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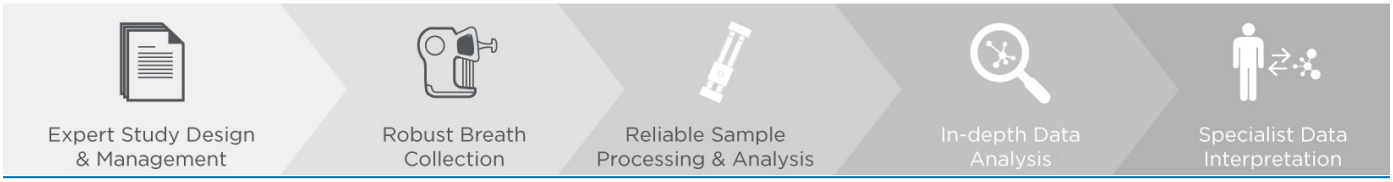
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cfDNA in exhaled breath condensate (EBC) and contamination by ambient air: toward volatile biopsies

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Altug Koc[1,2,3,6], Tuncay Goksel[4], Levent Pelit[5], Korcan Korba[5], Tugberk N Dizdas[5], Ertan Baysal[5], Umut C Uzun[5], Ozge Ozer Kaya[3], Berk Ozyilmaz[3], Yasar B Kutbay[3], Taha Resid Ozdemir[3], Ozgur Kirbiyik[3], Kadri M Erdogan[3], Merve Saka Guvenc[3], Gizem Calibasi Kocal[1] and Yasemin Basbinar[1]

[1] Department of Translational Oncology, Institute of Oncology, Dokuz Eylul University, Izmir, Turkey
[2] Department of Medical Genetics, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey
[3] Genetic Diagnosis Center, Izmir Tepecik Training and Research Hospital, Health Sciences University, Izmir, Turkey
[4] Department of Pulmonary Medicine, Faculty of Medicine, Ege University, Izmir, Turkey
[5] Department of Chemistry, Faculty of Science, Ege University, Izmir, Turkey
[6] Author to whom any correspondence should should be addressed

E-mail: draltug@gmail.com **and** altug.koc@deu.edu.tr

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Abstract

Exhaled breath is a source of volatile and nonvolatile biomarkers in the body that can be accessed non-invasively and used for monitoring. The collection of lung secretions by conventional methods such as bronchoalveolar lavage, induced sputum collection, and core biopsies is limited by the invasive nature of these methods. Non-invasive collection of exhaled breath condensate (EBC) provides fluid samples that are representative of airway lining fluids. Various volatile and nonvolatile biomarkers can be detected in volatile condensates, such as H_2O_2, nitric oxide, lipid mediators, cytokines, chemokines, DNA, and microRNAs. Studies have examined cell-free DNA (cfDNA) in plasma samples from non-small-cell lung cancer patients, offering to new insights and fostering development of the liquid biopsy. However, few studies have examined cfDNA in EBC samples. This study examined whether EBC is an appropriate source of cfDNA using housekeeping-gene-specific primer probes and quantitative real-time polymerase chain reaction in healthy subjects. Ambient (room) air is contaminated with DNA, so caution is needed. Preliminary studies indicated that volatile biopsies are becoming an important diagnostic tool in lung cancer.

1. Introduction

Therapies targeting somatic genetic alterations (driver mutations) of tumor tissue have opened a new era in clinical practice. However, genotype evolution as cancer progresses dictates continuous monitoring, which is difficult using conventional solid tissue biopsies. The solution is liquid biopsies. Exhaled breath condensate (EBC), also called volatile condensate, is a biological matrix that provides non-invasive access to lung epithelial lining fluid. The exhaled breath is cooled in a suitable condenser system during sample collection from the lungs. Different custom-made glass [1] and commercial devices, such as the RTube breath condensate collector [2], EcoScreen condenser system [3], TURBO-DECCS portable condensate device [4], and Anacon breath condenser device [5], have been designed for collecting EBC.

EBC is a biomarker-containing matrix that is equivalent to biological samples such as urine, sweat, tears, saliva, and blood [6]. The relationships between EBC and numerous diseases, including asthma, allergic rhinitis, cystic fibrosis, and chronic obstructive pulmonary disease, have been studied [7–9]. The major component of EBC is condensed water vapor, and only a small fraction of the condensate originates from respiratory droplets containing volatile and non-volatile molecules [10]. Adenosine, hydrogen peroxide, nitrogen oxides, ammonia, protons, and various ions have been found in EBC [11–13]. Recently, DNA and mutation analysis using EBC have attracted increasing attention [14].

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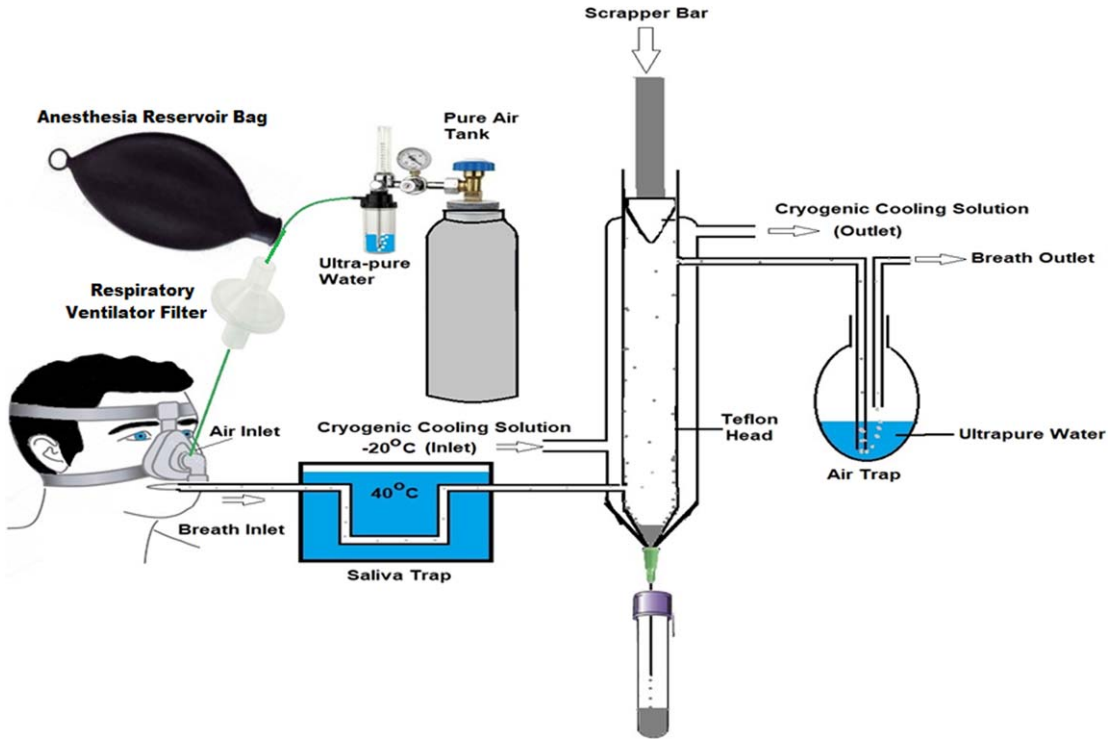


Figure 1. The EBC collection system.

Plasma-derived cfDNA is easy to access. The investigation of driver somatic *EGFR* mutations in plasma-derived cfDNA from non-small-cell lung cancer (NSCLC) patients has become a routine part of patient follow-up [15]. In addition to blood plasma, EBC is a potential source of tumor DNA [16, 17]. Recently, patients with *EGFR* mutations detected in breath samples have been reported [18, 19]. In some cases, volatile condensates are superior to plasma, and it has been proposed that EBC may be of particular benefit in metastatic disease confined to the chest, an area where the plasma sensitivity is lowest [20]. Hotspot mutations of cancer-related genes other than *EGFR* have been detected using next-generation sequencing, including *APC*, *BRAF*, *DDR2*, *ERBB4*, *FBXW7*, *FGFR1*, *FGFR3*, *KRAS*, *MAP2K1*, *MET*, *NRAS*, *PIK3CA*, *PTEN*, *RET*, *SMAD4*, *STK11*, *TP53*, and *p16* mutations [21–23]. Microsatellite alterations have also been investigated in patients with NSCLC [24–26] or idiopathic pulmonary fibrosis [27]. Besides cancer, clinical investigations targeting obstructive lung disease and bacterial DNA in EBC have been reported [28, 29]. To our knowledge, however, there is no standardized approach for extracting DNA from EBC. We hope to contribute to the literature on basic genetic investigations.

2. Material and methods

2.1. Study group

The study group consisted of 70 healthy males and females (35 each), 20–50 (mean 25) years old. The control condensate sample group included condensates of 15 ambient air and 10 filtered dry air samples. The control condensates were collected among the study samples, and the genetic laboratory did not know the source of the samples. The genetic investigation also included four positive DNA samples (positive template control; PTC) and four negative samples (negative template control; NTC).

2.2. EBC sample collection

The EBC samples were collected using a custom-made jacketed cooling borosilicate pipe (figure 1), which allows the non-invasive collection of condensable components of expired air. The condenser was 1.0 m long with an inner diameter of 3.0 cm. The expired air was condensed at –20 °C using a cryostat (Teknosem, THS-55).

Subjects came in the morning and were instructed to refrain from smoking, eating, or drinking for at least 8 h before EBC collection. The subjects rinsed their mouths with water before sample collection. Subjects were inhaled moistened dried air during the EBC collection step. For this purpose, pure dry air was moistened by passing through ultra-pure water. After that, some part of the moistened air was also stored in a reusable anesthetic bag to provide the excessive air demand of subjects. The humidified air was then passed through a single-use respiratory ventilator filter to avoid any contamination during the inhalation process. Then, the subjects breathed pure air via a nasal mask (KNGMED Medical, Turquoise nasal CPAP Mask) for 5 min. The subjects were asked to inhale

pure air through the nose and to exhale exclusively through the mouth into a mouthpiece connected to a U-shaped thermostatic glass trap (+40 °C) that also prevented saliva contamination via an integrated saliva trap (figure 1). If they felt saliva in their mouth, they were instructed to swallow it. They were asked to breathe at a normal frequency and tidal volume for 15 min. The EBC on the inner surface of the condenser's glass wall was swept from top to bottom with a Teflon scraper bar into a tube containing K_2EDTA (BD Vacutainer® EDTA tubes, USA). Approximately 1–1.5 ml of EBC was collected, and the total volume of EBC-containing solution in the tube was 3.5–4 ml. Then, the EBC sample was centrifuged at 1100 × *g*, and the supernatant (2 ml) was stored at –80 °C until cfDNA isolation.

Condensed samples of both ambient air and humidified pure air were collected for comparison with EBC samples. After passing through the filter, humidified air was directly transferred to the EBC condenser for the condensation of humidified air. On the other hand, ambient air was directly transferred to EBC condenser by a vacuum pump without using any filter for the condensation of ambient air. Sample collection step in filtered humidified pure air and ambient air were carried out until approximately 1.5 ml of sample volume was obtained.

2.3. Cleaning the EBC condenser

The glass EBC condenser was cleaned before and after collecting each sample. The EBC condenser was soaked in 1.0% (m/v) sodium hypochlorite solution for 15 min and then flushed three times for 10 min with ultra-pure water. Finally, the inner surface of the EBC condenser was dried completely by passing hot (200 °C) pure nitrogen through it for 5 min. The inner part of the glass condenser was isolated from ambient air until the next sample was collected to prevent the condensation of ambient contaminants. When a glass EBC condenser was used repeatedly, no carry-over effects from previous samples were detected.

2.4. cfDNA isolation and quantitative real-time PCR (RT-PCR)

The cfDNA was extracted from the EBC samples using a COBAS® cfDNA Sample Preparation Kit (Roche: 07247737190) according to the manufacturer's protocol and kept at –20 °C. For the RT-PCR procedure, the cfDNA was investigated using a COBAS z480 (Roche). Four housekeeping genes were studied using specific primer–probe sets: NGF (Chromosome 1), MYBPC3 (Chromosome 11), TP53 (Chromosome 17), and NAGK (Chromosome 2). The primers and probes (table 1) were designed using Primer3 and the PrimerQuest Tool (IDT).

They were checked using the blastn and UCSC *in silico* PCR tools. qPCR was performed using the manufacturer's recommended conditions and Roche LightCycler 480 Probes Master (04707494001), and 10 μl of cfDNA was measured in 25 μl final reaction volumes by qPCR in a 96-well optical PCR plate using a COBAS z480 instrument (Roche, USA). We prepared four PCR mixes containing 12.5 μl of Probes master mix (Roche, USA), 1 μl (10 μM) of the forward and reverse primers, 0.5 μl of 10 μM TaqMan probes, and 10 μl of the cfDNA sample. The results were analyzed using the absquan second-derivative Ct method.

2.5. Validation of RT-PCR

None of the NTC samples produced PCR curves, indicating that the laboratory environment and genetic test consumables were contamination-free. The respective PTC samples produced PCR curves for the *NGF*, *MYBPC*3, *TP53*, and *NAGK* genes, which showed that the PCR conditions were optimal. The NTC included PCR-grade nuclease-free water instead of sample DNA, and human genomic DNA (G3041 Promega; 2 ng μl^{-1}) was used as a PTC in the qPCR experiments.

3. Results

The measured concentrations of the extracted cfDNA were 0.15–0.8 ng μl^{-1} using a Qubit 4 fluorometer (Thermo, USA) and dsDNA HS Assay Kit (Q32854 Thermo, USA). All four locus-specific primer–probe sets were informative for cfDNA-containing samples. Table 2 summarizes the Ct values and copy numbers of the four housekeeping genes.

cfDNA was found in 81% of the healthy subjects. None of the control samples derived from filtered dry air contained cfDNA, but ambient (room) air was a major source of contamination, and 80% of these samples contained cfDNA. Table 3 shows the distribution of the cfDNA-positive and -negative samples.

4. Discussion

Initial studies demonstrated the concept of investigations using exhaled breath [3]. EBC is an exciting technique for nucleic acid research because of its non-invasive nature and its ability to obtain multiple biochemical volatile condensates simultaneously. There are interesting studies of its use in NSCLC patients with somatic driver mutations and cancer-associated mutations in healthy individuals [11]. These findings led us to explore EBC collection standards and examine whether cfDNA is always found in EBC samples and whether there are DNA contaminants in room air.

The majority of our samples (81%) contained detectable cfDNA. Simple ways to increase the cfDNA content of the condensate may include increasing the duration of exhalation using condensers with specific cfDNA-entrapment techniques. More sensitive

Table 1. Primer and probe sequences and concentrations.

Name	Sequence (5′−3′)	Type	Cont.
NGF-F	TTCTATCCTGGCCACACTGAG	Forward Primer	0.4 μM
NGF-R	TTCTGACTTGCCCTCTCAGGT	Reverse Primer	0.4 μM
MYBPC3-F	TGGTGTACGAGATGCGCGTC	Forward Primer	0.4 μM
MYBPC3-R	TCACCGATAGGCATGAAGGG	Reverse Primer	0.4 μM
TP53-F	TGTCCTTCCTGGAGCGATCT	Forward Primer	0.4 μM
TP53-R	CAAACCCCTGGTTTAGCACTTC	Reverse Primer	0.4 μM
NAGK-F	TGGGCAGACACATCGTAGCA	Forward Primer	0.4 μM
NAGK-R	CACCTTCACTCCCACCTCAAC	Reverse Primer	0.4 μM
NGF-Pr	5′-Fam- TTGCCAAGGTCCTCCCTCTGCAGCT-Tamra-3′	TaqMan Probe	0.2 μM
MYBPC3-Pr	5′-Fam- TCAACGCCATCGGCATGTCCAGG-Tamra-3′	TaqMan Probe	0.2 μM
TP53-Pr	5′-Fam- CAGCCCCCGGCTCCGCTAGA-Tamra-3′	TaqMan Probe	0.2 μM
NAGK-Pr	5′-Fam- TGTTGCCCGAGATTGACCCGGT-Tamra-3′	TaqMan Probe	0.2 μM

Note. Cont., concentration.

Table 2. Mean Ct values and copy numbers of the four housekeeping genes in four PTC samples.

	NGF	*MYBPC3*	*TP53*	*NAGK*
Mean Ct (range)	37.38 (22.15–41.42)	37.70 (22.85–43.76)	36.56 (21.39–40.17)	40.30 (20.66–48.8)
Mean copy number per ml (range)	12.31 (2.16–690.7)	23.77 (1.74–452.5)	12.06 (2.44–455.7)	14.06 (2.03–618.20)

Note. Ct, cycle threshold.

Table 3. Distribution of cfDNA-positive and -negative samples.

	Number of samples	**cfDNA positive**	**cfDNA negative**
Samples of healthy subjects	70	57 (81%)	13 (19%)
Control samples			
Ambient Air	15	12 (80%)	3 (20%)
Filtered Air	10	0	10
Control cfDNA			
NTC	4	0	4
PTC	4	4	0

Note. NTC, negative template control; PTC, positive template control.

detection methods like ultra-deep next-generation sequencing and digital PCR may be preferred.

Air containing DNA fragments may contaminate the condensates. Therefore, use of a face mask and a clean air source should be mandatory for sample collection. Most reports do not mention the contamination risk. Even some commercial EBC collection devices do not include a face mask to keep out ambient air [29]. To our knowledge, neither information on condenser cleaning nor disposable condensers are available. In fact, the contaminated ambient air contained similar amounts of DNA to exhaled breath in 80% of the control samples.

Without optimizing the EBC collecting protocols, the management of patients is impossible. For example, consecutive condensate sampling by inhaling ambient air may cause false-positive results due to the contamination of room air by a previous mutation carrier.

In conclusion, investigating DNA using EBC is a promising approach, but ways to identify the features of DNA fragments in ambient air and strict separation of exhaled breath from contaminant DNA by standardizing sample-collection techniques and using controls (air source, NTC, PTC) are needed before its routine diagnostic use will be feasible.

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ORCID iDs

Altug Koc https://orcid.org/0000-0002-8366-6806
Taha Resid Ozdemir https://orcid.org/0000-0003-4870-6945

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