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# DNA investigation in the exhaled breath condensate (EBC) in non-small cell lung cancer

**Emine Afsin[1],** iD  **Mehmet Polatli[2],** iD  **Gokay Bozkurt[3]** iD

[1]*Department of Chest Diseases, Bolu Abant Izzet Baysal University, Faculty of Medicine, Bolu, Turkey*
[2]*Department of Chest Diseases, Adnan Menderes University, Faculty of Medicine, Aydın, Turkey*
[3]*Department of Genetics, Adnan Menderes University, Faculty of Medicine, Aydın, Turkey*

## ABSTRACT

**Aim:** Exhaled breath condensate (EBC), one of the materials which is used to detect mutations in the early period, is collected by completely non-invasive a technique which has no risk for the patient. We aimed to investigate whether EBC samples are suitable for the detection of DNA or not in non-small cell lung cancer (NSCLC) and control patients.

**Methods:** 26 patients with NSCLC and 20 patients without lung cancer were included in the study. EBC procedure was performed by the help of Eco Screen- Jaeger device in 10-15 minutes during breathing at the tidal volume. DNA was isolated using tissue spin-column DNA isolation kit in the collected EBC.

**Results:** DNA amount was twofold high in the NSCLC group than non-cancer patients in spite of short time (*p*>0.05). However, in cancer group DNA amount was found lower in patients with endobronchial lesions than without endobronchial lesions (*p*>0.05). Although, there was no relationship between DNA amount and all of EBC collection time, collected sample amount and expiration air volume in the cancer group, a positive relations was detected between DNA amount and EBC collection time in the non-cancer group.

**Conclusıon:** This may be explained by the pathological changes which occur at the cellular level in the lungs during cancer development process. However, it may also result from relative decrease which develops from redundancy of EBC volume in the non-cancer group. The source of DNA in EBC may be considered to be pathological changes resulting from the systemic inflammatory response, apart from the localized lesion in the lungs.

**Key words:** Non-small cell lung cancer, exhaled breath condensate, DNA.

## Introduction

Many diseases caused by environmental effects are closely related to the response that occurs according to the characteristics of the genetic structure of the individual. Recently, there has been increasing data on the potential pathogenic effects of noxious environmental stimuli on the lungs. One of these diseases is lung cancer. The 5-year survival rate of lung cancer, a significant cause of mortality in men and women in the present day, has only 10-15% [1]. Inhalation of tobacco smoke, which is the most critical factor in the development of lung cancer, causes permanent changes in the respiratory tract epithelium. Studies on the early diagnosis of lung cancer have shown that early diagnosis cannot improve survival. This causes from the possibility of early metastasis of lung cancer, even if it is clinically detected in small size. As

a result, it is necessary to detect lung cancer while still in the preneoplastic stage [1]. It was suggested that some genetic changes in the bronchial epithelium form the basis of preneoplastic change in lung cancer.

Long-term exposure to carcinogens causes damage to the genetic structure and changes the genes that control cell proliferation. These changes are mutations at the DNA level and changes in the expressions of cancer-related genes at the RNA level [2]. When mutations develop in oncogenic genes (such as K-ras, cyclins) and/or tumor suppressor genes (such as p53, Retinoblastoma, p16), a further step is taken for tumor development [3].

Changes in the genetic structure are closely related to certain diseases. In this way, it can be predicted which diseases are more likely to occur in patients with genetic changes. However, it is not always possible to obtain suitable examples in which these changes can be demonstrated. For example, in lung cancer, tissue samples that directly represent the lungs can be obtained by invasive methods such as bronchoscopy or semi-invasive methods such as stimulated sputum. In recent years, an increasing number of studies have been published on the value of some biomarkers in the exhaled breath condensate (EBC) obtained by condensing the expiratory air to evaluate airway inflammation. What makes EBC interesting is that obtaining the material is entirely non-invasive and does not pose any discomfort or risk to the patient [4]. Since EBC can represent some markers showing changes in the lungs, it is thought that expiratory air does not only consist of water vapor but also plays a carrier role for some soluble and non-soluble substances. The limited number of studies evaluating EBC regarding genetic analysis is promising [5, 6]. This study aimed to investigate whether the EBC sample contains enough DNA for research in patient groups with non-small cell lung cancer (NSCLC) and without a cancer diagnosis.

**Materials and methods**

Twenty-six male patients diagnosed with NSCLC as a result of outpatient and/or inpatient examinations of Adnan Menderes University Practice and Research Hospital Chest Diseases clinic between 2009 and 2010, and 20 male patients of the same age group who did not have lung cancer or other organ cancer, were included in the study (Date:01.12.2018, no:2008/00275). The diagnosis of lung cancer was made after bronchoscopic biopsy, transthoracic lung biopsy, and, in some patients, surgery for resection of the nodule. Bronchoscopic findings were grouped by direct monitoring of the endobronchial lesion and mucosal findings (normal, hyperemia, infiltration, external repulsion). Cranial CT/MRI, bone scintigraphy, or PET-CT were used for staging lung cancer. TNM staging was used in bronchoscopic staging.



**Figure 1**. EBC collection instrument (Eco Screen- Jaeger).

All the patients were informed about the procedure to be performed next to the device.

Afterward, for the EBC collection procedure, the individuals were examined with the Eco Screen-Jaeger device while they were in a sitting position, and their noses were closed with a nose clip for approximately 10-15 minutes, while they were breathing at tidal volume (Figure 1). It was explained that during the EBC collection, patients could swallow by keeping their mouths apart from the device when needed in order not to cough, to breathe frequently and deeply, and to avoid contamination from salivary secretions. In the collected EBC, DNA was isolated with invitek tissue spin-column DNA isolation kit.

### *DNA isolation from exhaled breath condensate (EBC)*

110 µl Elution Buffer D was put at 55oC, and a 400 µl sample was obtained from EBC. 400 µl of lysis buffer G and 40 µl of Proteinase K were added to the samples taken into the Eppendorf tube and vortexed for 5-10 seconds. The samples were incubated at 55oC for 60 minutes in thermal shaking and then centrifuged for 2 minutes at maximum speed. The supernatant portion was transferred to a new 1.5 µl Eppendorf tube. 200 µl Binding Buffer T was added and vortexed for 10 seconds. The filter tube was placed into the 2.0 receiver tube. The lysate was transferred into the filter tube and incubated for 2 minutes at room temperature, then centrifuged for 2 minutes at 13000 x g. The filtrate was discarded, the filter tube was placed in the same 2.0 receiver tube, and 550 µl of Wash buffer was added to the filter tube. Afterward, a centrifuge was performed at 13000 x g for 1 minute, the filtrate was discarded, and the filtered tube was placed in the same tube. 550 µl Wash Buffer was added. Another centrifuge was performed for 1 minute at 13000 x g. The filtrate was discarded, and the filtered tube was placed in the same tube. The

sample was centrifuged for another 2 minutes at 13000 x g. The filter tube was placed in the 1.5 ml receiver tube. 50 µl of elution buffer D (55oC) was added. It was incubated for 5 minutes at room temperature and centrifuged at 8500 x g for 2 minutes. 50 µl elution buffer D (55oC) was added, centrifuged for 2 minutes at 8500 x g.

### *Statistical analysis*

SPSS 14.0 program was used for statistical analysis of the data. Results were presented as mean ± SD. Intergroup comparisons were evaluated using the Mann-Whitney U non-parametric method, the examination of the relationship between the variables was determined using Pearson correlation or non-parametric Spearman correlation methods. The results of the research were evaluated by accepting the $p<0.05$ value as significant in the 0.95 confidence interval.

### Results

The patients included in the study were between the ages of 38-79 years, and the mean age was 64.30 ± 8.76. The mean age was 63.96 ± 10.07 in the cancer group and 64.75 ± 6.92 in the non-cancer group ($p>0.05$). While no significant difference was found between the two groups in terms of age to start smoking, the smoking burden was higher in the cancer group ($p<0.05$) (Table 1).

**Table 1.** Demographic data.

| Parameters | NSCLC group (n: 26) | Control group (n: 209 | *p*-value |
|---|---|---|---|
| **Age** | 63.9± 10.0 | 64.7± 6.9 | *0.920* |
| **Age to start smoking** | 18.6± 6.2 | 20.6± 7.7 | *0.455* |
| **Smoking burden (pack-year)** | 43.6± 12.2 | 37.5± 17.8 | *0.043* |

**Table 2.** DNA and EBC data of cancer cases by bronchoscopic findings.

| Parameters | Normal mucosa (N: 8, 47.1%) | Extrinsic repulsion (N: 5, 29.4%) | Mucosal hyperemia (N: 1, 5.9%) | Mucosal infiltration (N: 3, 17.6%) | Endobronchial lesions (N: 9, 34.6%) |
|---|---|---|---|---|---|
| DNA amount (µg/ml) | 79.38± 60.68 | 81.00± 69.92 | 20 | 67.50± 66.14 | 59.44± 52.96 |
| EBC sample (ml) | 2.60± 0.86 | 2.04± 0.44 | 1.70 | 1.93± 0.15 | 2.12± 0.50 |
| EBC volume* (L) | 172.20± 62.99 | 154.60± 30.77 | 103.10 | 119.20± 4.15 | 139.83± 34.24 |

*Expiratory air volume made by the patient at the time of EBC collection.*

**Table 3.** Comparison of subgroups with and without endobronchial lesions in the cancer group.

| Parameters | Those with endobronchial lesions (n: 9) | Those without lesions (n: 17) | *p*-value |
|---|---|---|---|
| DNA amount (µg/ml) | 59.44± 52.96 | 74.26±60.0 | *0.766* |
| EBC amount (ml) | 2.12± 0.50 | 2.26±0.70 | *0.586* |
| EBC volume (L)* | 139.83± 34.24 | 153.61±50.29 | *0.483* |
| Duration of procedure (min.) | 12.14± 4.95 | 12.18±2.85 | *0.258* |

*Expiratory air volume made by the patient at the time of EBC collection.*

**Table 4.** EBC data in cancer and non-cancer group.

| Parameters | NSCLC (n:26) | Control group (n:20) | *p*-value |
|---|---|---|---|
| Age | 63.96± 10.07 | 64.75±6.92 | *0.920* |
| Age to start smoking | 18.65± 6.28 | 20.65±7.78 | *0.455* |
| Package-year | 43.65± 12.20 | 37.55±17.87 | *0,043* |
| EBC volume (L)* | 148.84± 45.15 | 205.21±41.98 | ***0.001*** |
| EBC amount (ml) | 2.22± 0.63 | 3.15±0.44 | ***0.001*** |
| Duration of procedure (min.) | 12.17± 3.61 | 16.31±2.85 | ***0.001*** |
| DNA amount (µg/ml) | 69.13± 57.03 | 32.63±9.12 | *0.096* |

*Expiratory air volume made by the patient at the time of EBC collection.*

EBC data of 26 cancer cases grouped by bronchoscopic findings were presented in Table 2 and a comparison of subgroups with and without endobronchial lesions in the cancer group in Table 3.

Two lung cancer cases were staged as Stage IA, one as Stage IB, five as Stage IIIA, eight as Stage IIIB, and ten as Stage IVB NSCLC. Nineteen patients (73.1%) in the lung cancer group had no family history of malignancy. Seven patients (26.9%) had a family history of lung, stomach, breast, and colon cancers. While there were no symptoms at admission in 6 patients (23.1%), the main symptoms were

dyspnea, cough, sputum, back and flank pain, and leg pain in the remaining 20 patients (76.9%).

In all cases included in the study, a significant negative correlation was found between mean DNA amount (μg/ml) and the amount of EBC sample collected from the patients (ml) ($r=$ -0.43; $p$=0.003), the procedure duration (min) of the patients ($r=$ -0.33; $p$=0.025) and the expiratory volume during this period (L) ($r=$ -)0.447; $p$=0.002). No significant relationship was found between the amount of EBC DNA and all of age, smoking load, and age of onset of smoking.

The relationship between the amount of DNA in the cancer group and the EBC collection time, amount, expiratory volume, and smoking burden was not statistically significant. In the non-cancer group, a significant positive correlation was found between the amount of DNA and the duration of the procedure ($r$=0.499, $p$=0.025) (Table 4).

## Discussion

Patients with lung cancer are generally at an advanced stage when diagnosed. In our study, the most common were Stage IV, followed by Stage IIIB and IIIA, which were consistent with the literature (38.5%, 30.8%, and 19.2%, respectively). The incidence of lung cancer reported by the Turkish Thoracic Society Lung and Pleural Malignancies group in Turkey is 40.4%, 32.1%, 14.2% for Stages IV, IIIB, and IIIA, respectively [7]. This is because the symptoms are often thought to be related to smoking by the patients, and the physician is not consulted. Indeed, while there were no symptoms at admission in 6 patients (23.1%) in the cancer group, the main symptoms were dyspnea, cough, sputum, back and flank pain, and leg pain in the remaining 20 patients (76.9%). In the study conducted by Beckles et

al., the main symptoms were reported as cough, weight loss, dyspnea, hemoptysis, and chest pain [8].

Considering the age distribution of our patients, although they were in the 38-79 age range, it is known that the incidence of lung cancer increases with age, and the most common age range is 50-70 [9]. In a study conducted in our country, the incidence of cancer was 56.7% in the age range of 46-65 years, and 31.6% after the age of 66 [7].

Age of onset of smoking, duration of smoking, and amount of cigarette smoked, which are considered the most critical risk factors for lung cancer, are crucial criteria in cancer development [10]. In our study, the smoking burden was higher in the group with lung cancer, and this result was statistically significant. Although the age of onset of smoking was younger in the cancer group, it was not statistically significant.

In the lung cancer group, while 19 patients (73.1%) had no family history of malignancy, 7 (26.9%) had a history of the lung (7.6%), stomach (7.6%), and breast (7.6%), and colon cancer (7.6%). In the study of Arınç et al., the most common presentation of gastrointestinal and breast cancer was reported in the relatives of patients with lung cancer [11].

The amount of EBC sample obtained in liquid form is proportional to the volume obtained by expiration. The sample obtained is not only the liquid obtained by the condensation of water vapor, but it is thought to play a carrier role for molten gas and particles coming from the lower respiratory tract.

Since non-volatile molecules such as some soluble mediators, protein, oxidation, and nitration products can be measured in EBC, EBC samples can be used in the differential diagnosis of airway diseases and the follow-up of treatment [12]. Among the oxidative stress

biomarkers, especially endothelin-1 and interleukin-6, were high in EBC of patients with lung cancer. In EBC, oxidative stress biomarkers and cytokines may help demonstrate recurrence in the follow-up period after tumor resection [13]. In NSCLC patients, increased hydrogen peroxide ($H_2O_2$) levels and decreased antioxidant capacity were found in EBC. This result indicates the imbalance in favor of oxidants between the oxidant-antioxidant levels in lung cancer. This indicator of oxidative stress may be a marker to lead to early diagnosis of lung cancer [14].

When the vascular endothelial growth factor (VEGF), 8-isoprostane, and TNF-alpha levels in EBC and serum of patients with lung cancer were compared, VEGF levels in EBC were found to be higher in T3-4 compared to the T1-2 group, and the correlation between serum and EBC levels was found to be statistically significant. TNF-alpha has also been shown to be at higher levels in EBC in patients with lung cancer. These findings suggest that some markers in EBC may be valuable in the follow-up of patients in the future [15].

Tumor markers have also been studied in EBC in patients with lung cancer. Cytokeratin 19 (CYFRA 21-1) and CEA were higher in EBC in patients with lung cancer [16]. In a study comparing biomarkers in bronchoalveolar lavage and EBC, the biomarker values (such as hydrogen peroxide, nitric oxide, and 8-isoprostane) obtained in EBC were found to be higher than BAL. This is because the biomarkers are diluted due to the isotonic liquid used while making BAL. Besides, inflammation occurs due to bleeding and irritation during the procedure, which affects the results [17]. The results obtained in our study show that there is detectable DNA in the fluid in both the cancer group and the control group. The fact that the amount of fluid

obtained in the cancer group was less may be related to the shorter EBC collection duration in the cancer group compared to the control group. On the other hand, although the mean DNA level detected in EBC did not differ statistically significantly from the control group, the DNA level was found to be approximately two times higher than in the non-cancer group. This can probably be explained by the pathological changes at the cellular level in the lungs during cancer development. However, it may also be due to a relative decrease in the volume of EBC in the non-cancer group. In cancer cases, when the groups with and without endobronchial lesions were compared, the amount of DNA was less in those with endobronchial lesions, contrary to expectations. This result suggests that the amount of DNA observed in EBC may be related to the level of DNA released from cells spilled into the airways rather than the tumor localization in patients. Indeed, during cancer development, the inflammatory response to environmental harmful gases and particles occurs not only in the area where the tumor arises but in all lung areas. Thus, the demonstration that inflammatory events in the lungs increase in parallel with the progression of COPD in pathological samples obtained from patients with COPD supports this hypothesis [18].

The fact that no correlation was found between the amount of DNA in the cancer group and EBC collection duration, the amount of sample acquired, and the expiratory air volume supports this view. On the other hand, in the non-cancer group, a positive correlation was found between the amount of DNA and EBC collection duration. Then, it can be thought that the source of DNA in EBC is mainly pathological changes due to diffuse inflammatory response rather than localized lesions in the lungs.

Molecular markers such as p53 and K-ras mutations in lung carcinoma may indicate cancer development in high-risk individuals. The p53 mutation is detected in 50–80% of NSCLC patients [2] and identifies premalignant lesions [19]. Genetic mutations can be detected in tissue, but bronchial biopsies are not suitable for screening because they require bronchoscopy. EBC, on the other hand, is a non-invasive test obtained with normal breathing for approximately 10 minutes. It is collected by passing through the cooled condenser and is mostly water. It also contains airway surface fluid at low concentrations and different molecules originating from the alveoli [20,21]. It is thought that EBC can provide information about the cancer development process in the airways. In the study of Gessner et al., DNA was amplified by PCR, and p53 mutation was investigated in EBC obtained from NSCLC patients and healthy subjects. While mutations were detected in 36.4% of the patients, no mutations were detected in the healthy patients. P53 mutation has been demonstrated in those with both peripheral and central lesions. Similar rates were found in adenocarcinoma and squamous cell cancer [5]. An interesting finding is that the same mutation could not be shown in the DNA of the tumor tissue in those with p53 mutation in EBC. This can be explained by the view that different genetic profiles can be found in the neoplastic process [22]. K-ras gene mutations have also been investigated in EBC in patients with lung cancer, and the presence of these mutations has been demonstrated [23]. Similarly, a higher rate of microsatellite instability has been demonstrated in NSCLC patients compared to the control group [24,25]. Microsatellite changes occurring in tumor suppressor genes play an essential role in tumor formation. It was found that microsatellite changes in DNA obtained from EBC show a very strong correlation with prognosis in NSCLC patients [6]. In the study in which 3p microsatellite changes in EBC and whole blood of NSCLC patients and healthy subjects were compared, microsatellite changes were detected in the NSCLC group in 53% and 10% in blood DNA ($p<1/1000000$). In the healthy group, 13% values were obtained in EBC-DNA and 2% in blood DNA. Smoking was found to be directly related to the number of microsatellite changes in EBC-DNA in NSCLC patients. In conclusion, EBC-DNA was highly sensitive in detecting microsatellite changes, and it was thought that it could be used in lung carcinoma screening and early diagnosis programs [24].

In another study of Carpagnano et al., 3p microsatellite changes in EBC, blood, and lung tissue were compared. It was shown that the DNA profile in EBC reflects the DNA in the tissue, and microsatellite changes in the NSCLC group are more frequent than in healthy subjects [25]. Carpagnano et al. concluded that the increased number of microsatellite changes detected in EBC in NSCLC might be a poor prognostic factor for patients [6].

In recent studies, it was suggested that the examination of EBC might be helpful in the follow-up and treatment of many respiratory diseases, especially asthma, COPD, cystic fibrosis, bronchiectasis, acute lung injury, and acute respiratory distress syndrome (ARDS) [26]. Currently, EBC has been found beneficial in the diagnosis of COVID-19 in patients with negative nasopharyngeal sampling but with high clinical suspicion by the possibility of sampling from the lower respiratory tract [27]. The limitations of our study are that it was a single-center study conducted between 2009 and 2010. It is necessary to continue the studies by expanding the patient sample.

## Conclusion

Since EBC samples contain DNA material at a level that can perform genetic examinations, it is considered a valuable and non-invasive method that can contribute to screening risk groups and/or the follow-up of diagnosed cases and evaluating their prognosis. If EBC is developed to detect many inflammatory and genetic markers simultaneously in the future, it may become a method that can be used frequently in the diagnosis and follow-up of diseases, similar to the pulmonary function test. Hereafter, in groups at risk for lung cancer, it may be possible to detect lung cancer while it is still in the preneoplastic stage by performing DNA analyzes in EBC for screening purposes. Thus, early diagnosis can be made, the disease can be treated before it progresses, and genetic treatments can be developed.

*Conflict of Interest: The authors declare that they have no conflict of interest.*
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