Online version at http://www.minervamedica.it

Minerva Pneumologica 2017 June;56(2):78-83
DOI: 10.23736/S0026-4954.17.01784-9

ORIGINAL ARTICLE

Expression of CK19-mRNA and CEA-mRNA biomarkers in pleural fluid of patients with non-small cell lung cancer

Narges MOSHREF BEHZAD [1], Naghmeh BAHRAMI [2, 3], Behrooz FARZANEGAN [4], Mohammad FATHI [5], Shohreh ZAREH KARIZI [6], Abdolreza MOHAMADNIA [7, 8] *

[1]Islamic Azad University Varamin Pishva Branch, Tehran, Iran; [2]Craniomaxillofacial Research Center, Tehran University of Medical Sciences, Tehran, Iran; [3]Oral and Maxillofacial Surgery Department, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran; [4]Tracheal Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran; [5]Anesthesiology Research Center, Anesthesia and Critical Care Department, Shahid Modares Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [6]Department of Biology, Islamic Azad University Varamin Pishva Branch, Tehran, Iran; [7]Virology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran; [8]Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding author: Abdolreza Mohamadnia, Virology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, 1956944413, Tehran, Iran. E-mail: mohamadnia.ar@gmail.com

ABSTRACT

BACKGROUND: Lung cancer is one of the most mortal diseases in the word. There are lots of researches in the world focusing on non-invasive laboratory tests for diagnosis and screening non-small cell lung cancer (NSCLC) in early stage that could help the oncologist and surgeon to select the best treatment panel for the patient. Most of these research trials are working on different type of biomarkers in biological body fluids in patients with NSCLC, but still a definite sensitive and specific biomarker has not reached common consensus yet. The purpose of this study was to evaluate the sensitivity and specificity of expression of the two mRNA biomarkers; carcinoembryonic antigen (CEA) mRNA and cytokeratin19 (CK19) m RNA in pleural effusion of patient with NSCLC.

METHODS: Forty patients with lung cancer and forty healthy individuals were studied in this paper. All samples were examined using the real-time RT-PCR method.

RESULTS: The expression of CEA-mRNA was positive in 30 out of 40 patients with NSCLC indicative for 75% sensitivity and 12 out of 40 of healthy individuals (30% false positive) indicative for 70% specificity. The expression of CK19-mRNA was positive in 26 out of 40 patients with NSCLC indicative for 65% sensitivity and 8 out of 40 healthy individuals (20% false positive) indicative for 80% specificity.

CONCLUSIONS: The evaluation of mRNA biomarker expression in pleural fluid of patients with NSLC can be used as a diagnostic or screening test for detecting NSCLC in early stages.

(*Cite this article as*: Moshref Behzad N, Bahrami N, Farzanegan B, Fathi M, Zareh Karizi S, Mohamadnia A. Expression of CK19-mRNA and CEA-mRNA biomarkers in pleural fluid of patients with non-small cell lung cancer. Minerva Pneumol 2017;56:78-83. DOI: 10.23736/S0026-4954.17.01784-9)

Key words: Carcinoma, non-small-cell lung - Carcinoembryonic antigen - Keratin-19 - RNA, Messenger - Biomarkers.

Lung cancer is one the most important causes of death in the world, with more than 1 million deaths per year. Every year lung cancer deaths are more numerous than breast cancer, colon cancer and prostate cancer deaths.[1]

Despite the advance in surgical techniques and combined treatment, lung cancer has an

unfavorable prognosis. Lung cancer with 5 years survival (13%) has the worst survival rate among other cancers. Interestingly if lung cancer is diagnosed in stage I, then the survival rate would increase to 85%.[2]

In most patients with advanced stages of NSCLC, surgical intervention is futile even in hands of expert surgeons and advanced surgical techniques will not be usually successful. According to the WHO classification, epithelial lung cancer is divided into two main categories with four main cellular sub-groups: small cell lung cancer (SCLC) 15%, non-small cell lung cancer (NSCLC) 85% including adenocarcinoma, squamous cell carcinoma (SCC), and large cell carcinoma.[3-5] Each one of them has a specific clinical, pathological and histological pattern. With developing the data about biology of tumor and genetic alteration and specific mutation, new classification is under survey. Knowing this molecular differentiation helps to lead more efficient treatment plans in future.[6, 7]

Many dynamic alterations in genome are known in non-small cell lung cancers including mutation in KRAS, PIK3CA, RB, P53, EGFR, EML4-ALK, loss of heterozygosity and tumor suppressor gene DNA mutilation.[8]

According to different genomic alterations and mutations in lung cancer, finding a reliable molecular biomarker to detect lung cancer in the early stages as a non-invasive screening and diagnostic exam could be very helpful.[9]

Biomarkers are the biological molecules in peripheral blood or pleural fluid or other bodily fluids which indicate a normal or abnormal process or a specific condition or a disease. There are different types of biomarkers such as protein biomarkers, DNA biomarkers and mRNA biomarkers. Cells are also recognized as biomarkers.[4, 10, 11]

In the late stages of cancers, circulating tumor cell (CTC) transfer to peripheral blood and specifically in lung cancer CTC could also enter into the pleural cavity, which is called malignant pleural effusion (MPE).[10, 12]

MPE has a close association with NSCLC. It is the first sign of malignancy in up to 15% of patients with lung cancer.[12] MPE is also seen in other malignancies like ovarian and gastric cancer and lymphoma.[12] There are different methods for evaluating the pleural fluid such as cytology and needle biopsy. Cytology is the most common method for evaluation of pleural fluid but there are lots of suspicious MPE cases with negative cytology reports, which could be related to the very small number of CTC in pleural fluid.[2, 13] Nowadays the molecular methods like real-time PCR are very useful for detecting the very small amount of CTC and their products (mRNA, DNA, mi-RNA) in different types of body fluids.[14-17]

We can follow the protein tumor markers which are produced by CTC with medical laboratory techniques like Eliza or Electerochemilominesecence or molecular technique such as real-time PCR for detecting the DNA or mRNA or mi-RNA.[2, 18]

In this study we elevated the expression of two mRNA biomarkers in pleural fluid of patients with NSCLC: carcinoembryonic antigen (CEA) mRNA and cytokeratin19 (CK19) mRNA.

CEA is a superficial glycoprotein tumor marker which has a role in cell adhesion. CEA is produced during fetal growth and its production is stopped just before birth. An increased level of CEA in serum gives us the prognostic data of disease process.[19]

CK19 is an intermediate filament protein. Cytokeratins are a large family group of proteins including 20 types of polypeptides which are divided into two main groups: the acidotic type (CK9-CK23) and the basic type (CK1-CK8). Cytokeratin 19 has an important role in holding and keeping the integrity of epithelial cells in addition to response to stress and apoptosis. CK19 is a very applicable marker in diagnosis and control of cancers.[20, 21]

Materials and methods

Forty patients with biopsy proven primary diagnosis of NSCLC, referred to MasihDaveshvary Hospital, were selected. The inclusion criteria were patients with stage I to III lung cancer (no evidence for distant metastasis) and also no history of any medical or surgical intervention.

For the control group, forty patients with suspected lung cancer referred to Masih-Daveshvary Hospital, but lung cancer was excluded by clinical exam, biopsy and pathological findings (healthy individuals).

This was a case-control study and the patients and the control group were matched according to gender and age.

A 10-cc pleural fluid was sampled from both groups. To reduce the risk of contamination with the skin epithelial cells, the first 2 cc of pleural fluid were removed.

RNA extraction

In this step we used RNeasy Midi Kit (qiagen Cat no.75144). The sediment acquired in the previous step was first lysed using RLT lysing solution present in the lysis kit. Next, 70% ethanol was added and centrifuged for 5 minutes. In the next step, RW1 and PRE solutions present in the kit were added to the column. Eventually the total mRNA was kept in buffer.

cDNA synthesis

In this step we usedViva 2-steps RT-PCR Kit (Cat no. RTPL12).

The purity and quantity of cDNA and RNA were measured by NanoDrop®.

Primer design software

We used AlleleID7 as a primer design software for each marker. After receiving the designed primer (lyophilization: freeze-drying). Primers concentrate 100 pmol/uL diluted with sterile distilled water and after that the primers keep in the freezer at -20 °C. The final concentration of primer that was used in the test was 10 pmol/uL (Table I).

Real-time RT-PCR

In this step, we used Sinaclon kit (Sinaclon, Tehran, Iran, Cat No. BT11101) that contained the EvaGreen® (Biotium, Hayward, CA, USA) fluorescent color, which attached to double strained DNA and then the fluorescent light could detect by real-time detector system. Real-time RT-PCR were performed on a total volume of 20 µL contains 2 µL of template cDNA, 4 µL Master Mix, optimal concentration of primer 10 pmol/uL and sterile distilled water to have a final volume of 20 µL.

The temperature and time of test was maintained according to the kit's protocol. At the end of every real-time RT-PCR reaction, the result was interpreted by amplification and melting curves. The ingredients, temperature and time of test were maintained so that the best result of markers was obtained, and prevented the error which could disturb the interpretation of the test (for example double primer).

Statistical analysis

The sample size was calculated using sample size estimation formula considering type one error of 5% and type two error of 20%. The data were analyzed using SPSS version 20. The mean values were measured between the two groups of test and control using *t*-test. Gene expression ratios in the two groups were statistically examined and compared using χ^2 Test. Level of significance was set at $P \leq 0.05$.

TABLE I.—*Characteristics of the primers used in real-time RT-PCR.*

	CEA	CK19	18s rRNA
Forward primer	ACCCTGGATGTCCTCTATGG	TCCGAACCAAGTTTGAGAC	GTAACCCGTTGAACCCCATT
Primer length	20	19	Ú20
Reverse primer	CAGGCATAGGTCCCGTTATTA	AATCCACCTCCACACTGA	CCATCCAATCGGTAGTAGCG
Primer length	21	18	20
Amplified fragment length	174	222	152
Annealing optimal temperature	61.2 °C	58.4 °C	53.5 °C

TABLE II.—*Comparison of the average age in lung cancer patients and healthy individual using t-test exam.*

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.

TABLE III.—*Comparison of the expression of 18s-rRNA in patinas with non-small cell lung cancer and healthy individuals.*

Main group	18s Ct value	
	Mean	SD
Patients (N.=40)	20.94	3.62
Healthy (N.=40)	20.12	2.88

P value=0.244, SD: standard deviation.

Results

The comparison with *t*-test between the patients and the control group based on their age showed no significant differences. Therefore the age factor in this study has no confounding effect (Table II).

Two cDNA vials of each sample were tested for expression of reference gene (18s-rRNA), CK19-mRNA and CEA-mRNA. Findings were interpreted by melting and amplification curves for each marker and comparing with 18s-rRNA.

In the case of a reference gene the result demonstrated no significant differences in both groups, which confirmed choosing the 18 s-rRNA as a reference gene (Table III).

Expression of Ck19-mRNA and CEA-mRNA

The findings demonstrated that the positive CEA-mRNAs were 30 out of 40 in patients with NSCLC, therefore the sensitivity for CEA-mRNA was 75%. In healthy individuals the positive CEA-mRNA were 12 out of 40, which indicated 30% false positive and 70% specificity. Statistical analysis for positive rate of CEA-mRNA biomarker in the two groups was performed using a two-sample binomial, and showed significant differences (P value=0.030).

The Ck19-mRNA were 26 out of 40 in patients with NSCLC which demonstrated sensitivity of 65% for Ck19-mRNA. In healthy individuals, the Ck19-mRNA positive were 8 out of 40, which indicated 20% false positive and 80% specificity. Statistical analysis for positive rate of CEA-mRNA biomarker in two groups using two-sample binomial exam also represents significant differences (P value=0.015).

Comparing the expression of Ck19-mRNA and CEA-mRNA in two groups of people in this study

The difference between the expression of Ck19-mRNA and CEA-mRNA markers by using ΔΔCT exam was measured and the ΔΔCT for CEA-mRNA was calculated -5.13, which mathematically if 2 to the power of ΔΔCT ($2^{\Delta\Delta CT}$) it demonstrated that the expression level of CEA-mRNA in patients with NSCLC was 35.02 times higher than in healthy individuals. The ΔΔCT for Ck19-mRNA was calculated -5.81, which mathematically demonstrated that the expression level of Ck19-mRNA in patients with NSCLC was 56.10 times higher than in healthy individual.

Discussion

The diagnosis of NSCLC in early stages is very important because more than half of the patients with NSCLC are in late stages when they are diagnosed. Biopsy is dependent on many factors including tumor availability, size and type of tumor and technical condition like having a good experience in bronchoscopy and pathology.[6, 22]

Cytology is the most common method to evaluate the pleural fluid, but there are lots of suspicious MPE cases with negative cytology reports, which could be related to the very small number of CTC in pleural fluid.[2] Nowadays the molecular methods like real-time PCR are very useful for detecting the very small amount of CTC and their products (mRNA, DNA, mi-RNA...) in different types of body fluids.[4, 23, 24]

Diagnostic and screening biomarkers can help the oncologist to detect lung cancer in

early stages in suspicious patients. Using these biomarkers and molecular data help oncologist to choose the best treatment for the patients.[10] Biomarkers are also very useful for monitoring the treatment process. Detecting CTC in blood (distant metastasis) and pleural fluid (tumor primary origin) could be a very helpful and non-invasive method for diagnosing of NSCLC in very early stages.[10, 20]

For detecting mRNA biomarkers, which are produced by CTC in process of tumor marker producing (CEA and CK19), a very specific and sensitive method, such as Real-Time Pcr, is required. As we discussed before due to difficulties in order to sample the pleural fluid, most of the studies for evaluating the biomarkers in NSCLC are in peripheral blood and only few of the studies are performed on pleural fluid.[11]

In a survey conducted by Yan *et al.* in 2015, expression of CK19, LUNX and KS1/4 mRNA in peripheral blood of patients with non-small cell lung cancer was evaluated and only 6.6% were positive, and CK19, LUNX and KS1/4 mRNA in peripheral blood of patients with NSCLC had more expression than patients with non-malignant pulmonary disease (P value<0.05), but the expression of CK19 was not correlated with clinical and pathologic index (P value>0.05).[25]

In another survey conducted by Zhu *et al.* in 2004, expression of CEA-mRNA and CK19-mRNA in peripheral blood of patients with NSCLC was 57% for CK19-mRNA and 40% for CEA-mRNA and 43% for both. They concluded that CEA-mRNA and CK19-mRNA are the appropriate markers for micrometastasis.[25, 26]

In a survey conducted by Feng *et al*. in 2004, the expression of CK19-mRNA in pleural fluid of the patient with NSCLC was significantly higher than in patients with non-malignant pulmonary disease (P value<0.05).[27]

In our study the expression of CEA-mRNA in pleural fluid of the patients with NSCLC was 75%, and CK19-mRNA in pleural fluid of the patient with NSCLC was 65%. A significant statistical difference was found between patients with NSCLC and healthy individuals (P value=0.030).

Conclusions

Our study confirms the role of tumor biomarkers in NSCLC as a diagnosis/screening test in diagnosis of early stages of lung cancer. Further studies with larger sample size are recommended.

References

1. Wender R, Fontham ET, Barrera E, Jr., Colditz GA, Church TR, Ettinger DS, *et al.* American Cancer Society lung cancer screening guidelines. CA Cancer J Clin 2013;63:107-17.
2. Xiao YF, Yong X, Fan YH, Lu MH, Yang SM, Hu CJ. microRNA detection in feces, sputum, pleural effusion and urine: novel tools for cancer screening (Review). Oncol Rep 2013;30:535-44.
3. Yan TD, Black D, Bannon PG, Mccaughan BC. Systematic review and meta-analysis of randomized and nonrandomized trials on safety and efficacy of video-assisted thoracic surgery lobectomy for early-stage non-small-cell lung cancer. J Clin Oncol 2009;27:2553-62.
4. Karimi S, Mohamadnia A, Nadji SA, Yadegarazari R, Khosravi A, Bahrami N, *et al.* Expression of two basic mRNA biomarkers in peripheral blood of patients with non-small cell lung cancer detected by real-time rt-PCR, individually and simultaneously. Iran Biomed J 2015;19:17-22.
5. Bahrami N, Gholami M, Jamaati HR, Mohamadnia A, Dargahi H, Kazempour Dizaji M, *et al.* Expression of two essential mRNA biomarker in the peripheral blood as possible biomarkers for diagnosis of nonsmall cell lung carcinoma. Minerva Pneumol 2016;55:31-6.
6. Network NCC. NCCN clinical practice guidelines in oncology, version 2.2013 [Internet]. Fort Washington: National Comprehensive Cancer Network; 2013 [cited 2013 Aug 22].
7. Johnson DH, Fehrenbacher L, Novotny WF, Herbst RS, Nemunaitis JJ, Jablons DM, *et al.* Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 2004;22:2184-91.
8. Maddaly R, Sahu B, Mohan DK. Genes Associated with Human Cancers: Their Expressions, Features, Functions, and Significance. Crit Rev Eukaryot Gene Expr 2015;25:209-38.
9. West L, Vidwans SJ, Campbell NP, Shrager J, Simon GR, Bueno R, *et al.* A novel classification of lung cancer into molecular subtypes. PLoS One 2012;7:e31906.
10. Bhatt AN, Mathur R, Farooque A, Verma A, Dwarakanath BS. Cancer biomarkers - current perspectives. Indian J Med Res 2010;132:129-49.
11. Mohamadnia A, Karimi S, Yadegar Azari R, Naji SA, Khosravi A, Bahrami N, *et al.* Expression Of CK19 Gene In Patients With Lung Cancer And Its Comparison With Carcinoembryonic Antigen In Peripheral Blood. Journal of Payavard Salamat 2016;9:459-68.
12. Kremer R, Best LA, Savulescu D, Gavish M, Nagler RM. Pleural fluid analysis of lung cancer vs benign inflammatory disease patients. Br J Cancer 2010;102:1180-4.
13. Ettinger DS, Akerley W, Borghaei H, Chang AC, Cheney RT, Chirieac LR, *et al.* Non-small cell lung cancer. J Natl Compr Canc Netw 2012;10:1236-71.
14. Grunnet M, Sorensen J. Carcinoembryonic antigen

(CEA) as tumor marker in lung cancer. Lung Cancer 2012;76:138-43.
15. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR. Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn 2005;5:209-19.
16. Karimi S, Bahrami N, Sharifi K, Daustany M, Baghbani-Arani F, Kazempour M, *et al*. Investigating gene expression level of MUC1 and CEA in pleural fluid of NSCLC lung cancer patients with real-time RT-PCR method. Minerva Pneumol 2017;56:18-24.
17. Jamaati H, Bahrami N, Abniki M, Tabarsi P, Farzanegan B, Doroudinia A, *et al.* Real-time RT-PCR Detection of HCN4 and ADAM8 genes in ventilator-associated pneumonia patients Hospitalized in intensive care unit. Journal of Cellular and Molecular Anesthesia 2016;1:163-7.
18. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008;3:1101-8.
19. Zaghloul MZ. Cytokeratin 19 (CK19) as a Tumor Marker in Pleural Effusion. Tropical Medicine & Surgery 2014;2015.
20. Zhang X, Xie J, Yu C, Yan L, Yang Z. mRNA expression of CK19, EGFR and LUNX in patients with lung cancer micrometastasis. Exp Ther Med 2014;7:360-4.
21. Zaghloul M. Cytokeratin 19 (CK19) as a Tumor Marker in Pleural Effusion. Trop Med Surg 3: e122. doi: 10.4172/2329-9088.1000 e122 Page 2 of 2 blood of patients with operable breast cancer: evaluation of their prognosti c significance. J Clin Oncol 2015;20:3404-12.
22. Cesario A, Ferri L, Galetta D, Cardaci V, Biscione G, Pasqua F, *et al.* Pre-operative pulmonary rehabilitation and surgery for lung cancer. Lung Cancer 2007;57:118-9.
23. Yadegarazari R, Hassanzadeh T, Majlesi A, Keshvari A, Monsef Esfahani A, Tootoonchi A, *et al.* Improved real-time rt-PCR assays of two colorectal cancer peripheral blood mRNA biomarkers: a pilot study. Iran Biomed J 2013;17:15-21.
24. Arikawa E, Sun Y, Wang J, Zhou Q, Ning B, Dial SL, *et al.* Cross-platform comparison of SYBR® Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. BMC genomics 2008;9:1.
25. Yan L, Yao Y, Wang LH, Wang ML, Fu XH. Detection of CK19, LUNX, and KS1/4 mRNA expression in the peripheral blood for diagnosis of micrometastases in patients with non-small cell lung cancer and their clinical implications. Genet Mol Res 2015;14:15090-5.
26. Zhu G, Liu D, Wang X, Chen J. [Detection of CK19 and CEA mRNA expression for the diagnosis of peripheral blood micrometastases in patients with non-small cell lung cancer]. Zhongguo Fei Ai Za Zhi 2004;7:226-9.
27. Xu F, Chen J, Shen HH, Wang XD, Shan J. The diagnostic significance of the detection of cytokeratin 19 mRNA by quantitative RT-PCR in benign and malignant pleural effusions. J Zhejiang Univ Sci 2004;5:1286-9.

Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Acknowledgements.—We sincerely thank the Faculty and staff members at both Universities and MasihDaneshvari Hospital for their cooperation. This research is a part of a master dissertation in Genetic of Islamic Azad University Varamin Pishva Branch, Tehran, Iran, performed in 2015-2016 in in ShaheedBeheshti University of Medical Sciences, MasihDaneshvari Hospital, Tehran, Iran.

Manuscript accepted: January 30, 2017. - Manuscript received: January 28, 2017.