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Original Article

Investigation of mRNA Levels of PFK-1, LDH-A, p53, and HIF-1α Genes Involved in Metabolic Reprogramming in Non-small Cell Lung Carcinoma

Fatemeh Shakeri[1], Mehdi Ebrahimi[2*], Shohre Zare Karizi[1]

[1]Department of Genetics, College of Biological Sciences, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran
[2]Department of Biochemistry and Biophysics, College of Biological Sciences, Varamin-Pishva Branch, Islamic Azad University, Varamin, Iran

Abstract

Background: It is expected that the expression of key enzymes of the glycolysis pathway, specially PFK-1, increases tumor cells and so enhances the function of this pathway. The p53 and HIF-1 proteins are regulators of the expression of PFK-1 and LDH enzymes. This study was performed to investigate changes in the expression of PFK-1, LDH-A, p53, and HIF-1α genes to identify metabolic changes in non-small cell lung carcinoma (NSCLC) samples.

Methods: A number of 30 tumors and their adjacent normal tissue samples from surgically approved NSCLC patients were used. Total RNA from each tissue was extracted. The changes in mRNA levels of PFK-1M, LDHA, p53, and hypoxia-inducible factor-1 (HIF-1α) genes were evaluated in tumor and normal tissues of all patients using the real-time polymerase chain reaction (PCR) method. Finally, statistical analysis was used to determine significant differences and the relationship between changes in mRNA levels.

Results: According to the results, there was no significant difference in the mRNA levels of these genes between tumor and normal tissues. A significant difference in the mRNA level of lactate dehydrogenase A (LDHA) between adenocarcinoma (AdC) and squamous cell carcinoma (SqCC) tumor types was observed ($P=0.014$). Also, the difference between the mRNA level of LDHA and HIF-1α in metastatic and non-metastatic samples was significant ($P=0.035$ and $P=0.046$ respectively). Age and male gender were directly associated with an increased risk of NSCLC.

Conclusion: The results of the present study revealed that the increase in mRNA level of PFK-1 and p53 may be involved in NSCLC initiation, and an increase in LDH-A and HIF-1a is associated with a metastatic phenotype.

Keywords: Lung cancer, Warburg effect, Phosphofructokinase-1, Lactate dehydrogenase, Hypoxia-inducible factor-1

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Introduction

Lung cancer, known as lung carcinoma, is a malignancy that originates from lung epithelial cells and usually spreads to other organs and adjacent tissues (1). According to the recent World Health Organization (WHO) report, lung cancer is the most important cause of cancer deaths. It has been estimated that lung cancer accounts for 11.6% of the total cancer patients and 18.4% of all deaths due to various cancers. Most people with lung cancer die, and the percentage of people who have a chance to survive for five years is about 19% (16% in men and 22% in women) (2).

Lung carcinomas are divided into the two main groups, non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC), based on cell size (3). NSCLC is also classified into four types adenocarcinoma (AdC), adenosquamous carcinoma, squamous cell carcinoma (SqCC), and large cell carcinoma based on tumor histology. AdC and SqCC account for about 80% of NSCLC cases of lung cancer (4,5).

Tobacco use, especially cigarette smoking, accounts for more than 90% of lung cancer cases worldwide. Environmental factors (such as cigarette butts, radon gas, and carcinogens present in the workplace), benign lung diseases, viruses, and hormonal and genetic factors account for the remained 10% (6,7).

Both normal and cancer cells are capable of modifying the speed and function of metabolic pathways in response to changes in their microenvironment. These metabolic changes are called metabolic reprogramming (8,9). Wide ranges of metabolic changes have been observed in cancer cells compared to the normal cells of the same type. Some of these changes such as up or downregulation of the speed of some pathways are simple but other changes

*Corresponding Author: Mehdi Ebrahimi, Email: ebrahimi@iauvaramin.ac.ir

require rewiring between metabolic pathways (10). One of the most important metabolic changes is the increase in glycolysis rates and the conversion of the majority of cellular glucose into lactate even under adequate oxygen conditions (normoxic), known as the Warburg effect (11).

Cancer cells are deficient in oxygen (hypoxia) and nutrients due to their high growth rate and limited angiogenesis. The production of ATP in hypoxia is restricted to the glycolysis pathway. Therefore, these cells mainly use glucose and produce lactate to adapt to hypoxic conditions. Although glycolysis efficiency in net ATP production is lower than oxidative phosphorylation, cancer cells increase the glycolysis rate to compensate the low efficiency of glycolysis (12).

Cancer cells use a variety of mechanisms such as the increase in the expression of key enzymes and Glucose transporters (especially Glut1) and allosteric effects to increase glycolysis rate (13). Phosphofructokinase-1 (PFK1) catalyzes the one-way reaction of fructose-6-phosphate (F6P) and ATP to fructose-1,6-bisphosphate (F1,6BP) and ADP. This reaction is the most important regulatory site in the glycolysis pathway. For this reason, several mechanisms are involved in controlling PFK1 activity, including control of PFK1 expression by oncogenes, allosteric regulation, and post-translational modifications (14). Lactate dehydrogenase (LDH) is another important enzyme that catalyzes the reversible reaction of pyruvate to lactate. Under hypoxia, which usually occurs in the central areas of tumor tissue, pyruvate is converted to lactic acid and provides the NAD^+ required to produce ATP in the glycolysis pathway. LDH has been identified as one of the key factors in the development and survival of various tumors and therefore has been considered as one of the important targets for designing anticancer drugs (15).

LDH is identified as one of the key factors in the development and survival of different tumors. Therefore, LDH has been considered an important target for the design of anticancer drugs. One of the tumor suppressor factors is p53, which mutates in most tumor cells. Inactivation of p53 by mutation can cause the cell cycle to continue in damaged cells (16). The p53 inhibits glycolysis by affecting TP-53-induced glycolysis and apoptosis regulator (TIGAR) and decreasing fructose-2,6-bisphosphate (PFK-1 negative effector) (17).

Hypoxia is one of the hallmarks of fast-growing tumors. Under these conditions, Hypoxia-inducible factor-1α (HIF-1α) induces changes in cells to adapt to hypoxic conditions. the most important change is the conversion of oxidative metabolism to glycolytic (18). HIF-1α is a key regulator of glucose metabolism and increases glycolysis in the cancer cells, resulting in a defective cell cycle. Moreover, lactate activates VEGF, TGF-β, IL-1, and HIF-1α even under normal oxygen conditions (19).

Some of the induced changes in cancer cell metabolism are dependent on the type of tumor tissue. These changes can be used as therapeutic targets for the selection of effective drugs. therefore, it is important to evaluate the change in transcription of key genes of these pathways in different types of tumors. This study was conducted to investigate the changes in the mRNA levels of HIF-1α, LDHA, p53, and PFK1 genes in non-small cell lung cancer tissue specimens, as well as to identify possible patterns of association of these genes in lung cancer.

Materials and Methods

NSCLC tissue sample collection

Tissue samples were obtained from patients only after their consent was obtained. Tumour tissue and its adjacent normal tissue (as normal tissue) were collected from 30 NSCLC patients at Masih-Daneshvari Hospital by a surgeon. The tissue samples were evaluated by a pathologist and used in this study after NSCLC confirmation. Patients enrolled in this study had not received any treatment related to the NSCLC. The tissue samples were separately collected in cryotubes and immediately transferred to a nitrogen tank and stored at -70°C. The present study was conducted under the permission of the Medical Ethics Committee of Islamic Azad University (IR.IAU.VARAMIN.REC.1397.039).

Total RNA extraction and cDNA synthesis

Total RNAs were extracted from the tissue samples based on the TRIzol method (20). Briefly, 100 mg of each tissue was homogenized. The tissue was transferred to DNase/RNase-free microtubes and 1 mL of TRIzol was added and vortexed vigorously. Samples were incubated at room temperature for 20 minutes. Then, 200 µL of chloroform was added to the microtubes and centrifuged at 12000 rpm for 15 minutes at 4°C. The same volume of Isopropanol was added to the aqueous phase and incubated at -20°C for one day. Samples were centrifuged at 12000 rpm for 4 minutes at 4°C. Afterward, the supernatant was withdrawn and residual sediment was washed with 2000 µL of cold ethanol (75% v/v). The sample was incubated at 55°C for 20 minutes and RNA was transferred immediately to -70°C. The cDNA synthesis steps were performed using a commercial kit (*GeneAll®*) according to the manufacturer's instructions.

Real-time qPCR method

Quantitative PCR was performed using a *Rotor-Gene® Q* (QIAGEN) real-time PCR cycler and a RealQ Plus 2x Master Mix Green High ROX™ solution (Ampliqon) containing fluorescent dye SYBR® Green according to the manufacturer instructions. Primers were designed using primer3 software based on the Intron-spanning method and analyzed with Oligo Analyzer-1.0.2 software (Table 1). Primers were synthesized by Sinaclon company. PCR steps and conditions for all genes were as follows:

Table 1. The sequence and PCR product lengths of designed primers for real-time qPCR

Gene		Sequence (5′→3′)	PCR Product (bP)
HIF-1α	Forward	CCAGCAGACTCAAATACAAGAACC	138
	Reverse	TGTATGTGGGTAGGAGATGGAGAT	
p53	Forward	CACTCCATCCACCTGAAGTCC	203
	Reverse	CTACTGACCGACACCTATTGC	
PFK-M	Forward	TTGGGGGCTTTGAGGCTTAC	118
	Reverse	CGAGGGAGATTGTTGGAGACTG	
LDH-A	Forward	GCGATTCCTGTGTGCCTGTATG	101
	Reverse	CCTCTCTTCCTTATCTTTATCAGTCCC	
GAPDH	Forward	CATCAAGAAGGTGGTGAAGCAG	120
	Reverse	GCGTCAAAGGTGGAGGAGTG	

denaturation at 95°C for 30 seconds, 40 cycles at 60°C for 60 seconds, and finally 15 seconds at 95°C. The GAPDH gene was used as a housekeeping gene and quantitative expression of gene expression was determined by the ΔΔCt method. Gene expression change was reported as fold change ($2^{-\Delta\Delta Ct}$).

Statistical analysis

Evaluation and statistical analysis of RT-qPCR raw data were performed using GenEx version 6 software (Trial version). Relative charts were drawn using Excel 2016 software. The normality of samples distribution was assessed by the Kolmogorov-Smirnov test. Significance of differences between healthy and tumor specimens was evaluated by the Wilcoxon test. The $P<0.05$ was considered a significant level of difference. Correlation analysis between groups of genes was done using the Spearman test.

Results

Demographic and pathological data of the evaluated samples

The NSCLC cancer and normal lung tissue samples were used to evaluate the changes in mRNA levels of HIF-1α, p53, PFK-M, LDH-A, and GAPDH genes using RT-qPCR. Demographic and pathological data of the evaluated samples are shown in Table 2. The mean age ± SD of the patients evaluated in this study was 59.1 ± 7.6 years and the highest distribution of ages was in the range of 50-70 years. In terms of gender, 73% of the samples were men and 27% were women. Histologically, 57% of the samples were AdC and 43% were SqCC. In terms of disease stage, the highest percentage belonged to stage IIIA and the lowest number to the stage IIIB with 30% and 3.3%, respectively. About 7% of NSCLC patients were smokers.

Fold change analysis of mRNA levels

The amounts of fold change in the studied samples were calculated based on the $2^{-\Delta\Delta Ct}$ equation and are shown in Figure 1. The mRNA levels of all genes increased in tumor tissue compared to normal. Increased mRNA levels for HIF-1α, p53, PFK-M, and LDH-A genes were 2.3, 1.08, 1.9, and 1.08, respectively.

Table 2. Demographic and pathological data of the evaluated samples

Variable	No. (%)
Age	
<40	1 (3)
41-50	1 (3)
51-60	14 (47)
>60	14 (47)
Gender	
Male	23 (73)
Female	7 (27)
NSCLC type	
AdC	17 (57)
SqCC	13 (43)
Stage	
IA	2 (6.7)
IIA	5 (16.6)
IIIA	9 (30)
IB	7 (23.4)
IIB	6 (20)
IIIB	1 (3.3)
Smoking	23 (73)

Analysis of RT-qPCR results using the Kolmogorov-Smirnov test showed that the ΔCt values in all of the genes did not have a statistically normal distribution. Therefore, Wilcoxon nonparametric test was used to investigate the ΔCt differences between normal and tumor tissue. The results showed that the differences in mRNA levels between normal and tumor tissues were not significant for all of the genes ($P>0.05$, Table 3).

The effect of tumor type, stage, metastasis, and smoking

Fold change of HIF-1α, p53, PFK-M, and LDH-A in smoking, tumor type, and tumor metastasis samples were evaluated using Mann-Whitney test. The fold change of the evaluated genes related to the type of NSCLC tissue is shown in Figure 2a. According to these results, significant differences between the two types of AdC and SqCC tissues were not observed in the mRNA level of HIF-1α, p53, and PFK-M genes, while the mRNA level of LDH-A gene in SqCC tissue was significantly more than AdC tissue ($P=0.014$).

The one-way ANOVA test was used to determine the significant fold changes of desired genes in NSCLC stages (Figure 2b). Results showed that there was no significant difference in mRNA levels of evaluated genes between stages of NSCLC. The mRNA levels of all genes

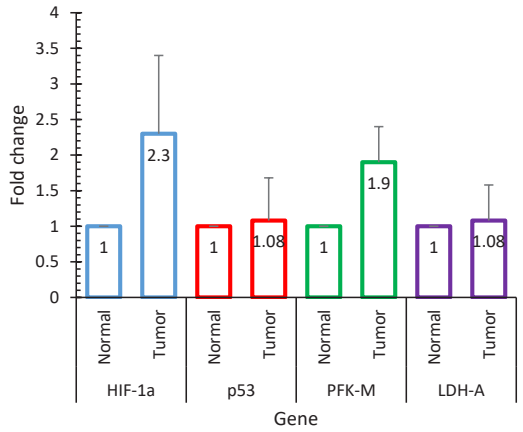

Figure 1. The fold changes of genes in the studied samples. Each box shows the average of fold changes of all samples for a gene. Each sample was evaluated in three replicates and the average was used for fold change calculation.

in metastatic NSCLC samples were increased compared to non-metastatic samples (Figure 2c). Increased mRNA levels of LDH-A and HIF-1α genes were significant in metastatic samples compared to non-metastatic ($P=0.03$ and $P=0.04$, respectively). Finally, there was not a significant difference between mRNA levels of genes and smoking (Figure 2d).

Correlation analysis

Spearman test was used to analyze changes in mRNA levels of the genes. The results showed a significant positive correlation between the changes in mRNA levels of p53, PFK-M, LDH-A, and HIF-1α genes (P value = 0) (Table 4). The positive correlation coefficient means that the changes in mRNA levels of genes are consistent. The higher coefficient represents a higher correlation. Accordingly, the highest correlation was observed between the mRNA level of PFK-M and p53 genes and the lowest correlation between p53 and LDH-A (Spearman coefficients were 0.69 and 0.49, respectively).

Discussion

Aerobic glycolysis in cancer cells requires an increase in glucose uptake and lactate production in the presence of oxygen. This increase in glycolysis activity could meet the urgent need of rapidly expanding tumor cells for energy and structural molecules (21).

The PFK1 is the regulatory enzyme in the glycolysis pathway. The PFK1 catalyzes the reaction of the committed step in glycolysis and is thus considered as the most important enzyme that regulates this pathway. The PFK1 activity is influenced by various factors, including the transcriptional rate of the respective gene and allosteric effects. Also, the PFK1 is a tetrameric isozyme composed of M, P, and L subunits (22). These properties make the regulation mechanism of this enzyme complex and variable in different tissues (23,24). In the present study, the mRNA level of the M isoform was evaluated. According to the results, the mRNA level of this enzyme in tumor tissue cells was 2.28 times that of normal lung cells of patients with NSCLC ($P>0.05$). Moreover, the mRNA level of PFK1 was similar in both AdC and SqCC tissue types. El-Bacha et al reported a 2 fold increase in PFK1 activity in breast tumor tissue compared to normal tissue. Moreover, in the breast tumor, the activity of PFK1 also increases with increasing tumor size (25). PFK expression was also increased in cervical tumor tissue samples compared to normal tissue (26). According to a study by Li et al, increased malignancy and progression of esophageal SqCC are also associated with increased PFK expression (27). However, according to the results of the present study, the mRNA levels of this gene in metastatic tissue were not significantly different from that of non-metastatic tissue. Also, the NSCLC stage did not affect the mRNA level of PFK1. Thus, the stage of NSCLC malignancy did not affect the mRNA level of PFK1. However, the results of a study by Wang et al show that the rate of glycolysis in breast cancer cells is dependent on the conversion of PFK1 from the L isoform to the P isoform (28). A comparison of smokers and nonsmokers shows that smoking does not affect the mRNA level of this enzyme. Thus, other mechanisms may be involved in enhancing PFK1 activity and thereby enhancing glycolysis in metastatic cells and different stages of NSCLC.

The function of the LDH enzyme is required to supply the NAD for the first reaction of the oxidative phase of glycolysis. Thus, LDH guarantees the glycolysis pathway to continue even under anaerobic glycolysis. The activity

Table 3. The fold change calculation and statistical analysis of the results

	HIF-1a		P53		PFK-M		LDH-A	
	Normal	Tumour	Normal	Tumour	Normal	Tumour	Normal	Tumour
ΔCt average	0.0007	-1.1957	-0.0003	-0.1073	-0.0007	-0.9220	-0.0003	-0.1173
STDEV	3.87	4.04	4.61	3.40	6.74	5.61	4.84	3.64
P value		0.25		0.92		0.57		0.92
ΔΔCt		1.20		0.11		0.92		0.12
Fold change		2.29		1.08		1.89		1.08

The ΔCt shows the difference between the average of all the samples in each group. The ΔΔCt shows the difference between the average of ΔCt of the tumor and normal groups. $P<0.05$ shows significant differences between normal and tumor groups. Fold changes were calculated according to the 2^(-ΔΔCt).

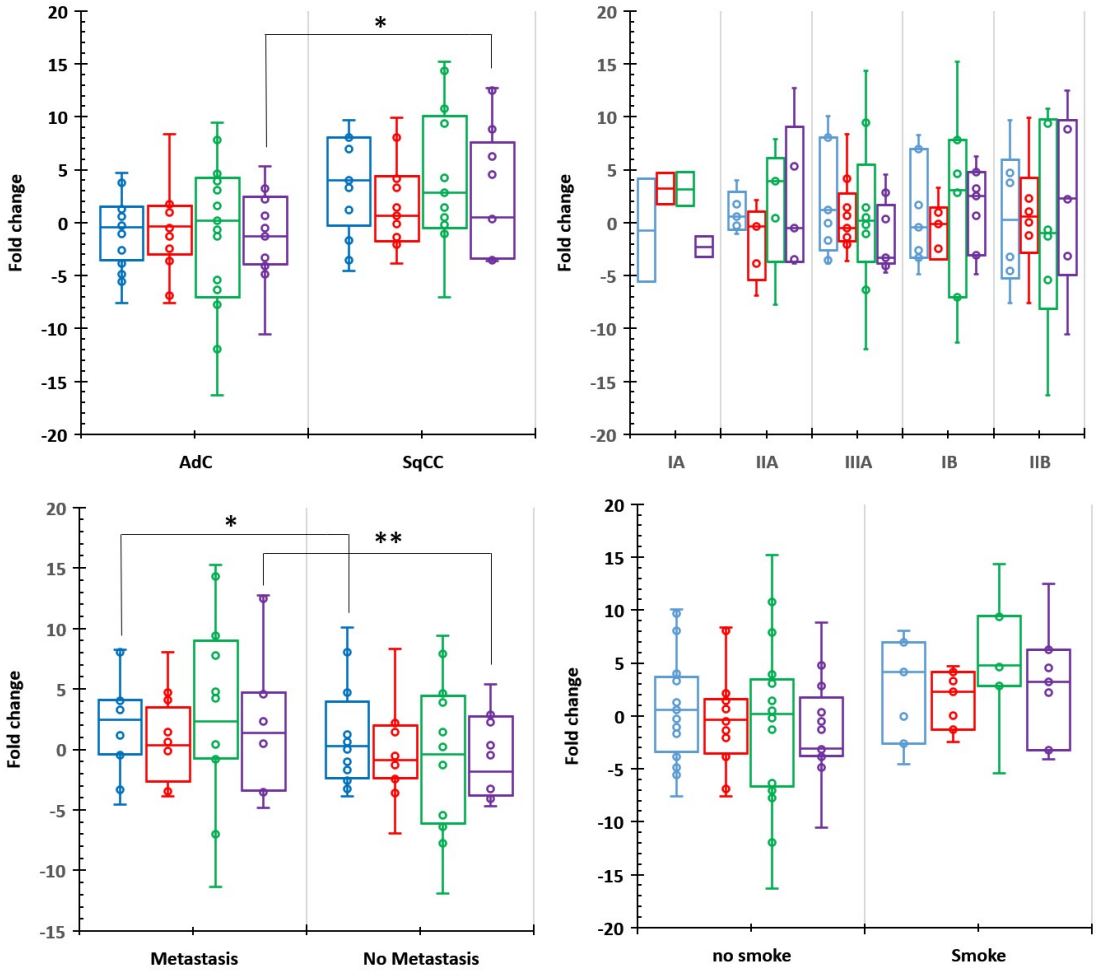

Figure 2. Fold changes of HIF-1α (blue box), p53 (red box), PFK-M (green box), and LDH-A (purple box) genes in response to tumor type (A), stage of NSCLC(B), tumor metastasis (C), and smoking (D). The mean of fold changes in each box was represented with a horizontal line. Each data point represents the average of fold changes given from three replication. The * and ** symbols represent significant differences between the related boxes ($P<0.05$).

Table 4. The results of the Spearman test for analysis of changes in mRNA levels of the genes

	HIF-1a		p53		PFK-M		LDH-A	
	Spearman	*P* value	Spearman	*P* value	Spearman	*P* value	Spearman	*P* value
HIF-1a	1	0.00	0.61	0.00000025	0.65	0.00000002	0.50	0.00005304
p53	0.61	0.00000025	1	0.00	0.69	0.00000021	0.49	0.00006623
PFK-M	0.65	0.00000002	0.69	0.00000021	1	0.00	0.68	0.00005547
LDH-A	0.50	0.00005304	0.49	0.00006623	0.68	0.00005547	1	0.00

of LDH enables glycolysis to provide the energy required for the growth and proliferation of cancer cells under hypoxia. Among different isoforms of this enzyme, LDHA is recognized as the dominant isoform of tumor cells. Therefore, in the present study, the expression of LDHA in NSCLC samples was examined. The results show that the mRNA level of the LDHA gene in tumor tissue was 1.08 times higher than normal tissue in patients with NSCLC ($P=0.92$). Additionally, the mRNA level of the LDHA gene was significantly higher in metastatic samples compared to non-metastatic samples ($P=0.035$), but there was no significant change in different stages of the NSCLC. Results of several studies show that LDH level in serum and its expression level increases with the increase in cancer cell malignancy. Thus, an increase in the LDH level is known as a negative prognostic factor in most types of cancers (29). Lactate produced by LDHA is transported out of the cytoplasm through a specific carrier located in the cell membrane and reduces the pH of the peripheral environment of the tumor. Lower pH in the tumor cell microenvironment is one of the factors contributing to the growth and metastasis of cancer cells.

The mRNA level of p53 gene in tumor tissue cells was 1.07 times higher than that in normal tissue ($P=0.92$).

p53 promotes DNA repair and survival of damaged cells. When DNA damage is broad and cannot be repaired, p53 activates the apoptosis or autophagy process and eliminates the target cell. The p53 protein induces transcription of the TP53 Induced Glycolysis Regulatory Phosphatase (TIGAR) gene. The TIGAR dephosphorylates fructose-2,6-bisphosphate as the major allosteric activator of PFK1. This function of the p53 reduces the rate of glycolysis pathway in tumor cells and consequently decreases their energy levels and has an inhibitory effect on the growth and survival of tumor cells. For this reason, p53 has a direct correlation with increased survival rates in NSCLC patients (30). There was no significant difference in mRNA levels of p53 at different stages of the NSCLC and also between metastatic and non-metastatic samples. Additionally, there was no significant difference in mRNA levels of p53 between smokers and no-smoking samples. However, studies have shown that smoking causes mutations in the p53 gene in people with NSCLC. Evaluation of the p53 expression in osteosarcoma specimens showed increased expression of the mutated variant of this protein (31). The p53 mutations are uniformly observed in all tumor types.

Nevertheless, there is no direct correlation between these mutations and p53 expression. However, some of the observed effects of this protein preclude a definitive comment on the effect of this protein on the glycolysis pathway. For example, p53 has been reported to activate the hexokinase-II promoter. Thereby, facilitating the entry of glucose into the glycolysis pathway and thus affecting tumor cell growth and survival (32).

The mRNA level of HIF-1α gene in tumor tissue of patients with NSCLC was 2.3 times higher than its level in normal tissue ($P>0.05$). In a similar study by Giatromanolaki et al, HIF-1α levels increased by 62% in NSCLC tumor samples compared to normal samples. Tumor cells are generally hypoxic due to the high growth rate and lack of angiogenesis. Under these conditions, HIF-1α is activated and then activates the transcription of a group of genes, including genes involved in glucose metabolism, which accelerate the anaerobic glucose metabolism. Because of the persistence of these conditions in tumor cells, these cells are expected to guarantee their energy supply through anaerobic glycolysis by increasing HIF-1α gene expression. In the present study, there were no significant changes in mRNA levels of the HIF-1α gene between the AdC and SqCC tissues. Additionally, there was no significant difference in the mRNA levels of the HIF-1α gene at different stages of NSCLC. A meta-analysis study reported a significant increase in HIF-1α expression in SqCC samples compared to AdC (33). In the present study, there was also a significant increase in mRNA levels of HIF-1α in metastatic versus non-metastatic samples ($P=0.046$). Increased expression of HIF-1α has been observed in the perineoplastic malignancies of the breast, colon, and prostate; whereas, it has not been reported in malignant breast and uterus tumors. Moreover, the association between HIF-1α and cancer progression in humans has been established.

The positive correlation between mRNA levels of p53, PFK-M, LDH-A, and HIF-1α genes suggests that there may be a common regulator between these genes, or they are mediated through intermediate signaling pathways. Exiting the cell from a regular cell cycle program is the first step in initiating the growth of a cancer cell and producing a cancer cell mass. Several factors contribute to cancer initiation, but commonly tumor suppressor factors, such as p53 inactivated due to mutations. In malignant tumor cells, both mutated and wild-type p53 are present. In normoxia, transcription of the wild-type p53 is increased. But, severe hypoxic conditions in tumors trigger mutation and accumulation of p53 in the nucleus and ultimately activation of its target genes (34).

After this stage, the cells proliferate in a disruptive fashion and tumor growth begins. To provide energy, the cancer cells reprogram their metabolic pathways to adjust their homeostasis. One of these changes is an increase in key enzyme PFK1 in the glycolysis pathway that results in the production of pyruvate. The NAD^+required to continue the glycolysis pathway increases when a normal cell becomes cancerous. This leads to an increase in transcription and activity of LDH and lactate production. Lactate transport out of the cell and reduces the pH of the cancer cell microenvironment. Rapid tumor growth exacerbates hypoxic conditions in tumor tissue resulting in activation of HIF-1α. HIF-1α is a key transcription factor that in turn activates glycolysis and reduces mitochondrial function under hypoxic stress conditions (35).

Conclusion

The incidence of lung cancer is higher in men than in women and increases with age. PFK-1 and p53 genes may be involved in the initiation of the NSCLC tumor while the metastatic phenotype may be due to an increase in the expression of the LDH-A and HIF-a genes. The relationship between PFK-1, LDH-A, p53, and HIF-a factors suggests that metabolic reprogramming may follow changes in the expression of genes involved in the onset and development of lung cancer.

Author Contributions

Conceptualization: Mehdi Ebrahimi.

Data curation: Fatemeh Shakeri, Mehdi Ebrahimi, Shohre Zare Karizi.

Formal Analysis: Fatemeh Shakeri, Mehdi Ebrahimi, Shohre Zare Karizi.

Funding acquisition: Fatemeh Shakeri.

Investigation: Fatemeh Shakeri, Mehdi Ebrahimi, Shohre Zare Karizi.

Methodology: Mehdi Ebrahimi, Shohre Zare Karizi.

Project administration: Mehdi Ebrahimi, Shohre Zare Karizi.

Resources: Fatemeh Shakeri.
Software: Fatemeh Shakeri.
Supervision: Mehdi Ebrahimi.
Validation: Mehdi Ebrahimi, Shohre Zare Karizi.
Visualization: Fatemeh Shakeri.
Writing – original draft: Fatemeh Shakeri.
Writing – review & editing: Mehdi Ebrahimi.

Conflict of Interests
The authors declared that there is no conflict of interest.

Ethical Approval
The present study was conducted under the permission of the Medical Ethics Committee of Islamic Azad University (IR.IAU.VARAMIN.REC.1397.039).

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