Turkish Journal of Molecular Biology & Biotechnology (2016) 1: 1-10

ORIGINAL ARTICLE

Turkish JMBB

The drug resistance may be the consequence of the increased cell viability by HIF-1α in Pancreatic cancer cell

Nilgün ÇETİN[1], Işınsu KUZU[2], Mehmet TAŞPINAR[3], Buse TÜREGÜN ATASOY[4], Fikret ŞAHİN[4]

[1] Department of Medical Biology, Cumhuriyet University School of Medicine, Sivas, Turkey
Departments of [2] Pathology and [4] Medical Microbiology, Ankara University, School of Medicine, Ankara, Turkey
[3] Department of Medical Biology, Yüzüncü Yıl University, School of Medicine, Van, Turkey

Keywords
Hypoxia-inducible factor-1 α, drug resistance, doxorubicin

Received: 08 March 2016
Accepted: 06 May 2016

ABSTRACT • Background and aims: Hypoxia-inducible factor-1 α (HIF-1 α) is an important factor in the adaptation of cells to hypoxic environments and significantly contributes to the aggressiveness and chemoresistance of a number of different tumours. In this study, we aimed to investigate the effects of HIF-1α gene overexpression in pancreatic cancer cell line (Pancl) on cell survival and drug resistance. **Materials and Methods:** For this purpose, HIF-1α cDNA was cloned into the pcDNA mammalian expression vector. Sequentially HIF-1α ectopically overexpressed in Pancl cell line and phenotypic changes including cell survive, and drug resistance were investigated. Cell survive, and drug resistance (doxorubicin) were tested with MTT assay. Colony forming assay was used as another test for the drug resistance analysis. Multiplex RT-PCR and western blot were utilized for the gene and protein expression analysis respectively. **Results:** Results showed that HIF-1α overexpression increases cell survival as much as 40% in three days. However, doxorubicin decreased cell survival at the same rate in both cells overexpressing HIF-1α and not. Expression analysis of the Akt-1, Bax, mTOR, cIAPI, cIAPII, Bcl2 and XIAP showed that neither of the genes' expressions was effected by the HIF-1α overexpression. **Conclusion:** As a conclusion the results of this study showed that HIF-1α increase the cell survival in Pancl cell and doxorubicin may act on the targets related to the cell survival.

Correspondence: Fikret ŞAHİN, Ankara University School of Medicine, Department of Medical Microbiology, Ankara, Turkey
Phone: +90 312 595 82 77 • Fax 0312 310 6370 • E-mail: fsahin29@hotmail.com

INTRODUCTION

The drug resistance may be the consequence of the increased cell viability by HIF-1α in Pancreatic cancer cell

Cancer is one of the most frequent causes of death in the world. Chemotherapy is one of the main treatment used to treat cancer. However, the development of resistance to chemotherapy treatment against cancer is one of the major problems. Hypoxia is oxygen deficiency condition, which is common in many forms of solid tumors (1-3). It occurs when the stromal vascular system can not send a sufficient amount of O_2 to the growing tumors. Consequently, tumor provides genetic changes to avoid from the hypoxia-related cell death including necrosis.

Responses to hypoxia are mediated by Hypoxia-inducible factor-1 (HIF-1), a protein heterodimer overexpressed in over 50% of solid tumors as a result of the hypoxic conditions inside the tumor (2, 4). Necrotic regions of tumors show high levels of HIF-1α, indicating HIF-1α levels are regulated by tumor oxygenation. In hypoxic conditions, HIF-1α gives the tolerance and adaptation to the stress caused by hypoxia in cells (5). Studies showed that HIF-1α contributes to the regulation vital biological processes required for tumor survival and progression (5). Accordingly, HIF-1α-positive patients in the clinic show significantly low 5-year survival rates compared to HIF-1α-negative patients (2). Controlled expression by hypoxia, HIF-1α plays a role in the regulation of many genes (6). The most of the genes are related to metabolic changes. HIF-1α regulates almost all the enzymes leading to glucose breakdown during glycolysis and regulates lactate production and lactate removal from cells by activating lactate dehydrogenase A and the lactate transporter MCT4 (7, 8).

In addition to the metabolic changes seen in response to HIF-1α, the HIF-1 heterodimer also triggers the transcription of gene products that are associated with resistance to anticancer agents (8).

In here, we aimed to investigate the effects of HIF-1α gene overexpression in pancreatic cancer cell line (PancI) on cell survival and drug resistance. For this purpose, HIF-1α cDNA was cloned into the pcDNA mammalian expression vector. Sequentially HIF-1α ectopically overexpressed in PancI cell line and phenotypic changes including cell survive, and drug resistance was investigated.

MATERIAL and METHODS

Cell and cell growth condition

PancI (a gift from Dr. G. H. Su, The Johns Hopkins University), a human pancreatic cancer cell line was used in these experiments. The cells were grown in Dulbecco's modification of Eagle's medium (DMEM) (Biochrom-Germany) supplemented with 10% (vol/vol) fetal bovine serum (Biochrom-Germany) Penicillin-Streptomycin 1% (Biochrom-Germany) in a 5% CO_2 atmosphere at 37 °C.

Drug and doses

Two different doses of doxorubicin HCl (ADRİ-BLAST the vial- Deva-Turkey) was applied. Dose 1 (D1) is twice of clinical dose (2,5 mg/L) and dose 2 (D2) is clinical dose of 1,25 mg/L.

Total RNA isolation

Total cell RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA quantification was carried out by spectrophotometry, and each sample was aliquoted and frozen at –80 °C until required.

cDNA Synthesis

The RNA samples were used as a template for reverse transcription with the RevertAid_First Strand cDNA Synthesis Kit according to the protocol supplied by the manufacturer (MBI, Fermentas, Germany).

Multiplex RT-PCR

Multiplex RT-PCR (MRT-PCR) was used first to determine the linear range of target genes. Second, the linear range of the internal standard was determined using actin primers. Conditions for MRT-PCR were optimized so that the target genes and internal control would be in the linear range. The PCR machine was programmed as follows; 94 °C for 2 minutes to denature template; 94 °C for 45 seconds for denaturation; at 60 °C for 45 seconds for annealing; and 68 °C for 1 minute for an extension. The PCR products were run in a 1.5% agarose gel, and the gel was stained with EtBr.

Primers used in the multiplex RT-PCR

All primers related to the genes and internal control were designed with the Primer3: WWW primer tool program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and are shown in Table 1. Primers were made by IDT, Inc. (Coralville, IA USA).

Antibodies

The antibodies used in immunoblotting for detection of HIF-1α, were HIF-1 (Cell Signalling), Akt-1 (C73H10-Cell Signalling-USA) and pIkB (Cell Signalling).

Immunoblotting

For western blotting, cells were lysed by addition of 1 ml of lysis buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF

Table 1 Primers used in the study

Primer name	Sequence	Used for
HIF-1α / HindIIIR	AAGCTTTAACTTGATCCAAAGCTCTG	Hif-1 cDNA cloning
Primer HIF-1α/ HindIIIF	AAGCTTAGACATCGCGGGGACCGATT	Hif-1 cDNA cloning
HIF-1-F	TGCTCATCAGTTGCCACTTC	Hif-1 expression
HIF-1-R	GTACTGTCCTGTGGTGACTT	Hif-1 expression
Akt1-F	ATGAGCGACGTGGCTAT	Akt1expression
Akt1-R	GAGGCCGTCAGCCACAG	Akt1expression
Bax F	CTGCAGAGGATGATTGCCG	Bax expression
Bax R	TGCCACTCGGAAAAAGACCT	Bax expression
mTOR F	GCACATTGACTTTGGGGACT	mTOR expression
mTOR R	CGCTTGTTGCCTTTGGTATT	mTOR expression
cIAPI F	GCACATTCATTATCTCCCACCTTG	cIAPI expression
cIAPI R	CATCATCCTTTGGTTCCCAGTTAC	cIAPI expression
cIAPII F	CCTCTCAGCCTACTTTTCCTTCTTC	cIAPII expression
cIAPII R	CATAGCATTATCCTTCGGTTCCC	cIAPII expression
XIAP F	TCAGCATCAACACTGGCACGAG	XIAP expression
XIAP R	TCTCTTGGGGTTAGGTGAGCATAG	XIAP expression
Bcl 2F	CGACGACTTCTCCCGCCGCTACCGC	Bcl expression
Bcl 2R	CCGCATGCTGGGGCCGTACAGTTCC	Bcl expression
Aktin F	GGTCAGAAGGATTCCTATGTG	Actin expression
Aktin R	GTCCACGTCACACTTCATGATG	Actin expression

and 0.01 mg of aprotinin per ml to the cell monolayer. Cell-free extracts prepared from $1x10^6$ cells were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to Hybond ECL membranes (Amersham; Arlington Heights, IL). Antibodies described above were used for immunoblotting.

CLONING OF THE HIF-1α GENE INTO THE pcDNA MAMMALIAN EXPRESSION VECTOR

Obtaining the whole cDNA of HIF-1α

The forward primer was chosen from the 5' site including ATG codon of the HIF-1α gen and Kozak sequence (Table 1). Reverse primers were chosen from the end of the HIF-1α including the stop codon. The PCR containing HIF-1α specific primers, proofreading enzyme and cDNA obtained from the PancI cell line was performed. PCR product runs on the 1% agarose gel. The PCR product corresponding to the HIF-1α cDNA was obtained from the gel using gene cleaning kit.

Cloning of the HIF-1α gene into the pcDNA

Since proofreading enzyme used in the PCR, the PCR product is blunt ended. pcDNA was cut with EcoRV enzyme and dephosphorylated using dephosphorylating enzyme (FastAP) (Fermentas-Germany) and pcDNA vector and HIF-1α cDNA product were ligated using T4 ligation enzyme (Fermentas-Germany) according the protocol.

Transformation

To obtain pcDNA/HIF-1α vector, pcDNA HIF-1α ligated vector was transformed into competent DH5α E.coli using heat shock transformation method (9).

Mini prep plasmid isolation

Alkaline lysis method was performed for to select the HIF-1α containing pcDNA/HIF-1α vector.

Transfection of pcDNA/HIF-1α vector into the PancI cell line

Panc-1 human pancreatic cancer cells were transfected when they reach a density of 70%. For transfection of the pcDNA/HIF-1α vector, ExGen 500 Transfection Reagent (Pure Extreme/Fermentas) was used according to the protocol. As a control, the same amount pcDNA empty plasmid was transfected into the cells.

Clonogenic (colony forming) assay

The cells transfected with pcDNA/HIF-1α or pcDNA were trypsinized after 42 hours. Cells were counted and the same amount of cell plated into the 100 mm plates with DMEM containing 10 ml 10% FBS, 1% L-Glutamine, 1% Penicillin-streptomycin. Twenty-four hours later, 0,4 mg/ml of G418 (Neomycin) was added into the each cell culture. Two weeks later, cells were washed with PBS and were fixed and stained with 1% methylene blue-methanol. A cluster of blue-staining cells was counted from the each cell plate.

Drug treatment

Two different doses of doxorubicin HCl (ADRİ-BLAST the vial- Deva-Turkey) was applied. Dose 1 (D1) is twice of clinical dose (2,5mg /L) and dose 2 (D2) is clinical dose of 1,25 mg/L. Twenty-four well plate were seeded with 5X104 of PancI and 24 hours after 6 well of plates were transfected with pcDNA/HIF-1α and another 6 wells were transfected with pcDNA as a control. 48 hours later medium from the cell was discarded. The medium containing doxorubicin HCl were added to the cell plates.

Cell survival assay (MTT)

MTT test was used for this purpose. MTT stock solution was prepared by diluting 25 mg MTT (3-(4,5-dimethyl diazole-2-yl) -2,5 diphenyl tetrazolium bromide) in 5 ml PBS. 72 hours after the transfection of the pcDNA/HIF-1α, cell medium was removed, and 100 µl medium containing 10% w/w MTT was added to the cells. Three hours after the

medium removed and lysis solution 1% SDS, 6% Acetic acid DMSO added. After 10-minute moderate shaking absorbance was measured at 570/630 with the spectrophotometer.

RESULTS

Overexpression of HIF-1α in PancI cell

To show the expression of HIF-1α cloned vector (pcDNA- HIF-1α), 72 hours after the transfection of pcDNA- HIF-1α, RNAs are obtained from the cell lines pcDNA- HIF-1α or pcDNA transfected, and then cDNA synthesis was completed. To show the RNA expression, PCR was performed from the cDNAs obtained from the cell transfected with pcDNA- HIF-1α or pcDNA. PCR products were investigated at the cycle of 15., 20, and 25 (Figure 1). While HIF-1α expression is observed at the cycle of 15 for the cell line transfected with pcDNA- HIF-1α, endogeneus expression of HIF-1α was not appeared until the cycle of 25.

To understand how much RNA converted into the protein, western blot was performed from the cell lines either transfected with pcDNA- HIF-1α or pcDNA (control) (Figure 2). The same amount of proteins were extracted from each cell and run in SDS agarose. Immunoblot was completed using HIF-1α specific antibody. At the same time, another immunoblot was completed using the Actin-specific antibody for the internal protein control. Immunoblot results showed that pcDNA- HIF-1α transfected cell have more than twice as much as HIF-1α compared to the pcDNA transfected cell. Both results of the PCR and Western blot showed that HIF-1α cDNA was correctly cloned into the pcDNA vector. Also, overexpression of HIF-1α occurs in the PancI cell.

Effect of HIF-1α on the cell proliferation

MTT test was used to investigate the cells proliferation. pcDNA- HIF-1α transfected and pcDNA transfected cell lines were investigated 72 hours later after the transfection. MTT result shows that HIF-1α overexpression increases cell survival as much as 40% in three days (Figure 3).

To investigate the protective effect of the HIF-1α against cytotoxic drugs

For this purpose, two different doses of doxorubicin were added into HIF-1α over-expressing or not HIF-1α over-expressing cells. 48 hours later,

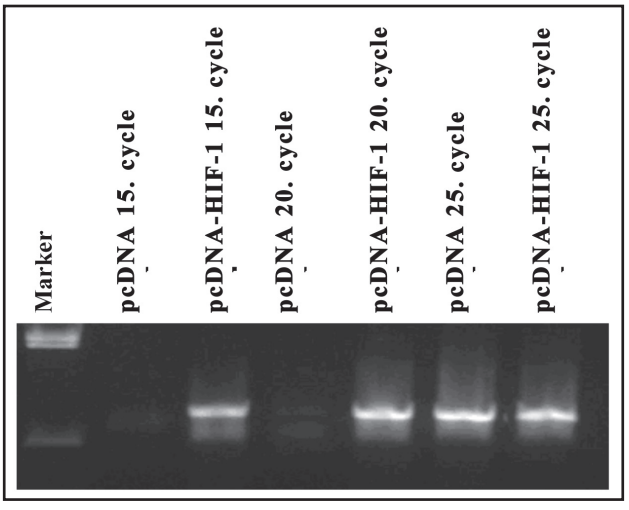

Figure 1 PCR products were investigated at the cycle of 15, 20, 25 of the cDNAs obtained from the PancI cell transfected with pcDNA (control) or pcDNA-HIF-1.

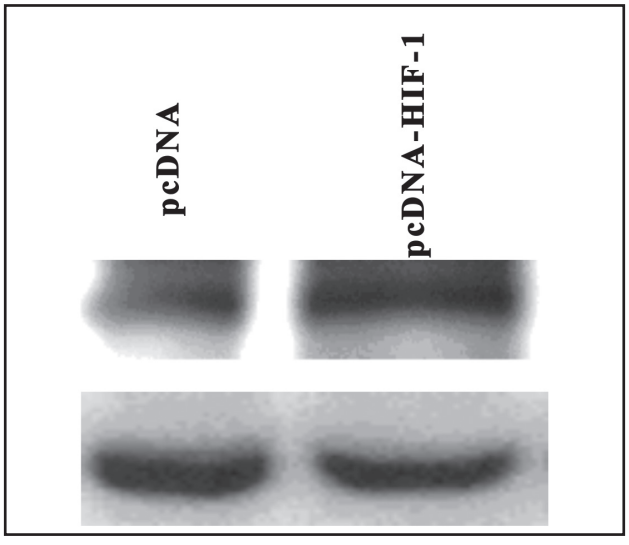

Figure 2 Western blot analysis of the HIF-1 protein expression obtained from the PancI cell transfected with pcDNA (control) or pcDNA-HIF-1.

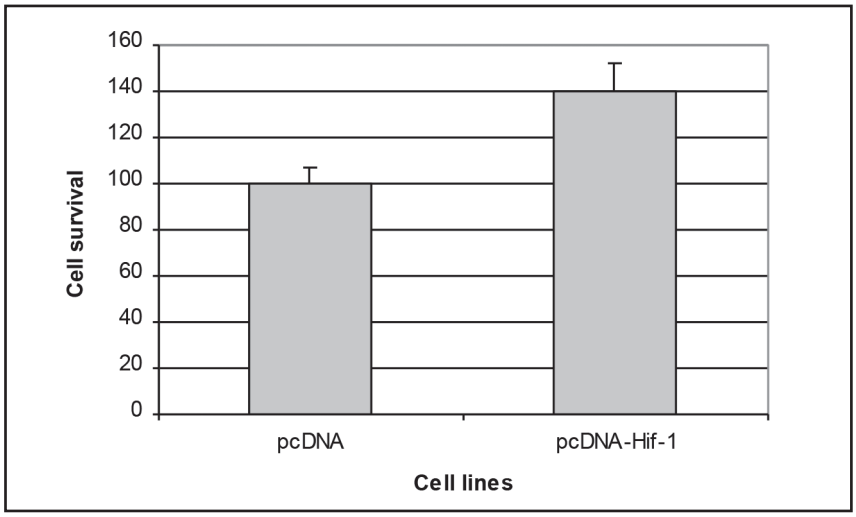

Figure 3 PancI cells transfected with pcDNA (control) or pcDNA-HIF-1 were analysed for cell survival. MTT test was used 72 hours after the transfection.

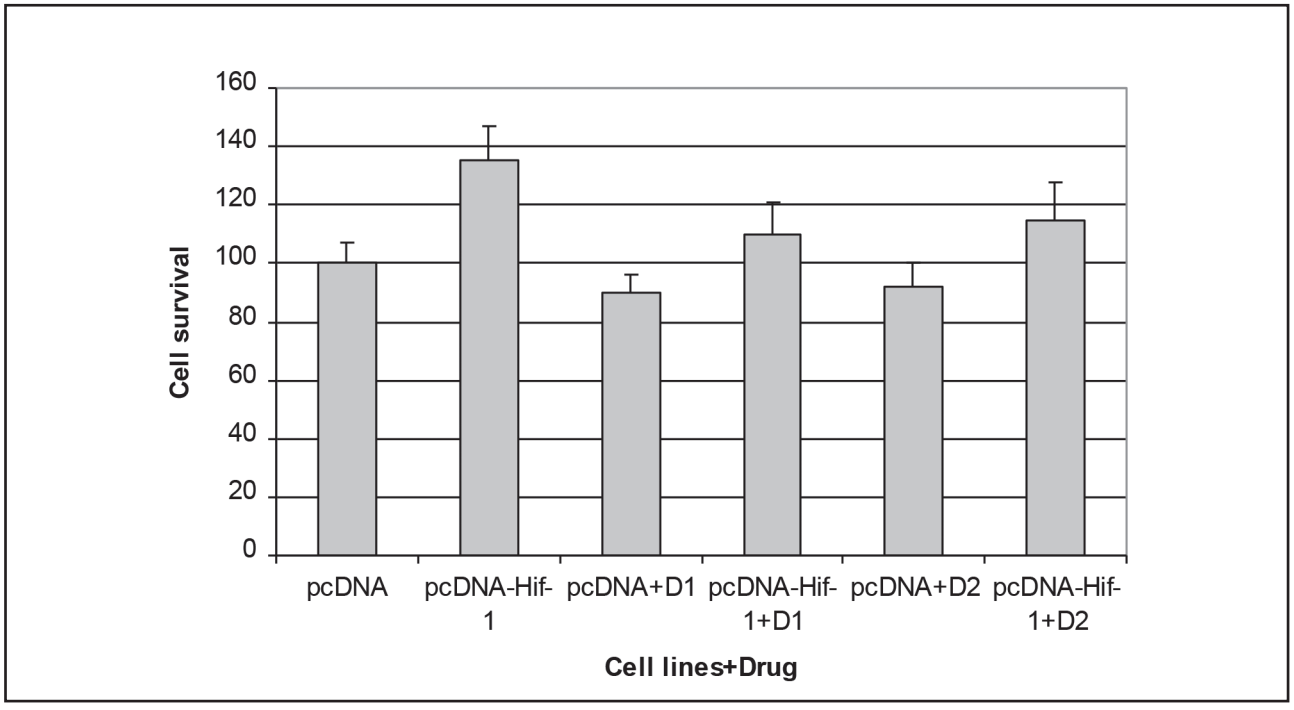

Figure 4 Two different doses of doxorubicin was added into HIF-1 over-expressing or not HIF-1 over-expressing cells. 48 hours later cell survival was investigated using MTT test.

cell survival was investigated using MTT test. The result of the MTT showed that doxorubicin decrease cell number around 20% depending on the drug dose in both HIF-1α overexpressing and control cells compared to the cells transfected with pcDNA- HIF-1α or pcDNA (no drug used) (Figure 4). In addition to doxorubicin, we also investigated the protective effect of the HIF-1α against G418. Commonly know as G418, Geneticin reagent is an aminoglycoside related to Gentamicin. The mechanism by which G418 exerts its slow toxicity in eukaryotes is thought to involve inhibition of pro-

tein synthesis by binding to 80S ribosomes. Colony forming assay was used to test the protective effect of the HIF-1α against G418. Geniticin was added into HIF-1α over-expressing or not HIF-1α over-expressing the cells. 24 hours after the transfection of the vectors medium was changed with medium containing geniticin. Two weeks later, colonies which are survived against the toxicity of geniticin were counted. Results of counting showed that HIF-1α overexpressing colonies 20-30% more colonies compared to the control cell (Figure 5).

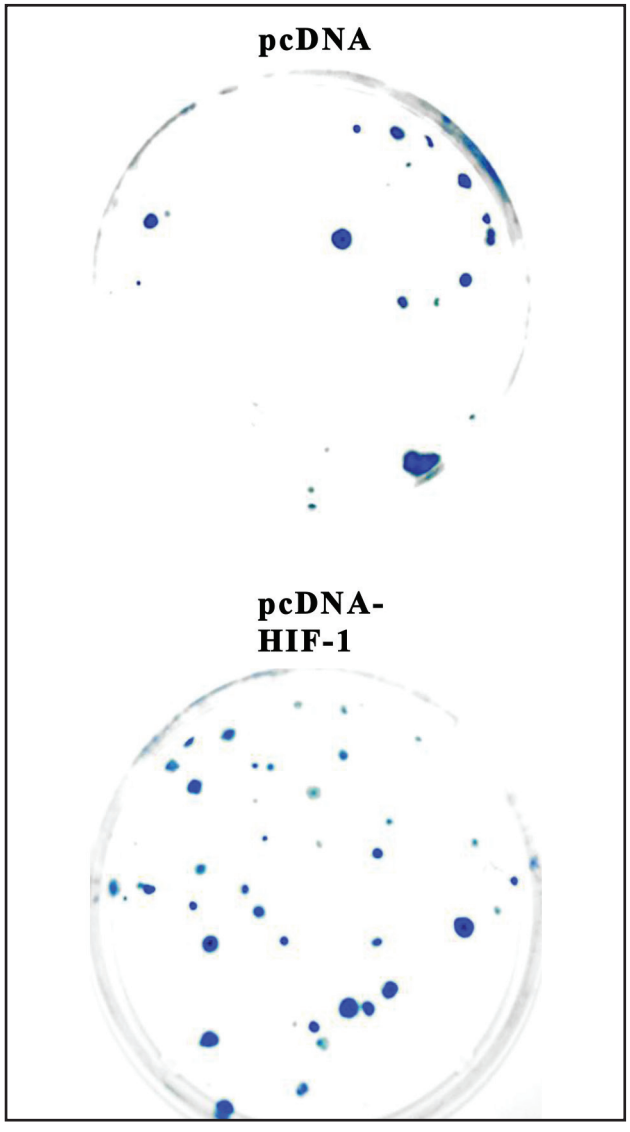

Figure 5 Colony forming assay of the cells HIF-1 over-expressing or not HIF-1 over-expressing in the presence of G418.

Investigation of genes' expressions that may cause proliferation

To understand the possible signal pathways related with HIF-1α, Akt-1, Bax, mTOR, cIAPI, cIAPII, Bcl2 and XIAP gene expression was investi-

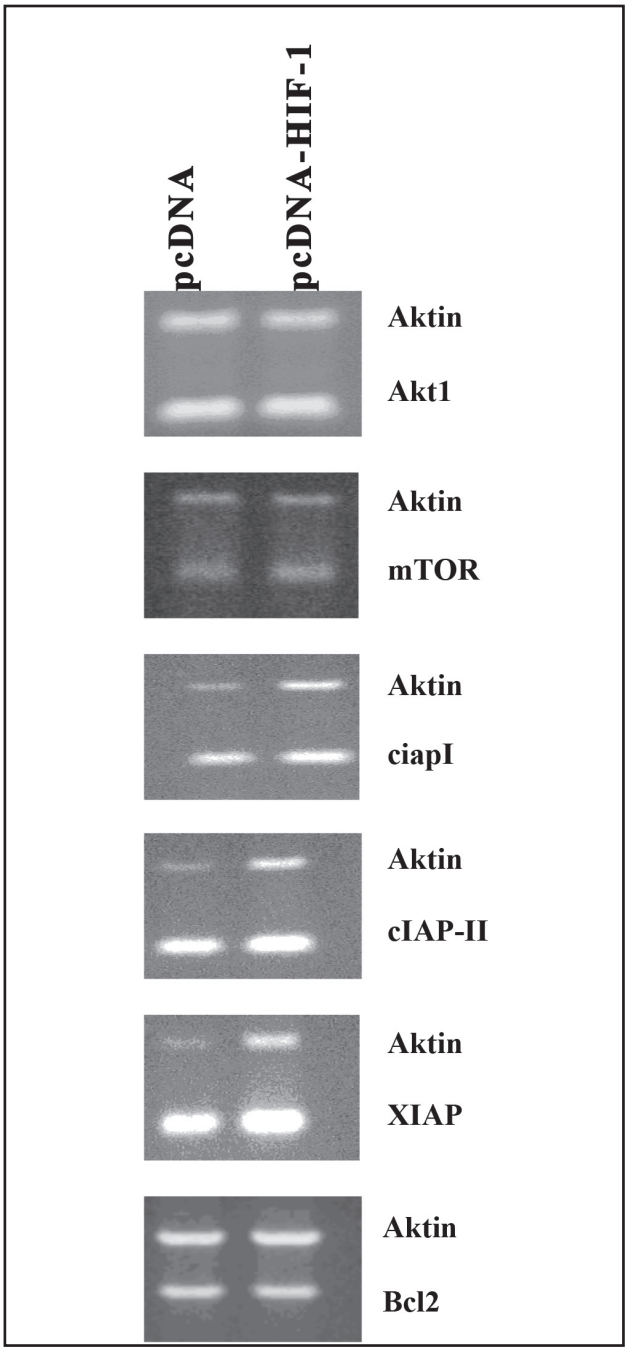

Figure 6 Akt-1, Bax, mTOR, cIAP-I, cIAP-II, Bcl2 and XIAP gene expression were investigated with Multiplex RT-PCR.

gated with Multiplex RT-PCR. In addition, Akt-1 and pIkB protein expressions were investigated with western blot. For Multiplex RT-PCR, first the linear range of target genes and the internal standard actin was determined, and then MRT-PCR was completed using specific primers for each gene. MRT-PCR results showed that none of the genes showed any difference in the cell expressing ectopic HIF-1α (Figure 6). Similarly, the western blot results showed that Akt-1 and pIkB protein expression was not significantly changed by the ectopic HIF-1α expression (Figure 7).

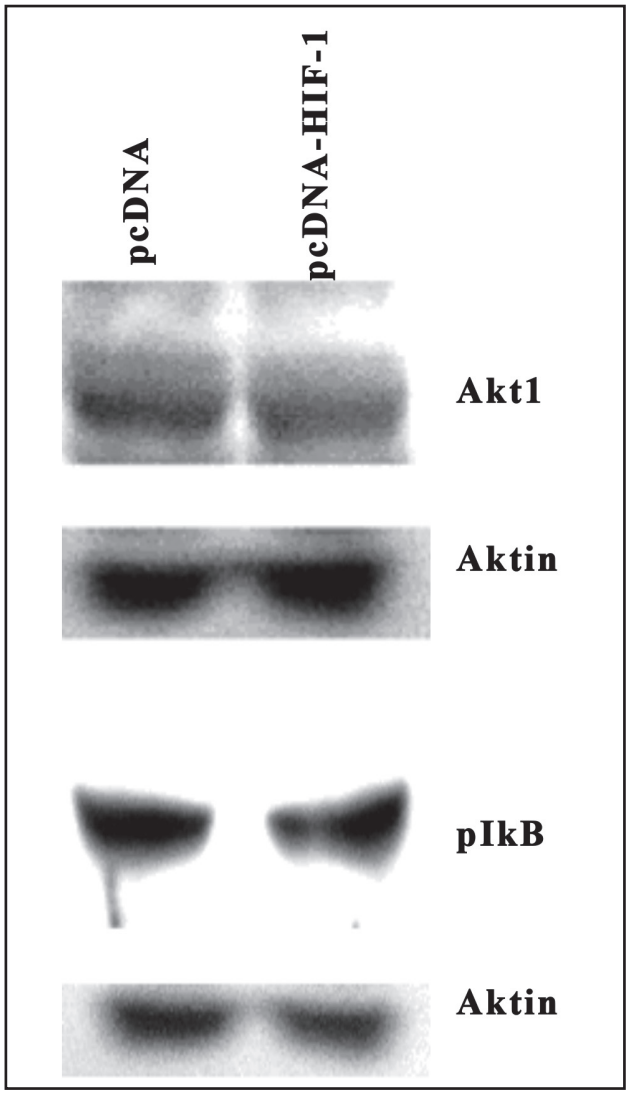

Figure 7 Akt-1 and pIkB protein expression were investigated with western blot.

DISCUSSION

In this study, we aimed to investigate phenotypic effects of the HIF-1α on the PancI cell survival and resistance to the doxorubicin. It was shown in different studies that HIF-1α contributes to the regulation vital biological processes required for tumor survival and progression (7). HIFs are DNA binding transcription factors that mediate cellular responses to reduced oxygen availability through transcriptional activation of a multitude of through transcriptional activation of over 100 downstream genes that encode proteins needed for oxygen delivery to tissues and energy metabolism (10, 11).

In this study, we showed that ectopic expression of the HIF-1α increased the tumor survival and progression. HIF-1α overexpressing cells growth almost 40% more in 72 hours compared to mock cells in pancreatic cell line PancI.

From the previous studies, HIF-1α-induced drug resistance in tumors has been reported for vincristine, doxorubicin, cisplatin, etoposide in different cells (2,4). Therefore, we next investigated roles of the HIF-1α for resistance to doxorubicin. We found that doxorubicin decreased cell survival at the same rate in both cells overexpressing HIF-1α and not. Initially, we assumed that if HIF-1α has a direct effect on the doxorubicin resistance we expected to see HIF-1α overexpressing cell would decrease less compared to the control cells. The result show that HIF-1α increase cell survival and doxorubicin just prevent cell survival in some part. Therefore, we assumed that the drug resistance may be the consequence of the increased cell viability by HIF-1α.

Drug resistance studies showed that HIF-1α transcriptional targets may induce drug resistance by affecting drug transporters, e.g. increased p-glycoprotein; drug targets, e.g. decreased topoisomerase II; or by changing the response to drugs, for instance by modifying drug-induced apoptosis, reducing drug-induced senescence, or inducing autophagy in response to drugs (2,7,12).

Related to the doxorubicin resistance a study conducted by Chen et al. showed that doxorubicin resistance caused by induction of the multi-drug-associated protein has also been demonstrated in many cancer lines which are one of the target genes of the HIF-1α in the glioma cell (13). Interestingly, another study conducted with pancreatic cancer showed that doxorubicin resistance caused by induction of survivin expression (14). Survivin has dual roles in promoting cell proliferation and preventing apoptosis (15). Therefore, in the presence of HIF-1α, doxorubicin resistance is not caused by drug exclusion or inactivation but increased cell survival which supports our results.

The resistance to hypoxia-induced apoptosis may be due to the increased expression of anti-apoptotic factors including the IAP3 and Bcl-2 family of proteins. phosphoinositol-3-kinase (PI3K) pathway, nuclear factor kappa-B (NF-κB), and STAT-3 are involved with the resistance to cytostatics in hypoxia, implying a role for these pathways in hypoxia-induced drug resistance (2, 16, 17). However, the degree in which they are dependent on functional HIF-1α is uncertain. NF-κB up-regulates the expression of anti-apoptotic genes such as Bcl-2, cellular inhibitors of apoptosis (cIAPs) (2, 18).

Activation of phosphatidyl inositol-4,5-bisphosphate-3-kinase (PI3K) can upregulate the HIF-1α protein translation. PI3K regulates protein syntheses through its target protein kinase B (Akt) and downstream component mammalian target of rapamycin (mTOR). mTOR mediates its action via phosphorylation of the eukaryotic translation initiation factor 4E (eIF-4E) binding protein (4E-BP1) disrupting the integrity of these two components, which is essential for inhibiting cap-dependent mRNA translation, resulting in enhanced HIF-1α protein translation (2,19).

Therefore to understand the possible signal pathways related with HIF-1α in PancI cell, we investigated expression of genes which are mainly in the PI3K pathway. Akt-1, Bax, mTOR, cIAP-I, cIAP-II, Bcl2 and XIAP gene expression were investigated with Multiplex RT-PCR. In addition, Akt-1 and pIkB protein expressions were investigated with western blot. MRT-PCR results showed that none of the genes showed any difference in a cell expressing ectopic HIF-1α. Similarly, the western blot results showed that Akt-1 and pIkB protein expression was not significantly changed by the ectopic HIF-1α expression. These results show that these genes' expression were not changed by the ectopic expression of the HIF-1α. However, since the used antibodies detected the amount of the protein only, results may not show the activation status of the protein. Therefore, further studies including phosphorylation status of the protein detection would give more data related to the activation of the protein.

As the conclusion, results of this study may indicate that HIF-1α increase the cell survival in PancI cell and doxorubicin may act on the targets related to cell survival.

Acknowledgments: This work was supported by The Scientific & Technological Research Council of Turkey (TUBITAK) with project number: SBAG-(104S249)

REFERENCES

1. Semenza GL. Targeting HIF-1 for cancer therapy. Nature reviews. Cancer 2003; 3:721-732.
2. Doktorova H, Hrabeta J, Khalil MA, Eckschlager T. Hypoxia-induced chemoresistance in cancer cells: The role of not only HIF-1. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia 2015; 159:166-177.
3. Ravizza R, Molteni R, Gariboldi MB et al. Effect of HIF-1 modulation on the response of two- and three-dimensional cultures of human colon cancer cells to 5-fluorouracil. Eur J Cancer 2009; 45:890-898.
4. Das V, Stepankova J, Hajduch M, Miller JH. Role of tumor hypoxia in acquisition of resistance to microtubule-stabilizing drugs. Biochimica et biophysica acta 2015; 1855:172-182.

5. Schnitzer SE, Schmid T, Zhou J, Brune B. Hypoxia and HIF-1alpha protect A549 cells from drug-induced apoptosis. Cell death and differentiation 2006; 13:1611-1613.
6. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proceedings of the National Academy of Sciences of the United States of America 1995; 92:5510-5514.
7. Masoud GN, Li W. HIF-1alpha pathway: role, regulation and intervention for cancer therapy. Acta pharmaceutica Sinica. B 2015; 5:378-389.
8. Semenza GL. HIF-1: upstream and downstream of cancer metabolism. Current opinion in genetics & development 2010; 20:51-56.
9. Cregg JMaKAR. Transformation methods. Methods in Molecular Methods Molecular Biology 1998; 103:27-39.
10. Brocato J, Chervona Y, Costa M. Molecular responses to hypoxia-inducible factor 1alpha and beyond. Molecular pharmacology 2014; 85:651-657.
11. Manalo DJ, Rowan A, Lavoie T et al. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 2005; 105:659-669.
12. Kallio PJ, Pongratz I, Gradin K et al. Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proceedings of the National Academy of Sciences of the United States of America 1997; 94:5667-5672.
13. Chen J, Ding Z, Peng Y et al. HIF-1alpha inhibition reverses multidrug resistance in colon cancer cells via downregulation of MDR1/P-glycoprotein. PloS one 2014; 9:e98882.
14. Chang Q, Qin R, Huang T et al. Effect of antisense hypoxia-inducible factor 1alpha on progression, metastasis, and chemosensitivity of pancreatic cancer. Pancreas 2006; 32:297-305.
15. Bache M, Holzapfel D, Kappler M et al. Survivin protein expression and hypoxia in advanced cervical carcinoma of patients treated by radiotherapy. Gynecologic oncology 2007; 104:139-144.
16. Li F, Huang L, Su XL et al. Inhibition of nuclear factor-kappaB activity enhanced chemosensitivity to cisplatin in human lung adeno-carcinoma A549 cells under chemical hypoxia conditions. Chinese medical journal 2013; 126:3276-3282.
17. Belaiba RS, Bonello S, Zahringer C et al. Hypoxia up-regulates hypoxia-inducible factor-1alpha transcription by involving phosphatidylinositol 3-kinase and nuclear factor kappaB in pulmonary artery smooth muscle cells. Molecular biology of the cell 2007; 18:4691-4697.
18. Lee BL, Lee HS, Jung J et al. Nuclear factor-kappaB activation correlates with better prognosis and Akt activation in human gastric cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 2005; 11:2518-2525.
19. Semenza GL. Hypoxia-inducible factors in physiology and medicine. Cell 2012; 148:399-408.