Methylation Assay for the Diagnosis of Lung Cancer on Bronchial Aspirates: A Cohort Study

Viola Schmiemann,[1] Alfred Böcking,[1] Marietta Kazimirek,[1] Alexandre Sherlley Casimiro Onofre,[1] Helmut Erich Gabbert,[2] Rainer Kappes,[3] Claus Dieter Gerharz,[4] and Hans Juergen Grote[1]

Abstract

Purpose: Recent studies have detected aberrant promoter methylation of *adenomatous polyposis coli promoter 1A* (*APC*), *cyclin-dependent kinase inhibitor-2A* ($p16^{INK4a}$), *retinoic acid receptor β2*, and *RAS association domain family protein 1* (*RASSF1A*) in bronchial aspirates and suggested their use as biomarkers for lung cancer diagnostics. The purpose of this study was to validate these candidate marker genes in a retrospective cohort study.

Experimental Design: Bronchial aspirates collected from a cohort comprising 247 patients with suspected lung cancer were investigated retrospectively regarding aberrant promoter methylation using a quantitative methylation-specific real-time PCR (QMSP).

Results: Eighty-nine patients were diagnosed with primary lung cancer, 102 had benign lung disease, and 56 showed miscellaneous other conditions. A panel consisting of *APC*, $p16^{INK4a}$, and *RASSF1A* emerged as useful combination. This panel detected aberrant methylation in bronchial aspirates of 22 of 35 (63%) and 21 of 44 (44%) centrally and peripherally located primary lung cancers, respectively. Bronchial aspirates also showed aberrant methylation in 5 of 7 (71%) patients with a recurrent lung cancer and in 8 of 30 (27%) cases without tumor recurrence. In contrast, only 1 of 102 patients with benign lung disease displayed a (false) positive test result. Rarely, aberrant methylation was found in patients with other malignancies (3 of 16). The QMSP assay correctly confirmed lung cancer in 8 of 12 (67%) cases with an ambiguous cytology. Moreover, it disclosed 9 of 26 (35%) of peripheral tumors lacking simultaneous cytologic or histologic diagnosis of malignancy.

Conclusions: Our findings suggest that the QMSP assay could be applied as a reflex test in cases of suspected lung cancer that defy a definite diagnosis by conventional methods. Thus, the assay could be a useful diagnostic adjunct especially regarding peripheral tumors.

Lung cancer is the most frequent cause of cancer-related death worldwide and accounts for 1.1 million deaths every year (1). Most lung cancers are detected late, with an estimated 5-year survival rate remaining below 17% and 7% in non–small cell lung cancer (NSCLC) and small cell cancer (SCLC), respectively (2). Flexible bronchoscopy is one of the main modalities used in diagnosis of suspected lung cancer. However, nondiagnostic results are not uncommon and lead to the use of additional invasive procedures (3). This is true especially with respect to peripherally located tumors that are now observed with increasing frequency. The application of molecular biomarkers on specimens from routine cytology represents a new approach to improve lung cancer diagnostics (4).

First attempts to use molecular alterations of exfoliated bronchial epithelia for diagnostic purposes were made already >10 years ago, when scientists focused on genetic changes like *KRAS* and *TP53* mutations as well as microsatellite alterations in sputum and bronchial aspirates of tumor patients (5–7). However, the use of genetic alterations as a biomarker for routine diagnostics was hampered by complex and time-consuming methodology, low prevalence of particular changes, and low sensitivity and/or specificity of the methods applied.

More recently, epigenetic changes emerged as a source for biomarker development (8). Aberrant methylation of gene promoter is a major mechanism of tumor suppressor gene inactivation and offers a new promising approach to improve lung cancer diagnostics (9–12). In previous case-control studies, bronchial aspirates of patients with lung cancer and benign lung disease were investigated with respect to promoter hypermethylation of multiple genes applying a quantitative methylation-specific PCR (QMSP) assay (13–15). Hypermethylation of *adenomatous polyposis coli promoter 1 A* (*APC*), *cyclin-dependent kinase inhibitor-2A* ($p16^{INK4a}$), *retinoic acid receptor β2* (*RARB2*), and *RAS association domain family*

Authors' Affiliations: [1]Institut fuer Cytopathologie and [2]Institut fuer Pathologie, Heinrich-Heine-Universitaet; [3]Klinik fuer Pulmologie, Florence-Nightingale-Krankenhaus, Duesseldorf, Germany; and [4]Institut fuer Pathologie, Ev. Krankenhaus Bethesda, Duisburg, Germany

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Requests for reprints: Hans Juergen Grote, Institut fuer Cytopathologie, Heinrich-Heine-Universitaet, Moorenstrasse 5, D-40225 Duesseldorf, Germany. Phone: 49-211-8118837; Fax: 49-211-8118402; E-mail: grote@med.uni-duesseldorf.de.

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protein 1 (*RASSF1A*) seemed to be useful biomarkers for lung cancer. In this retrospective cohort study, we wanted to validate our QMSP assay as an adjunct for lung cancer diagnostics on bronchial aspirates and to test whether combining *APC*, *p16^{INK4a}*, *RARB2*, and *RASSF1A* to marker panels may improve the diagnostic accuracy of the assay.

Materials and Methods

Clinical samples. The study was approved by the local ethics committee. Institutional guidelines do not require written permission for the use of anonymous archival specimens. Between the July 5 and October 31, 2002, all bronchial aspirates (bronchial washings, bronchoalveolar lavages) sent from the Department of Pulmonology of the Florence-Nightingale-Hospital, Duesseldorf, were collected continuously at the Institute for Cytopathology, Duesseldorf, Germany. Only the first entry of each patient was considered eligible for this retrospective cohort study. A subset of the cohort (50 patients with benign lung disease, 2 SCC, 1 adenocarcinoma, 1 $NSCLC_{others}$, and 5 SCLC) was part of previously published case-control studies (13, 14). The following criteria were used to distinguish cases with lung cancer from those without it: (*a*) histologic diagnosis of lung cancer done on a biopsy or resection specimen simultaneously to the index test (QMSP) or during follow-up served as standard (positive reference standard); (*b*) cytologic diagnosis of lung cancer was considered positive reference standard if this diagnosis was supported by clinical findings (imaging or bronchoscopy); (*c*) histologic and/or cytologic diagnosis of benign lung disease served as standard if it was in accordance with the overall clinical picture or if a follow-up of >6 months did not show malignancy (negative reference standard). Demographic data and information regarding smoking habits, occupational exposure to asbestos, tumor type, and staging according to the tumor-node-metastasis staging system were obtained from databases of the Institute of Cytopathology (Duesseldorf) and the Institute of Pathology (Duisburg) as well as from chart review at the Department of Pulmonology (Duesseldorf).

Immediately after bronchoscopy, bronchial aspirates were fixed in Saccomanno fixative (50% ethanol, 2% polyethylene glycol 1.500, and 60 mg/L rifampicin). For routine diagnostic purposes, an aliquot from each sample was used to prepare four conventional Papanicolaou-stained smears. Residual material was stored at 4°C for subsequent investigations.

Cell lines. The human cell line Wi-38 (fibroblast, lung) was grown and harvested according to the instructions of the American Type Culture Collection (Manassas, VA).

Bisufite treatment and quantitative methylation-specific real-time PCR. The genomic DNA of cell lines and cells in bronchial aspirates was isolated using the Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN). DNA extracted from Wi-38 cells was treated with SssI methylase (New England Biolabs, Frankfurt, Germany) according to the instructions of the manufacturer. One microgram of DNA per sample was modified by sodium bisulfite treatment according to Herman et al. (16). Promoter methylation analysis was done by fluorescence-based, real-time quantitative PCR using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). *Myogenic differentiation antigen 1* (*MYOD1*) was used as internal reference to control for input DNA. Primers, TaqMan probes, and annealing temperatures are given in Table 1. Genbank accession numbers are as follows: U02509, position 716 to 834 (*APC*); U12818, position 129 to 199 (*p16^{INK4a}*); X56849, position 951 to 1,095 (*RARB2*); AC002481, position 17,883 to 18,052 (*RASSF1A*); AF027148, position 4,860 to 5,021 (*MYOD1*). Wi-38 DNA treated with SssI methylase was used as positive control. A sample without DNA served as a negative control. The PCR products were additionally analyzed on 1% agarose gels to verify their correct size. Results were confirmed in 25% of arbitrarily chosen samples by repeated QMSP assays.

The promoter methylation level in a particular sample was calculated as ratio of the fluorescence emission intensity values according to the following formula: (gene of interest / internal reference) × 100. Considering *APC* and *RARB2*, a cutoff was set at ≥35 and ≥30, respectively, as described previously (13, 14). Regarding *p16^{INK4a}* and *RASSF1A*, all values >0 were evaluated as positive. Assessment of promoter hypermethylation was done blinded for the diagnosis of the underlying lung disease.

Statistical analysis. Observed frequency distributions were compared with a theoretical one by a two-sided χ^2 test. The level of significance was set to $P < 0.05$.

Table 1. PCR primers and TaqMan probes for QMSP

Gene	Primer sequences	Annealing temperature (°C)	CpGs (*n*)	Product size (bp)
APC	F: 5′-GAACCAAAACGCTCCCCAT-3′	58	8	74
	R: 5′-TTATATGTCGGTTACGTGCGTTTATAT-3′			
	TaqMan: 5′-6FAM-CCCGTCGAAAACCCGCCGATTA-TAMRA-3′			
p16^{INK4a}	F: 5′-TGGAGTTTTCGGTTGATTGGTT-3′	58	7	70
	R: 5′-AACAACGCCCGCACCTCCT-3′			
	TaqMan: 5′-6FAM-ACCCGACCCCGAACCGCG-TAMRA-3′			
RARB2	F: 5′-CGAGAACGCGAGCGATTC-3′	60	11	146
	R: 5′-GACCAATCCAACCGAAACGA-3′			
	TaqMan: 5′-6FAM-CCTTCCGAATACGTTCCGAATCCTAC-TAMRA-3′			
RASSF1A	F: 5′-GGTTTTGCGAGAGCGCGT-3′	61	10	169
	R: 5′-GCTAACAAACGCGAACCGAAC-3′			
	TaqMan: 5′-6FAM-GGAGGCGTTGAAGTCGGGGTT-TAMRA -3′			
MYOD1	F: 5′-CCAACTCCAAATCCCCTCTCTTTAT-3′	58	0	161
	R: 5′-TGATTAATTTAGATTGGGTTTAGAGAAGGA-3′			
	TaqMan: 5′-6FAM-TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC-TAMRA-3′			

Abbreviations: F, forward; R, reverse.

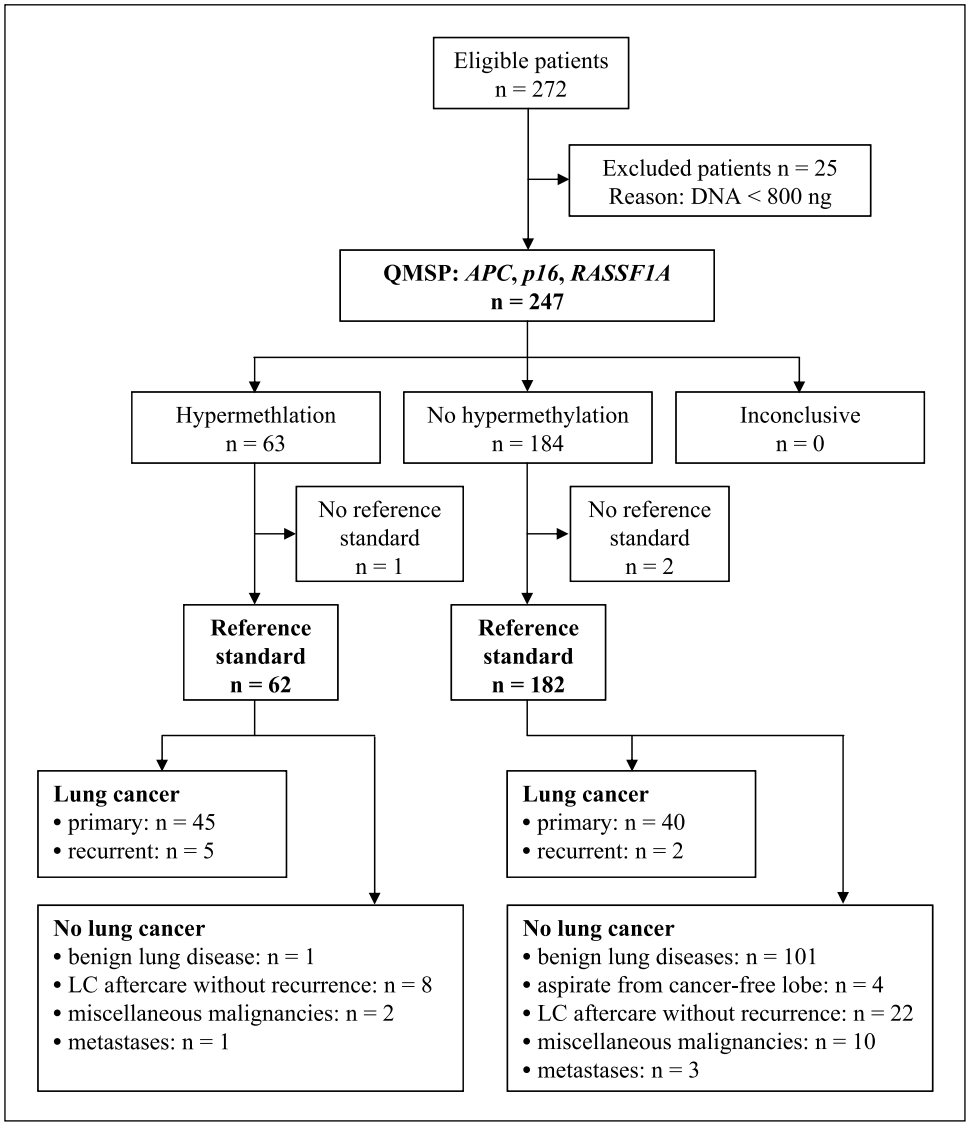

Fig. 1. Flow chart illustrating the progress of subjects through the retrospective cohort study. LC, lung cancer.

Results

Figure 1 shows the Standards for Reporting of Diagnostic Accuracy flow diagram for the study (17). There were 272 consecutive patients eligible for this retrospective cohort study. Specimens of 25 patients had to be excluded due to low amount (<800 ng) of DNA left after preparation of smears for routine cytology. Clinicopathologic characteristics of the remaining 247 patients enrolled in this study are compiled in Table 2. The group of patients with benign lung disease comprised cases with acute or chronic bronchitis (n = 48), pneumonia (n = 18), interstitial lung disorder (n = 12), asthma (n = 7), tuberculosis (n = 6), pulmonary thrombembolism (n = 3), pleuritis (n = 3), hypersensitivity pneumonitis (n = 1), chondroma (n = 1), aspergilloma (n = 1), bronchiectasis (n = 1), and atelectasis (n = 1). In 37 of 89 patients finally diagnosed with lung cancer, simultaneous histology was negative. In these patients as well as in three of seven lung cancer aftercare patients with a recurrent tumor, diagnosis of malignancy was made either by follow-up histology (n = 34) or by cytology alone (n = 6). In a 64-year-old male with a 25 pack-years history of smoking, upper lobe resection proved an aspergilloma and, thus, a false-positive cytologic diagnosis of an adenocarcinoma.

The methylation frequencies of each promoter detected in bronchial aspirates of patients diagnosed with different tumor types as well as in aspirates of patients with benign lung disease are listed in Table 3. Four cases with primary lung cancer were not considered as the aspirates were taken from a cancer-free lobe (i.e., not from the tumor site). *APC* preferentially detected patients with a NSCLC, being positive in 17% to 24% of corresponding bronchial aspirates. The $p16^{INK4a}$ promoter was a good marker for SCC showing a detection rate of 38%. *RASSF1A* detected 82% of cases with a SCLC. In contrast, no aberrant $p16^{INK4a}$ and *RASSF1A* methylation was detected in patients with benign lung disease and only one of these presented with an aberrant *APC* methylation.

The 85 patients diagnosed with primary lung cancer displayed promoter hypermethylation of 4 markers in 1 case, of 3 markers in 7 cases, of 2 markers in 20 cases, of 1 marker in 31 cases, and of 0 marker in 26 cases. No correlation between the number of positive markers and clinicopathologic characteristics of the tumors was found.

Methylation frequency was generally higher in smokers compared with never-smokers (smokers versus never-smokers: *APC*, 8% versus 0%; *p16*, 4% versus 0%; *RARB2*, 37% versus 1%; and *RASSF1A*, 21% versus 1%) and highest in smokers with >30 pack-years of exposure (≤30 pack-years smoked versus >30 pack-years smoked: *APC*, 12% versus 3%; *p16*, 7% versus 3%; *RARB2*, 38% versus 30%; *RASSF1A*, 26% versus 10%). However, this reached statistical significance only with respect to *RASSF1A* methylation and the amount of pack-years smoked during the lifetime ($\chi^2 = 4.36$, $P < 0.05$). An association between methylation and gender was found solely with *RARB2*, which was methylated significantly more frequent in males than females in the nontumor group even after adjustment for smoking habits (males: methylated *RARB2* in 29%; females: methylated *RARB2* in 7%; $\chi^2 = 8.17$, $P < 0.01$). There was no correlation between the methylation status of the investigated genes and age, asbestos exposure, or tumor stage.

Table 2. Characteristics of the patient population

	Benign lung disease* ($n = 102$)	Primary lung cancer ($n = 89$)	Others† ($n = 56$)
Age	63 (21-82)	64 (43-83)	66 (34-83)
Sex			
Female	39 (38)	31 (35)	29 (52)
Male	63 (62)	58 (65)	27 (48)
Pack-years‡	32 (3-100)	40 (15-120)	40 (15-100)
Smoking status§			
Current smoker	41 (40)	58 (65)	26 (46)
No smoker			
Ex smoker	31 (30)	22 (25)	20 (36)
Never smoker	6 (6)	1 (1)	1 (2)
Not specified	20 (20)	5 (6)	7 (13)
Location‖			
Central		37 (42)	
Peripheral		50 (56)	
Stage¶			
I		14 (19)	
II		4 (6)	
III		24 (33)	
IV		31 (42)	
Limited disease		5 (31)	
Extensive disease		11 (69)	
Histology			
AC of lung		32 (36)	4 (7)
SCC of lung		20 (23)	0 (0)
$NSCLC_{others}$		19 (21)	1 (2)
SCLC		17 (19)	2 (4)
cSCLC		1 (1)	0 (0)
DMM			7 (13)
Miscellaneous**			12 (21)

NOTE: Data are presented as median (range) or *n* (%).
Abbreviations: AC, adenocarcinoma; cSCLC, combined SCLC; DMM, diffuse malignant mesothelioma.
*See text.
†Other cases (37 lung cancer aftercare patients of whom seven had recurrent disease, four patients with metastases, seven diffuse malignant mesothelioma, and eight patients with miscellaneous conditions).
‡Patients with history of smoking.
§Smoking data lacking in nine patients.
‖Location not specified for two patients.
¶Percentages are calculated for NSCLC and SCLC separately.
**Miscellaneous: metastasis ($n = 4$), non-Hodgkin's lymphoma ($n = 1$), Hodgkin's lymphoma ($n = 1$), laryngeal carcinoma ($n = 1$), pulmonary blastoma ($n = 1$), Hodgkin's lymphoma and dysplasia grade 2 ($n = 1$), no reference standard ($n = 3$).

To improve the sensitivity of the QMSP assay as a molecular test for lung cancer, the marker genes tested were combined to a three- and a four-marker panel. A three-marker panel consisting of *APC*, *p16*, and *RASSF1A* yielded a sensitivity, specificity, positive, and negative predictive value of 53% (45 of 85), 99% (101 of 102), 98% (45 of 46), and 72% (101 of 141), respectively. The corresponding values were 71% (60 of 85), 78% (80 of 102), 73% (60 of 82), and 76% (80 of 105) if *RARB2* was added to the panel. Due to the limited specificity of aberrant *RARB2* methylation, only the three-marker panel comprising *APC*, $p16^{INK4a}$, and *RASSF1A* was considered subsequently. Hypermethylation of at least one promoter of the three-marker panel was found in 63 bronchial aspirates (Fig. 1). Primary lung cancer was detected in 52.9% (45 of 85) and recurrent lung cancer in 71% (5 of 7) of cases. In patients without a history of previously treated lung cancer, the specificity with respect to malignancy was >99%. One patient with benign lung disease (obstructive sleep apnea syndrome) showed aberrant methylation of the *APC* promoter. This patient showed no evidence for malignancy within a follow-up time of 14 months. Using the three-marker panel, 11 bronchial aspirates from patients without primary lung cancer presented an aberrant methylation of one of the marker genes. Among these were 8 of 30 lung cancer aftercare patients who had no evidence for tumor recurrence (methylated *APC*: $n = 4$; methylated *RASSF1A*: $n = 4$). The tumors of two of these cases recurred 14 (methylated *APC*) and 16 (methylated *RASSF1A*) months after the positive QMSP result. One patient listed in Fig. 1 as a miscellaneous malignancy was diagnosed with a pleural epithelial mesothelioma (methylated $p16^{INK4a}$). The other patient had a Hodgkin's lymphoma involving mediastinal lymph nodes as well as a dysplasia grade 2 of the tracheal epithelium (methylated *RASSF1A*). In one patient with a pulmonary metastasis of a rectal carcinoma, *APC* hypermethylation was detected in the bronchial aspirate. Conventional cytology showed multiple tumor cells. Four patients with a primary lung cancer were classified as target condition absent because the aspirate was taken from a cancer-free lobe (i.e., not from the tumor site).

The diagnostic yield of histology, cytology, and the QMSP assay was highly dependent on tumor location (Table 4). Although biopsy was successful in the vast majority of centrally located tumors (89%), the success rate was much lower considering peripheral tumors (40%). It is noteworthy that the QMSP assay was most useful with respect to the latter. The methylation assay disclosed 9 of 26 peripheral tumors with a negative histology and cytology. This result points out that the QMSP assay is able to detect lung cancer even in bronchial aspirates without morphologically visible atypical cells.

Altogether, conventional cytology was false-negative in 44 cases. Rescreening of these bronchial aspirates revealed one case of SCLC with unequivocal tumor cells and two cases with cells highly suspicious for NSCLC. Consequently, screening error accounted for 7% (3 of 44) and sampling error for 93%

Table 3. Frequency of aberrant methylation in bronchial aspirates of patients with primary lung cancer (n = 85) and benign lung disease (n = 102)

	Aberrant methylation in bronchial aspirates*			
	APC, % (n/n_{total})	*p16^{INK4a}*, % (n/n_{total})	*RARB2*, % (n/n_{total})	*RASSF1A*, % (n/n_{total})
SCC	19 (3/16)	38 (6/16)	53 (8/15)	25 (4/16)
AC	24 (8/33)	6 (2/33)	33 (11/33)	33 (11/33)
$NSCLC_{others}$	17 (3/18)	6 (1/18)	50 (9/18)	28 (5/18)
SCLC	0 (0/17)	6 (1/17)	75 (12/16)	82 (14/17)
cSCLC	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)
Benign lung disease	1 (1/102)	0 (0/102)	21 (21/102)	0 (0/102)

*Aberrant methylation was considered if methylation-level [(gene of interest / internal reference) × 100] exceeded the following thresholds: *APC* ≥ 35, *p16^{INK4a}* > 0, *RARB2* ≥ 30, and *RASSF1A* > 0 (13, 14).

(41 of 44) of cytologically false-negative bronchial aspirates, respectively. Using the QMSP assay with a three-marker panel, cases with a false-negative, doubtful, or highly suspicious cytology were correctly diagnosed as cancer in 45% (20 of 44), 25% (1 of 4), and 88% (7 of 8), respectively.

Figure 2 depicts the additive value of different diagnostic measures with respect to sensitivity of a single bronchoscopy. Cytology raised the sensitivity of histology by 9%. Applying a three-marker panel, the QMSP assay added up another absolute 13% to sensitivity of conventional morphology. As a result, the sensitivity of a single bronchoscopy for the diagnosis of lung cancer was 81%.

Discussion

The application of molecular biomarkers for lung cancer screening and early detection has attracted considerable attention (18). Thus far, only minor attention was focused on methylation markers as a diagnostic adjunct for clinically already suspected lung cancer and its surveillance (9, 15, 19, 20).

Applying a QMSP with a three-marker panel (*APC*, *p16^{INK4a}*, and *RASSF1A*) on bronchial aspirates from a cohort of patients with suspected pulmonary malignancy, we were able to detect 53% of cases with primary lung cancer. The test complemented histology and conventional cytology mostly with peripherally located cancers. In patients without a history of previously treated lung cancer, the specificity considering malignancy was >99%.

The marker genes *APC*, *p16^{INK4a}*, and *RARB2* were established and discussed in detail in our previous case-control studies on bronchial aspirates (13, 14). The methylation frequencies of single genes observed in this study confirmed the results published before. Recently, *RASSF1A* hypermethylation was detected in 30% to 39% of bronchoalveolar fluids of patients diagnosed with NSCLC (15, 21). With respect to SCLC, no corresponding data on bronchial aspirates are available but a hypermethylated *RASSF1A* was found in 72% to 79% of tumors (22, 23). Generally, cytologic specimens of controls showed *RASSF1A* hypermethylation at low frequency (0-4%; refs. 15, 24, 25). The present study yielded similar results.

Our strategy was to design a highly specific test that may guide the diagnostic procedure if conventional modalities encounter difficulties to prove a suspected lung cancer. The QMSP approach used in this study has the advantage that it reduces unspecific amplification because the PCR reaction needs specific binding of both the conventional methylation-specific primers and an additional TaqMan probe (26). Aberrant methylation of bronchial epithelia in absence of a tumor is another problem for the design of a highly specific methylation assay on bronchial aspirates. Frequently, aberrant methylation of normal appearing epithelia is identical to that in the tumor and, thus, may represent a field defect of preneoplastic changes that occur early in carcinogenesis (21, 24). Such a strong association between methylation in the bronchial epithelium and corresponding primary tumor was reported, e.g., for *p16* and *RASSF1A*. Although not transforming by itself, inactivation of these genes due to methylation is likely permissive for the acquisition of additional genetic and epigenetic changes leading to lung cancer. Alternatively, methylation in nonneoplastic epithelia may be different from the tumor or even occur in patients without malignancy (15, 21, 24, 25). In any case, a methylation assay on bronchial aspirates does not measure aberrant methylation exclusively of tumor cells. This may explain why the assay was positive in 21 of 48 (44%) patients diagnosed with lung cancer although cytology of respective bronchial aspirates did not show atypical cells. Methylation in patients without malignancy generally occurs at low levels (13–15). Therefore, a quantitative PCR reaction like that one used in the QMSP assay makes it possible

Table 4. Impact of tumor location on sensitivity

Method	Central tumors, % (n/n_{total})	Peripheral tumors, % (n/n_{total})
Biopsy	89 (31/35)	40 (19/48)
Cytology	54 (19/35)	35 (17/48)
QMSP		
Total	63 (22/35)	44 (21/48)
Cases with negative biopsy	25 (1/4)	41 (12/29)
Cases with negative biopsy and cytology	25 (1/4)	35 (9/26)

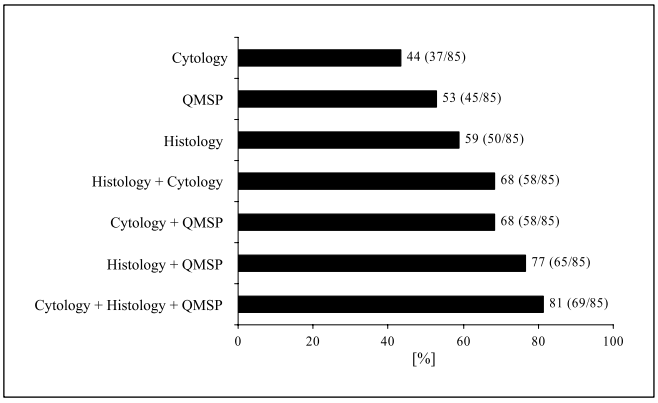

Fig. 2. Sensitivity of cytology, histology, and QMSP assay considering a single bronchoscopy. Columns, percentage and number of positive cases/total number.

to discriminate between malignant and nonmalignant cases by means of cutoff points. Nevertheless, *RARB2* was excluded from the marker panel due to its low specificity (79%). Whether methylated *RARB2* just indicates a damaged epithelium, as suggested by Zöchbauer-Müller et al. (25), or whether it may be a useful risk marker for lung cancer remains to be elucidated by future investigations on patients with long term follow-up.

In this study, a small number of cases showed aberrant methylation without evidence for a primary or secondary lung cancer. Only 1 of 102 nontumor patients displayed a false-positive QMSP assay presenting with an aberrant methylation of the *APC* promoter 1A. The remaining cases with a positive QMSP assay in the absence of lung cancer were each connected with malignancy. The majority of these had a history of treated lung cancer without evidence for tumor recurrence. This may again be explained by a field defect of respiratory epithelium (21, 24). Consequently, the QMSP assay is not applicable for the detection of tumor recurrence. Whether aberrant promoter methylation in the bronchial aspirate of a patient with treated lung cancer indicates an increased risk for tumor recurrence remains to be elucidated by long-term follow-up of our cohort. Another three patients with a positive QMSP assay presented with miscellaneous disorders. A moderate dysplasia of the tracheal epithelium was detected by chance in a patient with Hodgkin's disease. The QMSP assay showed an aberrant *RASSF1A* methylation. A similar finding was reported by Zöchbauer-Müller et al. (25) in sputa showing mild to moderate dysplasia. The second case presented with a tumor cell–positive bronchial aspirate due to pulmonary metastasis of a rectal carcinoma. Aberrant *APC* methylation detected in this cases is likely to originate from metastatic tumor cells because this alteration occurs in ~40% of metastases of colorectal cancer (27). Aberrant $p16^{INK4a}$ methylation was present in the bronchial aspirate of one of seven malignant mesotheliomas. As $p16^{INK4a}$ was unequivocally and clear-cut negative in all patients with benign lung disease we tested thus far, we assume some kind of connection between the epigenetic alteration found in this case and the epithelial mesothelioma. The exact mechanism remains speculative and may, e.g., relate to an intraparenchymal growth of a mesothelioma with $p16^{INK4a}$ methylation (28, 29).

Different settings can provide the indication for a QMSP assay. An ambiguous cytologic finding may prompt the cytopathologist to perform adjuvant methods to make a final diagnosis. In a subset of these cases, fluorescence *in situ* hybridization, DNA image cytometry or immunocytochemistry may be applicable (30, 31). The QMSP assay represents a highly specific alternative method, which, in this study, correctly confirmed lung cancer in 88% and 25% of cases with a highly suspicious or doubtful cytology, respectively. Furthermore, as the use of methylation markers does not require cells with abnormal morphology, a clinically suspected tumor that evaded from its pathologic proof will likely be the most relevant indication for the QMSP assay. In the present study, the QMSP assay yielded its major diagnostic surplus with respect to peripheral lung cancers, as the assay disclosed 9 of 26 (35%) peripheral cancers that presented with a negative simultaneous cytology and histology. Nevertheless, for the purpose of routine diagnostics, a higher test sensitivity would be desirable. Searching for complementary marker genes could be a successful strategy to achieve this aim.

Rescreening of cytologically false-negative cases revealed a very low screening error rate (7%) that contrasted with a moderate sensitivity of cytology (44%) when data observed in this study were compared with those given in the literature (3). The moderate average sensitivity of cytology is in part attributable to a high portion of peripherally located tumors present in the cohort. It is likely, however, that a more selective collection of bronchial aspirates would have resulted in an increased sensitivity of both cytology and the QMSP assay. Comparing cytology and the QMSP assay in terms of diagnostic yield, the latter was even superior regarding identification of lung cancer in patients with a false-negative biopsy. As a QMSP may be done on residual material of regular cytology within a maximum of 3 days, it is possible to integrate the methylation assay into routine diagnostics with little effort.

In conclusion, the QMSP assay on bronchial aspirates presented in this study could be useful as a reflex test in patients who are clinically suspicious for lung cancer but do not display a final cytologic or histologic diagnosis of malignancy. Further effort has to be made to improve the sensitivity of the assay. In view of the high or even rising prevalence of peripherally located lung cancer, there might be a considerable demand for such a diagnostic adjunct supplementing conventional modalities in the future.

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