

p16^{INK4A} Hypermethylation Detected by Fluorescent Methylation-specific PCR in Plasmas from Non-Small Cell Lung Cancer¹

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ABSTRACT

Purpose: The *p16^{INK4A}* tumor suppressor gene is inactivated in many solid tumors, including non-small cell lung cancers (NSCLCs), through promoter hypermethylation. Presence of *p16^{INK4A}* hypermethylation in precursor lesions of NSCLC and in body fluids of individuals at risk makes it a potential candidate for early disease detection. However, the current low sensitivity of *p16^{INK4A}* hypermethylation detection in plasma limits its consideration in a diagnostic grid.

Experimental Design: A fluorescent methylation-specific PCR assay (F-MSP) was established to evaluate *p16^{INK4A}* promoter hypermethylation in 35 NSCLC and paired plasma samples and in 15 plasmas from healthy donors. F-MSP sensitivity was investigated in combination with microsatellite alterations on 3p (evaluated by fluorescent PCR), *K-ras* mutations (determined by a mutant-enriched PCR), and quantification of circulating DNA. Assay results were analyzed by two-sided χ^2 or Fisher's exact tests.

Results: *p16^{INK4A}* promoter hypermethylation, detectable by F-MSP in 22 of 35 NSCLCs (63%) and in 12 of 22 (55%) plasmas from patients with methylated tumors, was independent of microsatellite alterations (detectable in 57% of tumors and 50% of paired plasmas), *K-ras* mutations (detectable in 31% of tumors but in no paired plasma), or amount of circulating DNA. *p16^{INK4A}* methylation in association with microsatellite alterations identified 62% (18 of

29) of plasma samples from patients presenting the same alteration in their tumors, and its sensitivity increased to 80% when combined with the amount of circulating DNA.

Conclusions: The establishment of F-MSP remarkably improved *p16^{INK4A}* promoter hypermethylation detection in plasmas from NSCLC patients. Microsatellite alterations, circulating DNA quantification, and *p16^{INK4A}* hypermethylation might contribute to a diagnostic grid for NSCLC.

INTRODUCTION

Delayed detection is the major factor responsible for lung cancer-related death in developed countries. In fact, although surgical resection still represents the best curative approach for this neoplasm, its efficacy often depends on the stage of disease presentation, and conventional screening techniques detect tumors at an advanced phase of clinical progression (1). In this respect, the identification of genetic and biochemical changes that characterize the malignant transformation of cells and the development of sensitive techniques able to detect molecular and cellular signatures of tumorigenesis in tissues but also in body fluids could contribute to improve early detection.

Recently, several studies have provided convincing evidence that tumor-derived DNA can be detected in sputum, bronchoalveolar lavage, and plasma (2, 3), and free-circulating DNA can be found in greater quantities in patients with NSCLCs⁴ than in healthy individuals (4). Moreover, molecular genetic signatures that characterize primary tumor DNA such as microsatellite instability, *p53* and *K-ras* mutations, tumor suppressor genes, and DNA repair gene hypermethylation, have been also identified by PCR-based methods in circulating DNA, thus confirming its tumor origin (5–7).

Aberrant methylation of CpG islands located in the promoter area of many genes is associated with their transcriptional inactivation and constitutes an alternative mechanism to the genetic loss attributable to deletion or mutation (8). The *p16^{INK4A}* tumor suppressor gene is one of the key regulators of the cell cycle and is often inactivated in many solid tumors. Hypermethylation of the promoter is one of the major causes of *p16^{INK4A}* inactivation, correlates with loss of protein expression, and represents a frequent event in lung cancer. It is detectable in a variable fraction of cases, from 30 to 85%, as a function of several patient-, disease-, or technical-related factors (3, 9, 10). This gene inactivation modality has also been found in plasma DNA from NSCLC patients, although with a limited sensitivity (3, 11). Moreover, the fact that *p16^{INK4A}* methylation has also been found in precursor lesions of lung carcinomas renders this

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⁴ The abbreviations used are: NSCLC, non-small cell lung cancers; 6-FAM, 6-carboxyfluorescein; MSP, methylation-specific PCR; STD-MSP, standard MSP; F-MSP, fluorescent MSP; LOH, loss of heterozygosity; p16M, *p16^{INK4A}* methylated; p16U, *p16^{INK4A}* unmethylated.

Table 1 $p16^{INK4A}$ promoter methylation status in tumor and plasma samples assessed by STD-MSP or F-MSP: basic analysis and comparison between the two detection assays

	STD-MSP		F-MSP			
	Tumor $p16^{INK4A}$ hypermethylation		Tumor $p16^{INK4A}$ hypermethylation		Healthy donor $p16^{INK4A}$ hypermethylation	
	Present	Absent	Present	Absent	Present	Absent
Tumor samples						
Total	20 (57%)	15 (43%)	22 (63%)	13 (37%)		
Plasma $p16^{INK4A}$ hypermethylation					0 (0%)	15 (100%)
Present	2 (10%)	0 (0%)	12 (55%)	0 (0%)		
Absent	17 (85%)	11 (73%)	9 (41%)	9 (69%)		
Not assessable	1 (5%)	4 (27%)	1 (4%)	4 (31%)		

marker an interesting candidate for early detection and risk assessment (2, 12).

To investigate the presence of $p16^{INK4A}$ methylation status in paired tumor and plasma samples from patients with NSCLC, we developed an optimized and reproducible detection technique for plasma DNA samples. We analyzed the association of $p16^{INK4A}$ promoter hypermethylation with other molecular markers such as microsatellite instability at various regions of 3p and *K-ras* mutations and circulating DNA levels to assess its contribution to a diagnostic grid that included information provided by the molecular alterations commonly present or clinically relevant in this tumor type.

MATERIALS AND METHODS

Case Series. Tumor specimens and paired blood samples were prospectively collected from a series of 35 NSCLC patients (28 males and 7 females, with a mean age of 64 years) who underwent curative pulmonary resection at the Istituto Nazionale Tumori di Milan during the period 1998 to 2000. This cohort of specimens represented a randomly selected fraction of a consecutive series of 84 cases previously investigated for various genetic abnormalities (4). These patients were radically resected for primary NSCLC without any previous systemic treatment and all of them had a confirmed histological diagnosis of NSCLC. Twenty-eight were stage I, 18 were classified as ADC and 10 as SCC. Blood samples were also obtained from 15 healthy blood donors. NSCLC patients and healthy donors gave their informed consent to be included in the study.

Sample Collection and DNA Isolation. Immediately after surgical resection, tumor specimens were sampled by the pathologist and stored at -80°C . Frozen tumors were not microdissected for molecular analyses but only those containing $>70\%$ neoplastic cells (assessed from previous H&E frozen control) were processed for the study. Peripheral blood obtained from each NSCLC patient on the day before surgery was immediately separated from the cellular fraction by centrifugation and processed as described previously (4). DNA was extracted from tissue, plasma, and WBC samples by using a QIAamp DNA Mini kit (Qiagen, Milan, Italy). Tumor and whole blood DNA concentrations were estimated by spectrophotometry, and quantification of the circulating plasma DNA (4) was performed by using a DNA DipStick Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

MSP. $p16^{INK4A}$ methylation status was determined by MSP (3, 13) as modified in Palmisano *et al.* (9). Briefly, 1 μg of DNA was modified with sodium bisulfite (Sigma, St. Louis, MO). Two hundred ng of bisulfite-treated DNA were used for a first round of PCR with primers specific for bisulfite-converted DNA but not discriminating between methylated or unmethylated sequences ($p16^{INK4A}$ forward: GAAGAAAGAGGAGGGGTGG; $p16^{INK4A}$ reverse: CTACAAACCCTCTACCCACC, 0.5 μM each). After a 40-cycle amplification (30 s at 95°C , 30 s at 60°C , and 30 s at 72°C) and a last incubation step of 10 min at 72°C , PCR products were diluted 20-fold and 2 μl were used for two nested PCRs with primers specific for p16M or p16U $p16^{INK4A}$ sequences (p16U forward: TTATTAGAGGGTGGGGTGGATTGT, p16U reverse: CAACCCCAAACCACAACCATAA; p16M forward: TTATTAGAGGGTGGGGCGGATCGC; p16M reverse: GAC-CCC GAACCGCGACCGTAA; 0.5 μM each), with an additional 40-cycle amplification (15 s at 95°C , 15 s at 70°C , and 15 s at 72°C). PCR products were then loaded onto 3% agarose gels and visualized by ethidium bromide staining. We defined this approach as STD-MSP.

To increase assay sensitivity, we successively investigated the possible improvements derived from the use of F-MSP targeting $p16^{INK4A}$ promoter sequence. For this purpose, the forward primers for the nested PCRs (PE-Applied Biosystems, Foster City, CA) were labeled with 6-FAM, and PCR products were separated electrophoretically on a 5% polyacrylamide gel and analyzed by laser fluorescence using an ABI Prism DNA Sequencer model 377 (PE-Applied Biosystems) equipped with GeneScan 2.1 software. The results obtained by STD-MSP according to Palmisano *et al.* (9) and F-MSP were comparatively analyzed on the same set of tumor and plasma specimens.

DNA from Raji (Burkitt's lymphoma) cell line was used as methylated control, whereas DNA from normal lymphocytes constituted unmethylated control. Water blanks were included in each experiment. Methylation status was defined as assessable whenever there was amplification of at least the unmethylated band.

Microsatellite Alterations. The analysis of microsatellite instability and LOH on a larger number of cases, including these samples, has already been published (4) and was performed by investigating microsatellite alterations at loci located at 3p14.2 (*D3S1300*, *FHIT* locus), 3p21 (*D3S1289*), 3p23 (*D3S1266*), 3p24.2 (*D3S2338*), and 3p25-26 (*D3S1304*), which

are hot spots of deletions in lung cancer. The sequences of nucleotide markers for microsatellite analysis are available through the Genome Database. LOH and the presence of allele shifts indicating genomic instability were recorded in the various samples and compared with the profiles obtained in DNA from normal peripheral lymphocytes. LOH was scored when a reduction of at least 30% of allele intensity in the experimental sample was seen following the guidelines for allelic imbalance established by Liloglu *et al.* (14). All DNA samples with microsatellite alterations were amplified at least twice to rule out PCR artifacts or sample contamination. In the presence of allelic imbalance in plasma, increasing amounts of plasma DNA were used in the PCR reaction to exclude unreliable allelotyping.

Detection of *K-ras* Mutations. Mutations at codons 12 and 13 of the *K-ras* gene were detected by using mutant-enriched PCR, as reported by Nollau *et al.* (15), with slight modifications. The method consists of two amplification steps (semi-nested PCR) in which artificial restriction sites are introduced by the use of mismatched primers. A restriction site, *Bst*NI or *Bgl*II for codon 12 or 13, respectively, introduced during the first PCR step, is localized in the immediate vicinity of the *K-ras* codon in analysis to distinguish between wild-type and mutant sequences. Wild-type amplicons were then digested by *Bst*NI or *Bgl*II restriction enzyme, whereas mutant products were enriched for a second round of amplifications.

For codon 12, the analysis was carried out in the following way: 2 μ l of DNA extracted from tumors or from serum were amplified in a volume of 50 μ l for 20 cycles (30 s at 95°C, 1 min at 50°C, and 1 min at 72°C), preceded by a cycle at 95°C for 3 min and followed by an incubation step of 3 min at 72°C. Primers used were: K-ras 3F: 5'-ACT-GAA-TAT-AAA-CTT-GTG-GTA-GTT-GGA-CCT-3'; and K-ras 10B: 5'-ACT-CAT-GAA-AAT-GGT-CAG-AGA-AAC-CTT-TAT-3'; 0.1 μ M each. Subsequently, 15 μ l of the PCR product were transferred to a new tube containing 1 \times NEBuffer2, 1 \times BSA, 5 units of *Bst*NI restriction enzyme (New England Biolabs, Beverly, MA), and incubated at 60°C overnight. One μ l of restriction digest was amplified in a second PCR reaction in a final volume of 50 μ l for 45 cycles. Each cycle consisted of 30 s at 95°C, 1 min at 54°C, and 1 min at 72°C. Primers used were K-ras 3F and K-ras 14B: 5'-TCA-AAG-AAT-GGT-CCT-GGA-CC-3'; 0.5 μ M each. Fifteen μ l of the amplified product were then digested under the same conditions used in the first digestion step.

The codon 13 was analyzed in the same way except that *Bgl*II enzyme (New England Biolabs) was used at 37°C for the overnight digestion. Primers for this codon were K-ras 9F (5'-ACT-GAA-TAT-AAA-CTT-GTG-GTA-GTT-GGC-CCT-GGT-3') and K-ras 10B for the first PCR and K-ras 9F and K-ras 4B for the semi-nested one. The final digestion products of codons 12 or 13 were electrophoresed on a 7% nondenaturing polyacrylamide, stained with ethidium bromide, and visualized by UV light. DNA-free PCR mix and DNA from serum of patients carrying a colorectal carcinoma with a codon 12 or 13 *K-ras* mutation were used in all PCR reactions as negative and positive control, respectively.

Statistical Analysis. The reproducibility of the STD-MSP assay in detecting *p16*^{INK4A} promoter hypermethylation was preliminarily assessed on DNA extracted from tumor specimens. After confirming that the unmethylated form of the gene was always detectable, we analyzed a randomly selected series

of 20 tumors by repeating PCR for the methylated *p16*^{INK4A} sequence five times on each sample amplified twice in the same assay. In this way, we obtained a run of 10 independent results for each tumor, which allowed us to determine reproducibility within the same PCR (the two repetitions of each sample) and among different PCRs. Results from this preliminary set of experiments were used to define criteria prospectively applicable for determining *p16*^{INK4A} promoter hypermethylation in plasma samples.

The association between *p16*^{INK4A} promoter methylation detected in tumor and plasma samples by STD-MSP and F-MSP was assessed by using Fisher's exact test. The association between *p16*^{INK4A} methylation status, in tumor tissue and in plasma, and other pathobiological features was tested by the χ^2 test, adjusted for continuity when appropriate. All *P*s were two sided.

RESULTS

Reproducibility of MSP to Detect *p16*^{INK4A} Promoter Methylation in Tumor Samples. Preliminary experiments carried out to assess STD-MSP reproducibility in tumor samples showed that among the 100 couples of generated data, one replicate was different from the other only in five samples, thus providing a 95% intra-assay reproducibility rate. Regarding interassay reproducibility, in 15 of 20 tumors there was a 100% concordance for all of the repetitions with the first PCR result, regardless of *p16*^{INK4A} methylation status. In 3 cases, there was just one repetition that differed from the others, and in 2 cases there was a consistent discordance among PCR results (4 of 10 replicates differed from the first result). After these observations, we set as a criterion to determine the *p16*^{INK4A} methylation status of a tumor a concordance in 2 of 3 independent PCRs. This criterion was used for all subsequent experiments and required PCR repetition in only 2 of the additional 15 tumors.

MSP to Detect *p16*^{INK4A} Promoter Methylation in Primary Tumors and Paired Plasma Samples. *p16*^{INK4A} methylation status was assessable by STD-MSP in all of the 35 tested tumor samples (Table 1, left) and hypermethylation was detectable in 20 tumors (57%). Conversely, *p16*^{INK4A} methylation status was assessable in 30 plasma samples, and hypermethylation was detectable in only 2 of the 20 plasma samples from patients with methylated tumors (Table 1). None of the 15 plasmas derived from patients with unmethylated tumors was positive for *p16*^{INK4A} methylation.

Given the scarce sensitivity of STD-MSP, we modified the approach by using 6-FAM-labeled fluorescent forward primers for the nested MSP and subsequently by loading the product onto a sequencing gel connected with a sequencer. The sensitivity of F-MSP, determined by serial dilutions of the methylated into the unmethylated control DNA, allowed detection of <1 methylated allele in >50,000 normal alleles (data not shown). Using such an approach (Table 1, right), *p16*^{INK4A} hypermethylation was detected in 22 of 35 tumor samples (63%). It is noteworthy that the 2 cases detected as methylated with F-MSP but not with STD-MSP were those exhibiting the least constant pattern among repeats (60% concordance) in the ethidium bromide analysis. Using F-MSP, *p16*^{INK4A} methylation was detectable in 12 of 22 plasma samples (55%) obtained from

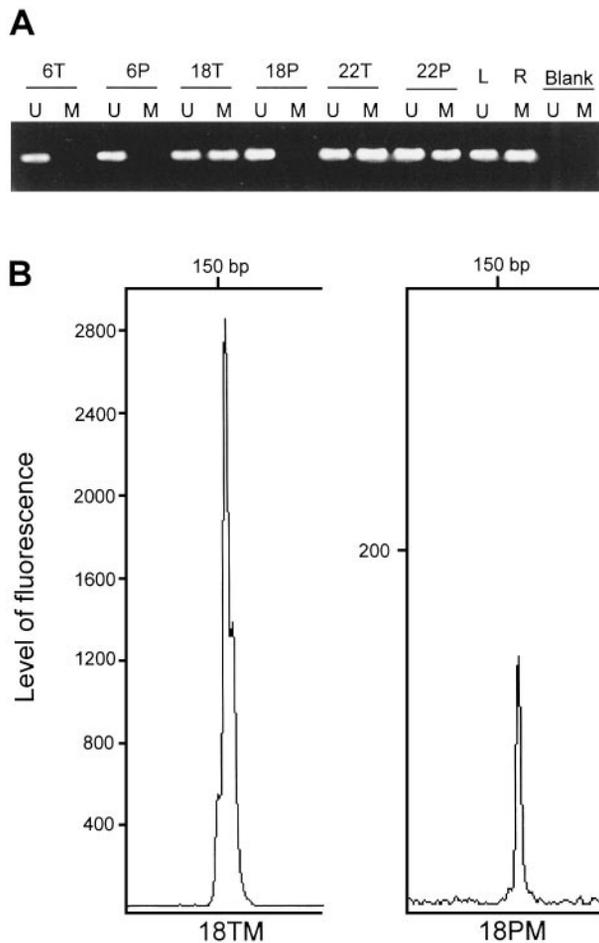


Fig. 1 Analysis of aberrant methylation of *p16^{INK4A}* gene in tumor (T) and plasma (P) of NSCLC patients. **A**, STD-MSP of tumor and plasma DNA of patients 6, 18, and 22. Products in Lanes U were obtained with primers for the unmethylated sequence (151 bp), whereas products in Lanes M were obtained with primers for the methylated sequence (150 bp). DNA from normal lymphocytes (L) and Raji lymphoma cells (R) was used as control for the unmethylated and methylated reactions, respectively. Water blanks are included. **B**, F-MSP of patient 18 tumor and plasma DNA amplified with 6-FAM-labeled primers for the methylated sequence.

patients with methylated tumors (Table 1). At the same time, none of the plasmas corresponding to unmethylated tumors was found positive for methylation, and the association of *p16^{INK4A}* methylation status in NSCLCs and paired plasma specimens was statistically significant (Fisher's exact test; $P = 0.004$), with an agreement in 25 of 35 cases (considering together negative and nonassessable samples). The high specificity of the assay was confirmed by the analysis carried out on 15 plasma samples from healthy donors, none of which was found positive for methylation.

Fig. 1 shows a representative example of the analysis of *p16^{INK4A}* promoter methylation, comparing results obtained by STD-MSP and F-MSP on tumor and blood specimens from the same patient for whom *p16^{INK4A}* methylation in the plasma sample was detectable by fluorescence but not by ethidium bromide analysis.

Relation between *p16^{INK4A}* Hypermethylation and Pathologic or Molecular Findings.

Table 2 reports the relationships between *p16^{INK4A}* methylation status, evaluated by F-MSP, and other pathologic and molecular features of the tumors. In this limited case series, *p16^{INK4A}* hypermethylation seemed to be unrelated to tumor histology or pathologic stage when evaluated on tumor ($P = 0.47$ for histology and $P = 0.35$ for stage) or on plasma ($P = 0.91$ for histology and $P = 0.60$ for stage) specimens. Similarly, no association was detected between *p16^{INK4A}* methylation and microsatellite alterations, which involved at least one of the five tested loci and were present in 20 of 35 tumors (57%) or in 11 of 34 plasma samples (32%) or *K-ras* mutations, which were detected in 11 of 35 tumors (31%) but in no plasma specimens.

In this cohort of cases, circulating plasma DNA levels were also determined. The results on this group as a part of the larger investigated series have been reported and analyzed in terms of sensitivity, specificity, relation with some molecular markers, and patient follow-up (4). No relationship was observed when tumor and plasma *p16^{INK4A}* methylation status was related to circulating DNA levels (Table 2). In fact, plasma DNA levels were similar in the 9 cases in which *p16^{INK4A}* was not methylated in tumor or in plasma specimens (DNA, ng/ml: median, 400; range, 5–500) as well as in the 9 cases in which only tumor *p16^{INK4A}* methylation was detected (median, 400; range, 5–1000) or in the 12 cases in which methylation was detected in tumor and plasma specimens (median, 325; range, 5–500).

Because the presence of *p16^{INK4A}* hypermethylation appeared to be independent of the other genetic alterations analyzed in this case series, we determined the fraction of NSCLCs exhibiting any of the alterations and investigated whether such a profile paralleled in plasma samples the molecular signature of the primary tumor. In tumor samples, *p16^{INK4A}* hypermethylation, *K-ras* mutations, and microsatellite alterations were present, singly, in 22 (63%), 11 (31%), and 20 (57%) of the 35 investigated cases, respectively. In association, alterations for at least one molecular-based assay were detectable in 32 tumor samples (91%). In plasma samples from the 32 patients whose tumors exhibited at least one altered molecular marker, *p16^{INK4A}* hypermethylation and microsatellite alterations were present, singly, in 12 of 32 (37.5%) and 10 of 32 (31.2%) cases, respectively, whereas in association, they were detectable in 18 of 32 cases (56%). Excluding from this analysis, *K-ras* mutations for the lack of detection of the alteration in DNA from plasma samples, *p16^{INK4A}* methylation or microsatellite alterations paralleled in 62% of plasmas (18 of 29) the same altered molecular profile exhibited in tumors. Similarly, circulating DNA amounts > 125 ng/ml [which corresponded to 100% specificity in discriminating NSCLC patients from healthy donors as computed by a receiver operating curve (4)] were present in 23 of 35 cases (66%). Such an identification rate increased to 80% when *p16^{INK4A}* methylation was also considered. In fact, plasma *p16^{INK4A}* hypermethylation was detectable in 5 additional cases in which circulating DNA levels were < 50 ng/ml. Superimposable results were obtained when we carried out all these analyses in stage I patients (data not shown).

Table 2 Relationship between *p16^{INK4A}* promoter methylation status in tumor or plasma samples and pathologic or molecular data

	Tumor <i>p16^{INK4A}</i> hypermethylation		Plasma <i>p16^{INK4A}</i> hypermethylation	
	No. assessable samples	No. methylated (%)	No. assessable samples	No. methylated (%)
Overall cases	35	22 (63)	30	12 (40)
Histology				
ADC ^a	18	12 (67)	15	6 (40)
SCC	10	7 (70)	9	4 (44)
Others ^b	7	3 (43)	6	2 (33)
Stage				
I	28	17 (61)	24	9 (38)
II	4	2 (50)	3	1 (33)
III	3	3 (100)	3	2 (67)
Microsatellite alterations				
Absent	15	9 (60)	19	8 (42)
Present	20	13 (65)	10	4 (40)
K-ras mutation				
Absent	24	15 (62)	10	4 (40)
Present	11	7 (64)	0	
Circulating plasma DNA (ng/ml)				
5–25			7	3 (43)
26–125			3	2 (67)
>125			20	7 (35)

^a ADC, adenocarcinoma; SCC, squamous cell carcinoma.

^b Others: bronchoalveolar ADC (5 cases) or SCC (2 cases).

DISCUSSION

In this study, we demonstrated a methodological improvement in the detection of *p16^{INK4A}* hypermethylation in plasma DNA samples from NSCLC patients using an F-MSP approach characterized by a satisfactory level of reproducibility and 100% specificity. Moreover, the simultaneous evaluation on plasma of *p16^{INK4A}* methylation status and unrelated molecular markers such as microsatellite alterations or DNA quantification was able to identify 62–80% of NSCLC patients.

The detection of tumor molecular signatures in body fluids may have powerful implications for the identification of high-risk subjects, patients with preinvasive or early stage lesions, and for monitoring the residual disease. Such an approach, which is characterized by a high specificity, presents a variable sensitivity, likely because of the presence of low tumor DNA quantities in plasma and in exfoliative materials and to high levels of contamination with normal DNA. Several approaches to improve assay sensitivity have been applied to tumor tissue, and then to plasma, sputum, stool, bronchoalveolar lavage, and brushings. Sensitivity of gene promoter hypermethylation detection has been improved over that of the original assay for MSP proposed by Herman *et al.* (13) at different methodological levels by performing a semi-nested MSP after a DNA preamplification step (2) or a nested two-stage PCR (9) or by using fluorescent primers for an automated quantification of MSP products by a DNA sequencer (16).

Our modification of the standard protocol of nested two-stage MSP by using fluorescent primers increased by ~6-fold the possibility to detect *p16^{INK4A}* hypermethylation in plasma from NSCLC patients (from 2 to 12 of the 20 cases with methylated tumors), with a detection level superior to that reported in the other two similar studies published thus far (3, 11). However, the still high percentage of plasmas in which the alteration present in the tumors was undetectable could be

because of a high contamination level of normal DNA as well as to the release of different DNA quantities from different tumor clones (6). In tumor tissues, conversely, the use of F-MSP did not improve the sensitivity in detecting *p16^{INK4A}* hypermethylation because its frequency was similar for F-MSP (63 ± 16%) and STD-MSP (57 ± 16%) in keeping with other published results obtained by using different approaches (9, 10, 12).

Promoter hypermethylation represents a major mechanism of *p16^{INK4A}* inactivation and a common alteration across many tumor types (17). In our limited series, *p16^{INK4A}* promoter hypermethylation was the most frequent alteration found in tumors and plasmas, and the methylation status was not associated to any other pathologic feature of the tumor.

No correlation was evident between *p16^{INK4A}* hypermethylation and molecular markers involved in partially unrelated pathways of lung tumorigenesis. The high prevalence of microsatellite alterations at various regions of 3p even in stages I and II of lung cancer, in this and in other series of patients (4, 5), suggests that also this feature is associated with the early phase of the disease and can thus be used as an independent marker, possibly improving the informativeness of tumor molecular signatures in plasma. Regarding *K-ras*, the frequency of alterations we found in tumors was in the range of that obtained by other authors (10), thereby validating the sensitivity of our approach. *K-ras* mutations have been demonstrated in sputum, bronchoalveolar lavage, pancreatic juice, bone marrow, and stool samples from patients with lung, colorectal, and pancreatic cancers (2, 10, 14, 18, 19), whereas *K-ras* analysis in plasma samples has been previously reported for pancreatic and colorectal cancers (7) but not for lung cancers. The lack of assessability of this alteration in our plasma series could be ascribed to technical and biological factors, previously mentioned for *p16^{INK4A}* methylation, or to the occurrence of *K-ras* mutations as a late event in lung carcinogenesis (2, 20), but also to a more

effective degradation of *K-ras* mutated alleles compared with the wild-type counterpart (19). This lack of detection would preclude, at present, the use of *K-ras* as a molecular marker in plasma-based molecular alteration detection for NSCLC.

For the involvement of multiple genetic abnormalities in the carcinogenesis process and the still unsatisfactory assay sensitivity for the different markers, it is unlikely that a single biomolecular marker will be able to detect all cancers of a given histotype. In contrast, the analysis of a panel of complementary and independent markers would enable the detection of a larger set of cancers and the identification of a larger number of patients. In our experience, combination of only two markers in plasmas, *p16^{INK4A}* hypermethylation and microsatellite alterations, raised the overall detection rate for NSCLCs from 30 to 40%, when singly considered, to 62%. Similarly, the use of *p16^{INK4A}* promoter methylation status in combination with circulating DNA level, which in a previous study from our group proved to discriminate between NSCLC patients and healthy individuals (4) enabled the detection of 80% of NSCLC patients.

Although our data need to be validated on a larger and independent case series, also including subjects at high-risk of developing NSCLC, they are the first, thus far, to support the consideration of *p16^{INK4A}* hypermethylation among the markers to be tested in a diagnostic grid for disease monitoring on plasma specimens. The enhancement of the sensitivity of *p16^{INK4A}* methylation detection in plasma, achieved through a fluorescent-based modification of STD-MSP, represents a methodological improvement for an objective, noninvasive, specific and rather sensitive molecular detection of NSCLC.

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