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Methylation profile in tumor and sputum samples of lung cancer patients detected by spiral computed tomography: A nested case–control study

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We evaluated the aberrant promoter methylation profile of a panel of 3 genes in DNA from tumor and sputum samples, in view of a complementary approach to spiral computed tomography (CT) for early diagnosis of lung cancer. The aberrant promoter methylation of *RAR*β*2*, *p16^{INK4A}* and *RASSF1A* genes was evaluated by methylation-specific PCR in tumor samples of 29 CT-detected lung cancer patients, of which 18 had tumor-sputum pairs available for the analysis, and in the sputum samples from 112 cancer-free heavy smokers enrolled in a spiral CT trial. In tumor samples from 29 spiral CT-detected patients, promoter hypermethylation was identified in 19/29 (65.5%) cases for *RAR*β*2*, 12/29 (41.4%) for *p16^{INK4A}* and 15/29 (51.7%) for *RASSF1A*. Twenty-three of twenty-nine (79.3%) samples of the tumors exhibited methylation in at least 1 gene. In the sputum samples of 18 patients, methylation was detected in 8/18 (44.4%) for *RAR*β*2* and 1/18 (5%) for both *RASSF1A* and *p16^{INK4A}*. At least 1 gene was methylated in 9/18 (50%) sputum samples. Promoter hypermethylation in sputum from 112 cancer-free smokers was observed in 58/112 (51.7%) for *RAR*β*2* and 20/112 (17.8%) for *p16*, whereas methylation of the *RASSF1A* gene was found in only 1/112 (0.9%) sputum sample. Our study indicates that a high frequency of hypermethylation for *RAR*β*2*, *p16^{INK4A}* and *RASSF1A* promoters is present in spiral CT-detected tumors, whereas promoter hypermethylation of this panel of genes in uninduced sputum has a limited diagnostic value in early lung cancer detection.

Key words: lung cancer; methylation; sputum; spiral CT

Lung cancer is the leading cause of cancer deaths in the world. Although surgical resection still represents the best curative approach for this neoplasm, its efficacy strictly depends on the stage of disease presentation. Earlier diagnosis of patients with lung cancer is expected to increase the number of potentially resectable tumors.[1] Several large prospective randomized trials have demonstrated that conventional sputum cytology and chest radiography are not effective in detecting early lung cancer and reducing lung cancer mortality.[2]

In this respect, over the last decade, spiral computed tomography (CT) has been tested as a powerful and fast imaging technique to detect tumors of less than 1 cm in diameter, with a proportion of detection of Stage I tumors greater than 80%, opening new possibilities for the early detection of lung cancer.[3,4] However, because of the complex algorithm of high-resolution CT employed in order to achieve the maximal performance of this approach[3] and to achieve the background noise created by the high detection rate of noncalcified nodules,[5] questions have been raised about the challenge of differential diagnosis, efficacy and costs of spiral CT screening.[6]

In 2000, a prospective trial of early lung cancer detection was launched in Milan, using repeated yearly low-dose spiral CT, selective use of positron emission tomography and analysis of molecular markers in a large cohort of 1,035 high-risk heavy smoker volunteers. The preliminary results by 2nd year of this program reported the detection of 22 tumors with complete resection in 95% of the cases, with a mean tumor size of 18 mm and a prevalence of pathological Stage I.[7] A second goal of this program is to assess whether biological markers are able to identify in this cohort individuals at higher risk of cancer and to improve the accuracy (sensitivity and specificity) of cancer detection by these imaging techniques. For this purpose, we collected peripheral blood, and separated white cells and plasma, from all volunteers and 1 day sample of uninduced sputa from 60% of the patients. More extended (3 days pooled sputum collection) and more invasive (induced sputum) biological sampling was excluded to achieve a high compliance rate in the recruitment of the volunteers (heavy smokers), and to limit the costs of the study.

Significant progress has now been made in the understanding of the genetic basis of lung cancer, which enables the use of molecular markers to detect lung cancer earlier in its natural history.[8]

Since ideal biomarkers should appear early in the course of disease and should be detectable in a biological fluid that can be obtained noninvasively, many studies have focused on the detection of genetic abnormalities in exfoliated cells from sputum or bronchoalveolar lavage (BAL) as well as in the circulating DNA found in serum or plasma.

The increased bronchial secretions of current and former smokers makes the analysis of sputum, containing exfoliated cells from the lower respiratory tract, an active area of research for markers development.[9] While conventional cytological analysis of sputum did not show predictive value in trials for early detection of lung cancer, molecular changes such as *p53* and *K-ras* mutations have been reported in the sputum samples of patients with NSCLC even before any clinical evidence of neoplasia,[10,11] and also in chronic smokers.[8]

Epigenetic silencing of tumor suppressor genes associated with aberrant methylation of normally unmethylated areas rich in dinucleotides Cytosine–Guanine(CpG), known as CpG islands, located in or near the promoter region of genes, has been frequently observed in many cancer types, including lung cancer. Hypermethylation offers the advantage of being a positive signal that can be observed even in a high background of normal cells (as in sputum samples), whereas other genetic changes such as loss of heterozygosity cannot be detected easily. Aberrant promoter methylation is an early event in tumor progression and may affect genes involved in cell-cycle control (*p16^{INK4A}*, *p15*, *Rb* and *p14*), DNA repair (*MGMT* and *hMLH1*), cell adhesion (*H-Cadherin* and *CDH-1*), signal transduction (*RASSF1A*), apoptosis (*DAPK* and *TMS1*) and cell differentiation (*RAR*β*2*).

p16^{INK4A}, DAP kinase, MGMT and RASSF1A promoter hypermethylation has been documented in sputum samples from lung cancer patients and from current and former smokers.[12,13]

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TABLE I – PRIMER SETS FOR MSP

Ref.	Primer set	Primer–F (5′→3′)	Primer–R (5′→3′)	Size (bp)	Cycle number	Annealing T (°C)
14	*p16^{INK4A}*	GAAGAAAGAGGAGGGGTTGG	CTACAAACCCTCTACCCACC	280	25	60
15	*RARβ2*	AAGTGAGTTGTTTAGAGGTAGGAGGG	CCTATAATTAATCCAAATAATCATTTACC	276	20	53
12	*RASSF1A*	GGAGGGAAGGAAGGGTAAGG	CAACTCAATAAACTCAAACTCCC	260	30	60
16	*p16^{INK4A}-M*	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	30	68
16	*p16^{INK4A}-U*	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	150	30	68
15[1]	*RARβ2-M*	GTCGAGAACGCGAGCGATTC	CGACCAATCCAACCGAAACG	146	30	66
15[1]	*RARβ-U*	ATGTTGAGAATGTGAGTGATTT	CTCAACCAATCCAACCAAAACA	146	30	64
13[1]	*RASSF1A-M*	GGGTTTTGCGAGAGCGCGT	GCTAACAAACGCGAACCG	169	30	68
13	*RASSF1A-U*	GGTTTTGTGAGAGTGTGTTTAGT	CACTAACAAACACAAACCAAACA	169	30	65

[1]We have modified primer sequences reported in literature to increase specificity of the amplified products.

In this article, we report a nested case–control study of 29 spiral CT-detected lung cancer patients and 112 cancer-free heavy smokers enrolled in a spiral CT trial. We investigated the frequency and the pattern of methylation of the 3 genes *RAR*β*-2* (retinoic acid receptor β2), *p16^{INK4A}* and *RASSF1A* (Ras association domain family 1A) in the tumor samples of 29 CT-screened lung cancer patients. In addition, in a subset of 18 CT-screened lung cancer patients, we could also analyze the methylation profile in the corresponding sputum samples. The latter was compared to that observed in 112 cancer-free heavy smokers enrolled in the ongoing spiral CT trial.[7]

We based our choice of the genes on the consideration that a panel of genes whose inactivation occurs at different stages of malignant transformation should be used in our longitudinal study. Thus, an early marker such as *RAR*β*2*, an early/intermediate marker such as *p16^{INK4A}* and a later marker such as *RASSF1* were evaluated.

The final goal of our study was to evaluate the possible diagnostic value of aberrant promoter hypermethylation in a panel of 3 genes in spontaneous sputum sample, as a complementary approach to spiral CT for early diagnosis of lung cancer.

Material and methods

Patients and collection of sputum and tumor samples

Thousand and thirty five volunteers, aged 50 or older, current or former smokers with a minimum pack/year index of 20, without prior history of malignant disease, were annually investigated at the European Institute of Oncology in Milan, with low-dose spiral CT for 5 years in a prospective lung cancer early detection study. Informed consent was signed by all the participants, and the study was approved by the institutional review board. The design and the 2 years results of the early detection trial have been reported in detail.[7] Concurrently, we conducted a case–control study that includes 29 tumor samples obtained from consecutive CT-detected and surgically resected lung cancer patients, of which 18 had tumor-sputum pairs available for the analysis, and 112 consecutive heavy smokers from the 1,035 study participants, who had a sample of sputum available and remained cancer free over the 4 years of the study. For each case, 4 cancer-free smokers were randomly selected from the cohort to obtain the best matching of control group by sex, age and smoking exposure (pack/years), with this order of priority.

The 18 uninduced sputum samples from patients who developed a CT-detected tumor were obtained at the time of first (baseline) annual CT examination, in a range of time from 1 to 30 months (median = 3.5 months) before surgery and stored at −140°C. In 5 of the CT-screened patients, the time of sputum collection anticipated the CT detection of the lung tumor of more than 1 year (range = 12.5–30 months). In the remaining patients, the sputum was collected at the time of cancer diagnosed.

To reduce the time and costs of biologic sampling and achieve the maximal compliance in the recruitment of high-risk volunteers, we decided to collect only 1 uninduced sputum sample from each patient. Since the volume of the collected sputa was less then 1 ml in 44.5% of the patients and considering that this amount would not be sufficient to carry out cytoinclusion and cytological smears as well as molecular analyses, we decided to use the entire sputum sample for DNA extraction to perform the methylation studies.

All 29 tumor samples were frozen in dry ice immediately after surgical resection. The uninduced sputum samples from controls were collected at the time of the first annual CT examination. All samples were stored at −140°C.

DNA isolation and methylation-specific PCR (MSP)

DNA from tumor and sputum was isolated using a QIAamp DNA Mini kit (Qiagen, Milan, Italy), and DNA concentration was estimated by spectrophotometry.

The methylation status in the promoter region of *p16^{INK4A}*, *RAR*β and *RASSF1A* genes was determined by methylation-specific PCR (MSP). DNA was modified by treatment with sodium bisulfite to convert unmethylated cytosines to uracil. Briefly, 1 μg of DNA in a volume of 50 μl was incubated at 95°C for 5 min and chilled on ice for 1 min. We used 1 μg of salmon sperm DNA as carrier for the samples where DNA amount was less than 1 μg. After denaturation by 3 M NaOH for 20 min at 37°C, samples were incubated in the dark with 3 M sodium bisulfite and 10 mM hydroquinone for 16 hr at 55°C. Modified DNA was purified using the Wizard DNA Clean Up System (Promega, Milan, Italy) and eluted into 100 μl of water prewarmed at 80°C. Purified DNA was subjected to desulfonization by incubation at 37°C for 20 min with 3 M NaOH, and then DNA was ethanol-precipitated and resuspended in 40 μl of H_2O. To eliminate unconverted DNA and to avoid overestimation of the DNA methylation level in the samples, we used a nested PCR assay. Primer sequences used for MSP of all genes were derived from previous references.[12–16] Number of cycles, annealing temperatures, and the expected PCR product sizes are summarized in Table I.

The first step of MSP uses a primer set that recognize the bisulfite modified template, but do not discriminate between methylated and unmethylated alleles. In the first PCR step, we used ~100 ng of modified DNA in a 50 μl volume. The PCR amplification protocol was as follows: 95°C (94°C for *RAR*β*2*) for 10 min, denaturation at 95°C (94°C for *RAR*β*2*) for 30 sec, annealing for 30 sec, extension at 72°C for 30 sec (*p16^{INK4A}* and *RAR*β*2*) or 45 sec (*RASSF1A*), followed by 10 min final extension. First step PCR products were diluted 20-fold and 2 μl were subjected to a second PCR in a 20 μl volume, with primers specific for methylated or unmethylated alleles.

The second step PCR comprised 30 cycles for all genes with 95°C (*p16^{INK4A}* and *RASSF1A*) or 94°C (*RAR*β*2*) for 30 sec, annealing for 30 sec (*RAR*β*2* and *RASSF1A*) or 15 sec (*p16^{INK4A}*) and extension at 72°C for 30 sec (*RAR*β*2* and *RASSF1A*) or 15 sec (*p16^{INK4A}*).

Ten microliters of PCR products were loaded onto 3% agarose gel, and visualized by ethidium bromide staining. Results were confirmed by repeating MSP analysis 3 times for all samples. Only

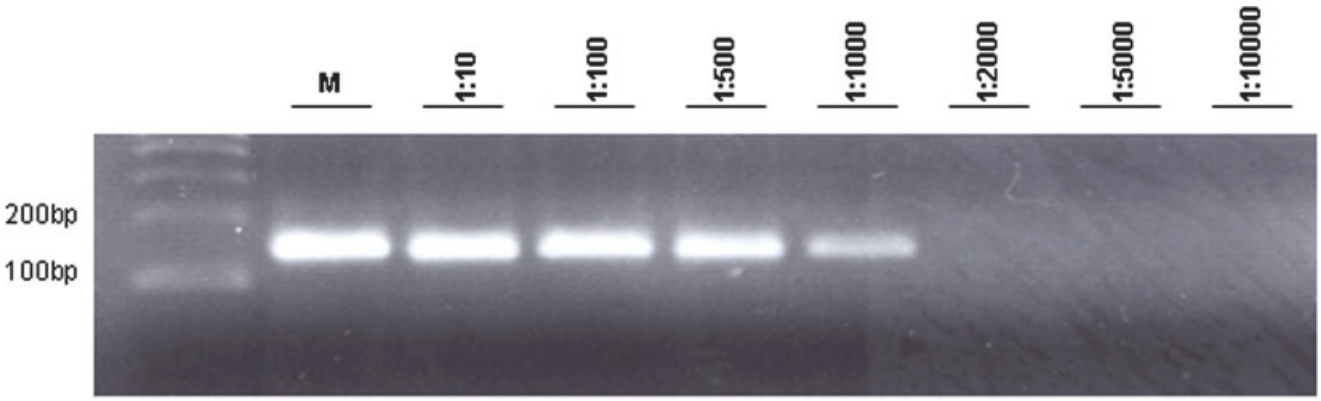

FIGURE 1 – Sensitivity of MSP for *p16^{INK4A}* gene. Sensitivity for detecting methylated alleles in a background of unmethylated DNA was determined by mixing DNA isolated from negative controls (lymphocytes) with in *vitro*-methylated DNA used as positive (methylated) control to achieve dilutions up to 1 in 100,000. The mixed DNA samples were subjected to bisulfite modification and subsequent analysis by the two step MSP approach for each gene. The sensitivity of our nested MSP assay ranged around 1/1,000 for all the 3 genes.

cases showing a consistent methylation product in at least 2 out of 3 triplicates were considered truly methylated. DNA from normal lymphocytes was used as unmethylated control, whereas *in vitro*-methylated DNA was used as methylated control in each experiment.

To validate the methylation status determined by MSP and to control the complete bisulfite modification, amplified PCR products (unmethylated and methylated) were purified using a QIAquick PCR purification kit (QIAGEN) and cloned. Several (3–6) individual clones were sequenced to determine the methylation status of the CpG islands within the amplified region.

Sensitivity for detecting methylated alleles in a background of unmethylated DNA was determined by mixing DNA isolated from the negative control (lymphocytes) with *in vitro*-methylated DNA used as positive (methylated) control to achieve dilutions up to 1 in 100,000. The mixed DNA samples were subjected to bisulfite modification and subsequent analysis by the two step MSP approach for each gene. The sensitivity of our nested MSP assay ranged around 1/1,000 for all the 3 genes.

Statistical methods

Simple descriptive statistics were computed and presented for continuous (mean, median, minimum and maximum value) and categorical variables (frequencies). Associations between categorical variables were tested by means of the Fisher exact test for 2 by 2 contingency tables, or its extension to higher order tables. All analyses were carried out using SAS® software.

Results

Clinical and pathological characteristics of the series

Twenty nine CT-detected lung cancer patients and 112 cancer-free heavy smokers were included in this study. Of the 29 patients (20 males and 9 females) 19 had stage Ia disease, 3 stage Ib, 2 stage IIb, 3 stage IIIa and 2 stage IIIb. There were 22 cases of adenocarcinomas (ADC), 1 bronchioalveolar carcinoma (BAC), 5 squamous cell carcinomas (SCCs) and 1 neuroendocrine tumor (NE). Median tumor size in patients was 12 (4–47) mm. The mean age of the CT-detected patients was 60.2 (51–74) years, and the median age was 59 years. As for the smoking habits, pack-years index distribution was as follows: up to 40 pack-years, 8 patients; 41–60 pack-years, 8 patients; above 60 pack-years, 13 patients.

The 112 controls included 29 females and 83 males. The mean and median age was 59 (50–76) years and the pack-years distribution was as follows: up to 40 pack-years, 53 patients; 41–60 pack-years, 29 patients and above 60 pack-years, 30 patients.

No significant difference in smoking exposure was observed between cases and controls ($p = 0.099$, Fisher exact test), even though an imbalance in smoking distribution was noted between patients and controls. In fact 45% of CT-detected patients fall in the category above 60 pack-years compared to 27% of the controls and, at the other side of the distribution, 28% of the patients were in the category up to 40 pack-years compared to 47% of the controls.

Validation of the sensitivity and specificity of MSP

For each of the 3 genes analyzed (*RAR*β2, *p16^{INK4A}* and *RASSF1A*), we have validated a nested PCR assay. Nested PCR was in fact suggested to allow better discrimination of efficiently bisulfite converted DNA, and thus to allow a more specific amplification of truly methylated DNA with respect to incompletely bisulfite converted DNA.[17]

Mixing experiments using DNA isolated from normal lymphocytes with *in vitro*-methylated DNA showed that the sensitivity of our nested MSP assays ranged around 1/1,000 for all the 3 genes (Fig. 1). In these experimental conditions, the normal lymphocytes used as negative control for methylation resulted always unmethylated. Moreover, sequencing analysis of at least 10 methylated and unmethylated PCR products for each gene from tumor and sputum samples of patients and controls revealed that all the C contained in the CpG dinucleotide remained unchanged only in the PCR products amplified with the primers for the methylated sequences of each gene, thus confirming the high specificity of the MSP assay conditions.

Promoter methylation patterns of RARβ2, p16INK4A and RASSF1A in tumor samples from CT-detected patients

Promoter methylation frequencies in tumor samples of CT-detected lung cancer patients are shown in Table II.

Methylation of RARβ2 was detected in 19/29 (65.5%) of the analyzed tumors. The CT-detected tumors, which displayed RARβ2 promoter hypermethylation, included 15/22 (68%) ADC and 4/5 (80%) SCC. p16^{INK4A} promoter methylation was present in 12/29 (41.4%) CT-detected tumors comprising 9/22 (41%) ADC and 3/5 (60%) SCC. Methylation of *RASSF1A* promoter genes was detected in 15/29 (51.7%) tumor samples, including 12/22 (54.5%) ADC and 3/5 (60%) SCC.

Twenty-three of twenty-nine (79.3%) of the tumor samples exhibited methylation in at least 1 gene, including 18/22 (81.8%) ADC and 5/5 (100%) SCC.

No significant association was observed between promoter methylation of the 3 genes and histologic type or tumor size. Rep-

TABLE II – FREQUENCIES OF PROMOTER METHYLATION OF *RARβ2, P16^{INK4A}* AND *RASSF1A* GENES IN 29 CT-DETECTED TUMORS, IN THE SPUTUM OF 18 CT-DETECTED PATIENTS AND IN 112 CANCER-FREE HEAVY SMOKERS[1]

Methylated genes	CT-detected patients' tumors	CT-detected patients' sputum	Heavy smokers' sputum
RARβ2	19(65.5)	8(44.4)	58(51.8)
p16^{INK4A}	12(41.4)	1(5.6)	20(17.9)
RASSF1A	15(51.7)	1(5.6)	1(0.9)
At least 1 gene	23(79.3)	9(50)	69(61.6)

[1]Values in parentheses are given in percentages.

resentative examples of MSP assays in tumor/sputum pairs are shown in Figure 2.

Promoter methylation frequencies in sputum from CT-detected patients

We investigated the presence of promoter methylation of the same panel of genes in DNA from the uninduced sputum samples in 18 patients with CT-detected lung cancer. The sputum samples were obtained at the time of baseline CT examination, in a range of time from 1 to 30 months before undergoing surgery (median time of sputum sampling from surgery was 3.5 months). In 5 of the CT-screened patients the anticipated time of sputum collection was more than 1 year (range = 12.5–30 months) after the CT detection of the lung nodule.

In Table II, the methylation frequencies for the 3 genes in sputum-tumors pairs are reported. In the sputum samples of the 18 patients, methylation was detected in 8/18 (44.4%) cases for RARβ2 and 1/18 (5.6%) cases for RASSF1A and p16^{INK4A}.

Nine of eighteen (50%) sputum samples exhibited methylation in at least 1 gene. Of the 18 CT-detected patients, with the available tumor and sputum, 6/12 patients showing RARβ2 methylation in the tumors had also methylation in the corresponding sputum sample, 4/6 patients without methylation in the tumor were negative also in the sputum and only 2/6 patients had RARβ2 methylation in sputum only. Thus, 10/12 (83.3%) patients were concordant for RARβ2 methylation status in tumor and sputum.

Each case with methylation of RASSF1A and p16^{INK4A} in the tumor had also methylation in the corresponding sputum. Representative examples of MSP assay in tumor/sputum pairs are shown in Figure 2. No significant association was observed between promoter methylation of the 3 genes in the sputum and histologic type or tumor size.

Promoter methylation patterns of RARβ2, p16INK4A and RASSF1A genes in sputum samples of CT detected cases and cancer-free heavy smokers

To investigate the diagnostic potential of promoter hypermethylation in the sputum for early lung cancer detection, we compared the methylation frequencies of *RARβ2*, *p16^{INK4A}* methylation and *RASSF1A* genes in sputum samples obtained from 18 patients with that observed in the sputum from 112 heavy smokers without cancer (Table II).

The unmethylated forms of the 3 genes were present in the sputum of all the patients, thus confirming the presence and integrity of DNA extracted from the sputum samples. RARβ2 methylation frequency was similar in the sputum of the 2 groups, 8/18 (44.4%) and 58/112 (51.8%) in sputum from CT-detected patients and from cancer-free heavy smokers respectively, suggesting that RARβ2 promoter hypermethylation could likely reflect an exposure to carcinogens, and thus behave as an indicator of smoke-damaged epithelium rather than as a tumor-specific marker.

p16^{INK4A} promoter methylation frequency was less frequent [1/18 (5.6%)] in the sputum of the CT-detected tumors with respect to the control group of cancer-free smokers [20/112 (17.9%)] even though this difference was not statistically significant ($p = 0.303$). Of interest, RASSF1A promoter methylation that, similar to p16^{INK4A} was detected in 1/18 (5.6%) of sputum sample from CT-detected patients, resulted very rare [1/112 (0.9%)] also in sputum samples from the heavy smokers, suggesting that RASSF1A methylation does not likely result from exposure to tobacco-smoke. Overall, no significant difference was observed between the methylation frequencies of the 3 genes (either individually or together) in the sputum of CT-detected patients and those detected in the sputum of controls.

We explored the association between smoking exposure and the methylation profile in the sputum of patients and controls by computing the methylation frequency for the 3 genes (either individually or together) across distinct smoking classes. No significant association was observed between promoter methylation of the 3 genes (either individually or together) in the sputum of patients and controls and smoking exposure.

Discussion

Because of the high sensitivity of spiral CT, a number of questions have been raised concerning the risk of lung cancer overdiagnosis that still remains a point of contention. A recent cDNA microarray analysis from our group showed a similar gene expression profile between spiral CT detected and symptomatic lung cancers.[18] However, the specific molecular signature of these tumors, in terms of the status of the major biological determinants of lung carcinogenesis, remains to be addressed. These molecular analyses could provide surrogate measures of the aggressive biological potential of the CT-detected cancers, a critical point given that definitive evidence of mortality benefit of lung cancer screening is lacking.

The observation of a high frequency of methylation of *RARβ2*, *p16^{INK4A}* and *RASSF1A* promoters in the CT-detected tumors analyzed in our study, as well as the presence of at least 1 methylated gene in such a large fraction (79.3%) of the samples, indicates that these early CT-detected lesions are not different from clinically-detected tumors in relation to the methylation profile. The frequencies of promoter methylation reported in our study are in fact similar to those reported in clinically-detected NSCLC patients. Depending on the different methodology used for methylation detection, the frequencies of promoter methylation reported in primary non CT-detected lung cancers, were around 43% for RARβ2,[19] ranged from 30 to 40%, for RASSF1A[20,21] and from 25 to 41% for p16^{INK4A}.[22,23] We have validated our MSP assays in terms of analytical sensitivity and specificity by using appropriate positive and negative controls. Our assay reached a sensitivity of 1 methylated out of 1,000 unmethylated molecules. In our hands, the use of one-step PCR with an elevated number of cycles in fact generates false-positive results, as revealed by sequencing of the PCR products, due to the mispriming of methylated primers to even small amount of unconverted DNA that is left after bisulfite conversion. Nested-PCR approach for each of the 3 genes (*RASSF1A*, *RARβ2* and *p16^{INK4A}*) allows better discrimination of efficiently bisulfite converted DNA in the first PCR cycle, and thus permits a more specific amplification of truly methylated DNA with respect to incompletely bisulfite converted DNA.[17]

In addition, for the initial purpose of our study that was to evaluate if biomarkers in sputum could enhance the specificity of spiral CT, a diagnostic imaging approach that detects very small nodules with many false-positive results, we were very cautious to set up MSP experimental conditions that could enhance specificity rather than sensitivity.

Concerning the potential diagnostic value of promoter methylation in sputum of CT-detected patients, our data are discordant from those reported in literature for non-CT detected cases. In fact hypermethylation of p16^{INK4A} gene has been detected by Palmisano *et al.*, in 10/10 (100%) sputum samples collected from patients with SCC of the lung, 5–35 months before clinical tumor detection.[14] The authors suggested that hypermethylation at this locus is associated with subsequent development of overt disease.

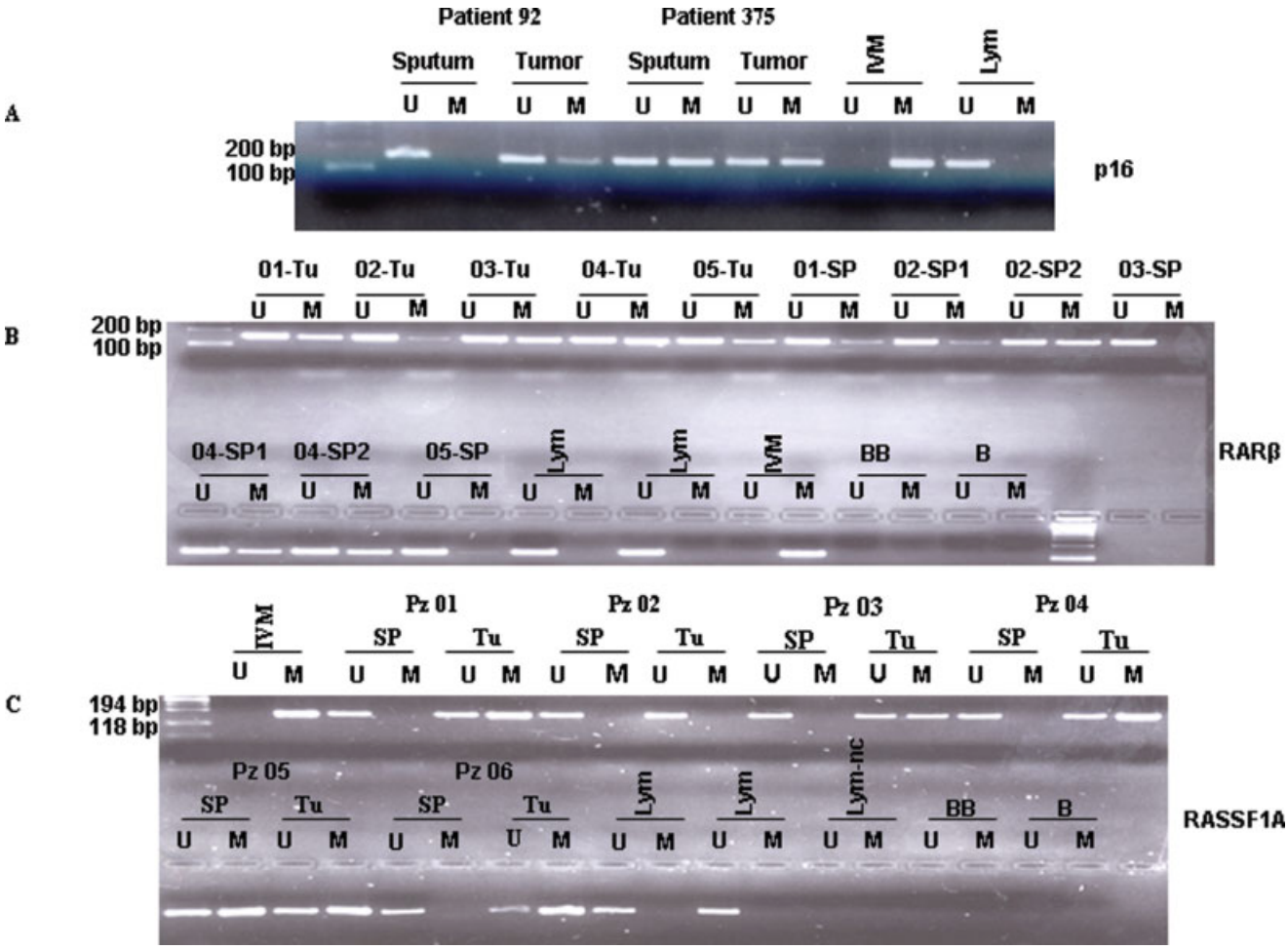

Figure 2 – Representative example of MSP assay in tumor\sputum pairs sample. (*a*) MSP for *p16*^{INK4A} promoter gene. (*b*) MSP for *RAR*β2 promoter gene. 02-SP1 and 02-SP2 sputum samples obtained from the same patient 1 year and 1 month before surgery, respectively. 04-SP1 and 04-SP2 sputum samples obtained from the same patient 8 months before the first surgery and 3 months before the second surgery, respectively. (*c*) MSP for *RASSF1A* promoter genes. Lym-nc, unconverted DNA from lymphocytes; IVM, *in vitro*-methylated DNA as positive control; Lym, DNA from lymphocytes as negative control; U, amplified product with primers recognizing unmethylated sequence; M, amplified product with primers recognizing methylated sequence; SP, sputum sample; Tu, tumor sample; BB, negative control (water) of nested PCR; B, negative control (water) of second step PCR.

However, p16^{INK4A}, RASSF1A and RARβ2 promoter hypermethylation has been documented in sputum samples not only from lung cancer patients but also from cancer-free current and former smokers.[12,13,24] In our study, methylation of *p16*^{INK4A} and *RASSF1A* genes in sputum from CT-detected patients was detected in a negligible number of samples (only 2 cases). Thus, the diagnostic potential of methylation analysis of these 2 genes in uninduced sputum for detecting this type of early cancer appears to be low. A major reason for this discrepancy could reside in the different clinical-pathological features of CT-detected tumors. In fact the prevalent tumor type of our CT-detected patients was Stage IA adenocarcinoma (15 cases) compared to SCC (2 cases), and it is likely that tumors of SCC type are more prone to release exfoliated cells in sputum than the ADC subtype.

However, we detected p16^{INK4A} methylation in the sputum of about 18% heavy smokers who remained cancer free over the 4 years of the spiral CT trial, thus suggesting that methylation of p16^{INK4A} may act as an exposure-marker. Similarly, high frequencies of p16^{INK4A} methylation were reported in cancer-free smokers by Palmisano (6/32, 18.8%), Belinsky (23/66, 35%) and Kersting (7/25, 28%).[8,12,14] Whether or not, as suggested by Belinsky *et al.*, p16^{INK4A} methylation in sputum of cancer-free smokers confers increased risk for lung cancer by permitting the acquisition of further (epi)genetic changes that ultimately lead to cancer is to be verified in long term longitudinal studies, with the analysis of a large panel of genetic and epigenetic markers.

RARβ2 methylation was equally present in the sputum of CT-detected patients and of 112 CT-screened heavy smokers without cancer, suggesting that RARβ2 methylation can also be interpreted as an exposure-related marker rather than a tumor-specific marker. These observations are in agreement with those already reported of elevated rate of RARβ2 methylation in sputum, bronchial biopsies and bronchial lavage of smokers without cancer.[25,26]

On the contrary, methylation of RASSF1A was detected in a high frequency of tumor samples from the CT-detected patients, but appeared in only one sputum sample from the 112 heavy smokers. These data suggest RASSF1A could more likely behave as a true tumor-associated marker. This hypothesis is supported by our analysis of RASSF1A promoter methylation in 15 sputum-tumors pairs from symptomatic (non-CT detected) tumors, where we detected RASSF1A promoter methylation in 53.3% tumors and in 62% of their respective sputum samples (data not shown). The significance of RASSF1A methylation as a tumor-specific marker should be investigated in larger studies.

Our results are different from those recently reported by Kim *et al.*[25] on BAL from 127 cancer-free patients who suggest that tumor-specific methylation of *p16^{INK4A}*, *RASSF1A*, *H-cadherin* and *RAR*β2 genes may be useful biomarker for the early detection of NSCLC in bronchial lavage. Our data showing a negligible fraction of p16^{INK4A} and RASSF1A methylation in the sputum of CT-detected patients, as well the presence of RARβ2 and p16^{INK4A} methylation in the sputum from 112 spiral CT-screened smokers who remained cancer free over the 4 years of the study indicate that these biomarkers are not related to the development of overt disease.

In conclusion our study does not support a potential role of this panel of methylation markers in the sputum for identification of early spiral CT-detected lung tumors. This may be due to the clinico-pathological characteristics of these tumors, making difficult the detection of such molecular changes in spontaneous sputum samples. Hypertonic saline sputum induction or 3 days pooled sputum collection can increase the sensitivity of molecular markers, but are more complex and expensive, and may prove difficult to apply to large scale early detection programs with spiral CT, where long-term compliance is directly related to the lowest possible invasiveness of screening procedures. Our data further support the concept that the prevalence of methylation markers in normal and tumor tissues is gene-specific, and suggest that a larger panel of genes should be analyzed in longitudinal prospective studies to determine the reliability of this molecular approach for early detection of lung cancer.

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