Molecular Alterations in Spontaneous Sputum of Cancer-Free Heavy Smokers: Results from a Large Screening Program

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Abstract

Purpose: The high mortality rate for lung cancer is likely to be reduced by the development of a panel of sensitive biological markers able to identify early-stage lung cancers or subjects at high risk. The aim of this study was to establish the frequency of *K-ras* and *p53* mutations and $p16^{INK4A}$, *RASSF1A*, and *NORE1A* hypermethylation in sputum of a large cohort of cancer-free heavy smokers and to assess whether these markers are suitable for a routine use in the clinical practice for the early diagnosis of pulmonary cancer.

Experimental Design: Sputum samples were collected from 820 heavy smokers. Inclusion criteria consisted of radiologic and cytologic absence of pulmonary lesions, age at least 60 years, male gender, and a smoking history of at least 20 pack-years.

Results: The analysis identified 56 individuals (6.9%) with one molecular alteration. *p53* mutation and $p16^{INK4A}$, *RASSF1A*, and *NORE1A* methylation frequencies were 1.9%, 5.1%, 0.8%, and 1.0%, respectively; no *K-ras* mutations were found. One patient with *p53* mutations was diagnosed with an early-stage lung cancer after 3-years of follow-up. The molecular analysis of bronchoscopy samples confirmed in half of the cases alterations present in sputum without revealing additional molecular changes.

Conclusions: Genetic and epigenetic abnormalities can be detected in cancer-free heavy smokers. Although the predictive value of the cancer risk is still to be established as it requires not less than 5 years of follow-up, *p53* and $p16^{INK4A}$ are more promising candidates than *K-ras, RASSF1A*, and *NORE1A* for the pulmonary molecular screening of heavy smokers healthy individuals.

Lung cancer is one of the most frequent malignancies worldwide and the leading cause of cancer-related death in male (1). The increasing incidence of non–small cell lung cancer (NSCLC) in the industrialized countries is mainly due to the expanding consumption of tobacco, directly associated with NSCLC etiology. Epidemiologic studies show that ~85% of the cases of NSCLC arise in current or former smokers (2). Interestingly, 50% of all newly diagnosed lung cancers occur in people who stopped smoking by at least 5 years, supporting that the damage to the respiratory epithelium is persistent over time (3).

Prognosis of NSCLC is largely dependent on the stage of the disease at the time of diagnosis: patients with stage I disease undergoing surgical resection have 65% to 75% 5-year survival rate, as opposed to <1% of stage IV patients (4). However, with standard methods (i.e., chest radiography and sputum cytology), it is often difficult to detect early-stage tumors; the development of an efficient diagnostic approach for the detection of early lung cancer may be able to substantially reduce the high mortality rate for this disease.

In the past few years, the presence of specific multiple genetic and epigenetic alterations has been documented in tumor tissue of patients with NSCLC (5). These abnormalities were also found in the respiratory epithelium of current and former smokers and they seem to be directly associated with cigarette smoking (6–11). Belinsky et al. reported that tumor suppressor gene promoter hypermethylation in biological fluids could be detected several years before a clinical diagnosis of lung cancer and suggested that they could approximate the lifetime risk for developing lung cancer (12, 13). These molecular markers could be useful not only for early detection but even for prevention of lung cancer in high-risk subjects.

An efficient and sensitive screening approach requires molecular markers to be present in biological samples easily

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available through noninvasive procedures. Many current and former smokers have increased bronchial secretions that result in the regular production of sputum containing exfoliated cells from the bronchial tree. This makes the molecular analysis of sputum of cancer-free current and former heavy smokers a promising area of research for biomarker development.

Among molecular alterations identified thus far in NSCLC and in corresponding sputum samples, there are *K-ras* and *p53* point mutations and $p16^{INK4A}$ and, more recently, *RASSF1A* and *NORE1A* promoter hypermethylation. *K-ras* has essential roles in controlling the activity of several crucial signaling pathways that regulate normal cellular proliferation. *K-ras* mutations cause persistent activation of the protein and are correlated with smoking (14). The vast majority of *K-ras* mutations affect codon 12 (>90%) and G→T transversion is the most common. *K-ras* mutations are rare in small cell lung cancer (15) but present in NSCLC with rates ranging up to 50%, both in tumor tissue and biological fluids of patients with cancer (6, 9, 16). Data obtained from analysis of cancer-free subjects are controversial: Ronai et al. documented *K-ras* mutations in sputum of cancer-free patients (17), but other studies did not (9).

The *p53* tumor suppressor gene prevents the proliferation of cells with genetic alterations and could be inactivated by both deletions and mutations. Somatic alterations of *p53* are found in NSCLC at frequency of ~50% (18–21); the presence of mutated *p53* has also been documented in premalignant lesions and in sputum from patients with or without cancer (9, 10).

More recently, transcriptional silencing of genes by CpG island methylation has been recognized as a crucial component in lung cancer initiation and progression (22). The $p16^{INK4A}$ tumor suppressor gene plays a key role in cell cycle regulation and is one of the most frequently methylated genes in NSCLC (23, 24), in early premalignant lesions (25, 26), and in sputum of patients with NSCLC or asymptomatic chronic smokers (6, 7). The COOH terminus of *RASSF1A* (Ras association domain family 1) is homologous to the mammalian Ras effector protein NORE1 and encodes a Ras association domain. *RASSF1A* promoter methylation is found in ~40% of NSCLC (27–31), whereas *NORE1A* methylation is less frequently documented (32, 33).

The aim of our study was therefore to determine the prevalence for *K-ras* and *p53* mutations and $p16^{INK4A}$, *RASSF1A*, and *NORE1A* promoter methylation in spontaneous sputum samples from a very large series of cancer-free current and former heavy smokers with available follow-up and to determine (*a*) the prevalence of the molecular abnormalities, (*b*) whether these abnormalities are persistent during follow-up or detectable with other sampling procedure (i.e., bronchoscopy), and (*c*) whether and which of these molecular abnormalities may antedate pulmonary cancer appearance. The final goal of the study is to select the most suitable markers to be included in a future screening program for the early detection of pulmonary cancer.

Materials and Methods

Subject enrollment and sputum processing. Three sputum samples were collected from 820 Caucasian current or former heavy smokers from metropolitan areas of northern Italy enrolled at our institution in an ongoing randomized trial for the early detection of pulmonary cancer with low-dose spiral computed tomography (34) between March 2001 and December 2004. Inclusion criteria were radiologic absence of pulmonary lesions and no cancer diagnosis within 10 y before the time of enrollment, age ≥60 y, male gender, and a smoking history of ≥20 pack-years, current or quitted <10 y before. All participants gave written informed consent and filled in a standardized questionnaire that described in detail their smoking history. Selected demographic features are summarized in Table 1. Subjects were provided with three sterile cups containing 70% ethanol and instructed to take a deep breath, cough deeply, and expectorate into the cup for 3 consecutive days.

Sputum samples were subsequently formalin fixed and paraffin embedded. For each sputum, three sections were stained with H&E and examined by an experienced cytologist. Cytologic diagnosis was the following: (*a*) inadequate for the absence of alveolar macrophages, (*b*) negative for cancer cells, (*c*) positive for cancer cells, and (*d*) cells with atypical features suspicious but nonconclusive for malignancy.

Molecular analysis of sputum samples. Five to 10 sections (10 μm thick) from each formalin-fixed, paraffin-embedded sputum cell block and 3 sections (5 μm thick) from the tumor sample were collected; sections from the same patient were collected in the same tube before xylene deparaffinization (Sigma). After deparaffinization, all samples were treated with proteinase K (Finnzymes) at 50°C overnight followed by standard phenol-chloroform extraction (Sigma) and ethanol precipitation.

Patients with molecular alterations of sputum have been enrolled in a follow-up screening with annual low-dose spiral computed tomography and, if they consented to it, a bronchoscopy with molecular analysis of bronchoalveolar lavages and biopsies.

Bronchoscopy. Bronchoscopy was carried out with the intent to identify mucosal alterations, if any, and to supply representative cytology samples from both lungs and random mucosal biopsies in an attempt to understand whether the identified molecular changes were specifically located or diffusely present. During bronchoscopy, two bronchoalveolar lavage samples and two random biopsies were collected from the left and the right side for the molecular analysis.

Flexible bronchoscopy was done under local anesthesia with 10% lignocaine spray and 5 mg i.v. midazolam for mild sedation in emotional subjects only. The larynx, trachea, and bronchial tree were inspected under white light, and bronchoalveolar lavage samples were obtained after injection of 20 mL sterile isotonic saline from the right and the left main bronchi. In the absence of any visible abnormality, mucosal biopsies were routinely obtained from the secondary carina between the upper and lower lobar bronchus on the left side and

Table 1. Summary of selected available demographic variables of the studied cohort

	All (*n* = 788)		Current (*n* = 469)		Former (*n* = 319)	
Age, y (95% CI)	64.2	63.7-64.6	63.3	62.6-64.0	65.4	65.0-65.9
Pack-years (95% CI)	48.2	46.1-50.2	49.0	46.6-51.4	46.8	43.1-50.5

Abbreviation: 95% CI, 95% confidence interval.

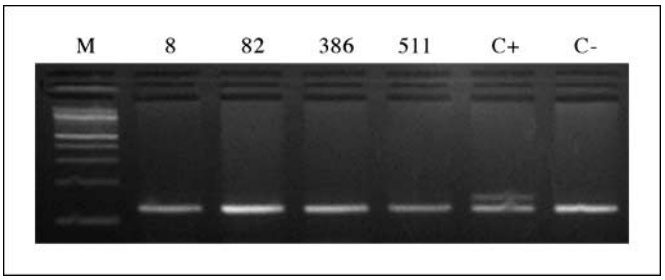

Fig. 1. Analysis of the mutational status of *K-ras* in sputum samples. *Bst*NI digestion of PCR products left intact only the PCR products derived from mutant alleles (157 bp). In contrast, from digestion of normal alleles derived two restriction products of 128 and 29 bp. M, molecular marker; C+, *K-ras* mutated control; C-, normal DNA.

between the upper and the intermediate bronchus on the right side. Any area of abnormal mucosa were biopsied as well. A separate informed consent was requested for this supplemental investigation.

The samples have been centrifuged at 4,500 × *g* for 10 min, the pellet was digested with proteinase K at 50°C overnight, and the DNA was extracted by standard phenol-chloroform method.

All the samples underwent molecular analysis. A subject was scored as positive when at least one of the samples was positive for the individual marker previously reported in sputum.

K-ras *mutation analysis.* *K-ras* codon 12 mutation analysis was done as previously described (6, 35) by using a mismatched primer, which introduces a *Bst*NI restriction site into PCR products derived from normal alleles. The PCR mixture contained 300 ng of genomic DNA, 0.2 mmol/L of nucleotide mix (Finnzymes), 0.5 mmol/L $MgCl_2$ (Perkin-Elmer), 0.2 μmol/L of each primer (Proligo), and 1 unit of Taq polymerase (Finnzymes). Primer sequences and PCR conditions are reported in Supplementary Data 1. PCR product (20 μL) was digested with 5 units of *Bst*NI (New England Biolabs, Inc.). The digestion was done for 4 h at 60°C under conditions recommended by the supplier. Positive (SW480 cell line) and negative (human placental DNA) controls were included in every experiment. Digested products (10 μL) were run on a 3% agarose gel and visualized by staining with ethidium bromide (Fig. 1).

p53 *mutation analysis.* *p53* mutations in exons 5 to 8 have been identified with single-strand conformational polymorphism analysis, running the previously denaturated PCR products on polyacrylamide gel and eventually by sequencing the samples with an altered migratory pattern (36).

PCR amplification was done in 50 μL reaction volume with a final concentration of 0.2 mmol/L deoxynucleotide triphosphates (Finnzymes), 0.2 μmol/L of each primer (Proligo), and 1 unit of Taq polymerase. Primer sequences and PCR conditions are described in Supplementary Data 1.

The PCR products, denaturated in a buffer containing 100% formamide (Sigma), were run at room temperature on 15% non-denaturing polyacrylamide gel (Sigma) for 1 h at 100 V and for 3 to 4 h at 250 to 300 V depending on the exon. DNA containing the wild-type *p53* (human placental DNA) was always included as control. Silver staining was done using PlusOne DNA Silver Staining kit (Amersham Biosciences Corp.) according to the manufacturer's instructions.

PCR products from samples with altered pattern were purified using DyeEx 2.0 Spin kit (Qiagen GmbH) and subjected to direct cycle sequencing using ABI PRISM Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems) and run on ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). An example of a sequence of mutated *p53* is represented in Fig. 2.

$p16^{INK4A}$, RASSF1A, *and* NORE1A *methylation-specific PCR.* As previously reported (6, 35), genomic DNA was modified by treatment with sodium bisulfite, which converts all unmethylated cytosines to uracil and then to thymidine during the subsequent PCR step. Modified DNA was purified using the Wizard DNA purification system (Promega) according to the manufacturer's instructions, precipitated with ethanol, and used immediately or stored at -20°C.

The methylation status of $p16^{INK4A}$, *RASSF1A*, and *NORE1A* was assessed by nested methylation-specific PCR method, as previously described (6, 35, 37). Modified DNA (5 μL) was amplified in a stage I PCR with a primer set that recognizes the bisulfite-modified template but does not discriminate between methylated and unmethylated alleles. Stage I PCR product (5 μL) was subjected to stage II PCR with primers specific to methylated or unmethylated template. All primer sequences and PCR conditions are reported in Supplementary Data 1.

All PCRs were done with positive and negative methylation controls: HT29 (human colorectal carcinoma cell line) completely methylated for $p16^{INK4A}$, Alex (human hepatoma cell line) completely unmethylated for $p16^{INK4A}$, HeLa (human cervical cancer cell line) completely unmethylated for *RASSF1A*, LOVO (human colon adenocarcinoma) hemimethylated for *RASSF1A* and completely unmethylated for *NORE1A*, and HCT 116 (human adenocarcinoma cell line) hemimethylated for *NORE1A*.

Examples of samples methylated and unmethylated for $p16^{INK4A}$ are shown in Fig. 3.

To confirm the specificity of the method, purified PCR product from positive and negative controls and from several sputum specimens was subjected to direct cycle sequencing using ABI PRISM Big Dye Terminator Cycle Sequencing kit and run on ABI PRISM 310 Genetic Analyzer (6).

Statistical analysis. The relationship between genetic data and age, pack-years, and smoking status was analyzed using multivariate logistic models.

One first model included both $p16^{INK4A}$/*p53* alterations as dependent variable, a second model included $p16^{INK4A}$ alone, and a third model included *p53* alone. Covariates were age, pack-years, and smoking status (current or former).

The Wald statistic, the regression coefficient estimates (with their *P* values based on Wald χ^2), and the odds ratio for every covariate were calculated for each model. Differences were considered significant for *P* values of <0.05. All statistical calculations were done using the Statistical Analysis System System, release 8.2, in Microsoft Windows XP environment.

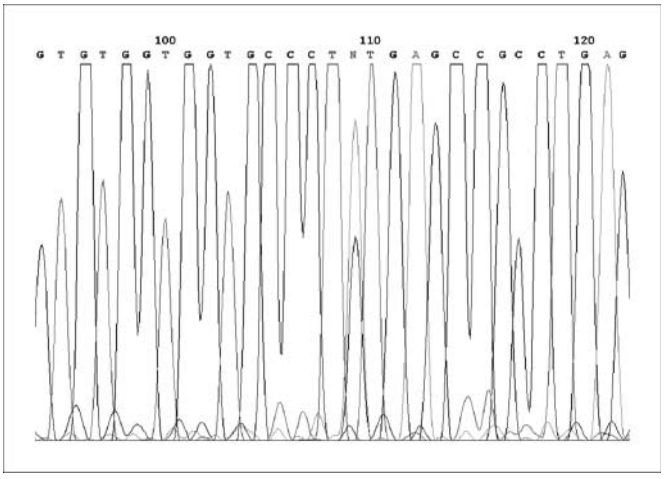

Fig. 2. Analysis of the mutational status of *p53* gene. Direct sequencing of a sample with altered running pattern on single-strand conformational polymorphism. In position 109, a double peak indicates the presence of a TAT→TGT mutation, then resulting in Tyr→Cys substitution.

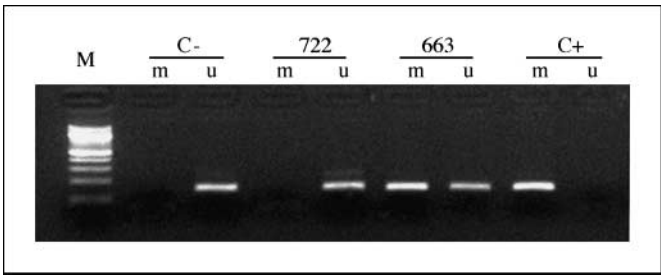

Fig. 3. Analysis of $p16^{INK4A}$ promoter methylation. The presence of a visible PCR product in the lanes marked "u" indicates the presence of unmethylated alleles; in the lanes marked "m," the presence of methylated alleles. C-, unmethylated control (Alex cell line); C+, methylated control (HT29 cell line).

Results

Overall cytologic profile in sputum samples. Eight hundred and twenty patients underwent cytologic analysis of sputum samples. Seventeen subjects (2.1%) had a prevalent pulmonary cancer at the enrollment but only 1 (5.9%) of these was identified on cytologic basis; the other 16 were diagnosed through imaging techniques. The overall results of the cytologic analysis are shown in Supplementary Data 2.

Only patients without cancer at the time of enrollment (n = 803) were then subjected to the molecular assay.

Overall molecular profile in sputum samples. Sputum samples from 803 patients have been analyzed with at least one marker. Genetic analysis identified 55 subjects (6.8%) carrying a single molecular abnormality and 1 patient with coincidental methylation of $p16^{INK4A}$ and *NORE1A* (Table 2).

Approximately 50% of *p53* mutations were in exon 8 and 50% were G→A transversions. One patient with a G→A transversion in codon 273 (exon 8) was diagnosed with an early-stage squamous cell carcinoma (pT1;N0) after 3-years of follow-up; surgical resection was carried out on November 2004, and to date, the patient is alive and in good health. This *p53* mutation was not documented in the resected tumor tissue. One patient with a C→T mutation in codon 283 of *p53* gene died of melanoma 1 year after the enrollment and one another with C→A mutation in codon 239 developed a colon adenocarcinoma after 4 years of follow-up. The overall mutational profile of *p53* is reported in Supplementary Data 2.

The most frequent molecular abnormality was $p16^{INK4A}$ promoter hypermethylation (5.1%). One of these patients reported a history of laryngeal carcinoma resected 11 years before and the same patient has now developed a bladder cancer.

RASSF1A and *NORE1A* promoter hypermethylation was studied in a subgroup of population: the observed molecular alteration rates were 0.8% (2 of 246) for *RASSF1A* and 1.0% (1 of 99) for *NORE1A*. The patient presenting coincidental methylation of $p16^{INK4A}$ and *NORE1A* is cancer-free after 53 months of follow-up.

Sputum with molecular alterations never showed cytologic abnormalities.

Molecular analysis of bronchoscopy samples. All the patients with molecular alterations documented in sputum (n = 56) were requested to undergo a bronchoscopy as a part of a general follow-up program, but only 18 of them agreed (5 with *p53* mutation and 12 with $p16^{INK4A}$ hypermethylation alone and 1 with a contemporary methylation of $p16^{INK4A}$ and *NORE1A*). Bronchoscopies were carried out after an average of 30 ± 10 months of follow-up.

Of five patients with *p53* mutations in sputum, two (40%) showed the same mutation in samples collected at bronchoscopy. Specifically, one patient showed the mutation in all the samples, whereas the other showed only in bronchoalveolar lavages from the right emisystem.

Of 13 patients with $p16^{INK4A}$ hypermethylation in sputum, 8 (61.5%) showed the same molecular change in bronchoscopy. Almost all the patients showed $p16^{INK4A}$ hypermethylation in one of four collected samples without elective topographical location. *NORE1A* promoter methylation has also been confirmed.

With the notable exception of a single case showing *p53* mutation and cancer development after 3 years, all the patients with molecular abnormalities in sputum are cancer-free after a mean follow-up of 45.2 months (±8.8 months).

Eighteen (2.2%) patients without molecular alterations in sputum at the enrollment developed a pulmonary cancer after a mean follow-up of 34.1 months (±13.2 months).

Statistical analysis. No statistically significant association between the presence of molecular alterations and age- or smoking-related characteristics, such as smoking status (current or former) and total exposure (as pack-years), has been observed in the cohort under study.

Discussion

One of the most important aims of modern oncology is to find an effective and sensitive method for early diagnosis of pulmonary cancer. Conventional techniques (i.e., radiologic imaging and cytology) are limited by low sensitivity. Indeed, the reported sensitivity of cytology is <1% of newly diagnosed cases (38); other studies have shown that the detection rate in large screening programs is ~4 of 1,000 screened subjects per year (39, 40). In our study, cytology identified only 1 of the cases with prevalent lung cancer (n = 17), whereas imaging techniques revealed the additional 16. Clearly, new molecular and biological tools are needed for an early diagnosis of this aggressive pathology.

To our knowledge, this is the largest study analyzing rates of abnormalities for *K-ras*, *p53*, $p16^{INK4A}$, and *RASSF1A* genes, and the first one investigating *NORE1A* gene methylation in spontaneous sputum of cancer-free chronic smokers. A statistically significant association between tumor tissue and

Table 2. Overall genetic and epigenetic alterations in sputum samples

Genes	No. analyzed cases	No. altered cases (%)
K-ras	506	0
p53	803	15 (1.9)
$p16^{INK4A}$	765	39 (5.1)
RASSF1A	246	2 (0.8)
NORE1A	99	1 (1.0)*
Total		56 (6.9)

*Patient with coincidental methylation of $p16^{INK4A}$.

Table 3. Prospective studies reporting molecular alterations in sputum

Reference	Alterations			Follow-up*
	p16^{INK4A}	*p53*	*K-ras*	
Ronai et al. (17)			5/40 (12.5%) †	
Belinsky et al. (25)	5/26 (19.2%) ‡			3 y: no tumor
Kersting et al. (9)	7/25 (28%) †‡	3/25 (12%)	0/25	1 y: 3 tumors
Palmisano et al. (13)	6/32 (18.7%)			6 y: no tumor
Wang et al. (10)		2/114 (1.7%) †		4 y: 1 tumor
Belinsky et al. (7)	32/66 (35%)§			
Zochbauer-Muller et al. (11)	4/73 (5.5%) †‡			
This study	39/765 (5.1%)	15/803 (1.9%)	0/506 (0%)	3 y: 1 tumor

*All the tumors reported in the follow-up column arose in patients with previous molecular alterations detected in sputum.
† Benign pulmonary disease.
‡ Induced sputum.
§ 25% with cellular atypia.

matched sputum was previously reported for *K-ras* (6, 9, 41), *p53* (10), and *p16^{INK4A}* (6, 42, 43). These studies have confirmed that the alterations observed in sputum are not due to a generalized epithelial damage but rather to the clonal expansion of the transformed cells.

We found no *K-ras* mutation in any of the cancer-free individuals. This result supports the findings of other groups who reported *K-ras* mutations in cancer tissue but not in the adjacent nonmalignant tissue or in sputum from healthy individuals (9). *K-ras* could therefore be considered as a putative marker for an already established malignancy rather than as a marker for high-risk pulmonary cancer patients. Nevertheless, a previous study reported *K-ras* mutations in sputum of cancer-free individuals (17). The discrepancy might be due to differences in the collection methodology, type of sampling, and manipulation procedures.

In the present study, we have identified a subpopulation of healthy subjects harboring *p53* mutations in the bronchial tree (1.9%), in agreement with the frequency (1.7%) reported by Wang et al. (10). Interestingly, one of our patient with exon 8 mutation repeatedly confirmed in sputum was diagnosed with an early-stage pulmonary cancer, although the mutation was not found in the resected tumor (bronchoscopy material was not available). We speculate that this *p53* mutation only represented the local setting of a genetic instability and the field for the sourcing of more severe carcinogenetic events.

p16^{INK4A} promoter hypermethylation was the most frequently detected molecular alteration (5.1%). Most of the few reports published in the last years suggested a higher methylation rate, ranging from 18% to 35% (7, 9, 13, 25), although in a single study (11) a frequency similar to that detectable in the present study was shown (5.5%). Likely, these differences are mainly due to selection criteria in the study population and/or in the methods used. Almost all the above-mentioned studies enrolled subjects already affected by pulmonary non-neoplastic diseases or at a very high risk for developing cancer for reasons unrelated to smoking (i.e., former uranium miners; ref. 13) and were also characterized by the small size of the series (<100 subjects) and by the collection of sputum obtained after induction procedures. A summary of data is reported in Table 3.

We deliberately decided to not collect induced sputum to avoid procedures that could not meet patient compliance in a large screening program. The present series of chronic smokers thus appears a cohort that for its size and inclusion criteria more closely reflects a general population at increased risk of developing pulmonary cancer. At present, none of the patients with *p16^{INK4A}* hypermethylation has developed a pulmonary cancer, after at least 2 years of follow-up (ranging from 2 to 6 years). It has been shown that *p16^{INK4A}* methylation is one of the earliest epigenetic changes seen in the lungs of smokers, so it could potentially anticipate tumor development by years (44). Recently, Belinsky et al. proposed a model in which the number of hypermethylated genes in sputum correlates with the relative risk of developing tumor. In this model, subjects with only one hypermethylated gene in their sputum develop neoplasia not less than 5 years of follow-up. To test this hypothesis on a subgroup of our population, we tested two additional epigenetic markers: *RASSF1A* and *NORE1A*. This analysis showed hypermethylation of these two markers at a very low rate (0.8% and 1%, respectively) with a single patient harboring both *p16^{INK4A}* and *NORE1A* hypermethylation. At the present time (after 3 years of follow-up), this individual is alive and disease-free. Only a few studies are available on *RASSF1A* in cancer-free individuals with discordant data: earlier articles report 3% to 4% hypermethylation frequency (7, 11), with 30.8% as a peak in a single report (8); recent data show a lower methylation rate (31), in keeping with our findings. NORE1A was not studied before in cancer-free individuals (32, 33).

In this study, we also evaluated whether the same molecular abnormality, once detected, can be persistently found over time in the bronchial tree. We documented that this is true in at least half of the patients for which we were able to carry out the analysis (13 of 18, 55.5%) because *p53* mutations and *p16^{INK4A}* methylation, found in sputum, were confirmed in bronchoscopy samples collected 30 ± 10 months after sputum analysis. This result may suggest that the number of cells harboring these changes is indeed very low but still above the threshold of detection in at least half of the patients, if not less than four different samples are collected at bronchoscopy.

An additional by-product of the present study was to evaluate the suitability of the DNA obtained from sputum compared

with bronchial lavage fluid or lung biopsies for monitoring genetic and epigenetic alterations in the bronchial tree. Although the quality of DNA from fresh samples, such as bronchoalveolar lavages and biopsies, was better than that from formalin-fixed, paraffin-embedded sputum, analysis of bronchoscopy samples did not reveal any additional alteration to those detected in sputum. These data suggest that the analysis of sputum samples offers a comparable, if not superior, level of detection compared with samples obtained by bronchoscopy. This finding is of clinical relevance because spontaneous sputum samples can be obtained in a relatively easy, noninvasive, and inexpensive way.

Molecular alterations in sputum did not correlate with age, smoking status (current or former), or smoke exposure (pack-years). Although the molecular abnormalities we investigated are likely related to a smoking habitus, no definite conclusions can be drawn because we decided not to recruit a control population of never smokers. Indeed, the yield and quality of spontaneous sputum in never smokers are poor, whereas the performance of invasive procedures in these individuals is not ethically justifiable. The use of spontaneous rather than induced sputum would make indeed this screening procedure more easily tolerated by healthy individuals.

In conclusion, the results of our study candidate *p53* and $p16^{INK4A}$ rather than *K-ras*, *RASSF1A*, and *NORE1A* for additional molecular studies on the risk of pulmonary cancer in healthy individuals. We retain, however, that more than 5 years of follow-up is likely needed to draw more solid clinical and biological conclusions on the predictive value of a panel of molecular markers in a cohort of cancer-free heavy smokers.

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References

1. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. CA Cancer J Clin 2002;52:23–47.
2. Hecht SS. Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst 1999;91:1194–210.
3. Wistuba I, Lam S, Behrens C, et al. Molecular damage in the bronchial epithelium of current and former smokers. J Natl Cancer Inst 1997;89:1366–73.
4. Harpole D, Herndon J, Young W, Wolfe W, Sabiston D. Stage I nonsmall cell lung cancer. A multivariate analysis of treatment methods and patterns of recurrence. Cancer 1995;76:787–96.
5. Forgacs E, Zochbauer-Muller S, Olah E, Minna J. Molecular genetic abnormalities in the pathogenesis of human lung cancer. Pathol Oncol Res 2001;7:6–13.
6. 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.
7. 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.
8. 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.
9. Kersting M, Friedl C, Kraus A, Behn M, Pankow W, Schuermann M. Differential frequencies of p16INK4a 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.
10. Wang B, Li L, Yao L, Liu L, Zhu Y. Detection of p53 gene mutations in sputum samples and their implications in the early diagnosis of lung cancer in suspicious patients. Chin Med J (Engl) 2001;114:694–7.
11. Zochbauer-Muller 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;107:612–6.
12. 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.
13. Palmisano WA, Divine KK, Saccomanno G, et al. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res 2000;60:5954–8.
14. Ahrendt S, Decker P, Alawi E, et al. Cigarette smoking is strongly associated with mutation of the K-ras gene in patients with primary adenocarcinoma of the lung. Cancer 2001;92:1525–30.
15. Wistuba I, Gazdar A, Minna J. Molecular genetics of small cell lung carcinoma. Semin Oncol 2001;28:3–13.
16. Husgafvel-Pursiainen K, Hackman P, Ridanpaa M, et al. K-ras mutations in human adenocarcinoma of the lung: association with smoking and occupational exposure to asbestos. Int J Cancer 1993;53:250–6.
17. Ronai Z, Yabubovskaya M, Zhang E, Belitsky G. K-ras mutation in sputum of patients with or without lung cancer. J Cell Biochem Suppl 1996;25:172–6.
18. Bodner S, Minna J, Jensen S, et al. Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. Oncogene 1992;7:743–9.
19. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994;54:4855–78.
20. Takahashi T, Nau M, Chiba I, et al. p53: a frequent target for genetic abnormalities in lung cancer. Science 1989;246:491–4.
21. Bennett W, Hussain S, Vahakangas K, Khan M, Shields P, Harris C. Molecular epidemiology of human cancer risk: gene-environment interactions and p53 mutation spectrum in human lung cancer. J Pathol 1999;187:8–18.
22. Jones P, Baylin S. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
23. Kurakawa E, Shimamoto T, Utsumi K, Hirano T, Kato H, Ohyashiki K. Hypermethylation of p16(INK4a) and p15(INK4b) genes in non-small cell lung cancer. Int J Oncol 2001;19:277–81.
24. Yanagawa N, Tamura G, Oizumi H, Takahashi N, Shimazaki Y, Motoyama T. Promoter hypermethylation of tumor suppressor and tumor-related genes in non-small cell lung cancers. Cancer Sci 2003;94:589–92.
25. Belinsky SA, Nikula KJ, Palmisano WA, et al. Aberrant methylation of p16INK4a is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci U S A 1998;95:11891–6.
26. Nuovo GJ, Plaia TW, Belinsky SA, Baylin SB, Herman JG. *In situ* detection of the hypermethylation-induced inactivation of the p16 gene as an early event in oncogenesis. Proc Natl Acad Sci U S A 1999;96:12754–9.
27. Chen H, Suzuki M, Nakamura Y, et al. Aberrant methylation of RASGRF2 and RASSF1A in human non-small cell lung cancer. Oncol Rep 2006;15:1281–5.
28. Wang Y, Yu Z, Wang T, Zhang J, Hong L, Chen L. Identification of epigenetic aberrant promoter methylation of RASSF1A in serum DNA and its clinicopathological significance in lung cancer. Lung Cancer 2007;56:289–94.
29. Liu Y, Gao W, Siegfried J, Weissfeld J, Luketich J, Keohavong P. Promoter methylation of RASSF1A and DAPK and mutations of K-ras, p53, and EGFR in lung tumors from smokers and never-smokers. BMC Cancer 2007;7:74.
30. Grote H, Schmiemann V, Geddert H, et al. Methylation of RAS association domain family protein 1A as a biomarker of lung cancer. Cancer 2006;108:129–34.
31. 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.
32. Irimia M, Fraga M, Sanchez-Cespedes M, Esteller M. CpG island promoter hypermethylation of the Ras-effector gene NORE1A occurs in the context of a wild-type K-ras in lung cancer. Oncogene 2004;23:8695–9.
33. Hesson L, Dallol A, Minna J, Maher E, Latif F. NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. Oncogene 2003;22:947–54.
34. Infante MV, Fabio LR, Cavuto S, et al. DANTE: a randomized study on lung cancer screening with low-dose spiral CT (LDCT): end of accrual and preliminary results. Chest 2006;130:114S–c–5.
35. Miranda E, Destro A, Malesci A, et al. Genetic and epigenetic changes in primary metastatic and non-metastatic colorectal cancer. Br J Cancer 2006;95:1101–7.
36. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A 1989;86:2766–70.
37. Herman JG, Graff JR, Myohanen S, Nelkin BD,

Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–6.

38. Gazdar AF, Minna JD. Molecular detection of early lung cancer. J Natl Cancer Inst 1999;91:299–301.

39. Berlin N, Buncher C, Fontana R, Frost J, Melamed M. The National Cancer Institute Cooperative Early Lung Cancer Detection Program. Results of the initial screen (prevalence). Early lung cancer detection: introduction. Am Rev Respir Dis 1984;130:545–9.

40. Kubik A, Parkin D, Khlat M, Erban J, Polak J, Adamec M. Lack of benefit from semi-annual screening for cancer of the lung: follow-up report of a randomized controlled trial on a population of high-risk males in Czechoslovakia. Int J Cancer 1990;45:26–33.

41. Zhang L, Gao W, Gealy R, et al. Comparison of K-ras gene mutations in tumour and sputum DNA of patients with lung cancer. Biomarkers 2003;8:156–61.

42. Wang Y-C, Hsu H-S, Chen T-P, Chen J-T. Molecular diagnostic markers for lung cancer in sputum and plasma. Ann N Y Acad Sci 2006;1075:179–84.

43. Chen J, Chen Y, Wang Y, Tseng R, Chen C. Alterations of the p16(ink4a) gene in resected nonsmall cell lung tumors and exfoliated cells within sputum. Int J Cancer 2002;98:724–31.

44. Belinsky S. Gene-promoter hypermethylation as a biomarker in lung cancer. Nat Rev Cancer 2004;4:707–17.

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