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 p16INK4A, RASSF1A, and NORE1A methylation 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 p16INK4A, 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 p16INK4A 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 smoking history of at least 20 pack-years.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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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 quittd <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 bronchi on the left side and

| Table 1. Summary of selected available demographic variables of the studied cohort |
|---------------------------------|--------|--------|--------|
| Age, y (95% CI)                 | Current (n = 469) | Former (n = 319) |
| All (n = 788)                   | 64.2   | 63.3   | 65.4   |
| Pack-years (95% CI)            | 48.2   | 49.0   | 46.8   |

Abbreviation: 95% CI, 95% confidence interval.
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 BstNI restriction site into PCR products derived from mutant alleles. The PCR mixture contained 300 ng of genomic DNA, 0.2 mmol/L of nucleotide mix (Finnzymes), 0.5 mmol/L MgCl2 (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 BstNI (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 cell line) 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 χ²), 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.
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

<table>
<thead>
<tr>
<th>Genes</th>
<th>No. analyzed cases</th>
<th>No. altered cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-ras</td>
<td>506</td>
<td>0</td>
</tr>
<tr>
<td>p53</td>
<td>803</td>
<td>15 (1.9)</td>
</tr>
<tr>
<td>p16(^{INK4A})</td>
<td>765</td>
<td>39 (5.1)</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>246</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>NORE1A</td>
<td>99</td>
<td>1 (1.0)*</td>
</tr>
<tr>
<td>Total</td>
<td>1,565</td>
<td>56 (6.9)</td>
</tr>
</tbody>
</table>

*Patient with coincidental methylation of p16\(^{INK4A}\).
Nevertheless, a previous study reported that as a marker for high-risk pulmonary cancer patients, p53 mutations in cancer tissue but not in the adjacent nonmalignant tissue or in sputum from healthy individuals (9). 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 carcinogenic events.

p16
\textsuperscript{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
\textsuperscript{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
\textsuperscript{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
\textsuperscript{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
\textsuperscript{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

Table 3. Prospective studies reporting molecular alterations in sputum

<table>
<thead>
<tr>
<th>Reference</th>
<th>Alterations</th>
<th>Follow-up*</th>
</tr>
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</table>
|           | p16
\textsuperscript{INK4A} | p53  | K-ras |
| Ronai et al. (17) | 5/26 (19.2%)\(\dagger\) | 3/25 (12%) | 5/40 (12.5%) \(\dagger\) | 3 y: no tumor |
| Belinsky et al. (25) | 7/25 (28%) \(\dagger\)\(\ddagger\) | 6/32 (18.7%) | 0/25 | 1 y: 3 tumors |
| Kersting et al. (9) | 2/114 (1.7%) \(\dagger\) | - | - | 6 y: no tumor |
| Palmasano et al. (13) | 32/66 (35%) \(\dagger\) | 4/73 (5.5%) \(\dagger\)\(\ddagger\) | 15/803 (1.9%) | 4 y: 1 tumor |
| Wang et al. (10) | - | - | 0/506 (0%) | 3 y: 1 tumor |
| Belinsky et al. (7) | - | - | - | - |
| Zochbauer-Müller et al. (11) | - | - | - | - |
| 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.

\(\dagger\) Benign pulmonary disease.

\(\dagger\) Induced sputum.

\(\dagger\) 25% with cellular atypia.

\(\dagger\) With complete data.

\(\ddagger\) With incomplete data.
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 bronchoscopic 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 p16INK4a rather than K-ras, RASSF1A, and NOREIA 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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37. Herman JG, Graff JR, Myohanen S, Nelkin BD, Wiestub, Lam S, Behrens C, et al. 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.

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