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ORIGINAL ARTICLE

A pilot study of CK20 mRNA, VEGF mRNA, VEGF protein in the peripheral blood of patients with non-small cell lung cancer

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ABSTRACT

BACKGROUND: Lung cancer is one of the most prevalent malignant diseases worldwide that nowadays has become one of the major problems for healthcare organizations in the field of diagnosis and therapy. Nowadays, studying the micro-environment around the tumor and investigating the genomic changes in different types of cancer have been recognized as the most important lines of study in cancer research. Investigating the expression of genes associated with cancer can be very important in determining the type of cancer and its prognosis.

METHODS: According to the estimation of the sample size, 40 patients with non-small cell lung carcinoma (NSCLC) were compared with 40 healthy individuals. The qRT-PCR (quantitative RT-PCR) technique was utilized to determine the expression levels of two biomarkers of CK20 mRNA and VEGF mRNA in peripheral blood of the patients and healthy individuals. Also, the VEGF protein was measured by ELISA (Enzyme-Linked ImmunoSorbent Assay) technique and the relationship of these biomarkers and tumor staging and cancer progression was evaluated.

RESULTS: Comparing these two groups using *t*-test, did not show a significant difference with regard to the mean age. Positive CK20 mRNA marker was observed in 18 out of 40 individuals with NSCLC and therefore, the sensitivity of this marker was determined as 45%. In healthy individuals eight out of 40 were reported positive. VEGF mRNA marker in the group of patients became positive in 31 out of 40 individuals, which was an indication of a sensitivity equal to 77.6% and in the group of normal individuals five of out 40 people were reported positive. Also, the serum levels of VEGF were measured using ELISA method. 28 out of 40 patients were reported positive, which was an indication of a sensitivity of 70% and in normal individuals group seven out of 40 people were reported positive.

CONCLUSIONS: Overall, it can be concluded that the result of this research, which is in the field of tumor markers in lung cancer (NSCLC), can be regarded as a diagnostic-screening test for the early detection of the disease in the early stages because in this study, the two VEGF mRNA and CK20 mRNA markers and the serum level of VEGF were evaluated with a good sensitivity.

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Key words: Carcinoma, non-small-cell lung - Biomarkers - Vascular endothelial growth factor A - RNA, messenger - KRT20 protein, human.

Lung cancer occurs when lung tissue cells proliferate abnormally and grow uncontrolled. There are various types of lung cancer, which the growth rate of some of them is more than others. In many countries in the world lung cancer is the most common cause of death due to cancer among men and women, and followed by breast cancer, it is the second leading cause of death among women worldwide. This type of cancer is usually caused by smoking. But many other factors affect its development.[1, 2]

Non-small cell lung carcinoma (NSCLC) includes between 5 to 90 percent of cases of the lung cancer, which involves an abnormal and uncontrolled growth of a wide range of lung cells. Most cases involved in this group of cancer, are associated with squamous cell carcinoma (epidermoid) and adenocarcinoma of the lung.[3, 4]

Smoking is one of the leading causes of the mortality in lung cancer, but is not the only reason. Breathing the air contaminated with smoke at home or in workplace causes an increase in the incidence of this type of cancer.[3]

Non-smoker individuals exposed to cigarette smoke such as the children and spouses of smokers are also at risk for lung cancer.[5, 6]

Lung cancer is one of the most prevalent cancers that cause many deaths each year and despite great advances made in early diagnosis and appropriate therapies, it is still the leading cause of death due to cancer.[6-8]

Although every day new approaches in dealing with lung cancer are presented, this disease still is a risk for many. Cancer is the disease of cells. The human body is a collection of cells, each responsible with a specific task. In order to be able to ensure the proper function and health of an individual, therefore, more attention needs to be paid to the micro-molecular structure and basics of cellular biology, so that it is possible to provide more extensive information about the development and morbidity of this fatal disease, with regard to the genesis of this disease from cellular levels.[9] A cell usually needs a series of mutations in both categories of genes, *i.e.*, tumor suppressor genes and proto-oncogenes, in order to transform into cancerous cells, which by the way will have extraordinary growth rates.[10]

Biomarkers are typically genes that experience structural or functional changes in the procedure of cancer and their expression changes. Therefore, they can have an important role in the diagnosis.[9, 11-13]

Nowadays, molecular methods are the most important methods to measure the markers, which are performed based on genomic content or transcriptomics.[14-16]

Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that causes the stimulation of tissues and blood vessels.[17]

When VEGF is expressed more, it leads to the disease. Cancers that are capable of VEGF expression can grow and metastasize.[18]

Cytokeratins are intermediate filaments found in intracellular skeleton of epithelial tissues.[17]

Cytokeratin 20 (CK20) is seen in some cancers such as colon and lung cancer. Based on studies, this biomarker can be used as a diagnostic marker for lung cancer.[18, 19]

Materials and methods

Forty patients who visited Masih Daneshvari Hospital were selected after diagnosis and before any therapy performed on them, and forty healthy individuals voluntarily participated in this study. This was a case-control study. The study was approved by the committee on research ethics at the institution in which the research was conducted and any informed consent from human subjects was obtained as required.

We included individuals whose disease was in stages one to three of the disease, meaning, they had no distant metastasis. No medical treatment (chemotherapy or radiotherapy) or surgery was performed on the patient. There was a definitive diagnosis by pathological confirmation. The patients and healthy subjects in this study were considered from similar age and gender groups.

Ten mL peripheral blood sample was taken from all individuals participated in the study. Then, this 10 mL sample, was divided into two

eight and two milliliter parts, the 8 mL part was added to Falcon tube containing anticoagulant EDTA and the 2 mL part was coagulated in order to extract the serum, and then, the resulting serum was stored in freezer at -20 ºC to measure the serum levels of VEGF. The uncoagulated 8 mL serum was immediately entered the RNA extraction phase.

The extraction phase was carried out using RNeasy Midi Kit (QIAGEN Cat no.75144). First, the red blood cells (if any) were lysed using lysing buffer and centrifuge was done and the resulting cell mass was entered the extraction phase and eventually the total mRNA was obtained.

cDNA synthesis was performed using Viva 2-steps RT-PCR Kit (Cat no. RTPL12). The main components for reverse transcription have been provided in the format of RT Primer Mix and reverse transcription.

Specific primers for each marker were designed by the help of AlleleID6 software and were ordered to be made. Table I shows the parameters and their amounts used in the final Real-time reaction.

Real-time RT-PCR was performed using HotTaq EvaGreen qPCR Mix kit (SinaClon company Cat No. BT11101). The components of Real-time RT-PCR consisted of: 1) sequencing template 2 µL; 2) master mix 4 µL; 3) primer according to the most proper concentration; 4) deionized distilled water with an amount so that the final volume of the reaction reaches 20 µL.

Temperatures and reaction times were set according to the instruction given in the kit (Table II). At the end of each reaction the interpretation of the results took place based on amplification curves and melting curve.

TABLE II.—*The temperatures and times of real-time RT-PCR reactions.*

Real-time step	Temperature	Duration
Initial activation	95 ºC	5 min
40 cycles		
Denaturation	59 ºC	15 s
Annealing	56-60 ºC	60 s
Extension	72 ºC	20 s

Also, the serum level of VEFG was determined using Bioassay Technology Laboratory kit (Cat No. E0080Hu).

Results

The study population consisted of the two groups of patients and healthy individuals, which according to the estimated sample size of the research, 40 individuals were placed in each group. Comparing these two groups using *t*-test showed no significant differences in terms of mean age, therefore, it can be argued that the age factor in studied groups has no confounding effect (P value=0.432). Also, no statistically significant difference was observed between the two groups in terms of gender (P value=0.66).

The amount of the expression of the reference gene of the study (18s-rRNA) can be obtained comparatively, from the Ct value measured for each sample. The resulting outcome indicated that there was no significant difference between the two groups, which confirms the accuracy of the choice of this marker as a reference gene of the study (P value=0.294).

After deriving the results of the real-time RT-PCR reaction, the number of people from each main group of the research who had

TABLE I.—*The specifications of primers used in real-time RT-PCR reaction. The number of each gene, sequence, size, and the amount of each primer consumed is specified separately.*

Parameters	CK20-mRNA	VEGF mRNA	18s rRNA
F primer	ACGCCAGAACAACGAATACC	GTGCCCCCTAGCAGTACCG	GTAACCCGTTGAACCCCATT
Length of primer	20	19	20
R primer	TTCAGATGACACGACCTTGC	GACGTGCCCCTACAAGTTGG	CCATCCAATCGGTAGTAGCG
Length of primer	20	20	20
Length of amplified fragment	208	123	152
Desired annealing temperature	61.2 °C	61.6 °C	53.5 °C

shown positive outcomes in terms of marker expression was determined. Positive VEGF mRNA marker in individuals with cancer was observed in 31 out of 40 people, and therefore, the sensitivity of this marker was determined as 77.5%. In healthy individuals group this rate was five out of forty people, which is an indication of 12.5% false positive cases. The statistical comparison of the positive level of this marker in patients and healthy people, that was done using two-sample binomial test, was an indication of a significant difference between these two groups (P value=0.034).

Marker CK20 mRNA was positive in 18 out of 40 patients in the group of patients, indicating a sensitivity of 45%, and among normal individuals this rate was eight out of forty people, indicating 20% false positive cases. The statistical comparison of the level of this marker becoming positive among patients and healthy individuals, performed using two-sample binomial test, was an indication of a significant difference between these two groups (P value=0.0.001) (Figure 1).

The relative difference between markers expression between patients and healthy individuals

The relative difference between markers expression between patients and healthy individuals was measured. This task was performed using ΔΔ CT method for VEGF-mRNA and CK20-mRNA.

ΔΔ CT for VEGF mRNA was measured as -3.54. Now, if we calculate the 2 to the power of -ΔΔ CT (expression fold change) the amount of the difference in marker expression is determined. The number of the initial copies of this marker in patients is in average 11.63 times as much as healthy individuals.

ΔΔ CT for CK20 mRNA was measured as -0.5, which mathematically shows that the number of initial copies of this marker in patients is in average 1.42 times as much as healthy individuals (Figure 2).

Measuring the serum level of VEGF protein

The serum level of VEGF protein was measured using ELISA technique. The average serum level of this biomarker in the group of patients was more than the group of healthy individuals. In 28 out of 40 patients the expression of this biomarker was reported as positive, indicating 70% sensitivity, and in the group of normal individuals seven out of 40 individuals were reported positive. Finally, the statistical comparison of this biomarker becoming positive between patients and healthy individuals took place using two-sample binomial test that indicated a statistical difference between these two studied groups (P value <0.001).

Discussion

Lung cancer is the most prevalent malignant diseases around the world that has turned into one of the major problems for healthcare organizations in the field of diagnosis and therapy.[1]

Different types of lung cancer include: small cell lung carcinoma (SCLC) and NSCLC.[5, 6]

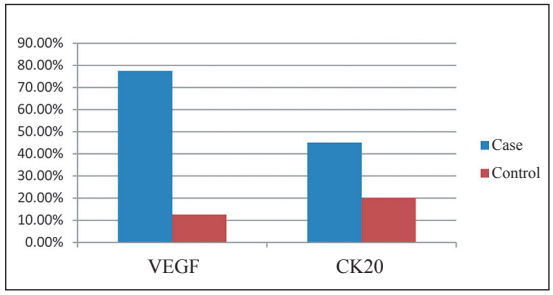

Figure 1.—The expression levels of VEGF mRNA and CK20 mRNA in peripheral blood from NSCLC patients and noncancerous persons.

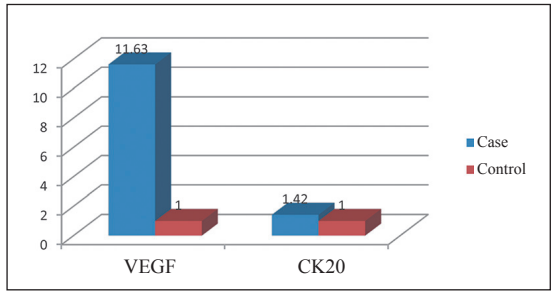

Figure 2.—Difference in expression of VEGF and CK20 genes at the case and control.

Various factors play a role in the incidence of lung cancer, the most important of which include: smoking, exposure to radon, asbestos, secondary immunodeficiency, air pollution, family history, etc.[9, 14, 19-21]

Despite the advanced surgical techniques and combined therapies, lung cancer remains a disease with weak and poor prognosis.[22]

Unfortunately, in many parts of the world, especially in developing countries, smoking is on the rise and along with it, lung cancer as well. While smoking is the leading cause of lung cancer worldwide but there are other important factors too.[9, 10]

More than half of the patients with lung cancer are at an advanced stage of the disease at the time of diagnosis. The majority of patients visit with symptoms, and impairment and abnormality in tests, which is associated with the primary lesion of the growth of tumor at the site of invasion by obstructing adjacent structures, growth at distant metastasis regions, or paraneoplastic syndrome.[23]

Biomarkers are biological molecules that play a key role in cancer progression as well as the fate of the tumor. Biomarkers are usually genes that have developed structural or functional changes during the progress of cancer and/or their expression or expression level has changed. Identifying biomarkers can be useful in determining the type of cancer and also in its prognosis.[9, 10, 14, 24]

A study was carried out in 2014 on MUC1 and VEGF gene expression and the efficacy of the Gefitinib drug on survival rate and gene expression. In this study, patients were sampled (55 patients) before and after the treatment with the drug and real time PCR was performed on the blood sample of the patients. The results of the study showed that the mRNA levels of MUC1 and VEGF genes before the start of treatment and four months after treatment were high.[25] In the present study, the expression level of VEGF mRNA and CK20 mRNA markers was also higher in patients compared to healthy individuals.

In a similar study, Adams *et al.* compared tissue and serum VEGF. In their study, there was a significant relationship between the VEFG amount in all groups of cancer (localized and metastatic).[26] In this study, a significant relationship was also observed between the two groups of patients and healthy individuals.

In 2009, in a study conducted on patients with colorectal cancer, 198 peripheral blood samples were obtained of which 169 cases had colorectal cancer and the rest were considered as healthy control group. The mRNA of the sample of the patients was extracted and then after preparing the cDNA the expression level of CEA, CK19, and CK20 were measured using real-time PCR. The results of this study showed that the expression levels of CK19, CEA, and CK20 in patients were 35.8%, 28% and 41.9%, respectively, and in non-patients in average it was three percent, which this difference was statistically significant.[27] In the present study there were also similar results between normal and cancer groups and a significant relationship was shown.

A study, in 2006, performed on CK20 mRNA expression in the pleural fluid of patients with lung cancer and of those with non-malignant lung diseases, showed that CK20-mRNA expression level in patients with malignant lung disease is significantly higher than those with non-malignant lung diseases (P value <0.05).[25] Thus, in this research, we choose CK20 mRNA as a suitable biomarker to distinguish malignant tumors from benign tumors of the lung.

Conclusions

Overall, the result of this research, which is on tumor markers of lung cancer, can be considered an initial introduction to a diagnostic-screening test for early discovery of the disease at early stages. However, to further prove the results of the research it is recommended that more comprehensive research to be done with larger sample sizes.

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