Three Markers in Cancerous and Healthy Cells of Patients with Non-Small-Cell Lung Carcinoma (NSCLC)

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Abstract

Introduction: Lung cancer is the most common cause of cancer-related death among males and females. The diagnosis of lung cancer is of great importance for clinical considerations and follow-up treatment. This study aimed to examine the expression of CEA, LUNX, and CK19 biomarkers in the cancerous and healthy tissues of patients suffering from NSCLC. Methods: In this study, 30 patients with NSCLCs referring to Masih Daneshvari Hospital in Tehran were voluntarily selected prior to taking any treatment. A tissue sample from the center and a sample of healthy tissues close to the cancerous masses were prepared by a specialist in the bronchoscopy sector and tested using real-time RT-PCR. Results: Positive CEA mRNA was observed in cancerous tissues in the center of tumors of 25 out of 30 cases. In the healthy tissue group, the same was found in 10 out of 30 cases (P<0.001). The markers CK19 and LUNX mRNAs showed to be positive in cancerous samples in the center of tumors of 15 and 22 out of 30 cases, and in the healthy tissue group, the expression was observed in 5 and 4 out of 30 cases, respectively (P<0.001). Conclusion: This study confirms that the aforementioned markers are the ones with a relatively appropriate sensitivity and specificity for the diagnosis of lung cancer.

Keywords: Lung cancer- biomarker- LUNX- CK19- CEA

Expression of Three Important Biomarkers...
screening tests that assist oncologists in the diagnosis of the disease at an early stage in patients with cancer; specifically, those with a higher risk of disease. Biomarkers are also used to screen the disease response to the treatment (Benediková et al., 2012).

CEA (carcinoembryonic antigen) is a cell surface glycoprotein and a known tumor marker for lung cancer. CEA plays a role in cell adhesion; it is normally synthesized during the development of embryo, and its synthesis stops shortly before birth (Grummet and Sorensen, 2012). The increased CEA level provides prognostic information on the disease process.

LUNX (lung-specific X protein) is a lung-specific gene, which is highly expressed in the NSCLC. According to a large number of evidence, the LUNX may be a diagnostic biomarker for lung cancer (Iwao et al., 2001; Wallace et al., 2005). Sequential analysis has indicated that LUNX may have a role in inherent safety (Mitas et al., 2003).

CK19 (cytokinin 19) belongs to a family of keratins, and its major role function is to maintain the integrity of the epithelial cell. This protein also has a critical role in the cell cycle of stress-response and apoptosis. CK19 is an extremely useful marker for the tumor diagnosis and control (Zaghloul, 2015).

18sRNA (18s subunit of ribosomal RNA) has widely been discussed as a house-keeping gene in similar studies and was used as a reference gene in the current study (Sardareh et al., 2014).

The main objective of this study was to determine the LUNX mRNA, CEA mRNA, and CK19 mRNA in the NSCLC tissue samples using the real-time PCR method and to examine the sensitivity and specificity of these markers in the patients suffering from NSCLC. In future, they could help to diagnose the disease at the early stage prior to metastases becoming apparent.

Materials and Methods

Method

In this study, 30 patients with NSCLC (with the mean age of 49 and standard deviation of 10.4) referring to Masih Daneshvar Hospital in Tehran were voluntarily selected prior to taking any treatment. After obtaining ethical code No. IR.SBMU.NRITLD.REF.1396.343., two tissue samples were prepared by a specialist in the Bronchoscopy Sector of …… and transported to the laboratory by a nitrogen tank. The first sample was procured from the center and the second one from healthy tissues close to the cancerous masses.

RNA extraction

For RNA extraction, RNA was first isolated from the samples by using the Cinma pure RNA kit (Cat no. PR891620-S, Sinaclon, Iran). Subsequently, a 20-25 mg piece of fresh tissue was prepared and grinded. The grinded tissue was mixed and homogenized with 400 μl of the lysis solution. The precipitation solution was then added to the homogenized mixture, and the resulting solution was transmitted to the extraction column and centrifuged. Finally, 50 nm μl of RNase-free water was added to the column, and the pure RNA in the column was extracted. The RNA quality was finally evaluated by a Nano-droplet device.

Complementary DNA (cDNA) preparation

To prepare the cDNA from the extracted mRNA, Viva 2-steps Reverse transcriptase kit (Vivantis, Cat no. RTPL12, Malaysia) was applied. As per manufacturer’s instructions, this kit has high sensitivity and specificity to reproduce any type of DNA from the RNA pattern. The specific primers of each marker were designed with the aid of AlleleID7 software and ordered to be prepared. Table 1 lists the parameters of the primers and their rates in the final real-time reaction.

Real-time quantitative reverse transcription (RT)-PCR

Real-time RT-PCR was performed with GeneAmp 5,700 sequence detection system (Foster City, CA, USA). The real-time RT-PCR reaction components were composed of (A) 2 μL of the template, (B) 4 μL of the Master mix, (C) primer with optimal concentration found in set up tests, and (D) denozified distilled water to reach a final reaction volume of 20 μL. Positive and negative controls were also used simultaneously for quality control and the detection of possible contamination. Initial step of RT-PCR was conducted at 50°C for 2 minutes, followed by a 10-minute hold at 95°C. Cycles (n = 40 first round) consisted of a 15-second melt at 95°C, accompanied by a 1-minute annealing/extension at 60°C. The final step was performed at 60°C incubation for 1 minute. All reactions were performed in triplicate, and a negative control lacking cDNA was included.

Relative differences between the expression of CEA mRNA, CK19 mRNA, and LUNX mRNA were measured for both the patients and healthy participants using the ΔΔ Ct method.

Statistical analysis

The sample size was estimated based on the positive biomarkers in the two groups with considering 5% and 20% type I and type II errors. Comparison of the mean of the two groups was done by t-test and comparing the markers positivity in the two groups using the two-sample binomial test using SPSS21 software. The statistical difference was considered significant at P≤0.05.

Results

The present work was performed using a tissue sample of 30 patients, of whom 8 were female and 32 were male. The participants’ mean age was 49 with a standard deviation of 10.4. There was no significant difference between the mean of samples obtained at the center and sides of the tumor (P = 0.058). Accordingly, the 18sRNA gene was selected as a reference gene and included in the study.
Expression analysis of studied markers

Following the extraction of the real-time RT-PCR reaction, the patients in each of the two groups who showed positive results for the expression of markers were determined.

The results showed the positive expression of the CEA mRNA. Such a positive expression was also observed for cancerous center tissue samples in 25 of 30 cases; therefore, the sensitivity of this marker was determined to be 83%. In the healthy side tissue group, the expression was found in 10 out of 30 cases. The comparative analysis of the gene expression between the two groups by two-sample binomial test represented a significant difference (P< 0.001).

The expression of CK19 and LUNX mRNAs were conducted in 15 and 22 out of 30 patients, respectively. The sensitivity of the two markers was determined to be 50% and 73%, respectively. However, in the healthy side tissue group, CK19 and LUNX mRNAs were detected in 5 and 4 out of 30 samples, respectively. The comparison of the gene expression, using two-sample binomial test, demonstrated a significant difference between the two groups of samples (P< 0.001; Figure 1).

Differences between the expression of markers in two sample groups

The ΔΔ Ct values for the CEA, CK19, and LUNX mRNAs were calculated to be -1.4, -1.2, and -0.8, respectively, which mathematically indicates that the number of initial versions for the markers in patients is 2.64, 2.30, and 1.74 times as much as that of healthy subjects, respectively (Figure 2).

Discussion

Lung cancer remains the most leading reason of cancer-related death among men and women. Smoking has been reported to be the main cause of lung cancer in the world (Thress et al., 2015), but in the United States, the second cause of lung cancer seems to be radon gas. Exposure to the radon gas along with smoking a cigarette significantly increases the likelihood of lung cancer (Rosell et al., 2009).

In this study, three biomarkers LUNX, CK19, CEA

| Parameters | CEA | CK19 | LUNX | 18s rRNA |
|------------|-----|------|------|----------|
| Accessing number | M29540 | NM_002276.4 | M29540 | NM_002276.4 |
| Primer F | ACCCTGGATGTCCTCTATGG | TCCGACCAAGTGTGAG | CCAACCGTCTCTATGTGACCA | GTCACCGTGAAACCCATT |
| Primer length | 20 | 19 | 20 | 20 |
| Primer R | TCCGAACCAAGTGTGAG | CCAACCGTCTCTATGTGACCA | GTCACCGTGAAACCCATT |
| Primer length | 21 | 18 | 19 | 20 |
| Product length | 174 | 222 | 211 | 152 |

Table 1. Specifications of Primers Used in Real-Time RT-PCR Reaction
were studied. It was shown that the expression of these biomarkers in cancerous tissue is significantly higher than healthy tissue. These results can help in the diagnosis and treatment.

Lung cancer mainly reveals no symptoms in the early stages; however, they emerge with the spread of cancer. The most frequent symptoms include respiratory problems such as cough, bloody sputum, systemic body complications like weight loss, etc (Marchetti et al., 2005).

Tumor markers are biochemical molecules of protein or glycoprotein generated in response to the emergence of cancer and can be detected in the blood, urine, and tumor tissues (Duffy, 2001; Thomas and Sweep, 2001). RNA markers can serve as the diagnostic tests, an advantage that persuaded the researcher to include them in the current study (Ghossein and Bhattacharya, 2000). Another advantage is detecting the small amounts of these markers by the use of the real-time RT-PCR, which is a high-sensitive method with great features. However, other protein markers, which are mainly in extremely small amounts, cannot be measured.

In a previous study, the expression of CK19 and CEA mRNAs was investigated in the peripheral blood of NSCLC patients. The rate of positive expression was indicated to be 57% for CK19 mRNA and 40% for CEA mRNA and 43% for both, suggesting that both mRNAs are suitable markers for the diagnosis of the micro-metastases (Zhu et al., 2004).

A number of studies have reported high embryonic carcinoma antigen in the serum levels of patients with NSCLC, as compared to those suffering from other types of cancer (Pastor et al., 1997; Oyama et al., 2003). This finding is in the same line with the findings of the present study. Wang et al., (2008) examined CK19 and LUNX mRNAs expression on tissue and lymph node samples of 90 NSCLC patients. The expression of each marker showed to be significantly higher in patients with NSCLC than those with benign lung cancers (Wang et al., 2008), which is in good agreement with our results. Likewise, Yu et al., (2014) evaluated the expression of LUNX, CEA, and CK19 mRNAs in 113 patients with lung cancer (case group) and 30 healthy subjects (control group). They found that the positive expression of LUNX mRNA in peripheral blood and CEA and CK19 mRNAs in serum was significantly higher in the case group relative to the control group (Yu et al., 2014). In a study by Karimi et al., (2015) on the expression of CEA mRNA and LUNX mRNA in 30 NSCLC patients, there were 24 cases with positive expression of CEA mRNAs, indicating 80% sensitivity, and 21 cases with positive expression of LUNX mRNA, suggesting the sensitivity of about 70%. These two biomarkers were suggested as two markers with an appropriate sensitivity and specificity for lung cancer detection, which supports the results of the present study.

It should be noted that there were limitations in doing this research, such as access to more numbers and limited resource use. However, good results were achieved.

In conclusion, to conclude, the result of this pilot study on tumor markers of lung cancer can be applied to introduce a diagnostic test for lung cancer disease. To proof this finding, further studies with a larger sample size are recommended.

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