemcitabine; sh1 α : shHIF-1 α). scale bar = 50 μ m. G-H, TUNEL positive cells in A549 (G) and H1703 (H) tumors were calculated in percentage and displayed as histograms. Cells were counted in 10 randomly selected fields in 6 tumor samples from each group. The values represent the mean $\pm$ SEM. ***P < 0.001.

Supplementary Files

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