ORIGINAL ARTICLE

Diagnostic value of LunX mRNA and CEA mRNA expression in pleural fluid of patients with non-small cell lung cancer

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ABSTRACT

BACKGROUND: Absence of diagnostic markers for early detection of lung cancer results in its progression. No specific biomarkers are currently available for accurate detection of lung cancer. Thus, detection of lung cancer in its early stages is challenging. This study sought to assess the expression of lung-specific X protein (LunX) and carcinoembryonic antigen (CEA) tumor markers in patients with non-small cell lung cancer (NSCLC) to evaluate their efficacy for detection of lung cancer in its early stages.
METHODS: This study was performed on pleural fluid of 80 individuals including 40 NSCLC patients and 40 healthy controls. RNA extraction was performed on pleural fluid samples. Using a specific kit, cDNA was synthesized from the mRNA and gene analysis was performed using real-time reverse transcription polymerase chain reaction (real-time RT-PCR).
RESULTS: The expression of LunX mRNA was positive in the pleural fluid samples of 90% of NSCLC patients (36 out of 40). The CEA mRNA marker was also positive in the pleural fluid samples of 82.5% of NSCLC patients (33 out of 40). The CEA mRNA marker was positive in 12 out of 40 healthy controls. None of the healthy controls was positive for LunX mRNA.
CONCLUSIONS: The LunX and CEA mRNAs in the pleural fluid can serve as promising biomarkers for detection of NSCLC.

(*Cite this article as:* Ghadimi K, Bahrami N, Fathi M, Farzanegan B, Naji T, Emami M, *et al.* Diagnostic value of LunX mRNA and CEA mRNA expression in pleural fluid of patients with non-small cell lung cancer. Minerva Pneumol 2017;56:90-5. DOI: 10.23736/S0026-4954.16.01773-9)

Key words: Human BPIFA1 protein - Carcinoembryonic antigen - Pleural cavity - Non-small cell lung carcinoma.

Lung cancer is the main cause of cancer mortality worldwide and is responsible for over one million deaths annually.[1] Lung cancer is classified into two types: small cell and non-small cell lung cancer (NSCLC).[2, 3] NSCLC accounts for 85% of the cases of lung cancer;

65-75% of patients diagnosed with NSCLC are in stages II to IV (metastatic).[2, 3] Advances in treatment of lung cancer have not been remarkable mainly due to the absence of specific markers for its early detection in the clinical setting.[4] Chemotherapy can increase the survival of patients to some extent;[5, 6] however, chemotherapy has limited efficacy and can rarely result in complete recovery of patients.[7] Thus, there is a clear need for more advanced therapeutic modalities.[8]

An early detection of lung cancer can result in faster and higher efficacy of treatment in patients. The use of tumor markers is a newly introduced technique for the detection of cancer. To date, no specific biomarkers have been proposed for detection of NSCLC. Thus, a combination of biomarkers must be evaluated for detection and accurate diagnosis of this type of cancer.[2, 3, 9, 10]

Real-time reverse transcription polymerase chain reaction (RT-PCR) is among the most efficient techniques for detection of diagnostic tumor markers.[11-13] Several prognostic and diagnostic biomarkers have been evaluated so far in cancer patients; the assessment of these biomarkers is not costly; thus, it is considered a suitable non-invasive technique for detection of cancer.[14-17]

The carcinoembryonic antigen (CEA) is a commonly used glycoprotein tumor biomarker. The expression of CEA mRNA can be assessed in body fluids such as the pleural fluid using real-time RT-PCR. According to previous studies, the expression of this biomarker increases in cancer patients.[18-23]

On the other hand, LunX is a novel human lung-specific gene. Evidence shows that it can be used as a diagnostic biomarker for the detection of micro-metastases of lymph nodes. Its expression can also be assessed in body fluids such as the peripheral blood or pleural fluid of NSCLC patients.[8, 24, 25] It has been suggested that LunX may play a role in innate immunity as well.[25] The exact morphologic function of this gene has yet to be fully elucidated but it plays an important role in the initiation of inflammatory reactions in response to upper respiratory tract stimulation.[26] Thus, determin-

ing its level of expression using real time PCR may be beneficial for the detection of NSCLC in its early stages.[26]

This study aimed to assess the level of expression of CEA mRNA and LunX mRNA as two novel tumor biomarkers in pleural fluid of patients with NSCLC. However, for a more accurate and early diagnosis of lung cancer, the assessment of a combination of biomarkers may be required.

Materials and methods

This study was conducted on the pleural fluid of 80 individuals including 40 patients with NSCLC and 40 healthy controls. The patient group included patients with pathologically confirmed NSCLC in stages I to III presenting to Masih Daneshvari Hospital. Plural fluid samples were collected from patients prior to chemotherapy. Control subjects were randomly selected among healthy individuals presenting for routine medical examinations. The two groups of patients and controls were matched according to age and sex. Also, both groups signed written informed consent forms and willingly participated in the study.

Pleural fluid sampling

Pleural fluid samples were collected and evaluated in this study. For this purpose, 10 mL of pleural fluid was collected from subjects; the first 2 mL of it were discarded due to the possibility of contamination with epithelial cells. The remaining 8 mL of the pleural fluid were used for RNA extraction, which was performed using RNeasy midi kit (Qiagen Cat No. 75144). In the extraction process, a series of commercial solutions namely RLT, RW1 and RPE were used and centrifugation was performed. Eventually, 250 mL of RNase-free water were added to the column and pure RNA was extracted and collected. Using NanoDrop, the quality of extracted RNA was assessed. The extracted RNA was immediately used for cDNA synthesis, which was performed using Viva 2-step RT PCR kit (Cat No. RTPL12). Of each RNA sample, three cDNA vials were syn-

TABLE I.—*The characteristics and amount of primers used in real-time RT-PCR.*

Property	CEA	LunX	18s rRNA
NCBI accession no.	M29540	NM 016583.3	X03205
Forward primer	ACCCTGGATGTCCTCTATGG	CCACCGTCTCTATGTCACCA	GTAACCCGTTGAACCCCATT
Primer length	20	20	20
Quantity used	15 pmol	10 pmol	10 pmol
Reverse primer	CAGGCATAGGTCCCGTTATTA	GCCAAGTCCATCAAGCAGA	CCATCCAATCGGTAGTAGCG
Primer length	21	19	20
Quantity used	10 pmol	10 pmol	10 pmol
Amplicon length	174	211	152
Optimized annealing temperature	61.2 °C	61.4 °C	53.5 °C

thesized and their quality was evaluated using NanoDrop. Next, 15 mL of RNA were used for the synthesis of cDNA according to the protocol provided in the kit. The synthesized cDNA was stored at -80 °C for later use for real time RT-PCR. The required primers were designed using allele ID7 software, controlled by several specialists and then synthesized. The amount and characteristics of the primers used in real time RT PCR are shown in Table I.

Real-time RT-PCR was performed using Hot Tag EvaGreen® qPCR Mix and the synthesized cDNA was entered into the reaction. The reaction components included the template (2 µL), master mix (4 µL) and 0.5 µL of each primer (R and F). The final volume was reached to 20 µL using deionized distilled water. The positive and negative controls were also included.

Statistical analysis

The data were analyzed using SPSS v.20 considering type one error of 0.05 and type two error of 20%. The mean and standard deviation values were compared between the two groups of patients and controls using *t*-test. The χ^2 test was applied to analyze the difference in biomarkers between the groups.

Results

A total of 80 subjects including 40 NSCLC patients and 40 healthy controls were evaluated. Of 40 patients, 31 were males and 9 were females. Of 40 controls, 32 were males and 8 were females. The mean age was 51.36±10.91

years in patients and 47.53±9.99 years in controls. The mean age was not significantly different between the two groups of patients and controls (P=0.539). No significant difference was found between the two groups in terms of sex either (P=0.754).

In this study, 18-s rRNA gene was selected as the reference gene and evaluated in the two groups. The C_t value of this gene was assessed using real-time RT PCR. The mean C_t was 20.50±3.72 in patients with NSCLC and 20.03±3.40 in the controls. Statistical analysis revealed no significant difference in this regard between the two groups (P=0.620), which indicated correct selection of the reference gene and that this gene was suitable for use as the reference gene.

Analysis of the expression of LunX mRNA and CEA mRNA

The CEA mRNA was positive in 33 out of 40 patients, showing 82.5% sensitivity. In healthy individuals, 12 out of 40 were positive for CEA mRNA, indicative of 40% false-positive rate.

Thirty-six out of 40 patients were positive for the LunX mRNA, indicative of 90% sensitivity. In healthy controls, no subject was positive for this biomarker.

The difference between the two groups of patients and controls was significant in terms of positivity for CEA mRNA (P<0.001).

To increase sensitivity, all tests were performed in triplicate and the results for both CEA mRNA and LunX mRNA for vials 1, 2 and 3 showed significant differences in the

TABLE II.—*Comparison of the positivity of vials 1, 2 and 3 in patients with NSCLC with general positivity for CEA mRNA and LunX mRNA.*

Vial #	CEA mRNA			LunX mRNA		
	Positive rate	Sensitivity	P value	Positive rate	Sensitivity	P value
1	66%	82.5%	<0.001	86%	90%	<0.001
2	80%	82.5%	<0.001	66%	90%	<0.001
3	63%	82.5%	<0.001	70%	90%	<0.001

CEA: carcinoembryonic antigen; LunX: lung-specific X protein.

positivity of each vial and the general positivity (Table II).

Differences in the marker expression in the pleural fluid

The difference in gene expressions in the two groups was assessed using $2^{-\Delta\Delta Ct}$. For this purpose, first ΔC_t and then $\Delta\Delta C_t$ were calculated between the two groups. The $\Delta\Delta Ct$ for the CEA marker was found to be -4.05. Using the formula $2^{-\Delta\Delta Ct}$, it was found that the frequency of primary transcripts of this marker in patients was 16.56 times the rate in healthy controls. The mean ΔC_t for the CEA marker was 8.33±4.68 in patients and 12.38±6.62 in controls. Comparison of the level of ΔC_t for the CEA mRNA revealed that significant differences existed in this respect between the two groups (P=0.048).

In patients, ΔC_t for LunX mRNA was calculated to be 8.04. Expression of this gene was not seen in the control group. Thus, this gene is exclusively expressed in NSCLC patients.

Discussion

Detection and definite diagnosis of lung cancer are challenging in some patients. Approximately 35% of lung cancer patients are diagnosed in the early stages of the disease and undergo surgery. However, 50% of these patients develop metastasis and die as a result of lung cancer.[4] About one-quarter of NSCLC patients who are diagnosed in primary stages of the disease and undergo surgery die due to tumor recurrence, which indicates the presence of undetectable metastasis at the time of surgery. These findings indicate that the currently used staging system does not have sufficient sensitivity for the classification of cancer patients.[27, 28]

Cancer cells are often detached from their primary location and are released into the bloodstream or body fluids; thus, they may be detectable and traceable in peripheral blood and pleural fluid of cancer patients.[26]

Therefore, searching for tumor markers in the peripheral blood, secretions or body fluids such as the pleural fluid can be a suitable technique for the detection of cancer at different stages of the disease.[26, 29]

In the current study, CEA mRNA and LunX mRNA were selected for further assessment among the currently used biomarkers for an early detection of cancer. Several methods are available for assessment of biomarkers. Real-time RT PCR is among the most commonly used techniques for this purpose, which was adopted in the current study. This method is ideal for the assessment of expression of genes and has relatively high sensitivity for this purpose.[23, 26]

The assessment of tumor markers in the pleural fluid is an alternative technique for detection and diagnosis of malignant tumors.[4] Iwao *et al.* showed an enhancement of LunX mRNA expression in 26 out of 31 patients with NSCLC.[24] Wallace *et al.* demonstrated increased expression of LunX in 15 out of 27 patients with NSCLC, which showed a sensitivity of 56% for this marker.[8] In 2008, Mincheng *et al.* stated that LunX was a specific marker for lung cancer cells in the peripheral blood and pleural fluid; they also added that CEA was another suitable marker in these patients.[4]

In the current study, real time RT PCR was used for assessment of LunX mRNA and CEA

mRNA and revealed that LunX mRNA was an exclusive marker for the detection of NSCLC. Also, a significant difference was noted in the expression of CEA mRNA between the patients and controls. Karimi *et al.* reported that LunX mRNA was exclusively expressed in the peripheral blood of NSCLC patients. Moreover, the expression of CEA mRNA was significantly higher in patients than in healthy controls since 24 out of 30 patients were positive for CEA mRNA.[26] Similarly, the difference in this regard between patients and controls was statistically significant in our study.

Conclusions

It may be concluded that CEA mRNA and LunX mRNA are optimal biomarkers for an early detection of NSCLC in its early stages and have an ideal sensitivity for the diagnosis of lung cancer. Further studies are required to better elucidate this topic and confirm the reliability of our findings in a larger population.

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