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Published in:
Journal of Clinical Pathology

DOI:
10.1136/jclinpath-2012-200712

Publication date:
2012

Prolonged sampling of spontaneous sputum improves sensitivity of hypermethylation analysis for lung cancer

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Accepted 9 February 2012
Published Online First 29 March 2012

ABSTRACT

Aims The adequacy of lung cancer diagnosis with sputum cytology depends on duration of sputum sampling. The aim of this methodological study was to determine whether the hypermethylation detection rate of RASSF1A, adenomatous polyposis coli (APC) and cytoglobin (CYGB) is influenced by the duration of sputum collection.

Methods Prospective sputum samples were collected from 53 lung cancer patients and 47 chronic obstructive pulmonary disease patients as controls. Subjects collected spontaneous sputum at home during nine consecutive days in three canisters I, II and III (ie, days 1–3, days 4–6, days 7–9, respectively). Quantitative methylation-specific PCR was performed to assess gene promoter methylation status of RASSF1A, APC and CYGB.

Results Analysis of each canister separately showed hypermethylation of RASSF1A, APC and/or CYGB in samples I, II and III, in 43%, 40% and 47% of cases, respectively. In control samples, these numbers were 4%, 2% and 4%, respectively. Cumulative analysis for days 1–6 and days 1–9 revealed an increase in sensitivity to 53% and 64%, and specificity of 94% and 91%, respectively.

Conclusion Sputum collected over multiple successive days results in a gain in sensitivity for the detection of lung cancer, at the expense of a small loss in specificity.

Condensed abstract Assessment of hypermethylation sensitivity of biomarkers in sputum collected over a prolonged period for the detection of lung cancer resulted in a promising gain in sensitivity, at the expense of a small loss in specificity.

INTRODUCTION

Lung cancer has a high mortality rate,[1] which is related to the fact that the majority of patients present with advanced stage at time of diagnosis. The mean 5-year survival is <15%,[2 3] while for stage IA lung cancer the 5-year survival rate is approximately 65%.[4] Sputum cytology is occasionally part of the diagnostic investigation of lung cancer. Prolonged sputum collection leads to more adequate sputum for cytological assessment, regardless of method of sputum collection.[5 6] Procurement of sputum, which can be self-collected in a non-invasive manner, is inexpensive and simple.

It is assumed that tumours shed cellular components such as free DNA, as well as dead cells in the bronchial epithelial lining fluid.[7] Highly sensitive molecular techniques applied on sputum are capable of detecting molecular tumour-related DNA alterations, such as mutations in KRAS and p53,[8–10] and DNA methylation.[9 11–17] Hypermethylation of the promoter of tumour suppressor genes results in gene silencing[18 19] and is considered to be a major event in the initiation and progression of lung cancer. In previous reports, promoter hypermethylation of tumour suppressor genes RASSF1A, adenomatous polyposis coli (APC) and cytoglobin (CYGB) have been identified as potential biomarkers for lung cancer detection in sputum.[11–13 16 20–23]

The aim of this methodological study was to investigate the sensitivity for molecular lung cancer diagnosis by prolonged sputum sampling. To this end, sputum samples were collected during nine consecutive days in three canisters I, II and III (ie, days 1–3, days 4–6, days 7–9, respectively) in both lung cancer patients and cancer-free controls, in which we examined gene promoter hypermethylation.

METHODS

Subjects

Prospective sputum samples were collected by patients diagnosed with lung cancer and patients with chronic obstructive pulmonary disease (COPD) as controls who have been enrolled in an on-going lung cancer screening study in the region of Amsterdam, The Netherlands. For this study, in a consecutive series, we selected the first 53 lung cancer patients and 47 controls who collected sputum over a period of nine successive days and had a positive control PCR (see below).

Sputum samples were collected between May 2009 and June 2010 at the departments of Pulmonology Diseases of VU University Medical Center Amsterdam, Amsterdam Medical Center, Antoni van Leeuwenhoek Hospital, Sint Antonius Hospital and Sint Lucas Andreas Hospital in the regions of Amsterdam and Nieuwegein, The Netherlands. The study protocol was approved by the Institutional Review Boards of participating hospitals and all subjects provided written informed consent. Lung cancer patients were considered eligible for inclusion in the study when they were diagnosed with lung cancer prior to treatment, or when progressive disease was evident after treatment. In the control group, individuals with spontaneous sputum

production and no history of lung cancer in the past 3 years were included. Controls diagnosed with COPD were classified according to the Global Initiative for Chronic Obstructive Lung Disease criteria.[24] Clinicopathological data were retrieved from medical records after a follow-up period of at least 1 year after enrolment in this study.

Sputum collection

Sputum was collected in Saccomanno's fixative (2% polyethylene glycol in 50% ethanol) as described before[25] with slight modification: patients were requested to collect spontaneous (non-induced) sputum at home on nine consecutive days, preferably in the morning, using three canisters in such a way that each single canister contained sputum expectorated during a period of three successive days. Canister I represented days 1–3, canister II days 4–6 and canister III days 7–9. After 9 days of sputum collection, the batch was returned to the VU University Medical Center Amsterdam. In lung cancer patients, sputum was sampled at time of cancer diagnosis, before cancer treatment or after recurrence.

DNA isolation, bisulphite treatment and quantitative methylation-specific PCR

Genomic DNA was extracted from the sputum aliquots by using the QIAamp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Subsequently, 2 μg of genomic DNA was subjected to sodium bisulphite treatment using the EZ DNA Methylation Kit TM (Zymo Research, Orange, California, USA). Bisulphite-converted DNA was used as template for quantitative methylation-specific PCR (MSP) analysis. The primers and probes used for RASSF1A, APC and CYGB were as previously described (table 1).[16 20]

In addition, a PCR for the bisulphite-converted housekeeping gene MYOD1 was tested as internal reference and quality control.[16] We designed two quantitative multiplex methylation-specific PCRs (QM-MSP), slightly modified from Shivapurkar *et al*.[16] The first was applied for simultaneous assessing promoter hypermethylation of RASSF1A and MYOD1 genes, and the second for hypermethylation of APC and CYGB genes. The performance of multiplex testing was equal to singleplex testing (data not shown). Both QM-MSPs were conducted on all samples. Per sample, amplification was carried out in a 12 μl reaction volume containing 200 ng of bisulphite-converted genomic DNA, forward and reverse primers (400 nM of RASSF1A, APC and CYGB primers, 200 nM of MYOD1 primers), probes (200 nM of each) and 2× QuantiTect Multiplex Master Mix (Qiagen, Westburg, Leusden, The Netherlands). The PCR protocol comprised the following steps: initial denaturation at 95°C for 15 min, followed by 45 amplification cycles of 94°C for 60 s and 60°C for 90 s. Specificity of each primer pair for bisulphite-converted methylated DNA was confirmed by absence of amplification of unmodified DNA to ensure that no amplification would occur in case of incomplete DNA modification. A standard curve of bisulphite-treated DNA of lung cancer cell line A549 (positive for gene promoter hypermethylation of all biomarkers) was included in each quantitative MSP. Water samples served as negative control. All QM-MSPs were performed on a TaqMan ABI 7500F Real-time PCR system (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

For each target, cycle threshold (Ct) values were measured at a fixed fluorescence threshold (ie, 0.01). To quantify level of methylation of the promoter of the genes RASSF1A, APC and CYGB in each sample, Ct ratios between the Ct values of the MYOD1 and target were used as calculated by the following formula: $2^{(Ct\ (MYOD1)\ -\ Ct\ (target))} \times 100$.

Ct values of more than 40 were considered to represent a non-methylated test result. All samples had Ct values below 27 for MYOD1, which indicates good DNA quality and recovery after bisulphite treatment. Samples were tested in duplicate and displayed good reproducibility. To relate the effect of cumulative testing of canisters I, II and III to repetitive (triplicate) testing on one canister, the multiplex assay was performed thrice on one of the canisters (ie, canister II).

Statistical analysis

As cut-off value for diagnostically relevant hypermethylation for a specific marker, we determined the 99th percentile of the methylation ratios of this marker in all canisters of controls. A sample was considered positive for promoter hypermethylation of the respective marker when its methylation ratio was equal to or above the cut-off. χ^2 tests were conducted to assess the ability of the test to discriminate between lung cancer and control samples. Cochran's Q tests were used for comparison among canisters I, II and III and for triplicate testing of canister II. κ Values were calculated for test reproducibility in triplicate testing of canister II. A two-sided p value of ≤ 0.05 was considered significant.

After establishing presence of hypermethylation in canister I, the percentage and exact binomial 95% CI of additional hypermethylation in canisters II and III in the negative group (negative for hypermethylation in canister I) were determined (SPSS V.15.0; SPSS Inc.).

RESULTS

Clinicopathological data of study participants are shown in table 2.

Promoter hypermethylation frequencies of RASSF1A, APC and CYGB genes stratified per canister (ie, I, II, III) and case-control status are shown in table 3.

Cochran's Q test did not show statistically significant differences in frequency of hypermethylation for all three markers among canisters I, II and III, both in cancer patients and control subjects. Hypermethylation frequency of RASSF1A, CYGB and the combination of RASSF1A/CYGB/APC was significantly increased in cancer patients over controls in all canisters.

Cumulative 6- and 9-day methylation frequencies of RASSF1A, APC or CYGB showed that sensitivity of lung cancer detection increased (table 4).

Table 1 Primer and probe sequences of genes RASSF1A, adenomatous polyposis coli (APC), cytoglobin (CYGB) and MYOD1 for quantitative real-time PCR assays

Gene symbol	Forward primer	TaqMan probe with fluorescence labels	Reverse primer
mRASSF1A	GCGTTGAAGTCGGGGTTC	*FAM*-ACAAACGCGAACCGAACGAAACCA-*TAMRA*	CCCGTACTTCGCTAACTTTAAACG
mAPC	GAACCAAAACGCTCCCCAT	*YAKIMA YELLOW*-CCCGTCGAAAACCCGCCCGATTA-*NFQ MGB*	TTATATGTCGGTTACGTGCGTTTATAT
mCYGB	CGAGGTCGATCGTTAGTTCGTTC	*FAM*- CGGCGGTCGTCGTGGATTTAGT	CCAACGACTAACTCGAAAACGCG
MYOD1	CCAACTCCAAATCCCCTCTCTAT	*CY5*-TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC	TGATTAATTTAGATTGGGTTTAGAGAAGGA

Table 2 Summary of clinicopathological data of study participants

Variable	Lung cancer patients (n=53)	Controls (n=47)	p Value*
Age (years)†	66.2±10.1	71.4±8.4	0.18
Gender (% male)	69.8	74.5	0.61
Smoking status (%)			
Current	35.8	42.6	0.43
Former‡	58.5	46.8	
Never	5.7	10.6	
Smoking history§			
Duration (years)	42.9	44.6	0.85
Mean smoking dose (pack-years)	50.3	42.4	0.07
COPD status (%)¶			
I	17.0	21.3	<0.001
II	26.4	31.9	
III	11.3	38.3	
IV	1.9	4.3	
No COPD	7.5	0	
Not available	35.8	4.3	

*Two-sided two-sample t test between lung cancer patients and controls in continuous variables/χ^2 test for differences between cases and controls in categorical variables.
†Mean±SD.
‡Defined by quitting smoking at least 1 year before study enrolment.
§Includes only current and former smokers.
¶According to GOLD classification.[24]
COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease.

Combined RASSF1A/APC/CYGB methylation analysis of sputum of canister I had a sensitivity of 43%, increasing to 53% when canister II is added and 64% when all three canisters were considered, with corresponding specificity of 96%, 94% and 91%, respectively.

To further explore the additive value of sampling sputum during more than 3 days, we determined the number of positive cases in the group who tested negative in canister I (table 5).

Of the 30 cases who tested negative for all three biomarkers in canister I, an additional 11 cases (36.7%; 95% CI 19.9% to 56.1%) showed gene promoter hypermethylation of RASSF1A, APC or CYGB in either canister II or III. These results indicate that roughly 30% more lung cancer cases are detected when sputum sampling is prolonged from 3 to 9 days.

Multiple testing of a single canister demonstrated high concordance (κ 0.73–0.94) and no significant differences (Cochran's Q test, p=0.21).

DISCUSSION

This is the first study examining the influence of duration of sputum collection on sensitivity of gene promoter hypermethylation analysis for lung cancer. Here, we show that cumulative 6- or 9-day methylation analysis substantially increases the chance of identifying lung cancer. Combined RASSF1A, APC or CYGB promoter methylation analysis demonstrated a sensitivity of 43% and specificity of 96% in sputum samples collected during three consecutive days, increasing to 64% (specificity 91%) for 9-day cumulative combined methylation analysis.

Our findings of improved sensitivity for lung cancer diagnosis when sputum is sampled over more than three consecutive days are in line with studies evaluating cytology in sputum specimens that were sequentially collected over several days.[5 26] The proportion of lung cancer diagnosis by means of sputum cytological examination was shown to increase with longer collection time. Apparently, the detection rate of aberrant tumour DNA and malignant cells may increase when sputum is investigated over a prolonged period of time.

Kennedy *et al*[6] have evaluated procedure of sputum collection on sputum adequacy by means of sputum cytology. Patients with COPD were randomised into two groups, the first to collect spontaneous sputum at home, followed by a single production of induced sputum at the hospital. The other group received the same instructions, but in reverse order. In both groups, the second sputum sample was demonstrated to provide best quality of sputum, irrespective of the sampling method. The authors concluded that in time a learning effect for sputum collection occurred in the patients which had a positive impact on the quality of the sputum for cytology reading. Although we investigated epigenetic alterations instead of cytological aberrations, we found no differences in number of positive samples detected by gene promoter hypermethylation between the canisters. Some subjects scored negative in canister I but positive

Table 4 Cumulative analysis of gene promoter hypermethylation of RASSF1A, APC and CYGB in sputum of lung cancer patients and controls based on qMSP analysis

	Canister			
	I and II		I, II and III	
Gene(s)	Lung cancer n (%)	Controls n (%)	Lung cancer n (%)	Controls n (%)
RASSF1A	19 (36)*	2 (4)	22 (42)*	2 (4)
APC	7 (13)†	1 (2)	9 (17)†	2 (4)
CYGB	15 (28)*	1 (2)	20 (38)*	1 (2)
RASSF1A, APC and/or CYGB	28 (53)*	3 (6)	34 (64)*	4 (9)

Canister I=days 1–3; II=days 4–6; III=days 7–9.
*χ2 test p value <0.001.
†χ^2 test p value <0.05.
APC, adenomatous polyposis coli; CYGB, cytoglobin; qMSP, quantitative methylation-specific PCR.

Table 3 Gene promoter hypermethylation of RASSF1A, APC and CYGB in sputum of lung cancer patients and controls based on qMSP analysis

	Canister						
	I		II		III		
Gene(s)	Lung cancer n (%)	Controls n (%)	Lung cancer n (%)	Controls n (%)	Lung cancer n (%)	Controls n (%)	p Value*
RASSF1A	15 (28)†	1 (2)	16 (30)†	1 (2)	15 (28)†	0 (0)	0.79
APC	4 (8)	0 (0)	7 (13)‡	1 (2)	5 (9)	1 (2)	0.22
CYGB	11 (21)‡	1 (2)	9 (17)‡	0 (0)	15 (28)†	1 (2)	0.10
RASSF1A, APC and/or CYGB	23 (43)†	2 (4)	21 (40)†	1 (2)	25 (47)†	2 (4)	0.44

Canister I=days 1–3; II=days 4–6; III=days 7–9.
*Cochran's Q test.
†χ2 test p value <0.001.
‡χ2 test p value <0.05.
APC, adenomatous polyposis coli; CYGB, cytoglobin; qMSP, quantitative methylation-specific PCR.

Table 5 Gene promoter hypermethylation of RASSF1A, APC, CYGB and all biomarkers combined in lung cancer cases who tested negative in the first 3 days (I-) with 95% CIs

	RASSF1A			APC		
	N I-	Positive % (n)	95% CI	N I-	Positive % (n)	95% CI
II+ I-	38	10.5% (4)	2.9% to 24.8%	49	6.1% (3)	1.3% to 16.9%
III+ I-	38	10.5% (4)	2.9% to 24.8%	49	4.1% (2)	0.5% to 14.0%
II/III+ I-	38	18.4% (7)	7.7% to 34.3%	49	10.2% (5)	3.4% to 22.2%
	CYGB			RASSF1A, APC and/or CYGB		
	N I-	Positive % (n)	95% CI	N I-	Positive % (n)	95% CI
II+ I-	42	9.5% (4)	2.7% to 22.6%	30	16.7% (5)	5.6% to 34.7%
III+ I-	42	14.3% (6)	5.4% to 28.5%	30	26.7% (8)	12.3% to 45.9%
II/III+ I-	42	21.4% (9)	10.3% to 36.8%	30	36.7% (11)	19.9% to 56.1%

(II+)=positivity in canister of days 4–6; (III+)=positivity in canister of days 7–9; (II/III+)=positivity in canister II and/or III.
APC, adenomatous polyposis coli; CYGB, cytoglobin.

in canister II and vice versa, but overall, this made no difference on the general outcome of positive hypermethylation. A likely explanation for the difference with the study of Kennedy *et al*[6] is the detailed instructions on the patient sputum information sheet, and the verbal instruction at inclusion used in this study, which may have overcome the learning effect.

In this study, we investigated gene promoter hypermethylation of RASSF1A, CYGB and APC. These markers have shown to be useful as markers for early detection of lung cancer. RASSF1A, which encodes a protein similar to RAS effector proteins, is involved in apoptosis and signal transduction.[27 28] RASSF1A is considered to be a tumour-associated biomarker.[21] RASSF1A is rarely hypermethylated in sputum of smokers, and more frequent hypermethylation is present in sputum of patients with invasive lung cancer.[16 21 29–33] CYGB is an emerging marker.[20] The function of this gene is not yet clear; it is distantly related to myoglobin and proposed to play a role in sensing and transport of oxygen and collagen synthesis.[34–36] It is still unclear how CYGB is involved in cancer pathogenesis, although its silencing in lung cancer supports a putative tumour-suppressor function. In vitro, restoration of CYGB in cancer cell lines has been shown to inhibit cancer growth, whereas silencing resulted in increased colony formation.[20] Last, the APC gene is mostly known because of its crucial role in the development of colon cancer in the adenoma-carcinoma sequence.[37] APC is a Wnt-pathway antagonist and regulates, through transcriptional activation, cell migration and adhesion. In sputum of lung cancer patients and smokers, APC is reported to be hypermethylated. In this study, results of RASSF1A (28%–30% in lung cancer patients) and CYGB promoter hypermethylation (17%–28% in lung cancer) are comparable with outcomes of previous studies,[11 16 20] while we found a lower frequency of APC promoter hypermethylation (6%–13%) compared with previous reports.[16 23]

In conclusion, sensitivity of gene promoter hypermethylation analysis in sputum for lung cancer increases when sputum is sampled during more than three consecutive days. This study suggests further research for establishing diagnostic performance of methylation markers on sputum samples of a larger cohort.

Take-home message

Assessment of hypermethylation sensitivity of biomarkers in sputum collected over a prolonged period for the detection of lung cancer resulted in a promising gain in sensitivity, at the expense of a small loss in specificity, which offers perspectives for improving lung cancer diagnosis.

Contributors The contributions of the authors to the study are as follows: AH, DH, ES, ET: involvement in the conception, hypotheses delineation and design of the study; AH, DH, GH, SB, PS, PK, HS, PP, BW, SD, PS, ES, ET: acquisition of the data or the analysis and interpretations. All authors have read and approved the manuscript.

Funding This study was funded by the Dutch Cancer Society (grant VU2008-4220).

Competing interest None declared.

Patient consent Patients signed an informed consent form not derived from *BMJ*, but according to the study protocol. The informed consent forms were approved by the Institutional Review Boards of all participating hospitals.

Ethics approval The ethics approval was provided by Institutional Review Boards of participating hospitals.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Ferlay J,** Shin HR, Bray F, *et al*. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;**127**:2893–917.
2. **Jemal A,** Siegel R, Xu J, *et al*. Cancer statistics, 2010. *CA Cancer J Clin* 2010;**60**:277–300.
3. **Sant M,** Allemani C, Santaquilani M, *et al*. EUROCARE-4. Survival of cancer patients diagnosed in 1995-1999. Results and commentary. *Eur J Cancer* 2009;**45**:931–91.
4. **Integraal Kankercentrum Nederland.** http://www.iknl.nl. http://www.cijfersoverkanker.nl/selecties/dataset_4/img4ed7645c7db74 (accessed 1 Dec 2011).
5. **Risse EK,** van 't Hof MA, Vooijs GP. Relationship between patient characteristics and the sputum cytologic diagnosis of lung cancer. *Acta Cytol* 1987;**31**:159–65.
6. **Kennedy TC,** Proudfoot SP, Piantadosi S, *et al*. Efficacy of two sputum collection techniques in patients with air flow obstruction. *Acta Cytol* 1999;**43**:630–6.
7. **Olaussen KA,** Soria JC, Park YW, *et al*. Assessing abnormal gene promoter methylation in paraffin-embedded sputum from patients with NSCLC. *Eur J Cancer* 2005;**41**:2112–19.
8. **Mao L,** Hruban RH, Boyle JO, *et al*. Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. *Cancer Res* 1994;**54**:1634–7.
9. **Destro A,** Bianchi P, Alloisio M, *et al*. K-ras and p16(INK4A)alterations in sputum of NSCLC patients and in heavy asymptomatic chronic smokers. *Lung Cancer* 2004;**44**:23–32.
10. **Somers VA,** Pietersen AM, Theunissen PH, *et al*. Detection of K-ras point mutations in sputum from patients with adenocarcinoma of the lung by point-EXACCT. *J Clin Oncol* 1998;**16**:3061–8.
11. **Belinsky SA,** Grimes MJ, Casas E, *et al*. Predicting gene promoter methylation in non-small-cell lung cancer by evaluating sputum and serum. *Br J Cancer* 2007;**96**:1278–83.
12. **Belinsky SA,** Klinge DM, Dekker JD, *et al*. Gene promoter methylation in plasma and sputum increases with lung cancer risk. *Clin Cancer Res* 2005;**11**:6505–11.
13. **Honorio S,** Agathanggelou A, Schuermann M, *et al*. Detection of RASSF1A aberrant promoter hypermethylation in sputum from chronic smokers and ductal carcinoma in situ from breast cancer patients. *Oncogene* 2003;**22**:147–50.
14. **Hwang SH,** Kim KU, Kim JE, *et al*. Detection of HOXA9 gene methylation in tumor tissues and induced sputum samples from primary lung cancer patients. *Clin Chem Lab Med* 2011;**49**:699–704.
15. **Kersting M,** Friedl C, Kraus A, *et al*. Differential frequencies of p16(INK4a) promoter hypermethylation, p53 mutation, and K-ras mutation in exfoliative material mark the

development of lung cancer in symptomatic chronic smokers. *J Clin Oncol* 2000;**18**:3221—9.
16. **Shivapurkar N,** Stastny V, Suzuki M, *et al*. Application of a methylation gene panel by quantitative PCR for lung cancers. *Cancer Lett* 2007;**247**:56—71.
17. **Tessema M,** Klinge DM, Yingling CM, *et al*. Re-expression of CXCL14, a common target for epigenetic silencing in lung cancer, induces tumor necrosis. *Oncogene* 2010;**29**:5159—70.
18. **Herman JG,** Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;**349**:2042—54.
19. **Esteller M.** Epigenetics in cancer. *N Engl J Med* 2008;**358**:1148—59.
20. **Shivapurkar N,** Stastny V, Okumura N, *et al*. Cytoglobin, the newest member of the globin family, functions as a tumor suppressor gene. *Cancer Res* 2008;**68**:7448—56.
21. **Cirincione R,** Lintas C, Conte D, *et al*. Methylation profile in tumor and sputum samples of lung cancer patients detected by spiral computed tomography: a nested case-control study. *Int J Cancer* 2006;**118**:1248—53.
22. **Belinsky SA,** Liechty KC, Gentry FD, *et al*. Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. *Cancer Res* 2006;**66**:3338—44.
23. **Konno S,** Morishita Y, Fukasawa M, *et al*. Anthracotic index and DNA methylation status of sputum contents can be used for identifying the population at risk of lung carcinoma. *Cancer* 2004;**102**:348—54.
24. **Gold PM.** The 2007 GOLD Guidelines: a comprehensive care framework. *Respir Care* 2009;**54**:1040—9.
25. **Van der Drift MA,** Prinsen CF, Hol BE, *et al*. Can free DNA be detected in sputum of lung cancer patients? *Lung Cancer* 2008;**61**:385—90.
26. **Risse EK,** van 't Hof MA, Laurini RN, *et al*. Sputum cytology by the Saccomanno method in diagnosing lung malignancy. *Diagn Cytopathol* 1985;**1**:286—91.
27. **Dammann R,** Li C, Yoon JH, *et al*. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 2000;**25**:315—19.
28. **Donninger H,** Vos MD, Clark GJ. The RASSF1A tumor suppressor. *J Cell Sci* 2007;**120**:3163—72.
29. **Baryshnikova E,** Destro A, Infante MV, *et al*. Molecular alterations in spontaneous sputum of cancer-free heavy smokers: results from a large screening program. *Clin Cancer Res* 2008;**14**:1913—19.
30. **Belinsky SA,** Palmisano WA, Gilliland FD, *et al*. Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. *Cancer Res* 2002;**62**:2370—7.
31. **Leng S,** Stidley CA, Willink R, *et al*. Double-strand break damage and associated DNA repair genes predispose smokers to gene methylation. *Cancer Res* 2008;**68**:3049—56.
32. **Stidley CA,** Picchi MA, Leng S, *et al*. Multivitamins, folate, and green vegetables protect against gene promoter methylation in the aerodigestive tract of smokers. *Cancer Res* 2010;**70**:568—74.
33. **Zöchbauer-Müller S,** Lam S, Toyooka S, *et al*. Aberrant methylation of multiple genes in the upper aerodigestive tract epithelium of heavy smokers. *Int J Cancer* 2003;**107**:612—16.
34. **Burmester T,** Ebner B, Weich B, *et al*. Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. *Mol Biol Evol* 2002;**19**:416—21.
35. **Oleksiewicz U,** Liloglou T, Field JK, *et al*. Cytoglobin: biochemical, functional and clinical perspective of the newest member of the globin family. *Cell Mol Life Sci* 2011;**68**:3869—83.
36. **Xinarianos G,** McRonald FE, Risk JM, *et al*. Frequent genetic and epigenetic abnormalities contribute to the deregulation of cytoglobin in non-small cell lung cancer. *Hum Mol Genet* 2006;**15**:2038—44.
37. **Markowitz SD,** Bertagnolli MM. Molecular origins of cancer: molecular basis of colorectal cancer. *N Engl J Med* 2009;**361**:2449—60.