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Gene expression alterations in hypoxic A549 lung cancer cell line

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Abstract. Human non-small cell lung cancer (NSCLC)is a very common disease with limited treatment options. Hypoxia is a characteristic feature of solid tumors associated with the resistance of cancer cells to radiotherapy and chemotherapy. Therefore, the expression changes in cancer-resistance genes may be biomarkers of hypoxia with value in targeted therapy. The aim of the present study was to examine the effect of hypoxia on gene expression and the changes that occur in relation to drug resistance in a human NSCLC cell line (A549). A549 cells were exposed to 72-h hypoxic episodes (<1% oxygen) for a total of 10 episodes (acute). The alterations in gene expression were examined using PCR array technology after 10 episodes of acute hypoxia and compared with normoxic cells. The chemoresistance of hypoxic cells toward doxorubicin was measured using a MTT cell proliferation assay. A549 cells were affected by acute hypoxia leading to induced doxorubicin chemoresistance. Evident changes in the gene expression level were identified following episodes of acute hypoxia. The most important changes occurred in the estrogen receptor 1 (ESR1) and Finkel-Biskis-Jinkins osteosarcoma (FOS) pathways and in different nucleic transcription factors such as aryl hydrocarbon receptor and cyclin-dependent kinase inhibitor. The present study showed that exposing cells to prolonged periods of hypoxia results in different gene expression changes. There was induction of chemo-resistance due to acute hypoxia. ESR1 and c-FOS are proposed as a potential hypoxia genes in lung cancer.

Introduction

Lung cancer is the most common type of cancer death globally with an estimated 1.6 million fatalities each year (1). Non-small cell lung cancer (NSCLC) constitutes ~85% of lung cancer cases (2). Doxorubicin is a chemotherapeutic frequently prescribed to treat a variety of solid tumors (3). An imbalance between the oxygen supply and demand for cancer cells is known as hypoxia (4). Hypoxia, or the lack of oxygen, is a characteristic of solid tumors that encourages genomic instability, increased aggressiveness, and metastasia; hypoxia plays a significant role in treatment resistance and poor survival (5,6).

A total of two types of hypoxia have been identified according to the duration of exposure: acute hypoxia and chronic hypoxia; each with distinct biological effects (7). Normal human lung tissue has an oxygen concentration of ~5.6%, whereas NSCLC has an oxygen concentration of 1.9-2.2% (8,9). Marhuenda *et al* (10) demonstrated that lung cancer cell growth is differentially enhanced by intermittent and continuous hypoxia regimes. Acute hypoxia is considered to be a sudden and quick exposure to short-term hypoxic circumstances when the blood vessel closure lasts for several minutes (11). Accordingly, A549 cells were subjected to continuous hypoxia *in vitro* for a few minutes to 72 h (12). Thus, hypoxia will lead to a number of adaptive processes such as autophagy that reduce oxidative metabolism to enable the cell to survive under these conditions (12). Notably, the resulting reactive oxygen species can lead to tumor cell survival and development (13).

Acute hypoxia makes tumors more aggressive because it promotes spontaneous metastasis (14). Acute hypoxia also results in genomic instability due to a delayed reaction to DNA damage (15). A rise in radiation and/or chemotherapy resistance is seen in both chronic and acute hypoxia. The increase in resistance may be brought on by hypoxia, which can shorten senescence, produce disorganized and dysfunctional blood vessels and promote metastasis (16).

Hypoxia can result in chemoresistance and thus it is crucial to investigate the genetics of hypoxic cells for an improved understanding of how the hypoxic microenvironment affects the evolution of genetic changes. Accordingly, the present study used a 96-well PCR array to determine the alterations in gene expression linked to the emergence of doxorubicin-resistant phenotype in non-small cell lung cancer cell line. The present study used doxorubicin as a model drug because the hypoxic phenotype is often resistant to doxorubicin as shown previously (17).

To the to the best of the authors' knowledge, this is the first study to measure how hypoxic cells develop resistance at the genomic level. The cell line was exposed to cyclical and intermittent hypoxia.

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Materials and methods

Cell culture NSCLC cell line A549 was purchased from the American Type Culture Collection. A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), high glucose (Euroclone SpA), supplemented with 10% (v/v) heat-inactivated bovine fetal blood (FBS) from Biowest, 1% 2 mM L-glutamine, and 100 U/ml streptomycin and 100 g/ml penicillin from Euroclone SpA. Cells were cultured at 37˚C in an incubator (NuAire, Inc.) with 5% CO_2 and 95% air. Under a class II biological safety cabinet (Heal Force Bio-meditech Holdings Ltd.), all cell culture processes were carried out sterile conditions. Prior to use, 76% ethanol was used to disinfect all used items including disposables.

Exposure to hypoxia. In order to create hypoxic conditions, an anaerobic atmospheric generator, Oxoid AnaeroGen Compact (Thermo Fisher Scientific, Inc.), was employed. To create and sustain anaerobic conditions, the system comprised a tightly packed bag (sealed with a plastic clip) and a gas-generating sachet. The vented culture flasks were located inside the plastic bags, and the paper sachets were taken out and put inside the bag before it was closed with a plastic zip. A549 cells were exposed to 10 hypoxia events, each lasting 72 h. The normoxic cells were cultured alongside the hypoxic cells as a control group.

Cell proliferation assay. Following the manufacturer's instructions, a cell proliferation assay was used to track the anti-proliferative effects of doxorubicin on A549 cells and discover the resistance pattern brought on by the hypoxia injections. Instead of a clinically comparable medicine, doxorubicin was employed in this work as an example because it is known to lose some of its activity throughout the development of the hypoxic phenotype (18).

The assay was a colorimetric experiment that relies on the reduction of MTT from a yellow tetrazole to a purple formazan, a process that takes place in the mitochondria of live cells. To summarize, a coated 96-well plate (Greiner Bio-One International GmbH) was seeded with ~$5x10^3$ cells per well. The hypoxic and normoxic cells were seeded in duplicate for at least 24 h. Then the wells were used to aspirate the media. Various concentrations of doxorubicin were added to each well. The cells were then cultured at 37˚C for 72 h. After that, each well received ~15 μl of MTT reagent. Then 100 μl of DMSO was added to each well after the plates had been incubated for 4 h at 37˚C. After that, plates were kept at room temperature in the dark overnight.

The absorbance was recorded using a 96-well plate reader (Elx808 Absorbance Microplate Reader; Biotek; Agilent Technologies, Inc.) at a wavelength of 570 nm. The results of the MTT cell proliferation assay were evaluated using GraphPad Prism 5.0 software (Dotmatics). From the logarithmic trend line of cytotoxicity graphs, the inhibitory concentration (IC_{50}) values, which is the drug concentration at which 50 percent of cells are viable, were calculated.

Ribonucleic acid sample preparation. Following the manufacturer's instructions, total RNA was extracted from normoxic cells and from A549 cells after 10 cycles of acute hypoxia using the TrizolLS Single-step RNA reagent; Bio Basic, Inc.) The RNA was kept at -80˚C to prevent degradation and the RNA concentration was measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Inc.). By comparing the optical densities of the samples at 260 and 280 nm (Elx808 Absorbance Microplate Reader; Biotek; Agilent Technologies, Inc.), the purity of extracted RNA was ascertained. For all samples, the optical density (OD) 260/280 ratio ranged from 1.9-2.2.

Complementary deoxyribonucleic acid synthesis. The RT2 First Strand kit (Qiagen GmbH) was used to create complementary DNA strands in line with the manufacturer's instructions. From each sample, aliquots containing 1 μg of total RNA were used. Peak resistance, which occurred at the 10th episode in the acute hypoxia model of the MTT colorimetric test, was used to select the RNA for cDNA production. For comparison, the RNA of normoxic cells was also used during the cDNA production process. A NanoDrop 2000c spectrophotometer (Thermo Scientific, Inc.) was used to assess cDNA quantity and quality.

Gene expression profiling using PCR. The effect of hypoxia on gene expression of A549 cell lines was investigated using a 96-well Real Time 2 Profiler PCR array (PAHS-004Z-D; Human Cancer Drug Resistance; cat. no. 330231; Qiagen, GmbH). (cat. no. 330231; Qiagen, GmbH) at 37˚C. In this array, 96-well plates contain primers assays for 84 genes known to respond to low oxygen concentration, in addition to 12 genes for quality control. Primers were provided by Qiagen GmbH (sequences not available). cDNA was mixed with RT2 SYBR green master mix (Qiagen GmbH) and nuclease-free water (Bio Basic, Inc.). Then, 20 μl mix was placed in every well and the plate was centrifuged (Hettich Holding GmbH & Co.) at 1,000 x g for 1 min at room temperature to remove air bubbles. qPCR was performed using the CFX (Bio-Rad Laboratories, Inc.) thermocycler as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Fold change is calculated by using the $2^{-\Delta\Delta Cq}$ method (19) β-actin, β-2-microglobulin, GAPDH, hypoxanthine phosphoribosyltransferase 1 and ribosomal protein lateral stalk subunit P0 (RPLP0) were used as a control for normalization.

The threshold for gene expression change was set at two folds of regulation of gene expression for consideration of significance. For the examination of the PCR arrays used in the present study, such a cut off is advised. Therefore, it was regarded to have a substantial effect if the level of gene expression/repression is increased/decreased by two folds.

Fold change calculation. Fold change was calculated by using the ΔΔCq method originally published by Livak and Schmittgen (19). It is the ratio of the relative gene expression of the control group to the test group. Numbers >1 indicate upregulated or increased gene expression, numbers between 0 and 1 indicate downregulated or decreased gene expression, and a fold change of 1 indicates no change.

First, ΔCq, also called the normalized raw data, for each gene is calculated by subtracting the selected normalization factor from the Cq value of each gene of interest. If one

Table I. IC_{50} of doxorubicin in acute hypoxic A549 cells measured at hypoxia episode 10.

IC_{50}	Mean	Standard deviation of the mean
Control normoxic A549 cells	0.059 μM	±0.0007
Acute hypoxia A549 cells (10 episodes)	8.164 μM	±2.0000
Fold change	138,372	

housekeeping gene is selected, then its Cq value is used as the normalization factor. The five housekeeping genes (ACTB, B2M, GAPDH, HPRT1 and RPLP0) was used as a control for normalization. The five different housekeeping genes are used in the fold of changes calculation to increase the accuracy of normalization.

ΔCq = Cq (Gene of Interest) - Cq (Housekeeping/Reference Gene)

Or+

ΔCq=Cq (Gene of Interest)-Average [Cq (Housekeeping/Reference Genes)]

Next, gene-specific ΔCq values in samples in the same group were averaged.

Average ΔCTq = [ΔCTq (sample 1) + ΔCq (sample 2) + ΔCq (sample 3) + … ΔCq (Sample n)]/n samples.

The ΔΔCq value was calculated by subtracting the ΔCq value of each test group from the control group.

ΔΔCq = ΔCq (test group n) - ΔCq (control group)

The fold-change value is then calculated by converting the ΔΔCq from a log2 scale to a linear scale using the following equation:

Fold change = $2^{-\Delta\Delta Cq}$ …. (5).

Statistical analysis. The replicate 2-Cq values for each gene in each treatment group were compared with the control group to get the P-values (two-tail distribution, equal variances between the two samples). RT2 Profiler PCR Array Data Analysis Webportal was used for the statistical analysis (https://geneglobe.qiagen. com/jo/analyze). It only calculates P-values for groups that have at least three samples (including the control group). The RT2 Profiler PCR Array Data Analysis Handbook contains a complete description of the analysis. Unpaired Student's t-test were been used were applicable.

The following formula was used to determine the viability of cells based on the MTT experimental findings.

[(B-A)/A]*100 = viability%

Where A is the absorbance under the negative control at 570 nm and B is the absorbance during the treatment at 570 nm. Data sets from MTT tests were examined using GraphPad Prism software version 9 (Dotmatics) to determine IC_{50} values.

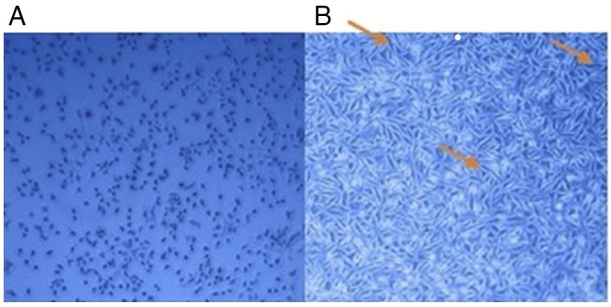

Figure 1. A549 cells epithelial mesenchymal transition under hypoxia. (A) Normoxic A549 cells. (magnification, x20) (B) Epithelial mesenchymal transition changes with elongated spindle-shape morphology in A549 cell line (magnification, x20), the arrows indicate the epithelial mesenchymal transition cells.

The data were represented as the mean of the calculated IC_{50} ± standard deviation. The experiments were performed in three different biological replicates. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of hypoxia on A549 cell line morphology and proliferation. After receiving acute doses of hypoxia, the A549 cell line underwent changes in morphology and proliferation relative to normoxic cells. The typical shape of the A549 cell line is depicted in Fig. 1. Acute hypoxia leads to a slower proliferation rate and increased mortality of NSCLC cells (17). The A549 cells developed an elongated spindle-shape morphology characteristic of the epithelial-mesenchymal transition (EMT) phenotype (Fig. 1). Longer hypoxic episodes that simultaneously caused growth arrest resulted in more significant morphological changes. Control cells doubled within 22 h, and the number of A549 cells were reduced when exposed to hypoxia.

Effect of hypoxia on A549 cells resistant to doxorubicin. The effect of doxorubicin on the growth of NSCLC cells was evaluated by determining cell viability using the MTT assay to test the development of drug resistance to the hypoxic phenotype. The IC_{50} increased after 10 episodes of acute hypoxia compared with normoxic cells (Table I). The viability of A549 cells was higher than the control cells after 10 episodes of acute hypoxia (Fig. 2).

Gene expression in A549 cells exposed to the acute hypoxia. For the purposes of the present study, a cut-off value of two-fold was chosen to demonstrate marked up- and down-regulation of the genes due to hypoxia. the role of hypoxia in altering the genetic expression and its relationship to drug resistance could then be assessed. A total of nine genes were

Table II. Genes upregulated in acute hypoxia-A549 cells.

Number	Gene Symbol	Gene description	Fold upregulation	Gene function (Qiagen, Inc.)	P-value
1	MSH2	MutS homolog 2, colon cancer, nonpolyposis type 1 (*E. coli*)	2.021	Protein Coding gene	0.001908
2	CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2	2.027	Drug metabolism	0.48241
3	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1	2.39	Regulator of cell cycle progression at G1	0.048339
4	BCL2	B-cell CLL/lymphoma 2	2.45	Anti- or pro-apoptotic regulators	0.032504
5	RELB	V-rel reticuloendotheliosis viral oncogene homolog B	2.8	Transcription Factor	0.000008
6	CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8	2.32	Drug metabolism	0.206225
7	RARB	Retinoic acid receptor, beta	3.81	DNA-binding transcription factor activity	0.001668
8	FOS	FBJ murine osteosarcoma viral oncogene homolog	3.96	Regulators of cell proliferation, differentiation, and transformation	0.002811
9	ESR1	Estrogen receptor 1	5.45	Hormone nuclear binding, DNA binding and activation of transcription	0.040855

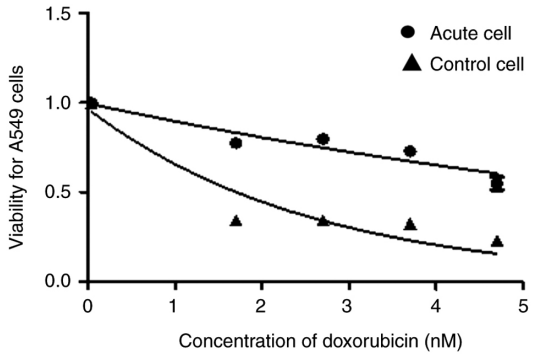

Figure 2. A549 cell viability in correlation with different concentration of doxorubicin.

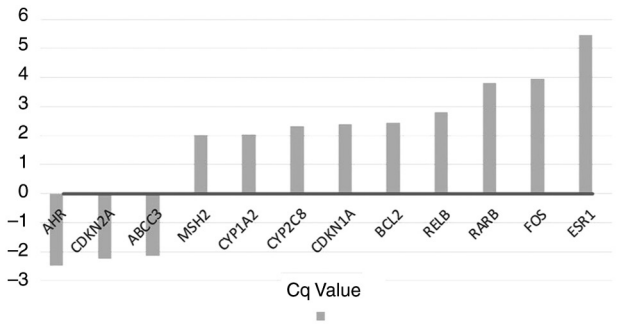

Figure 3. Plots of upregulated and downregulated genes with altered expression.

notably upregulated compared with normoxic cells (Table II and Fig. 3) after 10 sessions of 72 h of acute hypoxia; three were markedly downregulated (Table III and Fig. 3).

Discussion

The A549 cells were subjected to 10 cycles of 72 h of hypoxic episodes each in an effort to overcome cancer resistance under these conditions. Doxorubicin chemo-resistance was evaluated using the MTT proliferation assay. A gene expression profile was then performed after 10 sessions of acute hypoxia at which point resistance developed. The results confirmed that hypoxia was implicated in the chemo-resistant phenotype that developed in the lung cancer cell line.

Genes upregulated in acute hypoxia. The genes upregulated in acute hypoxia belonged to a number of crucial drug resistance mechanisms. Acute hypoxia increased the expression of two genes involved in the drug inactivation of the A549 cell line: CYP1A2 increased up to two-fold and CYP2C8 increased by 2.32-fold. These genes are responsible for the metabolism of hydrocarbon substances such as doxorubicin (19,20) and might be the cause of the reported doxorubicin resistance. The main way to activate CYP1A2 is via nuclear translocation and ligand-mediated transactivation of aromatic hydrocarbon receptors (21). The expression and activity of this gene have been linked to malignancies and chemotherapy resistance (22). The overexpression and polymorphisms of CYP2C8 were also significantly connected with the basal enzymatic activity, which can result in chemo-resistance (22). Similarly, a 1.88-fold increase in expression of CYP2B6 was observed.

The results of the present study showed that acute hypoxia caused the estrogen receptor alpha gene (ESR1) to be upregulated by 5.45-fold. This gene produces the estrogen receptor, a nuclear ligand-inducible transcriptional receptor (23) protein.

Table III. Genes downregulated in acute hypoxia-A549 cells.

Number	Gene symbol	Gene description	Fold downregulation	Gene function (Qiagen, Inc.)	P-value
1	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	-2.15	ATP-dependent drug efflux pump	0.01293
2	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	-2.26	Tumor suppressor gene	0.401826
3	AHR	Aryl hydrocarbon receptor	-2.49	DNA-binding transcription factor activity	0.061225

Upregulation of ESR1 has been linked to the development of EMT in lung cancer cells, which contributes to tumor resistance in immune-mediated cytotoxicity and lung cancer chemotherapy. Various studies have shown that ESR1 is dysregulated in cancer, which results in therapeutic resistance and metastatic biology (23,24).

A total of four genes were upregulated in the cell cycle and cell death inhibition mechanisms: cyclin dependent kinase inhibitor 1A (CDKN1A; also known as p21), c-Fos, BcL2, and RARβ. CDKN1A experienced a 2.39-fold increase in regulation. It functions as a cell cycle regulator and mediates cell proliferation, differentiation, death, and growth. CDKN1A is known to have a key inhibitory function in p53-dependent apoptosis (23). Cells that express it are protected from doxorubicin-induced apoptosis (25). The relevance of CDKN1A in tumor development and its role in mediating a drug-resistant phenotype, including chemotherapeutic medicines, tamoxifen and trastuzumab resistance, has been suggested previously (26).

c-Fos is a proto-oncogene that occurs in different cancer types. The main functions of the protein c-Fos are signal transduction, cell differentiation and proliferation. The role of c-Fos overexpression in boosting EMT state and CSC marker expression has been demonstrated in a number of studies. In head and neck squamous cell carcinoma (HNSCC) cells, the unique function of c-Fos has been suggested to be a regulator of EMT and cancer stem cell reprogramming (26). In mammary epithelial cells, overexpression of c-Fos has been shown to promote the development of a drug resistant phenotype resulting in cell polarity loss and EMT, which promotes invasive and metastatic growth (26). These results convincingly demonstrated that c-Fos promotes stemness in HNSCC cancer and plays a crucial role in tumor development (27).

The Bcl-2 family of proteins can either suppress or enhance apoptosis and is an important regulator of cell death (28). Bcl-2 proteins that are both pro-survival and pro-apoptotic are commonly up- and downregulated in various types of cancer cell. The Bcl-2 family plays a role in carcinogenesis and cancer cell resistance to anticancer therapies (29). The transcriptional expression of this gene is significantly increased in response to hypoxia in an HIF-1-dependent manner (28). These findings are consistent with the discovery of BCL-2 overexpression in doxorubicin-resistant A549 (30).

Lung cancer is characterized by promoter methylation, also known as hypermethylation, which is an early event in the carcinogenic process and inactivates tumor suppressor genes. The promoter methylation increases with neoplastic progression from hyperplasia to adenocarcinoma. Retinoic acid receptor β (RARβ) is frequently investigated in the context of promoter methylation in lung cancer (30). RARβ is a member of the RAR superfamily and is frequently suppressed in a variety of malignant tumors (30,31). Research has indicated that drug resistance may be caused by a lack of RARβ expression in clinical studies (32-34). Studies have revealed that the cholangiocarcinoma QBC939 cell line's reduced RARβ expression makes it much more resistant to chemotherapeutic drugs (32-34). Additional research demonstrates a strong correlation between elevated risk in NSCLC patients and hypermethylation of RARβ (35). A potential risk factor, diagnostic marker and potential therapeutic target for NSCLC is the inactivation of the RARβ gene brought on by RARβ methylation (36). Unexpectedly, the present study discovered that the RARβ gene was upregulated in A549 cells by a factor of 3.81. More research is thus needed to examine the role of RARβ in lung cancer. One explanation of such debate can be taken from the observation that RARβ can be re-expressed in dormancy models which is related to the hypoxic model represented in the present study (35).

The NF-κb family consists of five members: p105/p50 (NF-b1), p100/p52 (NF-b2), p65 (RelA), RelB, and c-Rel. These members regulate the transcription of target genes (37). Studies have investigated how RelB works in solid tumors and hematologic malignancies. Interleukin (IL)-8 regulation and the anti-apoptotic response in breast cancer cells are significantly regulated by RelB and the aryl hydrocarbon receptor (38). RelB is a helpful regulator in multiple myeloma cell survival (39). High RelB expression in NSCLC is associated with low differentiation, deep tumor invasion, positive lymph node metastasis, distant metastasis, and advanced clinical stages (35). These results suggest that increased RelB expression is essential for the development and metastasis of NSCLC tumors. Thus, resistance to chemotherapy and ionizing radiation have been associated with activation of the transcription factor NF-κB during treatment (40). These results are consistent with the findings of the present study, which showed that RelB was increased by 2.8-fold. This partly explains doxorubicin resistance and underscores the value of targeting NF-κB as a viable therapeutic approach to combat chemo-resistance and radiation-resistance in the treatment of cancer (41).

Genes downregulated in acute hypoxia. The present study identified three genes downregulated in acute hypoxia for NSCLC: CDKN2A by a factor of 2.26, AHR by a factor of 2.49, and ABCC3 by a factor of 2.15. A member of the family of ABCC3 is actively effluxed in a wide range of anticancer medications from tumor cells. This in turn contributes to multidrug resistance (17). The 2.15-fold downregulation of this gene in NSCLC suggests that it plays no part in the resistance mechanism.

An investigation into the role of CDKN2A in gastric cancer has revealed that CDKN2A depletion results in unchecked cell proliferation, which causes neoplastic transformation (42,43). This explains why CDKN2A was downregulated in the present study. However, there is still debate over CDKN2A's role in lung cancer and thus more research is required.

Limitations of the present study include the absence of histological analysis, the absence of data on additional cell lines, and the absence of data on clinically relevant drugs

In conclusion, the A549 lung cancer cell line exhibits alterations in gene expression levels after acute hypoxia. The results shed significant light on the potential pleiotropic pathways including the ESR1 pathway and nucleic transcription receptors such as CDKN1A, which are crucial in doxorubicin resistance induction. Targeting the modulated genes in NSCLC cases resistant to chemotherapy may have clinical value.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MZ conceived the present study. RA and MZ were responsible for methodology, formal analysis, and investigation. MZ and NA were responsible for resources. MS was responsible for writing the original draft preparation. MZ, MS and NA were responsible for project administration. All the authors were responsible for writing, reviewing and editing the manuscript and for supervision. RA, MZ and NA confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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