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Combined sputum hypermethylation and eNose analysis for lung cancer diagnosis

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ABSTRACT

Aims The aim of this study is to explore DNA hypermethylation analysis in sputum and exhaled breath analysis for their complementary, non-invasive diagnostic capacity in lung cancer.

Methods Sputum samples and exhaled breath were prospectively collected from 20 lung cancer patients and 31 COPD controls (Set 1). An additional 18 lung cancer patients and 8 controls only collected exhaled breath as validation set (Set 2). DNA hypermethylation of biomarkers *RASSF1A*, cytoglobin, *APC*, *FAM19A4*, *PHACTR3*, *3OST2* and *PRDM14* was considered, and breathprints from exhaled breath samples were created using an electronic nose (eNose).

Results Both DNA hypermethylation markers in sputum and eNose were independently able to distinguish lung cancer patients from controls. The combination of *RASSF1A* and *3OST2* hypermethylation had a sensitivity of 85% with a specificity of 74%. eNose had a sensitivity of 80% with a specificity of 48%. Sensitivity for lung cancer diagnosis increased to 100%, when *RASSF1A* hypermethylation was combined with eNose, with specificity of 42%. Both methods showed to be complementary to each other ($p \leq 0.011$). eNose results were reproducible in Set 2.

Conclusions When used in concert, *RASSF1A* hypermethylation in sputum and exhaled breath analysis are complementary for lung cancer diagnosis, with 100% sensitivity in this series. This finding should be further validated.

INTRODUCTION

As the leading cause of cancer mortality worldwide, lung cancer imposes a major disease burden on society.[1] Clinical presentation of disease occurs mainly at advanced stage, when treatment options are limited, resulting in poor 5-year survival rates, typically <15%.[2] Detection of early stage lung cancer may improve prognosis.[3] Up to now, no suitable screening method exists, although low-dose spiral CT (LDCT) may play a role in future.[4 5] Drawbacks of the latter approach are costs and a high false-positive rate in the National Lung Cancer Screening Trial (NLST).[4] Thus, a need exists for developing novel lung cancer detection methods, which are ideally non-invasive, simple and cost-effective. For the current study, two different methods for non-invasive lung cancer detection were integrated.

DNA promoter hypermethylation, resulting in transcriptional silencing of (tumour-suppressor) genes, is a cell control mechanism that enables cancer cells to become manifest.[6] These epigenetic tumour aberrations have been detected in sputum and can be used to identify lung cancer patients.[7 8] In previous research, we selected and evaluated one diagnostic biomarker (*RASSF1A*) and several risk biomarkers (*APC*, cytoglobin (*CYGB*), *3OST2*, *PRDM14*, *PHACTR3*, *FAM19A4*) in sputum for lung cancer diagnosis.[9 10]

In human breath, thousands of molecular volatile organic compounds (VOC) in gas phase have been identified that are endogenous and exogenous in origin.[11] Exhaled breath analysis is based on VOCs identified by gas chromatography and mass-spectrometry (GC-MS), or alternatively, by pattern recognition using composite nanosensors arrays ('electronic nose' technology; eNose[12]). The composition of VOCs provides information on metabolic processes in the human body, in particular in the lung, and is associated with pathological conditions such as lung cancer.[13–16]

To date, DNA hypermethylation biomarkers in sputum and exhaled breath analysis for detection of lung cancer suffer from insufficient sensitivity when used independently. Since both methods measure status of biological processes on different levels, we hypothesised that these methods may act in a complementary way to detect lung cancer. Hence, the aim of this study was to examine the diagnostic value of DNA hypermethylation in sputum in combination with exhaled breath analysis in lung cancer patients and controls.

MATERIAL AND METHODS

Subjects

This substudy was nested in the sputum study that prospectively collected sputum in a case-control design,[17] and comprised additional prospective collection of exhaled air samples of subjects who also submitted sputum. The substudy included 20 cases and 31 controls (Set 1). A second set (Set 2) consisted of persons (18 cases, 8 controls) of whom only eNose data were available (without collection of sputum). The latter group was used for validation of exhaled breath analysis and hence analysed separately. Cases consisted of symptomatic patients who were diagnosed with lung cancer prior to initiation of lung cancer treatment, or patients with progression of lung cancer during treatment. Controls were cancer-free individuals (ie, not diagnosed with lung cancer during time of follow-up), of whom the majority were diagnosed with chronic

obstructive pulmonary disease (COPD), according to the Global Initiatives for Chronic Obstructive Lung Disease (GOLD) criteria[18] (table 1).

One control developed lung cancer 18 months after enrolment and was at time of analysis placed in the lung cancer group. Participants were included between June 2009 and February 2013 by pulmonologists at Amsterdam Medical Centre (AMC), Amsterdam, The Netherlands. The study was performed in compliance with medical ethical regulations (synonym: Institutional Review Board) of AMC and VU University Medical Center (VUmc), Amsterdam. All subjects provided written informed consent. The research samples were not used to guide clinical management.

Sputum analysis

A detailed description of the sputum study, including the protocol for sputum collection during nine consecutive days in three canisters (each canister representing 3 days collected sputum) and follow-up procedures have been published previously.[17] For this study, only the first canister was used for analysis.

Cytology, histology, clinicopathological and methylation data of the sputum samples of the subjects included in the current study were retrieved from the sputum study database.[10] DNA hypermethylation data involved the biomarkers *RASSF1A*, *CYGB*, *APC*,[17] *3OST2*,[8] *PRDM14*, *FAM19A4* and *PHACTR3*[20] as assessed by quantitative methylation-specific PCR (qMSP) as described before.[10]

Exhaled breath analysis

Subjects collected exhaled breath during a single visit, according to a validated method as previously described.[21] Nose breathing was prevented by the use of a nose clip. Instructions were to breathe as normal through a mouthpiece that was connected to an inspiratory VOC-filter (A2, North Safety, Middelburg, The Netherlands), for a total of 5 min. Subjects were then asked to exhale a single expiratory vital capacity volume, after maximal inspiration, into a 10 L Tedlar bag (SKC, Eighty Four, Pennsylvania, USA). Subsequently, the Tedlar bag was connected to the eNose device for measurement of sampled air. This was performed within 10 min after exhaled breath collection, simultaneously with sampling from a Tedlar bag containing VOC-filtered ambient air for use as background reference.

The electronic nose applied in this study was a Cyranose 320 (Smiths Detection, Pasadena, California, USA). This is a portable chemical vapour detector, consisting of 32 composite carbon black polymer sensors. VOCs in exhaled air bind to the polymers, generating a reversible change in the electrical resistance of the sensors.[22 23] All 32 sensor deflections together form a specific fingerprint or 'breathprint'. Since initial measurements may yield deviant raw data ('first sniff effect'), data from the first measurements of each day were discarded and repeated, as instructed by the manufacturer.

To minimise influence of possible confounding variables, eating, drinking and smoking were not permitted during the 2 h prior to exhaled breath collection. Subjects were requested not to use inhalation medication on the day of the visit.

Data analysis

Clinical parameters were described as mean and SD or as median and IQRs depending on the distribution of continuous variables. To assess differences in distribution between cases and controls, either the Student t test or Mann–Whitney non-parametric test for skewed distributed continuous variables were applied. Contingency tables and χ^2 test were used for categorical variables. Fisher's Exact Test was used when the number of expected counts was below 5.

The raw data from the breathprints were restructured using principal component analysis (PCA) into six sets of principal components (PC) that covered 98% of the total variance within the dataset. The six PC sets were composed for all subjects that collected both sputum and exhaled breath (n=51). Only the fourth PC (PC4) showed a statistical significant difference between groups (p=0.04) and was selected for further analysis.

Receiver operating characteristic (ROC) curve was composed for PC4, and area under the curve (AUC) was calculated with corresponding 95% CI. To analyse the diagnostic accuracy of hypermethylation biomarkers and PC4, cut-off was determined based on Youden's J statistic for optimal discrimination between cases and controls. For hypermethylation biomarkers, predefined cut-offs were used.[10] Univariate analysis and multivariate logistic regression (with a forward selection procedure, p value for entry ≤0.05) with a binary outcome indicator of case and control were performed. McNemar tests were used to compare sensitivity and specificity between eNose and DNA hypermethylation. To estimate the additional diagnostic value of PC4 relative to DNA hypermethylation and vice versa, likelihood ratio (LR) tests were conducted in logistic regression analysis.

Validation of PC4 in Set 2 consisted of computing the ROC curve, its AUC and the sensitivity and specificity for this group. The cut-off was taken as determined on all subjects that collected both sputum and exhaled breath.

All statistical tests were two-sided with a significance level at 0.05 (p≤0.05). SPSS V.20.0 was used (IBM, Armonk, New York, USA).

RESULTS

Characteristics of subjects

Clinical characteristics of subjects are described in table 1. Median follow-up for cases was 12.5 months (range 1–33 months), for controls 19 months (range 0–43 months). No differences were observed between the groups for age, gender, smoking status and smoking history. No cytological aberrations were reported in sputum samples of both lung cancer patients and controls.

DNA hypermethylation analysis

DNA hypermethylation data are summarised in table 2.

RASSF1A, *3OST2* and *PRDM14* were able to distinguish lung cancer patients from controls (all p<0.005). *PHACTR3* was borderline significant (p=0.051). *RASSF1A* showed at highest specificity level observed in this series (87%), the highest sensitivity for lung cancer diagnosis, detecting 11 out of 20 lung cancer patients (sensitivity 55%; see online supplementary figure S1). Multivariate logistic regression analysis demonstrated that the combination of *RASSF1A* and *3OST2* had best prediction for lung cancer diagnosis, yielding a sensitivity of 85% (95% CI 62% to 97%) at a specificity level of 74% (95% CI 55% to 88%).

eNose analysis

PCA on the raw data from the 32 eNose sensors yielded six sets of PCs, of which the fourth set (PC4) was selected that showed best discriminatory performance: ROC curve of PC4 had an AUC of 0.66 (95% CI 0.51 to 0.81), and PC4 at the cut-off based on Youden's J statistics revealed a sensitivity of 80% and specificity of 48% (table 3; see online supplementary figure S1).

Table 1 Clinical characteristics of subjects

	eNose+sputum			eNose validation set		
	Lung cancer (n=20)	Control (n=31)	p Value*	Lung cancer (n=18)	Control (n=8)	p Value*
Age (yrs, mean±SD)	64.9±8.3	64.9±8.9	1.00	63.6±8.1	53.3±7.4	0.005
Males (%)	12 (60)	22 (71)	0.42	10 (56)	4 (50)	1.00
FEV1 (% pred, mean±SD)	81.0±27.1	52.7±21.5	0.002	87.6±21.4	50.8±27.8	0.001
Smoking						
Current (%)	7 (35)	12 (39)	0.89	10 (56)	4 (50)	1.00
Former (%)	12 (60)	19 (61)		8 (44)	4 (50)	
Unknown (%)	1 (5)	0		0	0	
PY (years, median (range))	38 (29–94)	38 (9–100)	0.37	40 (20–60)	30 (25–100)	0.35
Tumour histology (%)						
Squamous cell carcinoma	6 (30)			4 (22)		
Adenocarcinoma	7 (35)			7 (39)		
NSCLC NOS	4 (20)			0 (0)		
Small cell carcinoma	1 (5)			5 (28)		
Other	2 (10)			2 (11)		
Tumour stage (%)†						
I	1 (5)			4 (22)		
II	3 (15)			2 (11)		
III	9 (45)			5 (28)		
IV	7 (35)			7 (39)		

*Comparison between lung cancer cases and controls (per set).
†Tumour staging according to the 7th edition of UICC TNM system.[19]
FEV1, forced expiratory volume in one second; NSCLC NOS, non-small cell lung cancer, not otherwise specified; PY, pack years.

This was subsequently validated in Set 2, showing similar AUC and high sensitivity, but lower specificity (table 3). There were no statistical significances observed between sensitivity and specificity between both sets, indicating good reproducibility (p>0.11).

Combined analysis eNose and DNA hypermethylation

Multivariate logistic regression with hypermethylation markers and PC4 showed that PC4 was not included in the model. However, when examining the combination of *RASSF1A* hypermethylation with PC4, we observed a sensitivity of 100%, indicating that all cancer patients were detected. Thirteen of 31 controls had a positive test result, leading to a specificity of 42%. Considering *3OST2* hypermethylation as well, sensitivity remained 100%, but specificity reduced to 39%.

Comparing sensitivity and specificity between *RASSF1A* hypermethylation and PC4, no differences were observed for sensitivity, but specificity was significantly lower for PC4 (p=0.004). The additional diagnostic value of PC4 in relation to *RASSF1A* hypermethylation and vice versa was significant (p<0.001 and p=0.011, respectively), indicating that both tests are complementary to each other in reaching the 100% sensitivity.

DISCUSSION

This explorative study showed that DNA hypermethylation analysis in sputum and eNose technology are complementary in establishing lung cancer diagnosis. In our series, testing of *RASSF1A* hypermethylation in concert with eNose detected all lung cancer patients (sensitivity of 100%) with a specificity of 42%. This is a proof of principle study that encourages further investigation. The high sensitivity in symptomatic patients is a first step towards adequately capturing lung cancer by non-invasive tests.

Regarding DNA hypermethylation analysis alone, the combination of *RASSF1A* and *3OST2* yielded a sensitivity of 85% and

Table 2 Univariate analysis of DNA hypermethylation biomarkers in sputum in cases (n=20) and controls (n=31). 95% CI are provided

		Sensitivity			Specificity			
Sputum marker	Cut-off*	Pos/20	Percent	95% CI	Neg/31	Percent	95% CI	p Value†
RASSF1A	0.01083	11	55	32% to 77%	27	87	70% to 96%	0.001
APC	0.04556	12	60	36% to 81%	17	55	36% to 73%	0.30
CYGB	0.03194	11	55	32% to 77%	21	68	49% to 83%	0.11
3OST2	0.11533	10	50	27% to 73%	27	87	70% to 96%	0.004
PRDM14	124.611	13	65	41% to 85%	25	81	63% to 93%	0.001
FAM19A4	0.10877	15	75	51% to 91%	8	26	12% to 45%	0.95
PHACTR3	0.00000222	12	60	36% to 81%	21	68	49% to 83%	0.051
RASSF1A and/or *3OST2*		17	85	62% to 97%	23	74	55% to 88%	<0.001

*Predefined cut-off,[10] based on Youden's J index.
†Comparison between lung cancer cases and controls (χ^2 test).

Table 3 Exhaled breath analysis by eNose in subjects who also collected sputum (20 cases, 31 controls; 'learning set')

				Sensitivity				Specificity			
Set	Cut-off*	AUC	95% CI	n/N	%	95% CI	p Value†	n/N	%	95% CI	p Value‡
Learning set	−0.580	0.66	0.51 to 0.81	16/20	80	56% to 94%		15/31	48	30% to 67%	
Validation set				17/18	94	73% to 100%	0.34	1/8	13	0.3% to 53%	0.11

Principal component analysis on the raw data from the 32 eNose sensors yielded six sets of principal components, of which the fourth set ('PC4') was selected that showed best discriminatory performance. Cut-off was based on Youden's J index and validated in set of subjects without sputum collection (18 cases, 8 controls). Area under the curve (AUC) with corresponding 95% CI are provided.
*Positive score if PC4 ≥cut-off value.
†Comparison of sensitivity between learning and validation set (Fisher's exact test).
‡Comparison of specificity between learning and validation set (Fisher's exact test).
AUC, area under the curve.

specificity of 74%. Both markers were able to detect different histological lung cancer types, which is in line with previous research.[10] The markers have been examined individually in sputum before.[8 10 24–29] In this study, *RASSF1A* has relatively high sensitivity and low specificity when compared to previous studies.[8 25–29] Statistically, no differences were observed between the methylation results of this study population compared to our large sputum study,[10] indicating sample representativity. Therefore, the lower specificity is explained as coincidental finding. With respect to *3OST2*, this marker is assessed in two other studies, with slightly comparable sensitivity, but contradictory specificity results.[8 28] However, similar methylation frequencies were observed in the large sputum study, supporting our results.

eNose was positive in our study in 80% of lung cancer patients. Remarkably, specificity of eNose is lower (48% (95% CI 30% to 67%)) than reported before.[13–15 30 31] This may be explained by the small size of the study population, but also several confounders might be involved in this. Tobacco smoking influences breathprint composition.[32] Current smokers were asked to refrain from smoking in the 2 h before breath collection, but it cannot be excluded that smoking has long-lasting confounded results. Gender, environmental factors, dietary intake and use of cosmetics are also potential confounders,[33] for which we have not corrected in this study.

The choice for application of DNA hypermethylation and/or eNose depends on the setting. One may prefer the DNA hypermethylation panel *RASSF1A* and *3OST2*, or choose the combination of *RASSF1A* with eNose. The methylation panel shows best discriminatory power based on the logistic regression model, whereas the combination DNA hypermethylation with eNose has high sensitivity (100%). Especially, the latter findings provide the high negative predictive value that is required for exclusion of lung cancer by low-cost diagnostics.

A strength of the study is that the same set of subjects have been tested with long follow-up in most subjects using two methods, that act on different biological levels. Both methods have been examined in previous research and are suitable for high-throughput application. Furthermore, analysis was conducted blinded for subject status and outcome. Limitations of this exploratory analysis are: (1) the small study population, (2) cases were mainly advanced stage lung cancer and (3) variable *RASSF1A* hypermethylation frequency in different lung cancer types.[24]

From a technical point of view, there are also a number of strengths and limitations. Collection of spontaneous sputum is participant-friendly and can be accomplished at home, in contrast with induced sputum, which requires additional visiting to an outpatient clinic. With respect to DNA hypermethylation analysis, isolation of methylated DNA from sputum can be readily accomplished. A limitation of risk biomarkers is that these are not specifically indicative for lung cancer, since these are also found to be hypermethylated in controls.[9] Advantages of the eNose are that it is non-invasive, inexpensive, portable, easy to use, and allows for quick on-the-spot analysis. A limitation of the eNose is that test results by the presently used brand may differ between devices, even if the eNoses are from the same manufacturer. This makes it more difficult to generalise results between the eNoses. However, breathprint measurements are highly reproducible using the same device.[34] The reported moderate specificity of the exhaled breath analysis indicates that further refinement of the eNose technology is needed when aiming for the confirmation of lung cancer.[35]

As mentioned above, the strength of combining the two methods lies in their complementary effect, by examining different physical characteristics: gaseous phase of exhaled air and hypermethylated DNA in sputum. The high sensitivity offers interesting perspectives for application, as prescreening test, reducing the number of people for subsequent low-dose CT (LDCT) screening. LDCT has established high sensitivity for lung cancer detection, as reported in the NLST,[4 5] but has a high false-positivity rate of 97%. A reduction of 40% of the patients to be screened with LDCT, results in saving of costs, less radiation exposure, and minimised additional (invasive) diagnostic procedures and psychological anxiety. Of note, one lung cancer patient was diagnosed with lung cancer 18 months after collection of sputum and exhaled breath. This patient showed positive hypermethylation for *RASSF1A*, *APC*, *FAM19A4* and *PHACTR3*, and also tested positive for exhaled breath analysis. This suggests that both *RASSF1A* hypermethylation and eNose are able to detect preclinical disease more than a year before clinical diagnosis.

Regarding future perspectives, independent validation of the test combination is needed in a larger sample set and a non a priori diagnosed group ('intention to diagnose'). Standardisation of protocols is required and feasibility of both tests has to be examined for screening of a population at risk. Furthermore, insight is desirable about the metabolic origin of breath constituents for refinement of eNose technology to improve specificity.

In conclusion, we have demonstrated the potential of eNose technology in combination with DNA hypermethylation in sputum for the diagnosis of lung cancer. When applied in concert, sensitivity is higher than when used alone, while retaining a moderate specificity. Ultimately, using these detection methods as first screen of high-risk individuals prior to LDCT screen, could be one step forward towards a non-invasive means to reduce lung cancer mortality.

Take home messages

- When used in concert, *RASSF1A* hypermethylation in sputum and exhaled breath analysis are complementary for lung cancer diagnosis.
- In this series, a high sensitivity for lung cancer diagnosis was observed in symptomatic patients.
- The results of this explorative study warrant further investigation in high-risk individuals for lung cancer.

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Notes