Alteration in the expression of the chemotherapy resistance-related genes in response to chronic and acute hypoxia in pancreatic cancer

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Abstract. Pancreatic cancer is currently one of the least curable types of human cancer and remains a key health problem. One of the most important characteristics of pancreatic cancer is its ability to grow under hypoxic conditions. Hypoxia is associated with resistance of cancer cells to radiotherapy and chemotherapy. It is a major contributor to pancreatic cancer genetic instability, which local and systemic resistance that may result in poor clinical outcome. Accordingly, identifying gene expression changes in cancer resistance genes that occur under hypoxic conditions may identify a new therapeutic target. The aim of the present study was to explore the association between hypoxia and resistance to chemotherapy and determine the alteration in the expression of cancer resistance-related genes in the presence of hypoxia. Pancreatic cancer cells (PANC-1) were exposed to 8 h hypoxic episodes (<1% oxygen) three times/week for a total of 20 episodes (chronic hypoxia) or 72 h hypoxic episodes twice/week for a total of 10 episodes (acute hypoxia). The alterations in gene expression were examined using reverse transcription-quantitative PCR array compared with normoxic cells. Chemoresistance of hypoxic cells toward doxorubicin was assessed using MTT cell proliferation assay. Both chronic and acute hypoxia induced chemoresistance toward doxorubicin in PANC-1 pancreatic cancer cell line. The greatest changes occurred in estrogen Receptor Alpha Gene (ESR1) and ETS Like-1 protein (ELK1) pathways, in nucleic transcription factor Peroxisome proliferator-activated receptors (PPARs) and in a cell cycle inhibitor cyclin dependent kinase inhibitor 1A (CDKN1A). The present study demonstrated that exposing cells to prolonged hypoxia results in different gene expression changes involving pleotropic pathways that serve a role in inducing resistance in pancreatic cancer.

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Abbreviations: ARNT, aryl hydrocarbon receptor nuclear translocator; EGFR, epidermal growth factor receptor; HIF-1, hypoxia-inducible factor; RAR, retinoic acid receptor; RXRB, retinoid X receptor β; SULT, sulfotransferase; NAT2, N-acetyltransferase; NSCLC, non-small cell lung carcinoma; PPAR, peroxisome proliferator activated receptor; pO_2, oxygen partial pressure; RB, retinoblastoma; ROS, reactive oxygen species; TP53, tumor protein 53

Key words: expression, resistance genes, chronic and acute hypoxia, pancreatic cancer

Introduction

As the number of pancreatic adenocarcinoma cases not eligible for surgery is growing (1), along with a decreased effectiveness of chemotherapy (2), an effective treatment and a novel target for the treatment of this disease are required (2). Pancreatic cancer is the seventh most common cause of death from cancer, with 338,000 new cases diagnosed in 2012 worldwide (3). This cancer is typically fatal; despite notable declines in general cancer-associated mortality, prognosis of pancreatic cancer remains remarkably poor (4,5). Overall, median survival from the time of diagnosis is 4.6 months; in patients with metastatic disease, this is 2.8-5.7 months (6). Pancreatic cancer is clinically marked by local invasion, early metastasis and resistance to standard chemotherapy (7). In the majority of cases, the disease is diagnosed at late stages, because of the absence of early signs and symptoms and lack of markers that help to identify pancreatic cancer early (2).

One key characteristic of pancreatic cancer is its ability to grow under hypoxic conditions (8). Hypoxia is associated with an imbalance between oxygen consumption of the cancer cells and limited oxygen delivery (9). Such imbalance is more notable in highly proliferating masses of tumor cells that develop faster than the vasculature providing oxygenated blood (10). Hypoxia is the low delivery of oxygen or low oxygen partial pressure (pO_2). The pO_2 of normal healthy and hypoxic tumor tissue is 40.00-50.00 and 0.02-35.00 mmHg, respectively. However, other investigators define hypoxia as low amounts of oxygen that normally have pO_2 of 0.7-15.0 mmHg (11). A total of 50-60% of tumors show hypoxic regions. Nevertheless, there are differences between tumors *in situ*, degree of hypoxia and causes, resulting in complex spatial and temporal heterogeneities in tumor hypoxia (12). Depending on the empirical

observation, duration and pathophysiological process involved, the hypoxia within the tumor tissue is classified as chronic and acute types (13,14). Chronic hypoxia is present in 65-86% of tumor tissues (13,14). The causes of chronic hypoxia include poor diffusion due to the large distance between the hypoxic area and the blood vessels; poor blood delivery because of structural abnormalities in blood vessels around the tumor, such as perforation, blunt ends, tortuosity, sluggish flow and poorly perfused vascular branches; pressure within tumor due to solid stress of non-fluid parts or the interstitial pressure of the fluid parts and anemia. All these causes lead to a stable type of reduced delivery of oxygen, nutrients, growth factors and prevent delivery of anti-cancer and imaging agents (13,15).

Acute hypoxia is known as perfusion-limited or ischemic hypoxia where the blood flow to the tissue is abolished. Such temporary cessation of blood flow may be caused by vessel remodeling that causes development of blood vessels plugs and shunts, tumor cell aggregates that form physical obstructions that block the flow in the blood vessels or anemia. All of these causes result in a dynamic inhibition of delivery of oxygen, blood, chemotherapeutic and imaging agents, nutrients and growth factors (13,15).

Hypoxia has been associated with resistance of cancer cells to both radiotherapy and chemotherapy (16). Oxygen concentration alters the sensitivity of chemotherapeutic agents (17) and increased hypoxia is linked with increased resistance to apoptosis induced by gemcitabine (18). Hypoxia is a major element in empowering the pancreatic cancer genetic instability that increases local and systemic drug resistance, leading to poor clinical outcome following treatment (19).

The present study aimed to investigate the association between hypoxia and genetic instability that leads to chemotherapy resistance in pancreatic cancer. Furthermore, the present study aimed to investigate the effect of the hypoxic microenvironment on the development of genetic expression alteration, which is a precursor to therapy-associated resistance (19). The present study investigated the relationship between hypoxia and chemoresistance and the alteration in expression of cancer resistance-related genes.

Materials and methods

Cell culture condition. PANC1, a human pancreatic cancer cell line, was purchased from the American Type Culture Collection (USA). PANC1 cells were cultured in DMEM high-glucose medium (EuroClone SpA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; all HyClone; Cytiva). PANC1 cells were grown in 75 cm^2 attached-type, filter-cap culture flasks (Membrane Solutions, LLC) at 37˚C in a humidified atmosphere containing 5% CO_2. All cell culture procedures were performed in sterile conditions under a class II biological safety cabinet (Lumitos AG). All materials and disposables were disinfected with 76% ethanol before use and subculturing was performed twice/week at 80-90% confluence.

Exposure to hypoxia. An anaerobic atmosphere-generating system, AnaeroGen Compact (Oxoid Ltd.; Thermo Fisher Scientific, Inc.) was used to generate hypoxic conditions. The system is composed of a tightly sealed (by means of a plastic clip) bag and gas-generating sachet. The AnaeroGen sachet decreases oxygen levels to <1% within 30 min, as previously described (20,21). After attaching to the flask, PANC-1 cells at 70% confluency were subjected to hypoxia over 5 months. Normoxic PANC-1 cells were incubated alongside hypoxic PANC-1 cells. For chronic hypoxia, PANC-1 cells were exposed to 8 h hypoxia (1% oxygen) three times/week for a total of 40 doses at 37˚C; for acute hypoxia, cells received 72 h hypoxia once/week for a total of 20 doses at 37˚C. Chronic hypoxia is diffusion-limited with short episodes of hypoxia in the body, while acute hypoxia is perfusion-limited with long episodes of hypoxia in the body (20,21). PANC-1 cells were used because of their aggressive nature and poor responsiveness to cancer drugs such as doxorubicin, cisplatin and 5-fluorouracil (22).

Cell proliferation assay. The CellTiter assay using Non-Radioactive Cell Proliferation Assay kit® (Promega Corporation) was applied according to the manufacturer's instructions to detect the anti-proliferative effects of doxorubicin on PANC-1 cells and resistance resulting from the hypoxia shots. A total of $7x10^3$ cells was seeded into each well of a coated 96-well plate for 24 h at 37˚C (Greiner Bio-One GmbH). Both hypoxic and normoxic cells were seeded in triplicate in DMEM (without doxorubicin) for ≥24 h at 37˚C. The media were aspirated from the wells. Afterwards, doxorubicin was added to each well at descending concentrations starting with 100.000, 50, 25, 12.5, 6.25, 3.125. 1.56, 0.78, 0.39, 0.19, .095, 0.047, 0.023, 0.012, 0.06, 0.03 μM. Cells were incubated at 37˚C for 72 h. A total of 15 μl MTT reagent was added to each well. The plates were incubated at 37˚C for 4 h, after which 100 μl solubilization/stop solution was added to each well. Optical density at 570 nm wavelength was recorded 1 h later using a 96-well plate reader (Biotek ELx808™ Absorbance Microplate Reader). Measurements were performed in triplets. Results were analyzed using the GraphPad PRISM®5.0 software (GraphPad Software, Inc.; Dotmatics). The half-maximal inhibitory concentration (IC50) values, defined as the drug concentration at which 50% of cells are viable, were calculated from the logarithmic trend of the cytotoxicity graphs.

MTT proliferation assay was conducted for cells exposed to chronic hypoxia after the 10th, 20th, 30th and 40th hypoxic shots, while for cells exposed to acute hypoxia, it was conducted after 10th, 15th and 20th hypoxic shots. The MTT proliferation assay was also conducted in parallel for control PANC-1 cells incubated under normoxic condition. Although doxorubicin is not commonly used in treatment of pancreatic cancer, it has been used in many studies to illustrate the hypoxic resistance phenotype (21,23).

RNA extraction and cDNA synthesis. Total RNA was extracted from PANC-1 cells after every 10 shots of hypoxia as well as from control (normoxic) cells. All RNA samples were stored at -80˚C until cDNA synthesis. RNA was isolated using an RNeasy® Mini kit (Qiagen GmbH) following the manufacturer's instructions. RNase-free DNase (Qiagen GmbH) was used according to the manufacturer's instructions to ensure complete genomic DNA elimination. Purity of isolated RNA was determined by measuring ratio of the optical density of the

Table I. IC50 of doxorubicin in chronic hypoxic PANC-1 cells.

Group	Mean IC50, μM
Normoxia, passage 8	0.29±0.01
Chronic hypoxia (10 shots), passage 15	0.34±0.010
Chronic hypoxia (20 shots), passage 27	0.70±0.05
Chronic hypoxia (40 shots), passage 42	0.38±0.06

IC50, half-maximal inhibitory concentration.

Table II. IC50 of doxorubicin in acute hypoxic PANC-1 cells.

Group	Mean IC50, μM
Normoxia, passage 10	0.21±0.04
Acute hypoxia (10 shots), passage 21	1.40±0.15
Acute hypoxia (20 shots), passage 32	0.40±0.03

IC50, half-maximal inhibitory concentration.

samples at 260 and 280 nm. (Biotek ELx808™ Absorbance Microplate Reader).

cDNA strands were synthesized using RT2 First Strand kit (Qiagen GmbH), according to the manufacturer's instructions; aliquots containing 1 μg total RNA were used from each sample. The OD260/OD280 ratio was calculated for purity.

Reverse transcription-quantitative PCR. RNA used in cDNA synthesis was selected at the time points coinciding with the maximum resistance according to the MTT colorimetric test, namely, the 20th shot of the chronic hypoxia and the 10th shot of the acute hypoxia models. RNA of normoxic PANC-1 cells was also used for comparison. The effect of hypoxia on gene expression of PANC-1 pancreatic cancer cell line was studied using a 96-well Real time 2 Profiler PCR array (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, Inc.) 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 to remove air bubbles at room temperature. 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 ΔΔCq method (24) β-actin, β-2-microglobulin, GAPDH, hypoxanthine phosphoribosyltransferase 1 and ribosomal protein lateral stalk subunit P0 (RPLP0) were used as a control for normalization.

Statistical analysis. Data were analyzed using unpaired Student's t test (two-tail distribution and equal variances) for gene expression. Data are presented as mean and SD. Each group contained ≥3 samples. The analysis was performed using RT2 Profiler PCR Array Data Analysis Webportal (sabiosciences.com/pcr/arrayanalysis.php).

Results

Effect of hypoxia on PANC-1 cell resistance to doxorubicin. The resistance to doxorubicin was used to confirm induction of the hypoxic phenotype. IC50 values of doxorubicin against PANC1 cells increased >2-fold after 20 shots of chronic hypoxia compared with the normoxic cells (Table I). IC50 was similar to that of the control after 40 shots of chronic hypoxia.

Table III. Genes profoundly upregulated in acute hypoxia PANC-1 cells.

Gene	Upregulation (fold-change)	P-value
ESR1	8.520	0.016
CYP2E1	7.450	0.001
ABCB1	6.590	0.209
CYP1A1	5.160	0.005
CYP1A2	5.140	0.044
SULT1E1	4.850	0.405
CDKN2D	4.360	0.005
XPC	4.110	0.084
CDKN1A	4.080	<0.001
CYP3A4	3.560	0.055
ERBB2	3.370	0.012
FOS	3.250	<0.001
CYP2B6	3.060	0.054
PPARA	2.960	0.003
CYP3A5	2.770	0.161
NAT2	2.670	0.043
ELK1	2.590	0.406
BCL2L1	2.380	0.187
PPARD	2.350	0.077
ARNT	2.300	0.058
RXRB	2.290	0.107
CYP2D6	2.220	0.050
ABCC5	2.140	0.096

Following acute hypoxia of 10 cycles, the IC50 increased to ~6-fold that of the control and was similar to that of the control after 20 cycles of acute hypoxia (Table II).

Gene expression in PANC-1 cells exposed to acute hypoxia. To investigate the effect of hypoxia on gene expression and its association with development of resistance, an arbitrary cut-off point of 2-fold was selected to show notable up- and downregulation of genes. Compared with passage-matched normoxic cells, cells exposed to 10 episodes of acute hypoxia demonstrated that 23 genes were profoundly upregulated (Table III) and three were profoundly downregulated (Table IV). Most genes were significantly upregulated, except Xeroderma pigmentosum, complementation group C (XPC), ATP binding cassette subfamily B member 1 (ABCB1), retinoid X receptor β (RXRB), Bcl-2-like protein

Table IV. Genes profoundly downregulated in acute hypoxia PANC-1 cells.

Gene	Downregulation (fold-change)	P-value
ABCG2	-2.87	0.000
ABCC2	-2.20	0.000
PPARG	-2.18	0.002

Table V. Genes profoundly upregulated in chronic hypoxia PANC-1 cells.

Gene	Upregulation (fold-change)	P-value
ESR1	4.71	0.003
CYP1A2	3.97	0.109
SULT1E1	3.9	0.001
CYP2C19	2.91	0.002
CYP1A1	2.7	0.002
ABCB1	2.63	0.002
CYP3A4	2.54	0.002
CDKN1A	2.2	0.003
CYP2B6	2.09	0.002

1 (BCL2L1) and ELK1. Of the upregulated genes, ESR1 exhibited the greatest change (8.52-fold), while ABCG2 was the most downregulated gene (-2.87-fold;). A total of eight genes involved in drug inactivation were upregulated in acute hypoxia: CYP2E1 (7.45-fold), CYP1A1 (5.16-fold), CYP1A2 (5.14-fold), SULT1E1 (4.85-fold), CYP3A4 (3.56-fold), CYP2B6 (3.06-fold), CYP3A5 (2.77-fold) and CYP2D6 (2.22-fold).

Gene expression in PANC-1 cells exposed to chronic hypoxia. Compared with passage-matched normoxic cells, in cells exposed to 20 episodes of chronic hypoxia, nine genes were profoundly upregulated (Table V) and 11 were profoundly downregulated (Table VI). All genes were significantly upregulated except CYP1A2. Although CYP1A2 upregulated 3.97 fold, it did not reach statistical significance. Peroxisome proliferator-activated receptor D (PPARD), Erb-B2 Receptor Tyrosine Kinase 4 (ERBB4) and XPC were not significantly downregulated. ESR1 demonstrated the highest upregulation and ABC demonstrated the most marked downregulation.

Discussion

The extent and duration of oxygenation is not consistent between tumors and even in different areas of the same tumor (25). Oxygen concentration is not constant in the same area (26,27). It is proposed that numerous fluctuations in oxygenation occur in the tumor microenvironment. These fluctuations are complex and dynamic, changing within minutes to hours. These fluctuations are known as intermittent hypoxia (25,28). Hypoxia occurs in a transient and heterogeneous manner. The tumor microenvironment exhibits

Table VI. Genes profoundly downregulated in chronic hypoxia PANC-1 cells.

Gene	Downregulation (fold-change)	P-value
ABCC1	-2.810	0.034
ELK1	-2.750	0.226
XPC	-2.690	0.912
FOS	-2.690	0.009
ABCG2	-2.510	<0.001
RARG	-2.460	0.078
ERBB4	-2.440	0.421
ABCC3	-2.390	0.001
CDKN1B	-2.350	0.046
TP53	-2.190	0.042
PPARD	-2.180	0.076

frequent cycles of hypoxia and reoxygenation (29); therefore, the present study used two intermittent hypoxic models that may mimic the real cancer hypoxic microenvironment. Cells showed desensitization with continuous exposure to acute and chronic hypoxia. This may be because cancer cells adapt to hypoxia after several hypoxic episodes (21).

The increase of doxorubicin IC50 in PAN1 cells after 20 shots of chronic hypoxia and 10 shots of acute hypoxia is an unexplained phenomenon that has been reported in previous investigations (29-33). The upregulated genes in both acute and chronic hypoxia are associated with drug resistance mechanisms, namely, drug efflux, drug metabolism inactivation, drug targets and signaling transduction molecules, repair of drug-induced DNA damage and evasion of apoptosis. The present results showed that in acute hypoxia, there was a profound upregulation in genes associated with active drug efflux, namely, ABCB1 (6.59-fold) and ABCC5 (2.14-fold). This upregulation explains decreased intracellular doxorubicin concentration in resistant MCF-7 cells (30). ABCB1 gene encodes the P-glycoprotein, a broad-spectrum multidrug efflux pump that is considered to be a key contributor in the development of multidrug resistance (31,32). ABCB1 gene was upregulated 2.63 fold in chronic hypoxia. A previous study in G3361 melanoma cells showed that cells with higher expression of ABCC5 showed higher doxorubicin resistance (33). Another proposed mechanism of resistance is related to hypoxia and extracellular acidity. Such acidity has a direct effect on the activity and/or uptake of certain anticancer drugs. Drugs that are weak bases such as doxorubicin, have a greater proportion of molecules in the charged form under acidic conditions, which decreases their ability to cross the plasma membrane and to be taken up into the cell, leading to decreased activity (34,35).

A total of eight genes involved in drug inactivation were upregulated in acute hypoxia. CYP2E1 expression is correlated with increased reactive oxygen species (ROS) generation in breast cancer and other types of cells (32). ROS trigger autophagy, DNA damage, impaired protein-folding and chemoresistance in cancer cells that undergo hypoxic oxidative stress (36). ROS induce chemoresistance by

stimulating P-gp function and expression in human colon cancer Caco-2 cells (37). This is consistent with the present upregulation of the ABCB1 and ABCC5 genes involved in drug efflux and also with previous data that low levels of ROS downregulate P-gp expression whereas high levels of ROS result in upregulation of P-gp in multicellular prostate tumor spheroids (38). The oxidative state induced by hypoxia followed by reoxygenation and accompanied by accumulation of ROS increases peroxisome proliferator-activated receptor (PPAR) γ coactivator 1-α (PGC-1α) that induces chemoresistance by enzymatic deactivation; PGC-1α a protein acts symbiotically with PPARA and PPARD that are upregulated as a result (39).

CYP1A1 is a factor in the aryl hydrocarbon receptor (AhR)/CYP1A1 signaling pathway which is responsible for chemoresistance in cancer cells; CYP1A1 expression is induced by AhR, a helix-loop-helix transcription factor, and can bind a number of native or foreign ligands; AhR is upregulated by hypoxia (40). Combined used of AhR antagonist and 5-flurouracil increases sensitivity to chemotherapy. On the other hand, the effect of doxorubicin was not changed, leading to a suggestion that doxorubicin acts on cancer cells independently of the mechanism involving AhR despite the fact that an AhR antagonist decreases the expression of CYP1A1 (41).

CYP1A2 a is phase 1 metabolism enzyme highly expressed in human liver (42). To the best of our knowledge, there are no reported data about the role of CYP1A2 in chemoresistance. Here, CYP1A2 was upregulated in chronic hypoxia up to 3.97-fold.

CYP3A5 is highly expressed in pancreatic cancer cells. Its expression can be induced by paclitaxel or erlotinib; knocking down the CYP3A5 gene, notably increases response to drugs (43). CYP3A4 participates in chemoresistance in colon cancer stem cells (44). The interplay between CYP3A4 and P-glycoprotein as a factor that limits oral drug bioavailability is well-established, especially in the intestinal mucosa, which may reduce the drug concentration that reaches the cancer cell (45,46). P-glycoprotein can work in concert with CYP3A4 to increase drug metabolism by controlling the access of the drug to the intracellular metabolizing enzyme such as CYP1A1, CYP1A2, CYP3A4 and CYP3A5 (47). Here, CYP3A4 gene was upregulated up to 3.56-fold in acute hypoxia and up to 2.54-fold in chronic hypoxia, along with ABCB1, which indicates possible interplay between CYP3A4 and P-gp in development of resistance in pancreatic cancer cells.

The present study showed that CYP2B6 gene was upregulated in acute hypoxia up to 3.06-fold and in chronic hypoxia up to 2.09-fold. CYP2B6 enzyme is associated with tamoxifen activation and mutations in CYP2B6 are associated with poor response to cyclophosphamide-based therapy in patients with breast cancer, in addition to inhibiting the anticancer effect of tamoxifen (48). Another report suggested the role of CYP2B6 polymorphism in increasing relapse after treatment with cyclophosphamide in patients with lymphoma (49,50). CYP2C19 was upregulated in chronic hypoxia up to 2.91-fold, but not in acute hypoxia. This enzyme is responsible for cyclophosphamide and ifosfamide activation. CYP2C19 polymorphism affects pharmacokinetic profiles of nelfinavir in patients with locally advanced pancreatic cancer (51). A previous study reported hypoxia has no effect on the regulation of CYP2C19 gene while other studies suggest that hypoxia downregulates this gene in a normal liver rat model (51,52), in contrast to the present results.

The present study showed that ESR1 gene was upregulated 8.52-fold in acute hipoxia and up to 4.71-fold in chronic hypoxia. The high presence of estrogen receptors in pancreatic cancer cells was firstly described by Greenway *et al* (53). Konduri *et al* (54) suggested that the ratio of ESR-β/ESR-α may predict a response to estrogen-associated therapy in the treatment of pancreatic cancer. A previous study has demonstrated that ESR1 expression in breast cancer cells is correlated with therapeutic efficacy of chemotherapy (55). Chemoresistance is associated with high expression of ESR-1 in breast cancer cells (56,57). Apoptosis-related molecules or signal pathways such as BCL-2 and p53 may be involved in ESR1-mediated chemoresistance (58).

The present study also showed that SULT1E1 was upregulated in acute hypoxia up to 4.85-fold and in chronic hypoxia up to 3.90-fold. The present data are in alignment with induction of hepatic estrogen Sulfotransferase (EST) mediated by oxidative stress in mice (59). Overexpression of SULT1E1 in MCF-7 is associated with arrested cell cycle and apoptosis (60). Karle *et al* (61) demonstrated elevated activity of this enzyme in MCF7 cells resistant to doxorubicin. The SULT1E1 levels are correlated with the levels of Estrogen Receptor α (ERα) in ovarian cancer cells (62).

Among genes associated with cell cycle and cell death inhibition mechanisms, the most upregulated genes in acute hypoxia were CDKN2D (4.36-fold), RXRB (2.29-fold) and CDKN1A (4.08-fold), which was also upregulated in chronic hypoxia up to 2.20-fold. CDKN2D, upregulated in acute hypoxia, encodes cyclin-dependent kinase 4 inhibitor D protein, a member of the INK4 family of cyclin-dependent kinase inhibitors. These function as cell growth regulators that control cell cycle G1 progression, which may explain slow proliferation rate in pancreatic cancer after shots of hypoxia (63). RXRB, upregulated in acute hypoxia, encodes RXRs, nucleic receptor transcription factors. These factors bind retinoid, natural and synthetic molecules structurally and/or functionally associated with vitamin A and regulate cell differentiation, proliferation and survival (64). An association has been suggested between RXRB and activation of pancreatic stellate cells that induce chemoresistance (65). RXRB heterodimers with PPAR initiate biological responses including oxidative stress response, which supports the present results (66). CDKN1A gene, upregulated in both hypoxia models, is a major inhibitor of p53-dependent apoptosis (67); expression of this gene protects cells from doxorubicin-induced apoptosis (68). High cytoplasmic expression of p21 induces resistance to cisplatin in testicular cancer cell (68).

PPAR genes were upregulated in acute hypoxia: PPARA 2.96-fold and PPARD 2.35-fold. PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily (69). Activation of PPARA leads to inhibition of apoptosis and increased oxidative stress in hepatocellular cells (70). PPARA also serves as an inflammation inducer in cancer and a factor in providing a suitable

microenvironment for tumor growth. PPARA antagonist can inhibit growth in pancreatic cancer cells (70,71). PPARD is a component of the angiogenetic switch in pancreatic cancer and high expression levels are associated with tumor progression and distant metastases (72,73). In breast cancer cells, PPARD protein expression is increased in response to hypoxic and metabolic starvation; PPARD helps breast cancer cells to survive by decreasing oxidative stress and enhancing survival signaling responses (74). The present PPARD upregulation supports the aforementioned findings and the upregulation of this gene is an adaption to hypoxia.

XPC gene was upregulated in acute hypoxia up to 4.11-fold. Hypoxia and hypoxia-inducible factor 1α (HIF-1α) are actively involved in XPC regulation and XPC is associated with increased sensitivity to oxidative DNA damage (75,76). It was shown that activation of the ataxia-telangiectasia mutated (ATM) gene which is recruited to the damaged DNA through XPC prevents cisplatin-induced apoptosis via nucleotide excision repair (77). XPC is associated with chemoresistance in non-small cell lung carcinoma (NSCLC).

ErbB2 (also known as HER2 or neu) was upregulated in acute hypoxia. This gene encodes a 185 kDa transmembrane glycoprotein, which belongs to the epidermal growth factor receptor (EGFR) family (78). ErbB2 is commonly overexpressed in cancers, including pancreatic cancer (79,80).

FOS is a proto-oncogene that serves an important role in many cellular functions such as proliferation, apoptosis, angiogenesis, epidermal-mesenchymal transition (EMT), invasion and metastasis. It was shown that this gene is affected by hypoxia (81,82). In human ovarian carcinoma cells, cisplatin resistance is associated with upregulation of the c-FOS gene (82). The present upregulation in the FOS gene in acute hypoxia up to 3.25-fold is consistent with the aforementioned findings.

ELK1 acts as transcription activator (83) and was upregulated in acute hypoxia up to 2.59-fold. ELK1 mRNA expression is upregulated in chemoresistant specimens of patients with serous epithelial ovarian cancer; patients with high nuclear expression of ELK1 have significantly shorter survival time (83). One of the pathways responsible for chemoresistance in cancer cells is the Ras/Raf/MEK/ERK pathway. ERKs activate transcription factors such as ELK1 that are associated with drug resistance (84). ELK1 activation is required to induce FOS transcription after ERK stimulation; both of these genes were upregulated in the present study, hence ELK1 represents a key link between signal transduction pathways and initiation of gene transcription (85).

AhR nuclear translocator (ARNT) attaches to HIF-1α to form the HIF-1, a master regulator of oxygen homeostasis with pleiotropic effects, including inducing chemoresistance (86). ARNT overexpression is associated with drug-resistant properties of cancer cells by upregulation of MDR1, which prevents the action of many chemotherapeutic drugs such as doxorubicin and cisplatin in different types of cancer (87). These data are consistent with the present upregulation of ARNT up to 2.30-fold in acute hypoxia.

N-acetyltransferase (NAT2) is an enzyme that both activates and deactivates aryl amine, hydrazine drugs and carcinogens; here, it was upregulated up to 2.67-fold in acute hypoxia. This is consistent with a study that found significant changes in the activity and protein and mRNA expression of NAT2 in rats in high-altitude hypoxia as a result of a whole metabolism change in cancer cells (49).

Bcl2L1 is an anti-apoptotic protein that regulates the production of ROS and release of cytochrome C by mitochondria, both of which are key inducers of cell apoptosis (88). Bcl2L1 was upregulated up to 2.38-fold for acute hypoxia in the present study. A previous study found that Bcl2L1 expression confers resistance to chemotherapy-induced apoptosis resulting from treatment with cisplatin, paclitaxel, topotecan and gemcitabine in ovarian cancer cells (88).

Here, three genes were downregulated in acute hypoxia: ABCG2 by 2.87-fold, ABCC2 by 2.20-fold and PPARG by 2.18-fold. The role of PPARG in pancreatic cancer remains controversial but cellular studies have demonstrated that PPARG inactivation increases pancreatic cancer cell growth and attenuates their migration and invasive capacity (89-93). PPARG agonists could be a promising pharmacological approach for the treatment of colorectal cancer (93). Synergy has been demonstrated between PPARG ligands, a platinum-based agent in two NSCLC-derived cell lines (94). *In vitro* studies demonstrate that PPARG activation decreases pancreatic cancer cell growth and a number of ligands have been designed as potential drugs that can be combined with gemcitabine (95,96). Therefore, downregulation of this gene may explain increased resistance and growth in the pancreatic cancer cell line.

The genes encoding efflux pumps ABCG2 and ABCC2 propagate resistance to chemotherapy (97); the hypoxic chemoresistance effect is induced by the regulation of ABCG2 via activation of the ERK1/2/HIF1a pathway (97), which contradicts the present results. ABCG2 was also downregulated in chronic hypoxia -2.51-fold, along with ABCC1 to -2.81-fold. On the other hand, ABCC3 was downregulated -2.39-fold only in chronic hypoxia, which conflicts with the role of these pumps in the initiation of chemoresistance in pancreatic cancer (98).

By contrast with acute hypoxia, the following genes were downregulated in chronic hypoxia: ELK1 by 2.75-fold, XPC by 2.69-fold, FOS by 2.69-fold and PPARD by 2.18-fold. This suggests that the adaptive mechanism of resistance in chronic hypoxia is different from that in acute hypoxia.

Tumor suppressive gene TP53 was downregulated in chronic hypoxia -2.19-fold; this gene acts as a DNA repair tool. Although TP53 is not a clinical marker for drug resistance, studies have correlated overexpression of mutated p53 with reduced or abolished resistance to standard medications (99,100) in pancreatic cancer, and increased expression of mutated TP53 leads to chemoresistance (99).

CDKN1B was downregulated in acute hypoxia -2.35-fold; this gene encodes a protein that controls the cell cycle progression at G1 and decreases cell cycle proliferation rate and is considered as a tumor suppressor gene (100).

CDKN1B protein expression is reduced in ~60% of human cancer cases which is indicative of poor prognosis and chemotherapy resistance (101,102). Decreased expression of p27 is associated with acquired resistance to docetaxel in breast cancer cells (103), which is consistent with the present findings; further research is needed to understand the role of p27 in inducing chemoresistance in pancreatic cancer. Retinoic acid receptors (RARs) are nucleic receptor transcription

factors that bind retinoids, natural and synthetic molecules structurally and/or functionally related to vitamin A, and regulate cell differentiation, proliferation and survival (103). RARG mediates the growth inhibitory response of retinoids in numerous types of cancer cells (104). In colorectal cancer, RARG knockdown results in downregulation of MDR1 and suppression of the Wnt/β-catenin pathway, leading to sensitivity to chemotherapy, suggesting that overexpression of RARG contributes to the multidrug chemoresistance of colorectal cancer cells (105). Here, RARG gene was down-regulated -2.46-fold. To the best of our knowledge, no previous study has investigated the role of RARG in pancreatic cancer chemoresistance.

ERBB4 was downregulated -2.44-fold, which, along with increased cell proliferation, is in accordance with previous data indicating that the lack of HER-4 expression may increase the metastatic capacity of pancreatic cancer cells (106-108). HER-4 may also be of potential prognostic value and deserves further attention (106-108).

The present study provided evidence that exposing cells to prolonged periods of hypoxia results in different genetic expression changes. Pleotropic pathways, including ESR1 and ELK1 pathways, and nucleic transcription receptors such as CDKN1A and PPARs are involved in resistance in pancreatic cancer but more investigation of gene expression is needed to determine gene interactions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TH performed the experiments. MZ conceived the study. NB interpreted data, HH designed the study. All authors have read and approved the final manuscript. TH and MZ confirm the authenticity of all the raw data.

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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