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## **Exhaled breath condensate A non-invasive source for tracking of genetic and epigenetic alterations in lung diseases**

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**Running title:** Genetic and epigenetic markers in EBC

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## **Abstract**

### **Background**

Lung diseases have been recognized as an extensive cause of morbidity and mortality in the worldwide. The high degree of clinical heterogeneity and nonspecific initial symptoms of lung diseases contribute to a delayed diagnosis. So, the molecular and genomic profiling play a pivotal role in promoting the pulmonary diseases. Exhaled breath condensate (EBC) as a novel and potential method for sampling the respiratory epithelial lining fluid is to assess the inflammatory and oxidative stress biomarkers, drugs and genetic alterations in the pathophysiological processes of lung diseases.

### **Methods:**

The recent studies on the analysis of EBC from both a genetic and epigenetic point of view were searched from database and reviewed.

### **Results:**

This review provides an overview of the current findings in the tracking of genomic and epigenetic alterations which are potentially effective in better management of cancer detection. In addition, respiratory microbiota DNA using EBC samples in association with pulmonary disease especially lung cancer were investigated.

### **Discussion:**

Various studies have concluded that EBC has a great potential for analysis of nuclear and mitochondrial DNA alterations as well as epigenetic modifications and identification of respiratory microbiome. Next-generation sequencing (NGS) based genomic profiling of EBC samples is recommended as a promising approach to establish personalized-based prevention, diagnosis, treatment and post-treatment follow-ups for patients with lung diseases especially lung cancer.

**Keywords:** exhaled breath condensate, non-invasive, lung, genetic, epigenetic, *personalized medicine*

## Introduction

*Current global studies* introduced lung diseases as a major cause of *morbidity* and mortality worldwide.<sup>1,2</sup> Despite impressive advances in 5-years survival rates of other types of cancer, that of lung cancer remains low at 15.6% (compared to 66% for colon cancer, 90% for breast cancer, 94% for melanoma, and 100% for prostate cancer).<sup>3</sup> Lung cancer patients usually are late diagnosed because of non-specific symptoms which are mistakably attributed to aging or smoking. Therefore, available screening tools such as sputum cytology, chest X-ray, etc. failed to decrease the mortality rate. There are also significant overlaps between clinical manifestations of lung cancer and chronic respiratory diseases (CRDs).<sup>4, 5</sup> Patients with some lung diseases such as chronic obstructive pulmonary disease (*COPD*), idiopathic pulmonary fibrosis (IPF) and tuberculosis (TB) are at increased *risk* for the development of *lung cancer*.<sup>6-9</sup>

*The survival rate tends to be low because most lung carcinomas are diagnosed at an advanced tumor stage so, early diagnosis is the main goal to improve survival rate in lung cancer.*<sup>10</sup> Both radiological imaging (CT, PET) and histological examination (bronchoscopy, thoracic needle aspiration or thoracentesis) are expensive and invasive procedures for population screening for lung diseases.<sup>11</sup> Due to the risk and discomfort for the sample donors, the conventional methods are not suitable for early diagnosis of lung disease especially lung cancer.<sup>12</sup> Within this scenario, the access to noninvasive, efficient, repeatable, cost-effective and simple sources of genetic material is one of the major necessity which allows to perform *screening and follow-up tests regularly and widespread for at-risk individuals*.

Exhaled breath condensate (EBC) is a novel and potential method for sampling the respiratory epithelial lining fluid. EBC used to assess the inflammatory and oxidative stress biomarkers, drugs and genetic alterations in the pathophysiologic processes of lung diseases.<sup>13-15</sup> The compounds found in EBC could be classified into two groups of volatile and non-volatile compounds according to their boiling point and consequently their analytical follow-up tools. Volatile compounds are analyzed by gas chromatography equipped with detection mainly mass spectrometer. Non-volatile compounds could be analyzed using a liquid chromatography or other instrumental analytical devices. Both of these compounds are originated

from exogenous sources - inhaled air which contains environmental volatiles and also microparticles, *etc.*- , endogenous sources - compounds penetrated from serum or other biological fluids into lung lining fluid- and also from metabolism of the lung, oral or nasal micro-organisms.<sup>16-18</sup> The main problem with the analysis of the compounds in EBC is their high dilution (2000-10000 folds) with water vapor which is condensed in the EBC collection setup.<sup>13, 19</sup>

EBC attracted more attention in recent years and a number of published works reviewed its applications in various fields. Khoubnasabjafari *et al*<sup>13</sup> reviewed the main advantages and disadvantages of EBC as an alternative biological sample and its potential applications in drug monitoring studies. More detailed methodological aspects of the analysis of volatile<sup>20</sup> and non-volatile<sup>21</sup> compounds are also provided recently. Davis and his colleagues reviewed the potentials of EBC testing in clinical research with the main focus of its applications in pediatrics.<sup>17</sup> Dent *et al*<sup>22</sup> reviewed various aspects of EBC analysis of EBC for diagnosis of lung cancer. Lim and Thomas reviewed the findings until 2013 in the area of EBC analysis in patients with COPD or lung cancer.<sup>23</sup> Inage *et al*<sup>24</sup> reported the latest findings on early detection of lung cancer which includes a very brief review on EBC.

The successful tracking of *Tp53* gene mutations in EBC samples by Gessner opened new insights into many interesting future applications from the genetics point of view.<sup>25</sup> Followed by the increasing focus on this topic, researchers have *attempted* to develop an effective framework for genetic investigation using EBC samples over the last decade. As several studies in recent years have shown, the EBC is a *potentially valuable source* in the detection of genetic and epigenetic alterations such as *KRAS*, *EFGR* mutations and microsatellite *instability in lung cancer*<sup>26-30</sup>. *Genetic data produced via next-generation sequencing of the EBC samples provides significant benefits to defining genetic architecture of lung diseases. The synchronous analysis of the metabolites such as lipid and protein as well as the nucleic acid in exhaled breath can be so promising for disease early diagnosis, and monitoring the treatment efficiency of respiratory diseases such as lung cancer.*<sup>31, 32</sup>

The main purpose of this review is to summarize recent achievements in the analysis of EBC from the genetics point of view. Obviously, the molecular analysis of EBC can be a surrogate and promising *technique* for tracking genetic alterations and molecular profiling in lung diseases. Certainly, the molecular analysis of the patients in regular intervals can improve our current molecular knowledge about the multistep/multigene model of carcinogenesis in lung cancers. Also, optimization of EBC-based methods can take the opportunity for individuals to benefit from early diagnosis, regular screening and follow-ups. Alternatively, bio information obtained from EBC can be used in personalized medicine for *relevant treatment decisions* based on each patient's unique genetic makeup.

### **Search strategy**

In this review, the following electronic databases were searched in order to identify all published studies up to November 2019: PubMed, Embase, ProQuest, and Cochrane. Following the database search, all identified studies were loaded into Endnote Version X, and duplicates were removed. The titles and abstracts of the remaining studies were screened on the basis of title, abstract, and keywords, exhaled breath condensate and genetic and epigenetic features were included.

### **Human exhaled breath composition**

Human breath contains several volatile, nonvolatile substances and bio aerosols with various origins. Some of them are inhaled as atmospheric pollutants or ingested as food and drugs, others produced and metabolized by human cells or colonized by micro-organisms in the airways.<sup>18, 33</sup> The composition of these compounds varies between individuals.<sup>34</sup> Figure 1 shows the composition of human exhaled breath. Exhaled breath contains thousands of volatile such as oxygen, carbon dioxide, nitric oxide. Profile analysis of volatile organic compounds (VOCs) in breath can reveal crucial information that specifically related to a disorder. There is a significant association between some various VOCs with pathological states of systemic diseases including diabetes mellitus, cystic fibrosis, asthma, COPD and lung, breast, colon, prostate cancers.<sup>35-38</sup> Inorganic compounds particles in EBC play pivotal roles in biological

processes. For instance, hydrogen sulfide *has* been recognized as a biologically *signaling* molecule in synaptic transmission, vascular tone, and angiogenesis. *Also this* molecule together with the other volatile substances nitric oxide and carbon monoxide act as an anti-inflammatory transmitter in respiratory diseases.<sup>39-41</sup>

In addition, EBC contains several non-volatile organic compounds e.g., cytokines, leukotrienes, surfactant, prostaglandins, interleukins, nucleic acids, bacteria, viruses, drugs and etc., which are diluted in large amounts of water vapor.<sup>21, 42-45</sup> *It is* important to note that *some* non-volatile compounds such as pepsin, trimethylamine, and carcinoembryonic antigen *are* only found in the EBC samples from diseased *but not in healthy individuals*.<sup>46-48</sup> *Interestingly, there is evidence to suggest that EBC contains elements from the lower respiratory tract*.<sup>49</sup> *Figure 2 shows the conceptual illustration of bronchiole fluid film burst (BFFB) model, is currently the most accepted model for the mechanism of breath aerosol formation in the lower bronchioles following exhalation*.<sup>50, 51</sup>

### **EBC collection devices**

Condensation of exhaled breath is a possible approach to measure and analysis of compounds in the airway lining fluid. *Several commercially-available and lab-made sampling devices have been used by various research groups*. The rapid cooling of exhaled breath is the general basis for all of sample collection devices. The schematic illustration of the basic components of the EBC collection devices is shown in Figure 2. However, the importance of proper selection of sampling device is not negligible because there it has been proved that sampling temperature and coating material of sample collection tube -silicone, glass, aluminum, polypropylene, and Teflon- can affect the biomarkers' concentration in EBC.<sup>52</sup> The sample is held by surface tension to the condenser wall, so adhesive properties of condenser coatings can determine ideal instruments for analysis of each marker.<sup>53</sup> The Eco Screen (Erich Jaeger, Hoechberg, Germany) is the most common exhaled breath sampling device for *genetic and epigenetic studies using EBC sample* according to the recent publications (Table 1). This

device contains a saliva trap, which is useful for protecting the collected samples from contaminations and degradation of DNA by *salivary* DNase.<sup>16, 54</sup> In addition, there are several commercial and lab-made sampling devices such as RTube™, Turbo-DECCS and infant or children specific sampling device.<sup>49, 55-60</sup>

**Table 1.** Sampling, storage conditions and extraction methods.

Category	Collection device	Duration of collection (Minutes)	Storage temperature (°C)	Nucleic acids extraction method/kit	References
<b>Nuclear genes</b>	EcoScreen	20	NR	Phenol-chloroform method	25
	RTube™	15	-80	NR	30
	EcoScreen	25	NR	Guanidium-thiocyanate method	73
	EcoScreen	25	NR	ReliaPrep™ FFPE gDNA Miniprep System	28
	EcoScreen	20	-80	QIAamp Circulating Nucleic Acid Kit	75
	EcoScreen	15	-70	QIAamp Circulating Nucleic Acid Kit	31
<b>Microsatellite instability</b>	EcoScreen	20	-70	QIAamp DNA Mini Kit	81
	EcoScreen	20	-70	QIAamp DNA Mini Kit	82
	EcoScreen	20	-70	QIAamp DNA Mini Kit	84
	EcoScreen	NR	-70	QIAamp DNA Mini Kit	88
	EcoScreen	20	-80	QIAamp DNA Mini Kit	86
	EcoScreen	20	-70	QIAamp DNA Mini Kit	27
<b>Mitochondrial genome</b>	Custom-made glass condenser	20	-80	QIAamp DNA Mini Kit	29
	EcoScreen	NR	-70	Concentrated with Microcon-30 kDa Centrifugal Filter Unit	26
<b>DNA methylation</b>	RTube™	10-15	-20	QIAamp DNA Mini Kit using oligo dT	64
	EcoScreen	15-20	-70	QIAamp DNA Mini kit	98
<b>MicroRNAs</b>	TURBO-DECCS system	15	-80	miRVana miRNA isolation kit (Life Technologies)	105
	RTube™	10	NR	miRVana small RNA extraction kit (Ambion)	104
	EcoScreen	Over 20	NR	TRIzol reagent	106
	NR	NR	NR	NR	103
<b>Respiratory microbiome</b>	EcoScreen	20	-70	QIASymphony	108
	EcoScreen	NR	-70	QIASymphony	111

NR, not reported; gDNA, genomic DNA

## Sampling procedures and storage

EBC sample must be collected during normal resting tidal breathing (RTB) of subjects at room temperature, using a nose clip. The small droplets of the lung lining fluid and *breathing water vapor* are condensed to a liquid or solid phase depending on the condenser temperature.<sup>61</sup> Temperature is a potent stimulus for controlling salivary DNase activity so EBC samples should be kept on ice *immediately then frozen stored* at or below  $-20^{\circ}\text{C}$  until extraction (Table 1).

As mentioned above, the collection device, collector surface, salivary trapping ability, sample volume, sampling temperature, storage temperature, duration of storage and other study variables can affect the quality of investigated markers in the collected samples. Therefore, standardization of EBC sampling condition in uniform style is not recommended and it depends on the purpose of the *studies*.<sup>61</sup>

## Nucleic acids content and DNA extraction from EBC samples

Several *papers* have explored the presence of genomic DNA, RNA and mitochondrial DNA in the body fluids. So investigation of circulating cell-free nucleic acids *as biomarkers* in body fluids is an area of increasing interest in molecular pathology.<sup>62</sup> Apoptosis, necrosis or spontaneously releasing of the nucleic acids from pulmonary cells as a result of oxidative stresses, are the main phenomenon responsible for the presence of nucleic acid molecules in the exhaled breath of individuals.<sup>63</sup>

There are several commercial kits from numerous companies and standard protocols to *isolate* circulating *cell-free nucleic acids*. As shown in Table 1, QIAamp circulating nucleic acid kit and QIAamp DNA Blood Mini Kit have been used frequently in most of the EBC based research projects. Gessner *and colleagues* used the standard *phenol-chloroform* method for circulating DNA extraction, moreover, they confirmed that there is no difference between using extracted DNA from EBC and native breath

condensate sample in the efficiency of polymerase chain reaction (PCR)<sup>25</sup>. In addition, using DNA carriers such as oligo-dT can enhance template recovery and DNA yield.<sup>64</sup>

### ***Tracking of genetic and epigenetic alterations in free circulating nucleic acids from EBC***

*Lung carcinogenesis process is driven by the accumulation of genetic mutations and epigenetic deviations in the respiratory epithelium that lead to dysregulation of cancer driver genes. Epigenetic alterations, including chemical modifications of the DNA and histones changes in histone variants and non-coding RNA expression, play the pivotal role in the lung cancer pathogenesis.<sup>65</sup> Exhaled breath is a particularly promising noninvasive source of circulating nucleic acids for lung cancer screening. This review provides an overview of the current findings in the tracking of genomic and epigenetic alterations as well as respiratory microbiota DNA using EBC samples in association with pulmonary disease especially lung cancer (Table 2).*

**Table 2.** Genetic and epigenetic analysis of lung diseases using EBC

<b>Category</b>	<b>Disease(s)</b>	<b>Target</b>	<b>Detection method</b>	<b>References</b>
<b>Nuclear genes</b>	NSCLC	<i>TP53</i> mutations	Nested PCR, sequencing	25
	Squamous cell lung cancer	<i>EFGR</i> mutations	PCR	30
	NSCLC, benign lesions	<i>KRAS</i> mutations	mutant-enriched PCR	73
	NSCLC	<i>KRAS</i> mutations	mutant-enriched PCR	28
	NSCLC	<i>PI6</i> mutations	PCR, sequencing	75
	NSCLC, SCLC	504 hotspot mutations (including 22 genes)	NGS	31
<b>Microsatellite instability</b>	NSCLC	3p MAs	PCR	81
	NSCLC	3p MAs	PCR	82
	NSCLC	19q MAs	fluorescent PCR	84
	MPE	3p, 12p, 5q and 17p MAs	PCR	88
	IPF	8p, 17q MAs	PCR	86
	NSCLC	3p, 5q and 15q MAs	PCR	27
<b>Mitochondrial genome</b>	NSCLC	mtDNA mutations	PCR and capillary sequencing	29
	Asthma, COPD	levels of mtDNA/nDNA	real Time PCR	26

Category	Disease(s)	Target	Detection method	References
	and ACOS			
DNA methylation	NSCLC, SCLC	promoter methylation of <i>DAPK</i> , <i>RASSF1A</i> and <i>PAX5β</i> genes	multiplex PCR, (tBGS)	64
	NSCLC	<i>P16</i> methylation	F-qMSP	98
MicroRNAs	NSCLC	miR-21, miR-486	quantitative PCR	105
	Asthma	Full miRNome	quantitative PCR	104
	Asthma, COPD	miR-1248, Let-7a, miR-328, miR-133a, miR-21, miR-1291, miR-155	quantitative PCR, sequencing	106
	Asthma, COPD	pool of microRNAs	real-time PCR	103
Respiratory microbiome	NSCLC, SCLC	HPV DNA	PCR, pyrosequencing	108
	NSCLC, SCLC	EBV, CMV DNA	real-time PCR	111

NSCLC, non-small-cell lung carcinoma; SCLC, small cell lung cancer; MPE, malignant pleural effusion; IFP, idiopathic pulmonary fibrosis; COPD, chronic obstructive pulmonary disease, ACOS, asthma COPD overlap syndrome; MAs, microsatellite alterations; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; miR, micro RNA; HPV, human papillomavirus; EBV, Epstein-Barr virus; CMV, cytomegalovirus; PCR, polymerase chain reaction; NGS, next-generation sequencing; tBGS, tag-adapted bisulfite genomic DNA sequencing; F-qMSP, fluorescent quantitative methylation-specific polymerase chain reaction.

## Genomic alterations

Genomic alterations are permanent changes in the DNA gene sequence. Based on our current knowledge, genomic alterations are associated with susceptibility, initiation and progression of lung diseases such as COPD, IPF, cystic fibrosis (CF), and asthma and lung cancer.<sup>65-69</sup> So, tracking of these genomic alterations using efficient and noninvasive methods can be useful in the prevention, diagnosis, treatment, management and genetic counseling of individuals. *Several studies have examined the efficiency of EBC application in detection of genomic alterations. We summarized EBC applications in the tracking of genomic alterations in four categories including nuclear genes mutations, microsatellite instability, mitochondrial genome, and non-coding RNAs.*

- *Nuclear genes mutations*

Due to the emerging picture of the *genetic architecture of lung cancer*, researchers have mainly carried out studies on the detection of gene mutations in lung carcinoma using EBC samples. The tumor suppressor *TP53* is the most frequently mutated *gene* in lung cancer.<sup>70</sup> In 2004, EBC-DNA sequencing of mutational hotspots of *TP53* gene, exon 5-8, performed by Gessner and colleagues in lung cancer patients. In addition, they confirmed the effectiveness of PCR for the human beta-actin gene fragment in native as well as extracted EBC-DNA samples.<sup>25</sup>

Activating mutations in the epidermal growth factor receptor (*EGFR*) gene are common in lung cancer. The recent clinical evidence indicated the *effectiveness* of erlotinib monotherapy in the treatment of advanced non-small cell lung cancer by inhibition of the intracellular phosphorylation of tyrosine kinase associated with the EGFR.<sup>71</sup> The parallel analysis of PCR product in EBC and cancer tissue from a heavy smoker with squamous cell lung cancer led to the detection of deletion within exon 19 of *EGFR* gene.<sup>30</sup>

*KRAS* oncogene is also frequently mutated in the vast majority of patients with adenocarcinoma.<sup>72</sup> Kordiak and colleagues was performed a large-scale study for tracking of *KRAS* mutations in EBC-DNA using mutant-enriched PCR technique. There is a promising potential for determination of *KRAS* mutations using the EBC samples in the follow-up after surgical treatment of lung cancer.<sup>73</sup> Despite intratumor heterogeneity and inhomogeneous distribution of *KRAS* point mutations, the results of a recent study indicated that there was a high accordance between *KRAS* mutation status in EBC–DNA and cancer tissue in lung cancer patients. So, they suggested the usefulness of tracking *KRAS* mutations in EBC–DNA as a biomarker of pulmonary malignancy.<sup>28</sup>

p16 is a tumor suppressor protein with an independent prognostic value in lung cancer. It plays a vital role in regulating the cell cycle by *down-regulation of cyclin D–dependent protein kinases*.<sup>74</sup> Several substitution mutations were detected in the *p16* gene by sequencing of exons 1 and 2 in EBC-DNA samples of 58 patients with non-small cell lung cancer.<sup>75</sup>

*Next-generation sequencing (NGS)* technologies have begun to revolutionize our molecular knowledge about complex diseases.<sup>76</sup> In recent years, the *success* rate of *NGS* testing on EBC-DNA samples have opened a *new* era of genomics and molecular biology in lung cancer.<sup>31, 32</sup> Obviously, these findings could provide a promising non-invasive method for the determination of the genetic profiles of each tested individual.

- ***Microsatellite instability***

*Microsatellites* are short (1–6 bp) repeat motifs which present in both coding and non-coding regions of the genome. The major factors including, *motif length*, *motif region*, *motif composition* and the number of repeats can influence the rate of mutability in specific microsatellites.<sup>77</sup> The results of several studies have *confirmed the pivotal role of* microsatellite alterations (MAs) in the pathogenesis of benign pulmonary disease such as asthma, IPF, and COPD as well as *malignant lung tumors*.<sup>78-80</sup> *During the last decade, Carpagnano and colleagues have published a series of papers on the feasibility of using EBC in the detection of MAs. The analysis of microsatellite instability in relation to clinic-pathological characteristics and survival rate in lung cancer patients confirmed the prognostic value of exhaled microsatellite alterations at 3p and 19q.*<sup>81-85</sup> In another study, they have designed a panel using eight polymorphic microsatellite markers on chromosome 3p, 5q and 15q for detection of MAs in EBC for assessing survival in lung cancer patients.<sup>27</sup> Additionally, they have demonstrated the association between exhaled MAs and susceptibility to other benign lung diseases such as IPF, asthma and atopy.<sup>86, 87</sup> Another study has highlighted the considerable advantage of *combined cytological and circulating cell-free DNA analysis to improve the sensitivity of MAs detection in patients with malignant pleural effusion*.<sup>88</sup>

- ***Mitochondrial genome***

The pulmonary diseases are associated with chronic inflammation and imbalance of oxidant/antioxidant due to exogenous reactive oxygen species (ROS). Lung diseases linked with higher production of ROS result in oxidative stress and imbalances of pro-inflammatory cytokine which trigger cancer development by induced oxidative DNA damage, uncontrolled cellular proliferation, and apoptosis. Maintaining an optimal level of ROS in cells is essential for normal oxidative metabolism.<sup>89, 90</sup> Mitochondria recognized as the main cellular source of ROS, so mitochondrial dysfunction due to mutations or changes in mitochondrial DNA levels are associated with *elevated levels* of ROS.<sup>91</sup> These reasons can explain the importance of mitochondrial analysis as a vital component of medical research. The results of mutation analysis of mitochondrial DNA in EBC using a PCR sequencing approach indicated a *significant increase in D-loop* mutation rate in the lung cancer group compared to the control. The germline polymorphisms were excluded using parallel saliva sample analysis.<sup>29</sup> In another new study, the ratio between mitochondrial and nuclear DNA (mtDNA/nDNA) in EBC of patients with obstructive lung diseases - asthma, COPD and asthma-COPD overlap syndrome (ACOS) - was measured in comparison with blood samples by real-time PCR. The results showed the levels of mtDNA/nDNA in the EBC are increased in the investigated patients compared to healthy subjects. The parallel measurement of exhaled confirmed increasing nitric oxide (NO) level as a *marker of oxidative stress* in patients. The validation of mtDNA/nDNA ratio by comparing the results from EBC with paired blood samples demonstrated there are no significant differences. This positive correlation indicated that EBC can be a potential alternative for blood.<sup>26</sup> As we know, the imbalance in mtDNA level, as well as mutations, may serve as the potential for prevention, early detection, tracking disease *progression*. Therefore, EBC can propose as a completely noninvasive and promising source for investigation of the mitochondrial genome in the study of lung diseases.

## **Epigenetic alterations**

Epigenetics is the study of heritable changes in gene expression that cannot be explained by changes in DNA sequence.<sup>92</sup> Aberrant *DNA methylation and MicroRNAs expression* has been implicated as a *promising biomarker* in lung diseases such as asthma, atopy, IPF, COPD and lung cancer.<sup>93</sup>

- ***DNA methylation***

DNA methylation is a biochemical process which play critical roles in the *regulation of gene expression* in cells.<sup>94</sup> Accumulating evidence indicates that changes in DNA methylation contribute to the pathogenesis of lung cancer.<sup>95-97</sup> *Reproducible* analysis of the DAPK, RASSF1A and PAX5B genes revealed promoter methylation patterns in EBC from lung cancer patients and healthy controls. Comparison of two different methods, quantitative methylation-specific PCR (qMSP) and tag-adapted bisulfite genomic DNA sequencing (tBGS), confirmed their consentient results <sup>64</sup> Aberrant promoter methylation of the tumor suppressor gene, *p16*, was confirmed as a potential biomarker for diagnosis of lung cancer in a parallel analysis of various samples including cancer tissues, adjacent normal lung tissues, blood plasma, and EBC by fluorescence quantitative methylation-specific polymerase chain reaction (F-MSP).<sup>98</sup>

- ***MicroRNAs***

Non-coding RNAs serve as *post-transcriptional gene expression regulators in mammalian cells*. MicroRNAs are a class of small non-coding RNAs which mediate gene silencing by target mRNA decay or translational repression.<sup>99, 100</sup> *MicroRNAs play a key role* in the *pathogenesis* of lung diseases such as lung cancer, asthma, cystic fibrosis, IPF and COPD.<sup>101</sup> Exosomes are small membrane vesicles that contain proteins, mRNAs and microRNAs. They can release by various type of cells into the body fluids.<sup>102</sup> Analysis of *exosomal* markers (CD9, CD63 and CD81) demonstrated that there are controversial results about the presence of exosomes in EBC.<sup>103, 104</sup> However, the results of the several separate studies demonstrated a successful detection of micro RNAs in EBC by quantitative real-time PCR which can

provide *new clinical insights* into the non-invasive screening, early diagnosis and treatment of lung disease.<sup>103-106</sup>

### **Respiratory microbiome**

There is a positive association between lung microbiome- viruses, bacteria, and fungi- and pathogenesis of lung diseases.<sup>107</sup> *Bronchoscopy is currently the most commonly employed invasive procedure for tracking the changes in composition and diversity of the lung microbiome and thereby EBC can be a noninvasive alternative for lung microbiota sampling. Detection of human papillomavirus (HPV) genome by pyrosequencing in paired samples - EBC and bronchial brushing and neoplastic lung tissue- of lung cancer patients suggested that EBC can be also used for assessing the viral genome.*<sup>108</sup> HPV infection has also been associated with carcinogenesis by several molecular mechanisms, especially HPV *genome integration close to common fragile sites adjacent to oncogenes of the host genome.*<sup>81, 84</sup> *Chromosome 3p microsatellite allelotyping of lung cancer patients from EBC-DNA, blood and bronchial brushing specimens showed the significant prevalence of 3p microsatellite instability in HPV positive than HPV negative patient which might be involved in lung carcinogenesis.*<sup>109</sup>

There are also some promising evidence which suspected role of the Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) in the etiology of human cancers.<sup>88, 89, 110</sup> In another study, *the EBV and CMV DNA levels were detected in EBC samples by real-time PCR assay.*<sup>111</sup>

### **Implementation of NGS of EBC in personalized medicine**

Personalized medicine is a holistic approach for personalization of prevention, diagnosis, prognosis and therapeutic approaches based on current knowledge about the heterogeneity of diseases.<sup>112</sup> The heterogeneous and complex molecular basis of lung diseases makes diagnosis and treatment more

sophisticated. Optimizing the *therapeutic responses* for the individual patient is the long-standing challenge in treatment.

The principle of precision medicine is using genomic profiles of individual's to direct the decision-making process for choosing the right drug at the right dose at the right time for the right period of time.<sup>113</sup> Gene-oriented treatment in precision medicine can change the paradigm of lung disease therapy in asthma, COPD, IPF and lung cancer.<sup>114-117</sup> For example, erlotinib was proven to be the better choice for EGFR mutated lung cancer and crizotinib has shown promising results for the treatment of ALK-positive non-small cell *lung cancer*.<sup>118-121</sup>

High throughput NGS technologies have shown a great potential in detecting genetic mutations with high sensitivity and low allele frequencies.<sup>122</sup> Recently, Youssef and colleagues detected several known and novel hot spot mutations by NGS in EBC from patients with lung cancer and healthy controls.<sup>31, 32</sup> The successful use of EBC as a non- invasive source of testing material for NGS provides a valuable insight into the nature of lung diseases. NGS-based genomic profiling *has given a new perspective to personalized medicine by the screening of high-risk individuals, tracking diagnostic markers for early detection before the development of pathological symptoms and* determination of the most appropriate combination treatments for patients.

## **Concluding Remarks**

The accesses to a noninvasive, readily accessible, cost-effective and repeatable sampling method, EBC, provides a new perspective on the investigation of the genetic architecture of lung diseases. Apoptosis, necrosis and spontaneous cell death are the basic phenomenon responsible for the presence of circulating nucleic acids in body fluids such as EBC. Various studies have concluded that EBC has a great potential to analysis of nuclear and mitochondrial *DNA* alterations as well as epigenetic modifications and

identification of respiratory microbiome. NGS based genomic profiling of EBC samples has potential *applications* for many purposes which recommended it as a promising approach to establish personalized-based prevention, diagnosis, treatment paradigms and post-treatment follow-ups for patients with lung diseases especially lung cancer.

NGS-based genomic profiling analysis of EBC can provide abundant information for understanding molecular mechanisms of lung diseases. Despite the exponential increase in EBC-DNA studies, especially successful NGS based investigations, genomic studies on EBC in lung diseases are still in its infancy. Therefore, we need more time to standardize sampling procedures and solve the basic questions concerning its efficiency in prevention, early diagnosis, and follow-up in comparison with conventional methods. It is anticipated that these findings will discourage use of EBC as non-invasive source for management of lung and other systemic diseases in the future.

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### **Ethical statement**

Not applicable.

### **Conflict of interest**

The authors have no the conflict of interest.

## Authors Contribution

- Somayeh Kazeminasab: Writing and reviewing
- Babak Emamalizadeh: Data handling, writing and reviewing
- Maryam Khoubnasabjafari: Conceptualization, writing, reviewing and supervision
- Abolghasem Jouyban: Project administration and supervision

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