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The expression level of fibroblast growth factor gene in serum samples of lung cancer patients

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ABSTRACT

Lung cancer, one of the most deadly and dangerous types of cancer in the world, kills many men and women every year. Activation of fibroblast growth factor signals plays a role in the pathogenesis of several cancers, including lung cancer. Also, this factor may indicate prognosis and is related to the survival rate in patients with NSCLC. Therefore, this research investigated the level of fibroblast growth factor gene expression in the serum of people with lung cancer. In this research, 60 serum samples of healthy people and 60 serum samples of people with NSCLC were prepared, and personal and clinicopathological information of the studied people was collected by questionnaires. Then, plasma isolation, RNA extraction, cDNA synthesis, primers design, implementation, and changes in fibroblast growth factor gene expression in the serum of healthy and lung cancer patients were evaluated by the Real-time PCR method. REST software was used to analyze the results. The findings showed no significant difference in the expression of the fibroblast growth factor gene in the serum of people with the first to third stages of metastasis. However, in the serum of patients with the fourth stage of metastasis, the expression level of this gene was significantly decreased by 3.92 times compared to normal samples ($P<0.05$). According to the results of this study, it is possible to use the expression level of the fibroblast growth factor gene in people's serum to predict the metastasis stage of lung cancer.

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Introduction

Lung cancer results from the accumulation of several somatic genetic changes (10 to 20 mutations) that include vital genes, and their protein products control proliferation, differentiation, and apoptosis (1). These genes include proto-oncogenes (positive growth regulators), tumor suppressor genes (negative growth regulators), and genes involved in apoptosis control (2).

Lung cancer is the leading cause of cancer-related deaths, and its incidence is high in men and women worldwide. So the number of new cases diagnosed worldwide is 1.8 million cases and the number of deaths is 1.6 million (3). According to the available statistics, the incidence of lung cancer has continuously increased in Chinese men and women. Studies have shown that the incidence rate in Chinese men is 33.6 per 100,000 people, which is low compared to developed countries, and the incidence rate in Chinese women is 2.57 per 100,000, which is lower than in men (3,4). Histologically, 85% of lung cancers include non-small cell lung cancers (NSCLCs), and 15% include small cell lung cancers (SCLCs). SCLCs frequently metastasize. NSCLC has significant molecular heterogeneity (4).

The main types of NSCLC are Adenocarcinoma, which accounts for 32-40% of lung cancers, and Squamous cell carcinoma makes up 25-30% of lung cancers, and large cell carcinoma, 8-16% of lung cancers (5). Most lung cancer cases are caused by tobacco or other products containing nicotine. The risk of lung cancer increases 20 times in smokers compared to non-smokers. The fibroblast growth factor gene, fibroblast growth factor number 9, is located on the long arm of chromosome number 13 (13q12.11) (6,7). The fibroblast growth factor-9 is a fibroblast growth factor (fgr) family member (5). Fibroblast growth factors are a group of multifunctional peptide growth factors. Abnormal activation of fibroblast growth factor signaling pathways is required for homeostasis, tissue repair, angiogenesis, and organogenesis (8). It is also involved in carcinogenesis, indicating its potential role as a target for therapeutic intervention. Miss-expression of fibroblast growth factor-9 has been detected in various tumors such as breast, prostate, and lung cancer, which indicates its biomarker role in various cancers (7).

Approximately 40% of NSCLCs are diagnosed at an advanced stage of the disease, and the overall survival rate is approximately 15 in 5 years. Also, due to the high incidence and recurrence rates, it is necessary to investigate more important molecular changes in NSCLC for timely diagnosis and prognosis (8). Considering the ability to

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detect the expression of genes in serum and since serum samples are helpful for biomarker studies due to their easy access, therefore, in this study, the expression level of the fibroblast growth factor-9 gene was measured in the serum of patients with lung cancer.

Materials and Methods

To conduct this study, 60 serum samples of healthy people and 60 serum samples of people with NSCLC were prepared, and first, a consent letter was obtained from them to enter the present study. Sampling was done randomly, and patient samples were prepared from all stages of the disease (first to fourth stages of cancer). Then, the expression level of the fibroblast growth factor-9 gene in the serum of healthy people and those of people with lung cancer was quantitatively investigated. This study was done in a descriptive-analytical way.

Questionnaire design

After obtaining consent from the subjects to enter the present research, a questionnaire was designed to record personal and clinicopathological information. Information was recorded, including name and surname, date of referral, age, sex, occupation, smoking (type and duration), duration of illness, place of residence, type of tumor, stage of the tumor, family history of the patient, history of other diseases, primary tumor and tumor after treatment.

Blood Sampling

Blood was collected from patients and controls, and the 5ml of blood taken from the subjects was poured into an EDTA anticoagulant tube.

Plasma separation

Blood plasma was separated using a refrigerated centrifuge (IHANIL-South Korea) at 4°C for 15 minutes at 1900rpm, poured into RNase-free microtubes, and kept in the freezer until the experiments.

The miRNA extraction from plasma, quantitative and qualitative analysis of extracted RNA, cDNA synthesis

RNA was extracted from plasma according to the Qiagen kit protocol to check gene expression. After RNA extraction, spectrophotometry (Thermofisher, USA) and agarose gel electrophoresis were used for their quantitative and qualitative analysis. Then complementary DNA was synthesized from RNA samples using a Thermo cDNA synthesis kit. The primers used in this research were designed using the NCBI website and Allele ID software. The sequence of the primers used is shown in Table 1. In this study, the housekeeping gene was GAPDH.

Examining the efficiency of primers and performing Real-time PCR reaction

The efficiency of the designed primers was determined, and the standard curve was drawn for each primer. For this purpose, first, five dilutions were prepared from the prepared cDNAs. Then BIONEER Real-time PCR reaction (South Korea) was performed twice for these dilutions with each primer separately. In the end, the standard curve for each primer was drawn based on the C values obtained against the dilutions used. Using the slope of the obtained curve line (Slope) and ($E=10^{-1/slope}$), the reaction efficiency (E) was calculated for each primer of relation.

The measurement of fibroblast growth factor-9 gene expression was done by amplification by real-time PCR reaction based on the standard method and in a relative manner. The relative quantification in real-time PCR was done by measuring the increase in fluorescence radiation due to the binding of Eva Green dye using the TM device. Exicycler M 96 bioneer (South Korea) fixed type was done. The reaction components for each sample according to the Eva Green kit protocol) Universal RT microRNA qPCR Kit Yμl cDNA (20 ng/μl) Master Mix 10 μl, specific primers 10 pmol, and Sterilized al distilled water made by Exiqon Denmark to a final volume of 20 μl They were prepared and placed in the device with the same thermal program to multiply the fibroblast growth factor-9 gene and the reference gene.

Each reaction cycle included four stages of incubation: 95°C for 5 minutes, 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. After the reaction, the expression level was calculated by the ΔΔct method, and statistical calculation was done using REST software. In the real-time PCR method, the control group consisted of two groups: GAPDH, which is the reference cell line and is considered as a positive control, i.e., people with lung cancer, and negative control, i.e., healthy people who do not have lung cancer.

Results

The patients' information was extracted from the electronic system of the cancer department of the hospital, and the demographic and clinical information of the patients was extracted. Table 2 shows the demographic information of the patients.

The results of RNA extraction and determining its quantity and quality showed the quality of RNAs extracted from blood serum by agarose gel electrophoresis and the presence of ribosomal bands 185 and 285 for several samples in Figure 1. The extracted RNAs were of good quality. To check the quantity of extracted RNAs, the absorption ratio of 260 to 280 was calculated by the nanodrop device (spectrophotometer), and the concentration of RNA was obtained. The absorption ratio of 260 to 280 shows the purity of extracted nucleic acids. In our research, the ratio of 260 to 280 was between 1.8 and 2.

The results of the expression of the studied gene according to the stage of cancer

After checking and determining the efficiency of the primers used in this research, the conventional PCR

Table 1. Primer sequences, product length, and annealing temperature for *fibroblast growth factor-9* gene and reference gene.

Gene	Primer Sequence (5'-3')	Product length	Annealing temp.
fibroblast growth factor-9	F: CGCGTAATACGTGATCCAGTGGGA R: GTAGGCATATTACGGCCAGGGTAC	130bp	53°C
GAPDH	F: ATTCGGACGGCCCTGATATACCCGTG R: CGGGCATATACGCATCGTACCAAGT	184bp	62°C

Table 2. Demographic information of patients.

Characteristics	Variable	Frequency (percent)
Gender	man	42 (70%)
	woman	18 (30%)
Smoking	Yes	44 (73.33%)
	No	16 (26.67%)
History of cancer in the family	Yes	28 (46.66%)
	No	32 (53.34%)
Age	>60	29 (48.33%)
	<60	31 (51.67%)

Table 3. Expression of candidate genes related to stage IV samples.

Gene	Type	Reaction Efficiency	Expression	Std. Error	95% C.I.	P (H1)	Results
fibroblast growth factor-9 (Stage IV)	Target	0.96	0.198	0.031-1.799	0.010-6.015	0.027	DOWN
GAPDH (Stage IV)	Reference	0.93	1.000	-	-	-	-

Note: P(H1): Probability of alternate hypothesis that difference between sample and control groups is due only to chance.

method (BIOERE, China) was used, and their specificity was confirmed. After performing conventional PCR and observing the desired bands that showed the specificity of the primer, the qRT-PCR method was used, and the expression of the fibroblast growth factor-9 gene was investigated. This test was repeated three times for each sample. REST software was used to analyze the results.

According to Table 3, the expression level of the fibroblast growth factor-9 gene in the serum of patients with the first to the third stage of the disease was not significantly different compared to normal serum samples. Also, according to Table 3, the level of fibroblast growth factor-9 gene expression in the serum of patients with the fourth stage of the disease compared to normal serum samples with a significant level of $P<0.05$, there was a significant decrease in the level of fibroblast growth factor-9 gene expression in the serum of patients with the disease. In the fourth stage of the disease, there was a significant decrease of 3.92 times compared to normal samples ($P<0.05$).

Discussion

Decades of research have shown that lung cancer involves a multi-step process, where genetic and epigenetic changes cause DNA damage in healthy lung epithelial cells and turn it into cancer. It is unclear whether all lung epithelial cells undergo these changes or whether some are sensitive to these changes (8,9). In addition, the cells in which the tumor started may have a small number of mutations, but when the tumor grows, the cells may acquire additional mutations (10). Low- and middle-income countries account for more than 50% of lung cancer deaths yearly. In the United States, lung and bronchial cancer in men and women account for 14% and 12% of all diagnosed cancers, respectively (11).

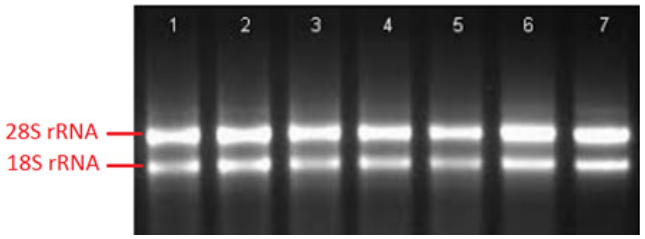

Figure 1. Agarose gel electrophoresis to check the quality of extracted RNA.

Several studies showed that fgfs and fibroblast growth factor receptors (fgfrs), including fgfr-1, fgf-9, fgf-1, and fgfr-2 are expressed in NSCLC cell lines and lung cancer patients. It has been reported that the inhibition of fgf/fgfr signals inhibits the proliferation of cancer cells. During lung development, fgf-9 is expressed in the epithelium and plays a vital role in the differentiation and proliferation of the epithelium (12). The fgf-9 is also necessary for the development of mesenchymal molecules (13). Due to the non-invasiveness of taking serum from people and considering that it is easy to isolate biomarkers and draw conclusions from it if we know which genes have changed (increased) or decreased (increased) in the serum of people with cancer, it can help to diagnose cancer in the future. Therefore, the studies aimed to use serum samples.

The real-time PCR method was used in this study, and the expression level of fgf-9 gene was investigated. The present study's data showed that the expression of the fgf-9 gene in the serum of people with first to third stages of cancer is not significantly different compared to normal serum samples. However, in the serum of patients with the fourth stage, the expression level of the fgf-9 gene was 3.92 times lower than that of normal subjects ($P<0.05$). Therefore, according to the results of the present research and by examining and confirming these findings in more samples, it is concluded that it is possible to suggest the expression of fgf-9 gene to predict the stage of metastasis in lung cancer. Inhibition of fgf-9 expression increases the proliferation and invasion of lung cancer cells.

The patients' demographic information showed a significant relationship between gender and smoking with lung cancer, so 70% of the 60 patients were male, and 73.33% of the 60 patients were smokers. At the same time, regarding having a family history of lung cancer, there is no significant difference between the history of this cancer among family members and the rate of lung cancer in an individual. Also, 48.33% of people with lung cancer were over 60. As a result, the incidence of this cancer is higher in old age. Wang et al. (14) showed that increasing the amount of fgf-9 gene significantly reduces the growth of

mitotic squamous lung cancer cells and their invasion. In the present study, the expression level of fgf-9 gene was significantly decreased (P<0.05) only in the fourth stage of the disease, which is the stage of metastasis, and this decrease increases the invasion and metastasis of lung cancer cells.

Gan and Hu (15) found that the expression of fgf-9 gene is significantly involved in the pathogenesis of colon cancer by reversing the tumor suppressor effects. Ren et al. (16) conducted a study in 2016 and showed that reducing the expression of the fgf-9 gene in gastric cancer leads to a decrease in cell growth and induction of apoptosis. Therefore, fgf-9 may have an essential oncogenic role in gastric cancer cells. As obtained from the present research results, the decrease in the expression of the fgf-9 gene causes the development of lung cancer. Sun et al. (17) found that the fgf-9 gene is an overexpressed novel growth factor in cancer-associated fibroblasts (CAFS) and increases the anti-apoptotic activity and invasiveness of gastric cancer cells. In our research, the results showed that the expression of the fgf-9 gene in the serum of people who were in the metastatic stage of lung cancer had a significant decrease (P<0.05).

Zhang et al.'s study (18) showed that signaling through fibroblast growth factors (fgf-2, fgf-9) and their receptors is an essential factor in the pathogenesis and progression of non-small cell lung cancer (SCLC). Ishioka et al. (19) found that induction of fgf-9 in adult lungs leads to the rapid development of adenocarcinoma in a specific mouse model. In our study, the decrease in the expression of the fgf-9 gene in the serum of the fourth stage of the disease caused the progression of the disease.

The studies of Ohgino et al. (12) showed that the fgf-9 gene is highly expressed in patients with lung adenocarcinoma, and changing the function of the gene, fgf-9, reduces the development and progression of lung adenocarcinoma. The findings show that the overall survival time is significantly shorter in patients with high expression of fgf-9 than in patients with low expression of fgf-9. The results of this research showed that a significant decrease (P<005) in the expression of the fgf-9 gene in the fourth stage of the disease increases the process of metastasis. Teishima et al. (20) found that the fgf-9 gene can be related to epithelial-mesenchymal transition and invasion in prostate cancer cells. In laboratory conditions, the fgf-9 gene increases proliferation and invasion properties in prostate cancer cells.

The presence of fgf-9-positive cancer cells shows a positive correlation with prostate cancer recurrence after surgery (17). Also, Korc and Friesel (21) showed that prostate cancer is associated with the expression of fgf-1, fgf-8, fgf-7, fgf-6, and fgf-9. According to the results of the present study, the expression of the fgf-9 gene in the metastasis stage had a significant decrease (P<0.05), which increases the metastasis process of lung cancer cells.

Yin et al.'s results (22) showed that the FGF9-FGFR3 signal is an early oncogenic pathway for lung adenocarcinoma. The expression of FGF9 in adenocarcinoma cell lines increases their invasive properties in the laboratory environment, and high expression of FGF9 has been associated with increased tumor stage and lymph node metastasis. FGF9, directly and indirectly, increases the proliferation of lung epithelium. Our research concluded that reducing fgf-9 gene expression in the metastatic stage of lung cancer could be significant. Schmid et al. (9) conducted a study in 2011 and found that fibroblast growth factor 9 (fgf-9) was overexpressed in endometriosis ovarian cancer samples. Our study observed that the expression of fgf-9 gene decreased in the fourth stage of cancer, which causes the disease to progress and increase metastasis. The findings of this study showed that the expression level of fgf-9 gene in the serum of people with first to third stages of lung cancer does not differ significantly, and they cannot be used for screening. However, in the serum samples of the fourth stage of metastasis, the expression of this gene has a significant decrease (P<0.05), and it can probably be used to check the treatment process and the amount of tumor tissue metastasis in patients.

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