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ORIGINAL ARTICLE

Investigating gene expression level of MUC1 and CEA in pleural fluid of NSCLC lung cancer patients with real-time RT-PCR method

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ABSTRACT

BACKGROUND: Lung cancer is one of the most common malignant diseases in the world that has turned to into a big problem for world health organizations in the diagnosis and cure field. Investigating the cancer related genes expression can have a significant role in early diagnosis and determining the kind of cancer. However, there is no specific biomarker to aid early clinical diagnosis. In this study, gene expression level of MUC1 and CEA were investigated among the non-small cell lung cancer (NSCLC) patients.

METHODS: In this study, 40 NSCLC patients were compared with 40 healthy people. Real-time RT-PCR method was used to determine the expression level of MUC1 mRNA and CEA mRNA biomarkers in pleural fluid of patients and healthy people.

RESULTS: Comparison of these groups with *t*-test showed no meaningful difference regarding age average. Positivity of CEA mRNA marker in NSCLC patients were observed in 30 of 40 patients and this marker's sensitivity was determined at 75%. In the healthy group, 11 of 40 had positive marker. MUC1 mRNA marker among patients were positive in 28 of them which shows 70% sensitivity and it was positive in 7 out of 40 people in the healthy group.

CONCLUSIONS: The results of this study can be considered a diagnosing-screening test for early discovery of the disease since MUC1 mRNA and CEA mRNA markers were evaluated at good sensitivity levels. For further validation of the research results, it is suggested to perform a more widespread study with more samples.

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Key words: Carcinoma, Non-Small-Cell Lung - Biomarkers - Gene expression.

Lung cancer is one of the most common malignant diseases in the world that has turned to into a big problem for world health organizations in the diagnosis and cure field.[1] This disease is one of the most lethal cancers in the world that after heart diseases, and sometimes

at the same level with them, stands at the top of the death causes list in most of the societies. Non-small cell lung cancer (NSCLC) is one of the most severe kinds of lung cancers which shows a weak response to chemotherapy.[2]

Different types of lung cancer include small cell lung cancer (SCLC) and NSCLC.

In the SCLC type which is so lethal and has a high death rate, cancerous cells grow in a very quick and uncontrolled way and spread all over the lung and hit other organs in a short time. Although this type shows a better response to chemotherapy, its growth and metastasis speed is higher compared to other types of lung cancer. This type of lung cancer only includes 10-15 percent of total lung cancers.[3, 4]

NSCLC type has a very quick and uncontrolled growth of a wide of lung cellules, and includes 85 to 90 percent of the lung cancers. The growth speed of this type is low and has a lower metastasis rate compared to SCLC. Most of the involved cases in this group are related to the carcinoma of squamous cell (epidermoid) and lung adenocarcinoma.[5, 6]

Biomarkers are biomolecules which play a role in cancer advancement and small changes in tumor environment. Usually, biomarkers are genes that had structural or functional changes in the cancer process or their expression or expression level has changed.[7] Different cancers can have different biomarkers and some biomarkers are in common in a lot of cancers. Diagnosis and identification of the biomarkers can be useful in early diagnosis and determining the kind of cancer.[8] One of the biomarker types are mRNA biomarkers which by evaluating their expression, cancerous cells can be identified and examined in specific tissues.[8] There are various methods to analyze the markers including real-time RT-PCR which is a sensitive and ideal method.[9]

MUC1, cell surface associated with mucin 1 or polymorphic mucin protein which has higher expression in mucosal layers of the body, is a transmembrane glycoprotein and is known as one of the important proteins of mucin family. O-glycosylation of this polypeptide has significance in determining its three dimensional structure and protein's function is highly dependent on this subject. The alpha chain of this protein is the main responsible part for cellular connections that can be attached to adhesive and non-adherent molecules. Mucin family proteins trap pathogens especially bacteria by forming a slimy mucous layer and rich of viscous polysaccharides and are considered the first defense line of the body in the mucous tissues.[10]

The beta chain exists at the end of its C terminal and causes induction of signal activity. MUC1 expression is different in normal and cancerous conditions. This protein has a complex relationship with epithelial growth factor receptor and changes its expression and translocation in epithelial cell.[11]

CEA is glycoprotein from CEA family that is observed in numerous variants due to different splicing of its gene which is located in chromosome 19. It has immunoglobulin repeats and can act as an adhesive molecule. CEA antigen has been identified as a cancer marker in many tumors of the abdominal area. This protein is consisted of 702 amino acids and its identification has a high importance in general.[12]

It should be noted that in evaluating the genes' expression level by methods like Real-time RT-PCR, reference genes must be used too and compare the results with their aid. In this study, 18s rRNA was selected as the reference gene.

Widespread researches have been conducted on lung cancer markers, however, no single marker with the worthiness of clinical usage for early diagnosis and prognosis has been found. In this study, investigation of MUC1 and CEA genes expression level in NSCLC patients at the early stages was investigated as the lung cancer early stages predicting biomarker. However, further studies with more samples is suggested.

Materials and methods

Forty patients of Masih Daneshvari hospital were selected after diagnosis by specialist and before any kind of treatment and 40 healthy people after medical examination voluntarily

took part in this study. The control sample included those who had normal results after lung cancer examinations.

The patient sample included those who were in 1 to 3 stage of the disease, which means no distant metastasis. No medical treatment (chemotherapy or radiotherapy) or surgery was performed on the patient. Patients and healthy people of this study were considered in similar age and gender groups.

From every participant 10 mL of pleural fluid was taken. The first 2 mL was thrown away due to the probable pollution with epithelial cells. In laboratory, samples immediately entered the RNA extraction. This volume of pleural fluid is calculated considering the mentioned sensitivity for real-time RT-PCR reaction. 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.

RNA extraction

This stage was done by using RNeasy Midi Kit (qiagen Cat no.75144). At first, red blood cell (if existed) were lysed by lysis buffer and centrifuge was done and the resulting cellular mass, washed twice by PBS, entered the extract stages. Finally, total mRNA resulted from each column was solved in preservative buffer and prepared for the next stage.

cDNA production

It was done by using Viva 2-steps RT-PCR Kit (RTPL12. Cat no). The main components are presented in the kit for Reverse Transcription in the form of RT Primer Mix. Quality and quantity of RNA and cDNA were analyzed and controlled at the end of each stage by Nanodrop Spectrophotometer (Bio-TeK, Winooski, VT, USA) device.

Designing exclusive primers by using AlleleID6 software

Exclusive primers of each marker were designed by the aid of AlleleID6 software and ordered for production. Table I shows the parameters and their usage amount in the final Real-time reaction. After receiving the produced primers from the company (that were presented in the form of lyophilized powder), primer solution with the density of 100 pmol/μL was prepared by adding autoclaved distilled water to the powder and this solution was kept in -20 centigrade degree before usage. For final use of the primer, this solution was diluted and entered the final reaction with 10 pmol/μL density.

Performing real-time RT-PCR by using HotTaq EvaGreen qPCR Mix

EvaGreen has been used in the kit of Sinaclon Company (Cat No. BT11101) that is a fluorescence color and emits fluorescent light after sticking to double-stranded DNA and is measurable by real-time device. Necessary parts for performing the reaction in mastermix were supplied. Reactive components of real-time RT-PCR were:

— pattern sequence for 2 μL;
— master mix for 4 μL;

TABLE I.—*Specifications of the used primers in the Real-time RT-PCR reaction.*

Parameters	CEA-mRNA	MUC1 mRNA	18s rRNA
F primer	ACCCTGGATGTCCTCTATGG	GTGCCCCCTAGCAGTACCG	GTAACCCGTTGAACCCCATT
Primer length	20	19	20
R primer	CAGGCATAGGTCCCGTTATTA	GACGTGCCCCTACAAGTTGG	CCATCCAATCGGTAGTAGCG
Primer length	21	20	20
Amplified fragment length	174	123	152
Annealing optimum temperature	61.2 °C	61.6 °C	53.5 °C

TABLE II.—*Temperatures and times of real-time RT-PCR reaction.*

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

— primer by considering the most proper density found in the set up experiments;

— deionized distilled water in an amount which makes the final reaction 20 μL.

Temperatures and times of the reaction were set according to the kit instructions. After each reaction, result interpretation was done according to amplification and melting curves. Components and temperatures of the reaction at the set up stage of the research were set in a way that achieved the best results for the markers and avoid errors that problematize result interpretation (like primer dimer formation) (Table II).

Statistical analysis

Comparison of the averages between the two groups was done by *t*-test statistical examination and comparison of positivity percentage of the markers was done by the aid of Two-sample binomial test in spss20 software. Statistical difference was considered at the meaningful level of $P \leq 0.05$.

Demographic information of participants

The study population consisted of two groups of patients and healthy people that 40 of each were put in groups according to the research sample size. Comparison of these groups with t-test showed no meaningful difference regarding age average, therefore it can be stated that age factor does not have a confounding effect in studied groups (Tables I-III).

TABLE III.—*Comparison of age average between lung cancer patients and healthy people by using t-test.*

Main group	Age (years)		
	Range	Mean	SD
Patients (N.=40)	25-70	45.95	10.86
Healthy (N.=40)	24-70	48.85	12.36
P value=0.442 (SD: standard deviation)			

Results

Real-time RT-PCR

After conducting the real-time RT-PCR reaction, reaction results of those who had shown positive marker expressions in both groups was revealed. Positive CEA mRNA marker among lung cancer (NSCLC) patients was observed in 30 out of 40 and therefore this marker's sensitivity was set at 75%. Among the healthy people, it was 11 out of 40 people, which shows 27.5% of false reports and therefore property is 72.5%. Statistical comparison of marker's positivity among patients and healthy people that was done by Two-sample binomial test showed a meaningful statistical difference between the two groups (P value=0.029).

MUC1 mRNA marker was positive in 28 of 40 patients which shows 70% sensitivity and in the normal group it was 7 out of 40 that shows 17.5% false report, therefore its property is 82.5%. Statistical comparison of marker's positivity among patients and healthy people that was done by Two-sample binomial test showed a meaningful statistical difference between the two groups (P value=0.001).

Analyzing the marker expression difference in the two groups

The relative marker expression difference between patients and healthy participants was measured. This was done by ΔΔ CT method in MUC1-mRNA and CEA-mRNA cases.

ΔΔ CT for CEA-mRNA was calculated at -4.42. Now if we power 2 to -ΔΔ CT, then marker expression difference value is revealed. The number of early releases of this marker among the patients on average was 21.41 times more than healthy people.

ΔΔ CT for CEA-mRNA was calculated at -5.5, which mathematically shows that the number of early releases of this marker among the patients on average was 45.26 times more than healthy people.

Discussion

Lung cancer is one of the most common malignant diseases in the world that has turned to into a big problem for world health organizations in the diagnosis and cure field. This disease is one of the most lethal cancers in the world that after heart diseases, and sometimes at the same level with them, stands at the top of the death causes list in most of the societies.[1]

Lung cancer occurs when lung tissue cells amplify abnormally and without control. Different types of lung cancers with various growth speeds exist.[2]

Different types of lung cancer include small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).[5]

In the SCLC type which is so lethal and has a high death rate, cancerous cells grow in a very quick and uncontrolled way and spread all over the lung and hit other organs in a short time.[6]

NSCLC type that is very quick and uncontrolled growth of a wide of lung cellules includes 85 to 90 percent of the lung cancers. The growth speed of this type is low and has a lower metastasis rate compared to SCLC. Most of the involved cases in this group are related to the carcinoma of squamous cell (epidermoid) and lung adenocarcinoma. 40 percent of NSCLC cancers are lung adenocarcinoma. Lung adenocarcinoma is formed in the bronchus cells. These cells are responsible for producing mucous in the bronchus.[5, 6]

Various factors cause lung cancer such as: smoking, radon exposure, asbestos, secondary immunodeficiency, air pollution, family history.[13-16]

Diagnostic methods of lung cancer are done in clinical examinations and tests which include: physical examination, X-ray photography, sonography, scan, bronchoscopy, biopsy.[17-19]

In spite of advanced surgery technics and combined therapies, lung cancer still has poor and weak prognosis.[20]

Unfortunately in many parts of the world, especially in the developing countries, smoking is increasing and lung cancer is increasing too. While smoking is the top cause of cancer in the world, more than 60% of new lung cancer cases are seen in those who have never smoked.[7]

Generally in NSCLC lung cancer patients, the average mutation rate among smokers is 10 times more than non-smokers.[8]

Lung cancer is one of the most common cancers which causes a high death rate annually and in spite of many advances in its diagnosis and treatment field, still tops the cancer deaths charts.[8]

More than half of lung cancer patients are at advanced level at the time of diagnosis. Most of the patients visit with signs and disorders in the tests which are related to primary lesion tumor growth in the invasion area with obstruction of adjacent structures in distant metastasis or paraneoplastic syndrome.[21]

Biomarkers are biomolecules which play a key role in cancer advancement and tumor destiny. Usually, biomarkers are genes that had structural or functional changes in the cancer process or their expression or expression level has changed. Diagnosis and identification of the biomarkers can be useful in determination of the cancer type and its prognosis. Also, tracking the biomarkers' status can be very important in patient's response to treatment.[8, 22, 23]

Biomarkers can be proposed in various levels of existing molecules in cellules, including genes, mRNAs, proteins and other cellular molecules.[7, 24]

Meanwhile, relatively proper characteristic of RNA markers that enables them to stand among the disease's diagnosis tests made this group of markers to be selected for the research. On the other hand, the ability to discover their very low values with Real- time RT-PCR method that has high sensitivity and top characteristics, is considered as the advantage of this marker over protein markers that are mainly not measurable at very low amounts.[25]

A study was conducted in 2014 on MUC1 and VEGF gene expression and gefitinib drug effect on the life span and expression of the genes. In this study, before and after the drug treatment, patients gave samples (55 patients) and Real Time PCR was conducted on the patients' blood samples. The results of this study showed that mRNA level of MUC1 and VEGF

genes was high before the treatment and 4 weeks after it among the patients.[26]

A study conducted in 2015 by Shirin Karimi *et al.* in Shahid Beheshti University which measured the mRNA of two LUNX and CEA genes in NSCLC patients by Real Time PCR method. The results of this study that was conducted on 30 patients of Masih Daneshvari hospital showed that CEA and LUNX expression level were respectively positive in 80 and 70 percent of the patient and at a high level.[9]

In a study in 2011, an analysis was done on 178 samples of NSCLC patients regarding the expression of MUC1 gene. This study was done by immunohistochemistry method and the results were reported in semiquantitative form. The results of this analysis showed that MUC1 has expression increase in 87% of lung adenocarcinoma patients and 39% of squamous cell carcinoma patients and 74% of other NSCLC patients.[27]

In a research on lung cancer in 2011, researchers investigated the probability of metastasis and angiogenesis by manipulating the expression of MUC1. Increase of MCU1's expression was observed in this research.[28]

Jan Kulpa *et al.* stated in a study that CEA's expression in squamous cancer cells has a meaningful relationship with other cancer-causing genes and its results showed that CEA tumor marker has a high expression among the patients.[29]

In this study, positivity of CEA mRNA marker among the NSCLC patients was determined at 75% and positivity of MUC1 mRNA marker among the NSCLC patients was determined at 70% and a meaningful statistical difference between the two groups of patients and healthy people was observed (P value=0.029).

Conclusions

The results of this study can be considered a diagnosing-screening test for early discovery of the disease since MUC1 mRNA and CEA mRNA markers were evaluated at good sensitivity levels. For further validation of the research results, it is suggested to perform a more widespread study with more samples.

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