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Assessing abnormal gene promoter methylation in paraffin-embedded sputum from patients with NSCLC

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Abstract

Aberrant methylation of CpG islands is an important pathway for regulation of gene expression. Recent data suggest that epigenetic abnormalities may occur very early in lung carcinogenesis. We studied the promoters of the four genes, *HOX A9*, $p16^{INK4a}$ (*p16*), *MAGE A1* and *MAGE B2* by methylation-specific PCR in matched normal tissue, tumour, and cytological negative sputum samples obtained from 22 patients with non-small cell lung cancer (NSCLC). We further report methylation abnormalities in sputum samples from 56 smokers with differential cytology readouts (negative, inflammatory changes, suspicious, and cancer). Our method was successfully performed on formalin-fixed and paraffin-embedded (FFPE) samples, and was fit to study only few cells obtained by a convenient non-invasive sputum collection and handling. The promoters of *MAGE A1* and *MAGE B2* had abnormal methylation patterns in, respectively, 50% and 41% of the cytologically negative sputum samples from NSCLC patients, whereas methylation abnormalities of *p16* was observed in 27% of negative sputum samples. Interestingly, 95.5% (21 of 22) of the cytologically negative sputum samples from NSCLC patients had abnormal methylation in at least one of the four genes indicating a high sensitivity of this marker system. Moreover, a higher frequency of methylation abnormalities was observed in sputum samples from smokers with high cytological grade compared to low cytological grade. We conclude that the identification of abnormal gene methylation of a limited number of markers in FFPE sputum samples is feasible and may be investigated as a potential system for population-based screening of early stages of lung cancer.

Keywords: *p16*; *HOX A9*; *MAGE A1*; *MAGE B2*; Screening

1. Introduction

Lung cancer is the most common cause of cancer death in western countries, accounting for more deaths than those caused by prostate, breast and colorectal cancer combined [1]. The prognosis for patients with lung cancer is strongly correlated to the stage of the disease at the time of diagnosis. Whereas patients with clinical stage I disease have a 5-year survival rate of approximately 60%, the clinical stages II–IV disease have a 5-year survival rate ranges from 40% to less than 5% [2]. This poor prognosis is largely attributable to lack of efficient diagnostic methods for early detection and the inability to cure metastatic disease. Additionally, clinical population-based screening assays for

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early detection of lung cancer had failed to improve survival [3–5]. Thus, further efforts aimed at early identification and interventions in lung cancer are of the highest importance. Sputum samples might contain pre-malignant epithelial cells in a cancer field throughout the airways and could therefore carry valuable genetic information allowing earlier diagnosis of lung cancer.

The activation of proto-oncogenes and/or the inactivation of tumour suppressor genes are essential events in lung tumour genesis. A major mechanism of this gene deregulation is epigenetic changes in the promoter region [6,7] through abnormal methylation. Several studies have demonstrated that promoter hypermethylation of the $p16^{INK4a}$ (*p16*) tumour suppressor gene is an early and frequent event in non-small-cell lung cancer (NSCLC) [8–12]. Further, many cancers, such as leukemia, colon, skin, prostate, breast and ovary cancers, exhibit an altered expression of homeobox genes [13]. *HOX* gene clusters are also hotspots of *de novo* methylation of CpG islands in human lung adenocarcinomas [14]. In particular, it has been shown that *HOX A9* is not or weakly expressed in the normal adult human lung [15,16], but is frequently up regulated in lung cancer cell lines and invasive tumours [17]. Finally, the melanoma antigen (MAGE) genes, *MAGE A1* and *MAGE B2*, have in common their absence of expression in normal tissues, except in testis and in a wide range of human neoplastic tissues via abnormal demethylation of their promoter CpG islands [18–21]. We, and others, have previously demonstrated that the activation of *MAGE A1* and *MAGE B2* genes occurs early in lung carcinogenesis [22–26].

Thus, the *p16*, *HOX A9*, and *MAGE A1* and *MAGE B2* genes are interesting candidates as biomarkers for early detection of lung cancer. The development of the highly sensitive methylation-specific PCR (MSP) technique has simplified the study of promoter methylation [27] making it possible to study multiple markers in relatively few cells, such as epithelial cells in formalin-fixed paraffin-embedded (FFPE) sputum samples [28,29]. In the present study, we aimed at investigating the sensitivity and specificity of methylation abnormalities in *p16*, *HOX A9*, *MAGE A*1, and *MAGE B2* promoters to be used as a diagnostic marker system applied to sputum samples. By studying sputum from confirmed NSCLC patients who had all given negative sputum samples (negative cytology), we show that testing such methylation abnormalities is feasible from FFPE samples and might be potentially sensitive enough for further investigations as a diagnostic system. Additionally, specificity was estimated by screening multiple methylation abnormalities in sputum collected from a high-risk population who had different cytological readouts.

2. Materials and methods

2.1. Study population

Twenty-two patients with resected NSCLC (stages I–IIIa) and matched tumour/normal tissues were studied for methylation abnormalities. These sputum samples were collected before surgery. Patient population characteristics are given in Table 1 (left part). All 22 patients gave sputum samples with complete absence of cancer cells by cytopathologic examination. Secondly, sputum samples from 56 smokers were studied for whom the cytological examination of the sample was rated by the pathologist as ''normal cytology'', ''inflammatory changes'', ''suspicious for cancer'', or ''cancer cells''. (These are pure cytological diagnoses. Smokers enrolled in this study are confirmed by biopsy or resection. ''Suspicious for cancer'' or ''cancer cells'' groups were all clinically diagnosed as cancer). Their clinical characteristics are given in Table 1 (right part).

2.2. Sample collection and DNA extraction

Resected primary NSCLC tumours were obtained as formalin-fixed paraffin-embedded blocks. The tissues were cut into 4-μm sections and hematoxylin-eosin stained for microdissection. Tissue microdissection was performed manually under a stereomicroscope using a 25-gauge needle. Sputum samples were spun down, alcohol-fixed and embedded in paraffin blocks, and cut into

Table 1
Study population characteristics

Variable	Study populations	
	NSCLC patients ($n = 22$)	Smokers ($n = 56$)
Age (years): mean (range)	65 (48–75)	60 (27–80)
Gender (*n*)		
Male	16	41
Female	6	15
Smoking history (*n*)		
Yes	18	56
No	4	0
Unknown	0	0
Mean smoking dose (pack-years)	49	47
Histology of tumours (*n*)		
Squamous cell carcinoma	10	16
Adenocarcinoma	5	8
Others	7	6
TNM stage (*n*)		
No cancer	0	22
I	12	3
II	8	9
IIIa	2	11
IIIb	0	5
IV	0	5

Table 2
PCR primers sequences, annealing temperatures, and PCR product sizes used for MSP

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Size (bp)
HOX A9 – M[a]	TCGTTCGTTCCGATTTTCGCGC	AATTCTCTCCTTAACGACGACG	215
HOX A9 – U[b]	GTTTTGTTTGTTTGATTTTTGTGT	AAATTCTCTCCTTAACAACAACA	215
p16 – M	TTATTAGAGGGTGGGCCGGATCGC	GACCCCGAACCGCGACCGTAA	150
p16 – U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151
MAGE A1 – M	GTTTAGGTTGAGACGTTTTTTCGC	TAAAAAACGACGTAAAATTCGACCG	177
MAGE A1 – U	GTTTAGGTTGAGATGTTTTTTTGT	TAAAAAACAACATAAAATTCAACCA	177
MAGE B2 – M	TAGTAACGTTAGAATAGTGATCTTC	ATCAAAATAAACCACATCCGCTCG	132
MAGE B2 – U	AGTAATGTTAGAATAGTGATGTTT	CAAAATAAACCACATCCACTCA	132

[a] M represents methylated-specific primers.
[b] U represents unmethylated-specific primers.

4-μm sections. For DNA extraction, the tissue fragments and sputum samples were processed using the QIAamp DNA extraction kit (Quiagen, Germany) according to the manufacturer's instructions.

2.3. Bisulfite treatment and MSP

Genomic DNA (1 μg) was treated with sodium bisulfite in a 50 μl reaction volume. Briefly, DNA was denatured with 2 M of NaOH, followed by treatment with 10 mM of hydroquinone and 3 M of sodium bisulfite (Sigma Chemical Co., St. Louis, MO). After purification in a Wizard® SV column (Promega, Madison, WI), the DNA was treated with 3 M of NaOH, then precipitated in ethanol and diluted in water. The primer sequences used for methylation-specific PCR amplification of each gene promoter region are specified in Table 2. The PCR conditions were 95 °C for 15 min, 40 cycles of 94 °C for 30 s, 54–66 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 5 min in a volume of 12.5 ml with 0.5 U Hotstar Taq Polymerase (Quiagen, Germany). Finally, the amplification products were separated on a 2.5% agarose gel and stained with ethidium bromide.

2.4. Statistical analysis

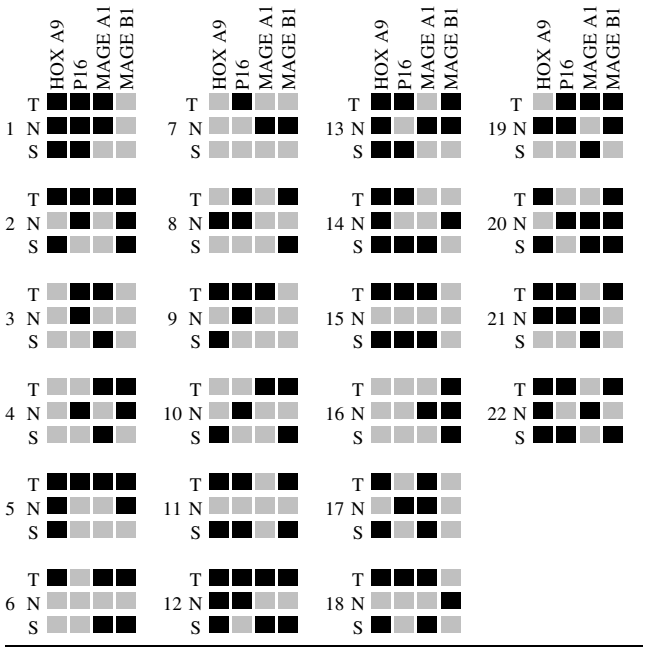
Statistical analysis was performed using the χ^2 test or Fisher's exact test for correlation among genes and for correlation between gene status, histological type and gender. The Wilcoxon rank-sum test was used for differences in median of age, and pack-years. All tests were given with $P < 0.05$ as the limit for statistical significance.

3. Results

3.1. Methylation abnormalities in cytologically negative sputum samples from patients with primary NSCLC

Tumours from 22 patients were matched with both normal adjacent tissues and negative (*i.e.*, absence of malignant cells by microscopic examination) paraffin-embedded sputum samples. The methylated versions of *HOX A9* and *p16* promoters and the unmethylated versions of *MAGE A1* and *MAGE B2* promoters were considered as abnormal. A methylated *HOX A9* promoter was considered abnormal because methylation was significantly more frequent in the NSCLC tissues (15 of 22; 68.2%) compared to normal lung tissues (9 of 22; 40.9%; $P = 0.05$; Table 3). Additionally, methylation abnormalities at the CpG island of *HOX A9* were observed at a significantly higher frequency in smokers' sputum with cancer cells than in smokers with negative sputum ($P = 0.03$, see Section 3.2 that follows). Briefly, 64% (14 of 22) of the cytologically negative sputum sam-

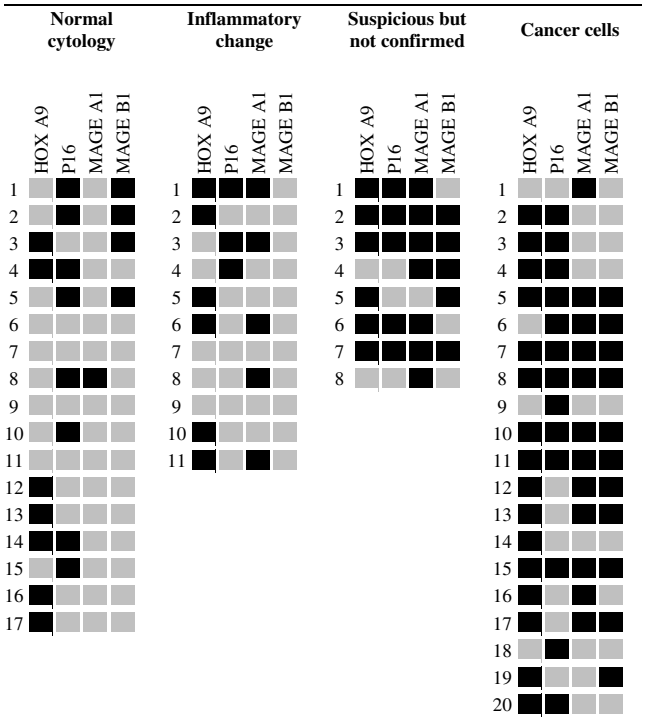
Table 3
Distribution of methylation abnormalities of *p16*, *HOX A9*, *MAGE A1* and *MAGE B2* promoters in 22 matched resected tumour, normal adjacent tissue and negative sputum samples



T stands for tumour tissue, N for normal tissue, and S for Sputum. Gray boxes represent samples that have normal methylation status in the promoter for a given gene; black boxes represent samples that have a methylation abnormality in the promoter for that given gene.

ples from NSCLC patients had abnormal methylation in *HOX A9*, whereas 50% (11 of 22) of *MAGE A1*, 41% (9 of 22) of *MAGE B2*, and 27% (6 of 22) of *p16* promoters displayed abnormal methylation in the negative sputum samples (Table 3). Furthermore, 95.5% of the negative sputum samples from NSCLC patients had abnormal methylation in at least one of the four genes tested, whereas 32% (7 of 22) had abnormal methylation in two genes, and 27% (6 of 22) had abnormality in three genes. None of the sputum samples with negative cytology had abnormal methylation in all of the four genes, in contrast to 14% (3 of 22) of the resected tumour tissues (Table 3). Furthermore, an identical pattern (presence or absence of methylation abnormality in the same gene) between sputum and tumour tissues was found in 86% (19 of 22) of cases for *HOX A9*, 77% (17 of 22) for *MAGE B2*, 64% (14 of 22) for *MAGE A1*, but in only 55% (12 of 22) of cases when only the *p16* methylation pattern were considered. We further found that the completely resected tumour tissue samples had methylation abnormalities of *HOX A9* and *p16* that were statistically correlated ($P = 0.05$, Fisher's exact test), whereas no such correlation could be found in the cytologically negative sputum samples.

Table 4
Distribution of methylation abnormalities of p16, *HOX A9*, *MAGE A1* and *MAGE B2* promoters in sputum samples from smokers, and classified according to the cytological grade of the sample



Gray boxes represent samples that have normal methylation status in the promoter for a given gene; black boxes represent samples that have a methylation abnormality in the promoter for that given gene.

3.2. Frequency of methylation abnormalities in sputum samples from smokers

To investigate the specificity of methylation abnormalities according to the cytological grade of sputum, we studied the sputum samples from 56 smokers with different cytological diagnosis: normal sputum cytology, inflammatory changes, suspicious, or presence of cancer cells (Table 4). The percentages of abnormal methylation in the *HOX A9* and *p16* promoters in normal-appearing sputum samples were 41% and 47%, respectively. The number of methylation abnormalities in sputum increased for all four genes with the cytological grade. When "normal" and "inflammatory" sub-groups were pooled into a group called "negative cytology", and "suspicious" and "cancer cells" were pooled into a second group called "cancerous cytology" (Table 5), a significant difference was found in the frequency of methylation abnormalities between these two groups for *HOX A9*, *MAGE A1* and *MAGE B2* ($P = 0.03$, $P = 0.001$, and $P = 0.002$, respectively, Fisher's exact test). The frequency of *p16*promoter methylation in smokers' sputum samples tended also to increase with the presence of suspicious/cancer cells, but it did not reach statistical significance ($P = 0.10$, Fisher's exact test). The two *MAGE* genes assured the highest specificities (only 21% and 14% of false positives), whereas *HOX A9* suffered from 47% false-positives (Table 4). Further, only 3.6% of the sputum samples from patients

Table 5
Percentage of cases with methylation abnormalities in *p16*, *HOX A9*, *MAGE A1* and *MAGE B2* promoters in sputum samples from smokers

	HOX A9 (%)	*p16* (%)	*MAGE A1* (%)	*MAGE B2* (%)
Normal cytology ($n = 17$)	41	47	6	24
Inflam. change ($n = 11$)	55	27	45	0
Suspicious for cancer ($n = 8$)	55	63	83	63
Cancer cells ($n = 20$)	80	65	60	55
Negative cytology ($n = 28$)	46	39	21	14
Cancerous cytology ($n = 28$)	79	64	68	57
Fisher's exact test	$P = 0.03$	$P = 0.10$	$P = 0.001$	$P = 0.002$

In the bottom part of the table, the "normal" and "inflammatory" sub-groups were pooled into one category called "negative cytology". The "suspicious" and "cancer cells" sub-groups were pooled into another category called "cancerous cytology".

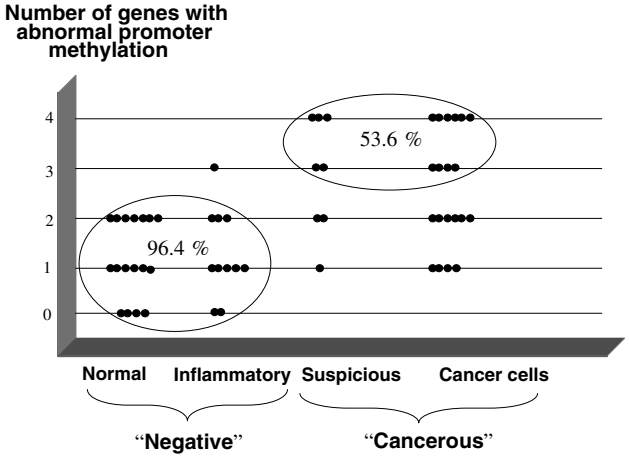


Fig. 1. Distribution of abnormal methylations in sputum samples according to its cytological status. By pooling "normal" and "inflammatory" into one group, and "suspicious" and "cancer cells" into a second, there were 96.4% of the normal/inflammatory sputum samples that had abnormal methylation in the promoter of 0–2 genes. On the other hand, 53.6% of the sputum samples with suspicious/cancer cells were abnormally methylated in 3–4 genes ($P = 0.0001$).

with negative cytology showed abnormal methylation in the promoter of more than two genes. A concomitant methylation of two or three genes was observed in 40% of smokers with negative cytology. Finally, no sputum with negative cytology had aberrant methylation in all four genes (Fig. 1). In contrast, no sputum with cancer cells showed a complete absence of abnormal methylation (Fig. 1).

3.3. Clinico-pathological correlations

No correlation between methylation data and clinicopathological parameters could be found in this study, except a gender-related difference for the *MAGE A1* gene in smokers' sputum, regardless of the cytological grade (methylation abnormality found in 3 out of 15 women (20%) against 22 out of 41 males (54%, $P = 0.02$). Additionally, the number of methylation abnormalities was not correlated with the stage of the patients, neither within nor between the study populations, which was mainly because of small sample size.

4. Discussion

The present study evaluates the presence of aberrant promoter methylation in four cancer-specific genes in negative sputum samples from NSCLC patients, then in sputum samples from smokers with normal, inflammatory, pre-neoplastic or neoplastic cytology. This simple molecular characterization of four different genes offers additional information compared to cytology alone. The high sensitivity obtained by considering at least one abnormal methylation shows the potential power of such multiple screening tests. Several reports have evaluated the frequency of aberrant promoter methylation of multiple genes in invasive NSCLC lesions or their exfoliate material [12,28–30]. Many of these studies have dealt with the *p16* tumour suppressor gene because it has been well demonstrated that promoter hypermethylation of the *p16* promoter is an early and frequent event in NSCLC [8,29,31,32], and that it is associated with poor survival in stage I adenocarcinomas [33]. But other genes are also highly interesting. In particular, we have previously demonstrated that *MAGE* genes that are regulated through promoter hypomethylation are frequently expressed in NSCLC [22]. Recently, *MAGE A1* was proven overexpressed in both SCLC and NSCLC cell lines [34]. The latter study also reported the expression of MAGE A proteins in 44% of 187 NSCLC clinical samples (stages I–III). Considering these and the present study, there are increasing arguments for using MAGE genes as markers for lung carcinogenesis, which can be based on sputum sampling. Remarkably, if the two MAGE genes were studied here alone, they would together represent some 77% of sensitivity (Table 2). Furthermore, an identical pattern (presence or absence of methylation abnormality in the same gene) between sputum and tumour tissues was found in 86% of cases for *HOX A9*, 77% for *MAGE B2*, 64% for *MAGE A1*, and in only 55% of cases when only the *p16* methylation pattern were considered. These observations might support that *HOX* and *MAGE* genes escape normal methylation regulation earlier than *p16* during cancer genesis. We further found that the completely resected tumour tissue samples had methylation abnormalities of *HOX A9* and *p16* that were statistically correlated, whereas no such correlation could be found in the cytologically negative sputum samples. Those negative sputum samples (no microscopic malignancy) might well contain variable numbers of pre-malignant epithelial cells in a cancer field throughout the airways that could explain such results. Some patients also displayed a methylation abnormality in sputum that could not be found in their tumour (for instance patients 14, 20, and 21 for *MAGE A1* in Table 3), which probably reflects the "field defects" and polyclonal nature of these lung tumours. More generally, there is increasing evidence that clonal evolution of tumors from pre-malignant lesions is a complex process that involves multiple genetic abnormalities, which do not necessarily have a linear progression. Thus, effective detection of pre-neoplasia or identification of individuals at high-risk is likely to mandate the use of a panel of molecular markers, spanning the various genetic alterations that might be present, rather than being feasible with single markers alone. Our data are also consistent with previous publications suggesting a possible pathway of global hypermethylation in cancer, which is characterized by the simultaneous methylation of multiple CpG islands,

including several known genes, such as *p16*, *hMLH*1, and *THBS*1 [35]. This notion of "pan-methylation" as a global marker for high risk of lung cancer is also a reason why multi-gene assessment of promoter methylation is more interesting than single gene tests.

Cancer-related methylation could also be found in smokers who are considered to be at high risk for lung cancer development. We observed a significant difference in the frequency of methylation abnormalities between the two groups "negative cytology" and "cancerous cytology" for *HOX A9*, *MAGE A1* and *MAGE B*2 but not for *p16* promoter methylations. This lack of statistical significance concerning *p16* is to be put into perspective with existing data. Kersting and colleagues [36] reported a 28% rate of *p16* hypermethylation in exfoliate material from symptomatic chronic smokers. Moreover, Palmisano reported that *p16* methylation could be detected in the sputum of 18.5% of cancer-free smokers [37], whereas Belinsky reported *p16* methylation in 19.2–38.9% of cancer-free smokers [8,38]. A larger study has shown methylation of *p16* in 17 out of 100 (17%) bronchial brush samples from former heavy smokers [31]. All these studies highlight the importance of early epigenetic changes of the *p16* gene in lung cancer development, especially in smokers, even if its specificity is probably low in the present study (35% false positives).

More generally, the overall mean frequency of one abnormal methylation was 38.6% in the sputum samples from smokers with negative cytology. This percentage of false-positives might seem high, but we do not know if all those cases are real false-positives or not. This population could actually be of an even higher risk for cancer development than smokers with no methylation abnormality (statistically, approximately 20% of these negative cytology smokers are expected to develop cancer one day). This issue can only be addressed with a lengthy follow-up of such patients. Further, only 3.6% of the sputum samples from patients with negative cytology showed abnormal methylation in the promoter of more than two genes. A concomitant methylation of two or three genes was observed in 40% of smokers with negative cytology. These patients might also have a higher risk for lung cancer than patients with only one methylated gene, if a dose-effect should be considered [39]. More generally, independently of belonging or not to a risk group, it is interesting to speculate upon the consequences on clinical practice with individuals having methylation abnormalities and a negative cytology for malignancy (false positives). Do they have premalignant cells in the airways, and are simply to be placed in a follow-up group for later explorations? One might also consider that simple microscopic evaluation is insufficient and that molecular testing is superior to the eye. But of course, the debate on the power of molecular tests in contrast to simple microscopic evaluation is still open. An interesting parallel to these concerns is the use of low-dose spiral computerized tomography (CT) for the screening of occult lung cancer, which has been evaluated recently. In terms of the actual presence of lung cancer, many false-positive results are the rule, and actual cancer detection rates using spiral CT are below 1% [40]. Therefore, such innovating diagnostic imaging approaches could potentially be more useful if combined with the determination of for instance methylation abnormalities in specific genes from exfoliated cells (sputum, bronchial brush, and bronchoalveolar lavage).

Our findings demonstrate that using multiple markers can significantly raise the specificity of such tests. For instance, a very high specificity for the presence of cancer cells in smokers' sputum could be reached by the nearly absence of false-positives (1 out of 28; 96.4%) if choosing arbitrarily at least three genes with abnormal methylation as a cut-off for positivity (Fig. 1 and Table 6). However, Table 6 illustrates the difficulties in ensuring specificity and sensitivity in screening. Actually, for population-based cancer screening purposes, it is often more relevant to increase the sensitivity, as would be achieved by choosing a less stringent cut-off for a positive test, which is visualized in Table 6. More generally, increasing the number of gene candidates to be tested in sputum samples could also contribute to increase the sensitivity of such tests. The question is rather to identify the most relevant candidate markers. Ultimately, the best choice of gene candidates would depend on whether the epigenetic change of a candidate gene happens early or late in the carcinogenic process. However,

Table 6
Is molecular testing superior to the eye?

Cut-off (number of genes with methylation abnormality)	Sensitivity (%)	Specificity (%)	Positive predictive accuracy (%)	Negative predictive accuracy (%)	Pearson's correlation (%)	*P* value (Pearson's χ^2 test with Yate's correction)
Negative test (0) vs. positive test (1–4)	100	21.4	56	100	34.6	0.03
Negative test (0–1) vs. positive test (2–4)	82.1	60.7	67.6	77.3	43.9	0.003
Negative test (0–2) vs. positive test (3–4)	53.6	96.4	93.8	67.5	55.3	0.0001
Negative test (0–3) vs. positive test (4)	32.1	100	100	59.6	43.8	0.004

Overview of the calculated sensitivities/specificities if the molecular assay tested only for the presence of cancer cells in smokers' sputum samples. The assay determines the number of genes (out of 4) having a methylation abnormality. Several cut-off values (number of genes with a methylation abnormality) to distinguish positivity from negativity are proposed.

our data does not aim at providing the optimal set of genes to be screened for methylation nor the absolute specificity or sensitivity of this approach, but rather to provide a proof of principle that analysis of methylation in FFPE sputum samples is feasible and potentially appealing. Another potential limitation of our study is the lack of a proper control population, which should have permitted to assess with more certainty if our findings could lead to early diagnosis of NSCLC. Nevertheless, our simple molecular characterization of four different genes offers additional information compared to cytology alone.

By combining the methylation status of the *HOX A9* CpG island with those of the three other genes studied, we were able to create an informative NSCLC detection system that highlights the sensitivity and power of using MSP techniques. Additionally, emerging data strongly suggest that sputum is a good alternative to bronchial brush samples, because obtaining sputum samples does not require an invasive bronchoscopy, and sputum seems to offer a level of detection comparable to other sample types [36,37]. Therefore, we suggest that multiple biomarkers in sputum should be tested in clinical practice.

Conflict of interest statement

None declared.

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