Short Communication

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Investigating the Expression of EGFR and FGFR4 Genes in Patients with Lung Cancer

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ABSTRACT

BACKGROUND AND OBJECTIVE: Lung cancer is a disorder that is caused by genetic and epigenetic changes and activates oncogenes and inactivates tumor suppressor genes. The aim of this study is to quantitative evaluation of EGFR and FGFR4 genes expression level in blood samples of lung cancer in compare with normal people to investigate the role of these two genes as biomarkers during lung cancer diagnosis and screening.

METHODS: This case-control study was performed on 50 blood samples of lung cancer patients compared with 50 normal controls.. Total RNA from Blood samples were extracted and cDNA is synthesized. The specific primers for detection of markers are designed and expression level of BRIP1, PALB2 in presence of gene GAPDH by using Real Time PCR method was quantitatively studied.

FINDINGS: Significant increase was observed in the expression of target biomarkers in cancer patients compared to control population. Results showed quantitative increase of FGFR4 and EGFR genes with 4.46 and 3.03 fold respectively for lung cancer in compare with normal samples (p=0.003). Also, there was a significant relationship between grade of the disease and biomarkers expression level, so that with increasing the stage and degree of severity of cancer, the expression of biomarkers increased (p=0.003).

CONSLUSION: Based on this study results we could predict the expression level of (EGFR, FGFR4) gens in suffered patients quantitatively which could use as biomarker indicator during screening of lung cancer samples.

KEY WORDS: *EGFR, FGFR4, Real Time PCR, Marker, Lung Cancer.*

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Introduction

Lung cancer is a disease characterized by the uncontrolled growth of the cell in the lung tissue. If the disease is not treated, cell growth can spread to the outside of the lung during metastasis and reach the surrounding tissues or other organs (1). Non-small cell carcinoma is the most common type of cancer and consists of three types of squamous cell carcinoma, adenocarcinoma and large cell carcinoma. 70% of patients with lung cancer are currently in advanced stage of the disease (stages III and IV) (2).

Lung cancer is due to different causes. Smoking causes 90% of lung cancers. Cigarette smoke is a complex combination of chemicals containing 73 known carcinogens. 90% of the deaths from cancer in men and 70% in women in 2000 were due to smoking. Cigarette smoking can increase lung cancer by 20 times (3). Lung cancer is a common cancer with first rank in men and fourth in women. The incidence of lung cancer may be related to air pollution caused by industrialization and increased use of cars in cities (4). Studies in Iran show that the incidence of non-cigarette-dependent lung cancers in Iran is higher than that of other countries. The researchers believe the potential cause of this difference is the pollution of air in the big cities of Iran.

According to statistics, about 1 to 2 percent increase the risk of lung cancer (5). Contrary to some common cancers, lung cancer does not occur in the classical family. However, there are many indications that in people with a history of lung cancer, even if they are not smokers, the risk of developing lung cancer increases, but a small percentage of lung cancer cases are due to hereditary factors (6).

In general, the vast majority of genetic factors are modified genes coding proteins which are involved in the signaling of tyrosine kinase receptors (RTKs) and cause the spread of non-small cell lung cancer (NSCLC) (7). Several genetic changes have been identified in lung cancer. Mutations were identified in a number of proto-oncogenes, including PI3K, BRAF, EGFR, KRAS, MEK, HER2 (8). The mutation in the KRAS oncogene also accounts for 10% to 30% of the lung cancers (9). The mutation in the PTEN gene or deletion causes about 20-15% of the squamous cell carcinoma (10-12). Several studies have also been performed on biomarkers involved in lung cancer. Pao et al. performed studies at the genomic analysis to determine the possible biomarkers involved in lung cancer, and a number of biomarkers were identified (13). Asgari et al. also examined the role of expression changes and the development of lung and prostate cancer, and identified 19 genes as probable biomarkers (14). EGFR is an oncogene belonging to the ERbb family, and its gene is located on the short arm of chromosome 7 at positions 9 and 12 and consists of 28 exons with 118 Kbp. EGFR (epidermal growth factor receptor) plays a vital role in regulating normal cell proliferation, apoptosis, and cellular function. Approximately 10% of patients with NSCLC in the United States and 35% in East Asia are associated with EGFR mutation (11). The structure of EGFR has the nature of tyrosine and plays a role in the activation of various pathways of PI3K / AKT (phosphoinositol 3 kinase) and RAS / RAF as well as MAPK (12).

The most common mutation known in this gene is in exons 19 and 21, containing 89% of mutations (15). EGFR mutation in SCLC patients is lower than NSCLC, and only about 4% of SCLC patients exhibit this mutation. Fibroblastic growth factor receptor family (FGFR) is associated with four members that play a crucial role in tyrosine kinase receptors in tumor cell proliferation, angiogenesis, migration, and survival. The FGFR family consists of four receptors of tyrosine kinase after binding with the activation of MAPK protein kinase and phosphorus 3 inositol kinase (PI3K) are placed on the cancerous pathway (17,16). The Gly388Arg polymorphism in FGFR4 is generally a hot spot for changes in lung cancer. It responds to more than 20 types of known ligands and activates the MAPK pathway (16).

Recent studies have shown direct correlation between the expression levels of SATB1 and Ki-67 and the mechanism of non-small cell lung carcinoma (19, 18). Due to the increasing prevalence of lung cancer, the design of a non-invasive technique, with a little early detection of biomarkers associated with lung cancer, can play a significant role in prognosis, diagnosis, response to treatment with targeted drugs and monitoring cancer treatment. The aim of this study was to examine the quantitative expression of EGFR and FGFR4 genes in blood samples of lung cancer patients compared to healthy controls and to investigate the role of these two genes as biomarkers for screening.

Methods

Sample collection: In this case-control case, after approval of ethics committee of Islamic Azad

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University of Tehran with a code of 340/22, 50 blood samples of adenocarcinoma lung cancer and 50 blood samples were obtained from control group whose radiological and laboratory findings were negative for three consecutive 1-year examinations. In addition to the questionnaire and satisfaction letter, according to the ethics rules in the Experimental Medical Studies of Helsinki, and following the relevant guidelines, samples were obtained from the pathology department of the Masih Daneshvari Hospital. After collecting, the blood samples were transferred to the nitrogen tank and -80 C freezer according to principles of transferring and maintaining. All ethical guidelines for keeping and using Human specimens were considered.

RNA extraction and cDNA synthesis: Total RNA extraction from blood samples was performed using a GeneAll kit. Samples extracted by Thermo Scientific (Germany) were quantitatively evaluated by horizontal electrophoresis on a 1% agarose gel. For the synthesis of cDNA, samples with absorbance ratio of 280-260 nm between 1/8-2 were used. 1 µl of RNA was used in the Applied Biosystem (ABI) using the cDNA synthesis kits, Revert Aid First Strand cDNA Synthesis Kit, # K1622, and Thermo Fisher.

Primer design for PCR: Version 3 Primer Express software (Applied Biosystems, Austin, TX, USA) was used to design the primers. In addition NCBI site was used. primers are shown in Table 1. The sequence of GAPDH gene was used as internal control.

Real Time PCR: Real Time PCR was performed using 480 Master mix, SYBR Green I (Roche Applied Science) with thermal program as the initial denaturation of DNA template at 95 °C for 5 minutes, the second stage was alternately performed over 40 cycles at 95 °C for 15-30 seconds and 61 °C for 30 seconds and 72 °C for 30 seconds. Fluorescence ratio was measured by the 6000 rotor-genes device manufactured by Corbet Corporation in Australia, and the reproduction and separation curves were plotted and analyzed using software. To confirm the correctness of the PCR reaction and the replication of the components, the Real Time PCR reaction product was evaluated by the nanodrop device and on a 2% agarose gel and the accuracy of the product size was evaluated. The results of amplification confirmation on the agarose gel are shown in Fig 1.

Data analysis and statistical analysis: Raw data from Real Time PCR was analyzed using software. After amplification, the CT (cyclic threshold) of the samples was determined and the PCR efficiency value was determined. Based on the Relative Quantization (RQ) and 2-ΔΔCt formula, a number of expressions of EGFR and FGFR4 genes were expressed and compared with the internal GAPDH genes in the adjacent normalized equilibrium. The statistical tests of Kolmogrov-Smirnov test (for evaluation of age parameter) and T_TEST were used to examine the gene expression in healthy indivituals and patients and Pearson test was used to obtain the relationship between quantitative parameters (age and gene ΔCT) and $p<0.05$ was considered significant.

Table 1. Primer sequence of EGFR and FGFR4 genes with GAPDH primer.

		Sequence
EGFR	F	5-AGGCACGAGTAACAAGCTCAC-3
	R	5- ATGAGGACATAACCAGCCACC-3
FGFR4	F	5- GGTGGCTGAAAAACGGGAAG-3
	R	5- AGATGGGACCACACTTTCCAT A-3
GAPDH	F	5 -CTCTCTGCTCCTCCTGTTCG-3`
	R	5 -ACGACCAAATCCGTTGACTC-3

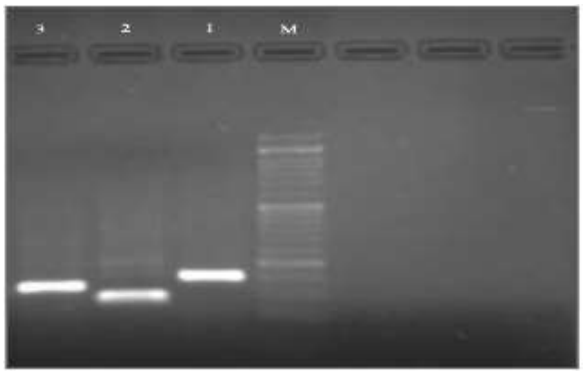

Figure 1. The well 1 is the product of the PCR of the GAPDH gene, the length of the fragment is 203 bp. The well 2 is a product of the EGFR gene, the length of this fragment is 104 bp. The well 3 is the product of FGFR4 gene, whose length is 177 bp. The well M is a DNA marker.

Results

The results of extracted RNA was investigated by the nanodrop device. In order to investigate the specificity of primers, the quality of SYBR Green fluorescence color, ensuring the replication of specific parts and the absence of non-specific components and absence of primer dimer in the PCR product, melting curve for EGFR, FGFR4 and GAPDH genes was separately drawn by Rotor gene Q Real time PCR. The results were obtained as single-hump, which is a single PCR product (Fig 2). The PCR product was also

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placed on the gel and each of the reactions performed by the specific primers had only one specific bond, which confirmed the specificity of the PCR results in our samples. The results of the cycle of gene expression amplification are plotted in Fig 3.

Study population: The mean age of patients in this study was 45.59±11.25 and the healthy subjects were 49.12±12.49 years.

Study of relative expression of EGFR and FGFR4 genes: Comparing the relative expression changes in patients with lung cancer compared to healthy subjects showed that the relative changes in EGFR and FGFR4 genes (p=0.003) in patients with lung cancer were 3.34±0.19 and 4.0±20.74, respectively. Also, the expression ratio of EGFR and FGFR4 genes in healthy control samples (p=0.003) was 1.10±0.23 and 0.94±0.19, which indicates an increase in the expression of EGFR and FGFR4 genes compared to healthy control that for the EGFR gene of 3.03 fold increase in expression than healthy subjects and for the FGFR4 gene, the expression is 4.46 times higher than healthy subjects. The results show that quantitative expression of EGFR and FGFR4 genes in the blood sample of patients with lung cancer is a marker for the detection and screening of lung cancer. In this regard, by identifying these two genes from the blood, results showed that the expression of EGFR and FGFR4 genes in blood samples of people with lung cancer was significantly higher than those of normal people.

Investigating the stage of disease : In this study, out of 50 patients, 26 (52%) had cancer cells in stage 3 and 24 persons (48%) were in stage 4 of lung cancer. In the study of the relationship between stage and severity of disease in patients, it was found that there is a significant relationship between these two parameters and the distribution of the frequencies in the stage and in the degrees is the same and with the increase of stage and degree, the severity of the cancer also increases .

Investigating the relationship between age and biomarker expression in patients and healthy subjects: The results of this study showed that there is a significant relationship between age and ΔCt of EGFR and FGFR4 genes (p=0.01).

Investigating the relationship between stage and degree of disease with the amount of EGFR and FGFR4 biomarkers: for this purpose, data were first divided into control and patient groups. The results showed a significant relationship between the disease stage and ΔCt (p=0.001). The same test was performed on the degree of disease, and the results showed that the degree of differentiation of cancer cells or grade of the disease was significantly correlated with expression level (EGFR and FGFR4) (p=0.003). Also, the results indicate that the expression of EGFR and FGFR4 is directly related to the increase in the stage of the disease.

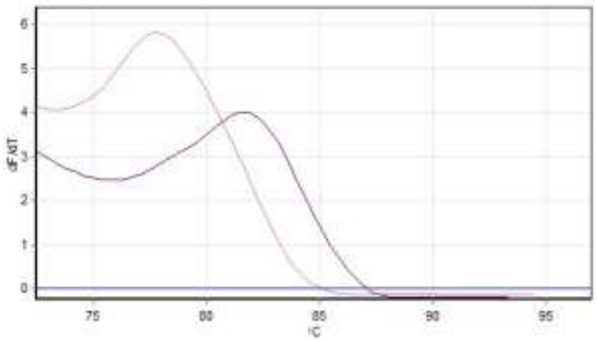

Figure 2. Melting curve of EGFR and FGFR4 genes in lung cancer cell line. The EGFR melting curve is at 81.5 ° C and the FGFR4 melting curve is at 77.8 ° C. The vertical axis indicates the fluorescent derivative to the time derivative and the horizontal axis represents the temperature at ° C. The single-peak in the diagrams indicates the absence of a primer dimer and a non-specific bond.

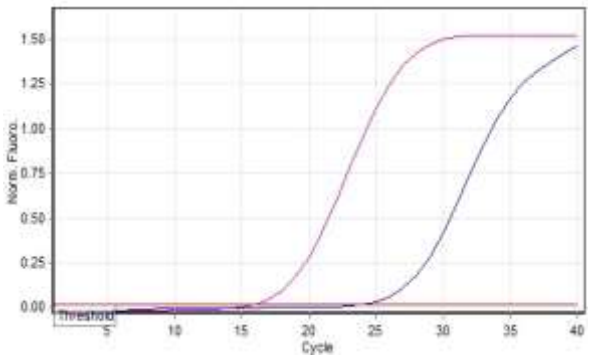

Figure 3. Biomarker amplification cycle - The horizontal axis is the number of cycles and the vertical axis is the fluorescence amount. The amount of CT for the EGFR gene is 24/10 and for the FGFR4 gene is 16.01.

Discussion

The results of this study showed significant changes in the expression of FGFR4 and EGFR gene expression at 4/46 and 3.03 times in lung cancer samples compared to normal ones. Also, the results showed a significant relationship between severity and degree of disease and increasing the expression of biomarkers. Therefore, the design of a non-invasive

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method with early diagnostic capabilities to investigate and identify biomarkers associated with lung cancer can play a significant role in prognosis, diagnosis, response to treatment with targeted drugs and monitoring cancer treatment. The results are consistent with studies on biomarker variations in multiple cancers, and in many cases the results of previous studies have shown a significant increase in probable biomarkers involved in lung cancer (21, 20). Liang et al. analyzed RBM5, EGFR and KRAS gene expression in NSCLC compared to normal tissue samples, and RBM5 expression in NSCLC was reduced compared to normal tissue, while the EGFR and KRAS genes in NSCLC increased compared to normal tissues (22). Also, the results of the study by Willemi et al. showed a positive correlation between the expression of FGFR1 and FGFR2 expression (23), in which the expression of FGFR1 and FGFR2 were significantly related to lung cancer, and also confirmed the association between the two genes as a biomarker for lung cancer. (24).

In other studies conducted by Fengli et al., the levels of expression of EGFR and COX-2 were associated with a significant increase, and EGFR was identified not only as an independent predictor of overall survival but also as a predictor of the recipient of radiotherapy. But there is no relationship between COX-2 expression and overall survival in patients (25). In a study conducted by Willemi et al., a positive correlation was found between the expression of FGFR1 and FGFR2 (23), which is consistent with the simultaneous results of biomarkers in the recent study, which may increase the simultaneous expression of the two genes in the cancer. FGFR1 and FGFR2 were significantly associated with squamous cell, and also the high expression of FGFR1 was observed with OS (23). In a study by Che et al. to investigate the expression of FGFR genes in cervical cancer, the results confirmed that expression of FGFR3, FGFR2 and FGFR4 increased and could be an important indicator of cervical cancer. (26). Since FGFR4 and EGFR proteins play different roles in multiple biological activities as growth factor receptors in cellular differentiation, cell growth and proliferation, the increased expression of this biomarker can have a protooncogenic role and may alter the signaling in the cell control pathways and probably leading to cancer progression (27, 19). Also, due to the simultaneous increase in the expression of the two genes in all cancer samples, it is likely that there is a direct correlation between the two genes and the progression of lung cancer. The results of numerous studies on lung cancer and other cancers show a simultaneous increase in the expression of FGFR4 and EGFR. In addition to simultaneous meaningful increase, there is a significant relationship between the rate of expression and severity and degree of disease. Therefore, a quantitative study of these biomarkers can be used as a potential marker for prognosis, screening and monitoring of lung cancer (28, 14). Considering the significant increase in the expression of EGFR and FGFR4 genes in blood samples from cancer patients compared to normal indivituals, it can be used as a potential biomarker for non-invasive screening in early diagnosis of lung cancer. Additional studies will be carried out in the future to determine the relationship between the expression of these genes and the histopathologic and clinical characteristics of the patients .

Conflict of Interest: No conflicts of interest.

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