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Application of a methylation gene panel by quantitative PCR for lung cancers

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Summary

Detection of lung cancer at early stages could potentially increase survival rates. One promising approach is the application of suitable lung cancer-specific biomarkers to specimens obtained by non-invasive methods. Thus far, clinically useful biomarkers that have high sensitivity have proven elusive. Certain genes, which are involved in cellular pathways such as signal transduction, apoptosis, cell to cell communication, cell cycles and cytokine signaling are down-regulated in cancers and may be considered as potential tumor suppressor genes. Aberrant promoter hypermethylation is a major mechanism for silencing tumor suppressor genes in many kinds of human cancers. Using quantitative real time PCR, we tested 11 genes (*3-OST-2, RASSF1A, DcR1, DcR2, P16, DAPK, APC, ECAD, HCAD, SOCS1, SOCS3*) for levels of methylation within their promoter sequences in non-small cell lung cancers (NSCLC), adjacent non-malignant lung tissues, in peripheral blood mononuclear cells (PBMC) from cancer free patients, in sputum of cancer patients and controls. Of all the 11 genes tested *3-OST-2* showed the highest levels of promoter methylation in tumors combined with lowest levels of promoter methylation in control tissues. *3-OST-2* followed by, *RASSF1A* showed increased levels of methylation with advanced tumor stage ($P<0.05$). Thus, quantitative analysis of *3-OST-2* and *RASSF1A* methylation appears to be a promising biomarker assay for NSCLC and should be further explored in a clinical study. Our preliminary data on the analysis of sputum DNA specimens from cancer patients further support these observations.

Keywords

Real time PCR; Tumor suppressor gene; Non-small cell lung cancer

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1. Introduction

Lung cancer is the leading cause of cancer-related deaths in the world [1]. The survival rates for lung cancers have changed little over the past two decades [2]. A major factor in the high mortality of lung cancer patients is the presence of metastases in approximately two-thirds of patients at the time of diagnosis [3]. It has been estimated that detection of lung cancer at earlier stages could potentially increase survival rates by 10–50-fold [4]. Lung cancer screening by chest X-ray and sputum cytology have proven ineffective in increasing patient survival [3,5], leading to the search for more sensitive and specific tests [6]. One promising approach is the identification of lung cancer-specific biomarkers and non-invasive methods for the detection of these biomarkers at an early stage. However, there is lack of a sufficient number of biomarkers especially for NSCLC (~80% of the total lung cancer cases) that are both cancer specific and highly sensitive. Thus, investigations into identifying biomarkers that can effectively separate cancer cells from normal cells with high sensitivity are needed.

Aberrant promoter hypermethylation is a major mechanism for silencing tumor suppressor genes in many kinds of human cancers [7]. Gene promoter hypermethylation potentially provides a non-invasive screen for early cancer detection and offers opportunities to develop novel therapies for reversing aberrant methylation [8]. The development of the real time methylation specific PCR has simplified the study of the genes inactivated by promoter hypermethylation in human cancer [9,10]. It has the advantage of increasing specificity and sensitivity (attributable to the use of an internally binding fluorogeneic hybridization probe for each gene) over conventional PCR and of great importance, provides quantification (qPCR). Recent publications have demonstrated the presence of promoter hypermethylation of various genes in clinical specimens containing exfoliated tumor cells (such as malignant effusions, sputum, serum, etc.) [11–14]. We believe that markers that are tumor specific, with high levels of methylation in tumor but low or absent methylation in adjacent non-malignant tissue and in blood mononuclear cells will prove to be clinically more useful. There is severe shortage of markers with high frequency and high levels of methylation for NSCLC that can also separate cancerous from adjacent non-malignant tissue.

Certain genes, which are involved in cellular pathways such as signal transduction (*3-OST-2*), apoptosis (*DcR1*, *DcR2*, *DAPK*), adhesion (*APC, E-CAD, H-CAD*), cell cycle (*P16*, *RASSF1A*), cytokine signaling (*SOCS1, SOCS3*), etc. are epigenetically inactivated in cancers and may be considered as potential tumor suppressor genes (Table 1) [15–32].

In an effort to identify new clinically useful markers for lung cancer detection, we used qPCR assays to determine the methylation frequencies of 11 genes implicated in critical cellular pathways, for their ability to separate lung cancer from normal cells. In a preliminary study, we applied our panel of markers to sputum specimens from patients suspected of having lung cancer.

2. Materials and methods

Surgically resected non-small lung cancers (NSCLC, n=40) and their adjacent normal lung tissues were obtained from MD Anderson Cancer Center, Houston TX. Institutional review board permission and informed consent were obtained from all patients. Tumor staging was performed according to the International Union Against cancer (UICC) TNM classification. Peripheral blood mononuclear cells (PBMC) were obtained from 40 healthy individuals with a family history of cancer. Three day pooled preferably morning sputum samples (uninduced) were collected in saccomanno's fixative (2% polyethylene glycol in 50% ethanol). Sputum samples were obtained from 13 patients with NSCLC and 25 individuals without lung cancer (COPD patients and patients treated for NSCLC, subsequent sputum

collection and follow-up of minimal 1 year without signs of lung cancer, i.e. normal controls, without malignancy) in the Canisius Wilhelmina Hospital, Nijmegen, the Netherlands (Table 2).

3. DNA extraction and methylation methods

Genomic DNA was extracted from cell lines, primary tumors and non-malignant cells by using a DNA extraction kit (Serological Corporation, Norcross, GA) [33]. Chemical modification of unmethylated (but not methylated) cytosines to uracil within CpG islands using sodium-bisulfite treatment was performed as described previously [34]. The modified DNA was used as a template for qPCR analysis. Genomic DNA was extracted from sputum samples according to the previously proposed protocol [35]. Dithiothreitol was used to dissolve mucus. Genomic DNA was extracted from sputum samples by using the QIAamp DNA blood mini kit (QIAgen, Venlo, the Netherlands). The DNA was bisulfite treated as described above [34]. The modified DNA was used as a template for qPCR analysis.

4. Semiquantitative real time PCR analysis

qPCR analysis was performed using the Chromo4 MJ Research Real time PCR system (Waltham, MA). Sodium bisulfite-treated genomic DNA was amplified by fluorescence-based real time MSP using TaqMan technology as described previously [9,10]. In brief, oligonucleotide primers were designed to specifically amplify bisulfite-converted DNA within the region of the test genes that correlates with loss of gene expression [27,36,37] and a probe designed to anneal specifically within the amplicon during extension (Table 3). The identities of the amplicons were confirmed through direct sequencing in forward and reverse directions. The primers used for *APC* and *DAPK* and *E-CAD* and *RASSF1A* were as previously published [9,13,14]. PCR assays are performed in a reaction volume of 25 μl. The final reaction mixture contained the forward and reverse primers (600 nmol/l of each); the probe (200 nmol/l); each of the four nucleotide phosphates (200 (mol/l for each); 5.5 mmol/l $MgCl_2$; 1XTaqMan Buffer A; 1 U of Hot Star Taq DNA polymerase (Qiagen, Valencia, CA) and 1 μl bisulfite-converted genomic DNA (corresponds to ~50 ng initial DNA used for conversion). PCR was performed under the following conditions: 95 °C for 15 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. The non-methylated form of *MYOD1* was used as an internal reference standard [9]. Amplification of *MYOD1* is independent of its methylation status, whereas the amplification of test genes is proportional to the degree of cytosine methylation within the amplicon. Serial dilution of the Sss1 treatment (which converts all cytosines to their methylated forms) of human genomic DNA from normal human lymphocytes was used to create a standard curve. The fluorescence emission intensities (threshold cycle or Ct values) for the biomarker genes and *MYOD1* were calculated using the intercept and the slope of the standard graph. The methylation ratio is defined as the ratio of the fluorescence emission intensity values for the PCR products of the biomarker gene to those of PCR products of MYOD1 multiplied by 100. The ratio is a measure for the relative level of methylation in an individual sample. Fig. 1 illustrates representative fluorescence curves generated from real time PCR analysis of lung tumors and adjacent normal lungs for *m3-OST-2, mRASSF1A* and *MYOD1*. The figure also illustrates standard curves for *m3-OST-2* and *mRASSF1A*. The standard curves demonstrate a linear relationship between fluorescence intensity (Log Quantity) and cycle number (Ct cycle) over more than three logs. Similar standard curves were obtained for all genes analysed in this study including *MYOD1* and they demonstrated good linear correlation (data not shown). Ct values from three to four independent runs of the same reactions differed by less than 1 cycle. To confirm the reliability of the assay system some of the tumors and adjacent non-malignant lungs were analyzed in a blinded fashion. In addition, a second, independent bisulfite treatment was performed on DNA from some of the

tumors and adjacent non-malignant lungs and they were analyzed in a blinded fashion. These data were used to confirm the reproducibility of the assay. The data were found to be reproducible with both high and low methylation level samples analysed in our study. All the sputum DNA samples were coded and shipped from Canisius Wilhelmina Hospital, Nijmegen, the Netherlands and analysed in our laboratory in a blinded fashion.

5. Statistical analyses

Sensitivity and specificity of a given gene are measured as the fraction of tissues whose methylation level exceed a predetermined threshold value (test positive) among all lung cancer tissues and the fraction of tissues test negative among all non-malignant lung tissues. For this study, we had no prior cutoff value for calling a biomarker positive, therefore, we applied the receiver operating characteristic (ROC) curve analysis to determine the cutoff values. The ROC curves, a plot of the sensitivity vs. specificity across all possible cutoff values, were used to identify the accuracy of a marker in discriminating lung cancer from non-malignant lung or adenocarcinoma from squamous carcinoma. A marker with perfect discriminatory capacity (100% sensitivity and 100% specificity) has an ROC plot that pass through the upper left corner, and the higher the ROC curve the higher the accuracy of the test. To measure the accuracy of discrimination, we considered the area under the ROC curve (AUC). An AUC not significantly different from 0.5 indicates that the test is neither sensitive nor specific. The AUC and 95% confidence interval (95% CI) were calculated by a non-parametric method [38]. To combine several markers, we first fitted a logistic regression with binary outcome (cancerous vs. normal tissue, or adenocarcinoma vs. squamous carcinoma) and used a panel of markers as covariates. The resulting linear predictive score from the regression model was then used as a composite marker and the ROC curves and corresponding AUCs were calculated. Cross-validation and permutation were used to assess the accuracy summaries in this case to correct for over fitting.

We also correlated quantitative methylation data for different genes with tumor stage using the Mann–Whitney *U*-test, which does not require parametric assumptions on the distribution of quantitative methylation. *P*-values<0.05 were considered significant.

6. Results

We determined the methylation profile of 40 NSCLC tumors (22 adenocarcinoma and 18 squamous carcinomas) and adjacent non-malignant lung tissues using qPCR. Additionally, PBMCs from 40 healthy subjects recruited for genetic epidemiology studies were also quantitatively analyzed. Fig. 2 shows the quantitative methylation data for 6 representative genes of the 11 genes tested. We found aberrant methylation of at least 1 gene in 39 of 40 (98%) of primary tumors. Frequencies of methylation in primary tumors ranged from 36 of 40 (90%) for *3-OST-2* to 16 of 40 (40%) for *DcR2.* Frequencies of methylation in adjacent non-malignant tissue ranged from 23 of 40 (58%). for APC to 1 of 40 (2.5%) for *DAPK. 3-OST-2* had the highest median QR, 14.19 (0–975)) followed by *APC*, 4.1 (0–331.13). However, *3-OST-2* methylation had the lowest median QR, 0 (0–4.1) in adjacent non-malignant tissue while *APC* had one of the highest median QR, 0.29 (0–13.95) in adjacent non-malignant tissue. Thus, *3-OST-2* methylation appeared to be both highly tumor specific with a high degree of sensitivity. For all the genes, we set the highest methylation level in non-malignant tissues as the cutoff value to determine methylation positive samples for lung cancer tissues. With this cutoff, the frequency of aberrant promoter methylation in tumors ranged from 70% for *3-OST-2* to 23% for DcR1 (Table 4). For *SOC3* and *E-CAD* the cutoff values were too high for them to be useful tumor markers. The median QRs for all the genes were 0 in PBMCs. However, *H-Cad*, *E-CAD*, *SOCS1* and *SOCS3* did show methylation in some cases (<25%) although at much lower levels than in tumors. Interestingly, *APC* was

unique in the sense that it showed high levels of methylation in non-malignant lung but showed no methylation in PBMCs from cancer-free individuals.

Fig. 3 shows the ROC curve and AUC (95% CI) which examine the accuracy of the markers to separate cancer from the adjacent non-malignant tissue. The data show that except for *SOCS3* and *E-CAD* all other genes have the discriminatory capacity at separating cancer from adjacent normal lung tissue. Additionally, the combination of *3-OST-2, DAPK* and *DcR1* best separated cancer from corresponding normal tissue. The accuracy of each gene at separating cancer from PBMCs of cancer free subjects is assessed with a ROC curve and the corresponding AUC (95% CI) (Fig. 4). The data show that all the genes separate cancer from PBMCs, indicative of no or negligible methylation of all the genes in PBMCs. Fig. 5 shows ROC curve and AUC (95% CI) which demonstrates the ability of the genes at separating adenocarcinoma from squamous carcinoma. The combination of *DcR1* and *3-OST-2* best separated adenocarcinoma from squamous cell carcinoma.

We also correlated quantitative methylation data for different genes with tumor stage using the Mann–Whitney *U*-test. For *3-OST-2* increased methylation levels (QR) were observed with increase in stage (stage 1–2, P=0.03), stage 1 and 3, (P=0.0002), stage 1 and 2 combined to stage 4 (P=0.0003) (Table 5). Besides *3-OST-2,* increased methylation levels (QR) were also observed to increase with stage for *RASSF1A* (stage 1 and 2 (P=0.05), stage 1 and 3 (P=0.03), stage 1 and stages 2–4 combined (P=0.03) (Table 6). No statistically significant correlation between methylation levels tumor stage observed for other genes.

Additionally, we analysed 38 sputum DNA samples (13 from patients with cancer and 25 from patients without lung cancer) for methylation of the 11 genes using quantitative PCR. Our data showed that, for 4 of the 11 genes, analysis of sputum DNA for methylation may have significant potential as biomarkers in cancer patients (Table 2) (Fig. 6). Analysis of sputum DNA for methylation did not show any significant potential as biomarkers in cancer patients for other genes (data not shown). This may be attributed to very small tumor cell population that might be present in sputum samples. Further, studies may be required with sputum as well as other clinical specimens containing exfoliated tumor cells (effusions and bronchoalveolar lavage) to evaluate the potential of these markers. Fig. 6A shows the quantitative methylation data of *3-OST-2*, *RASSF1A*, *P16* and *APC* for sputum samples from cancer patients (n=13) and normal controls (n=25). Cutoff ratios were established based on the highest QR values in control groups. Based on the cutoff values, methylation was detected in 4, 5, 3 and 3 cancer patients for *3-OST-2*, *RASSF1A*, *P16* and *APC*, respectively (n=13). Thus, based on the same cutoff values none of control samples showed methylation in any of the four genes. When the above quantitative data were analysed for methylation of either *3-OST-2* or *RASSF1A* 7 of the 13 cancer patient sputa (54%) and 0 of 23 normal controls (0%) (P<0.0002) showed methylation of either of the two genes. When the same quantitative data were analysed for methylation of any of the four genes, 8 of the 13 cancer sputum (62%) and 0 of 23 normal controls (0%) (P<0.0001) showed methylation of atleast one of the four genes. Fig. 6B shows the ROC curve and AUC (95% CI) which examine the accuracy of the markers to separate cancer sputum from the normal sputum. The data show that combination of *m3-OST-2, mRASSF1A, mP16, mAPC* have excellent discriminatory capacity at separating sputum of cancer patient from that of normal control.

7. Discussion

Methylation specific PCR allows one to specifically assay for methylation at a common region within a gene promoter that correlates with loss of transcription [7]. Thus, aberrant methylation of tumor suppressor genes has formed basis in development of biomarkers for early detection of lung cancer [8]. One might conceptualize that markers that show high

levels of methylation in tumor but very low methylation in adjacent non-malignant tissue and in blood mononuclear cells will prove to be clinically more useful. Since such assessment needs quantitative assay, in this study we used qPCR we quantitated promoter methylation for 11 genes in NSCLC. The presence of hypermethylation of one or more genes was found in 98% of the NSCLC. Initially, we tested the ability of the panel of markers to separate lung tumors from adjacent non-malignant lung, tumors from PBMCs and adenocarcinomas from squamous carcinomas. We found nine genes (*3-OST-2, RASSF1A, P16, DcR1, DcR2, SOCS1, APC, DAPK, H-CAD)* whose methylation levels are potentially useful as biomarkers in NSCLC. To our knowledge this constitutes the first report, which determined if levels of methylation of genes such as *3-OST-2, DcR1, DcR2, SOCS1* and *SOCS3* could be used as potential biomarkers.

It appears from our data that levels of *3-OST-2, DcR1, DcR2* and *SOCS1* methylation may have significant potential as biomarkers in NSCLC. Additionally, we compared the quantitative methylation data of previously untested genes with previously tested genes such as *RASSF1A*, *APC* and *p16* [11]. *RASSF1A* was shown to be methylated at high levels in NSCLC (although at a frequency less than 50%) while present at low levels in adjacent non-malignant lung [14] and was considered a promising biomarker. *P16* was shown to be methylated at intermediate levels but was also methylated at low levels in adjacent non-malignant lung [14]. *APC* was shown to be methylated at a high levels and at a high frequency in NSCLC while methylation was also found in high frequency (but at lower levels than tumor) in adjacent non-malignant tissue [11,12]. Our study indicated that methylation of *3-OST-2, DcR1, DcR2, SOCS1* and *H-CAD* in tumor tissues were one to two orders of magnitude higher than adjacent non-malignant tissue. Thus, the previously untested markers appear to be highly tumor specific. The assay system used here may also serve as a more stringent model for realistically comparing the potential of biomarkers to detect lung cancer in a clinical setting.

Most interestingly, *3-OST-2* showed the highest levels of promoter methylation in tumors (frequency of 70% based on the highest QR in adjacent normal lung tissue as a cutoff) combined with lowest levels of promoter methylation in adjacent normal tissue and also with lowest levels of promoter methylation in PBMCs from non-cancerous individuals. Furthermore, *3-OST-2* promoter methylation levels, and *RASSF1*A methylation correlated with tumor grade. Generation of polysaccharide chains and enzymatic modification of them to yield the specific saccharide sequences are critical for cell surface heparin sulfate proteoglycans in ligand binding and signal transduction. *3-OST-2* is the enzyme involved in the last of the three sequential steps each transferring a sulfate group to sugar residues [15,16] and of critical importance in functional integrity of the heparin sulfate proteoglycans. Quantitative analysis of *3-OST-2* methylation along with *RASSF1*A methylation appears to be a promising sensitive and specific biomarker assay for NSCLC and should be further validated in a clinical setting. Based on the preliminary data reported in this manuscript quantitative analysis of *3-OST-2, RASSF1A, P16 and APC* methylation in sputum specimens may have excellent potential as a biomarker assay for patients with lung cancer. Methylated DNA has also been found in other diagnostic samples containing exfoliated tumor cells and may vary in the DNA fragment size, levels of PCR inhibitors, relative percentage and types of non-malignant cell types. Our preliminary findings of the application of a methylation panel for the diagnosis of malignancy in sputa need to be expanded. In addition, studies are in progress to evaluate the potential of the methylation gene panel in detection of lung cancers, in malignant effusions, serum and bronchoalveolar lavage samples of lung cancer patients.

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Abbreviations

MSP	methylation specific PCR
NSCLC	non-small cell lung cancer

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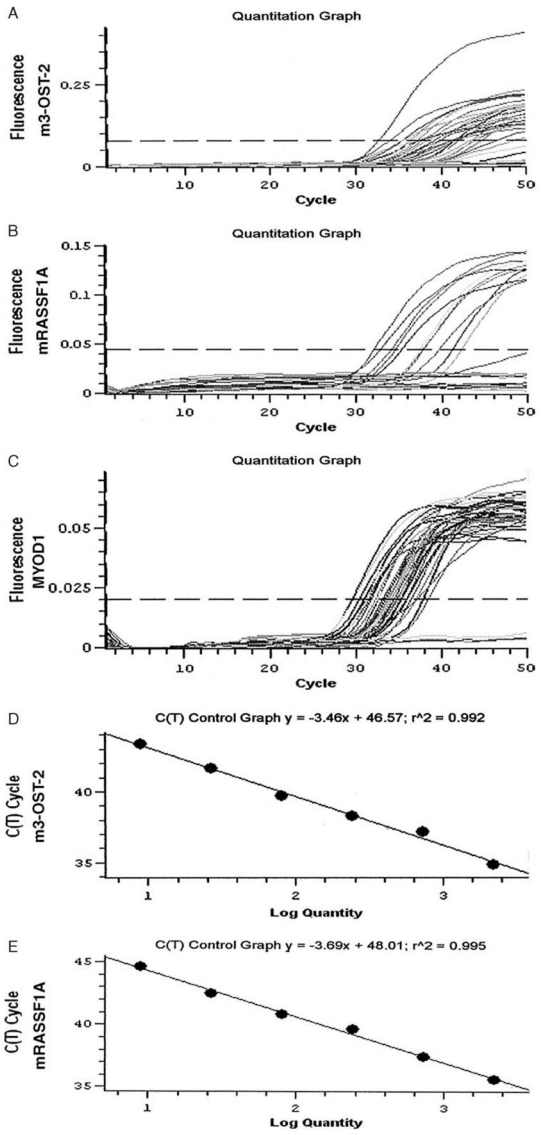
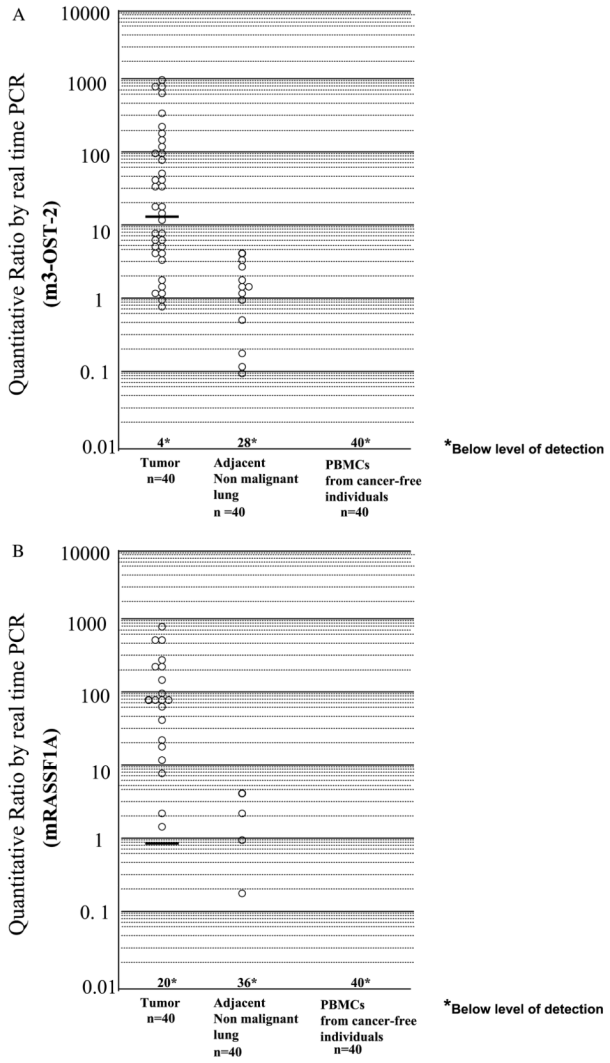
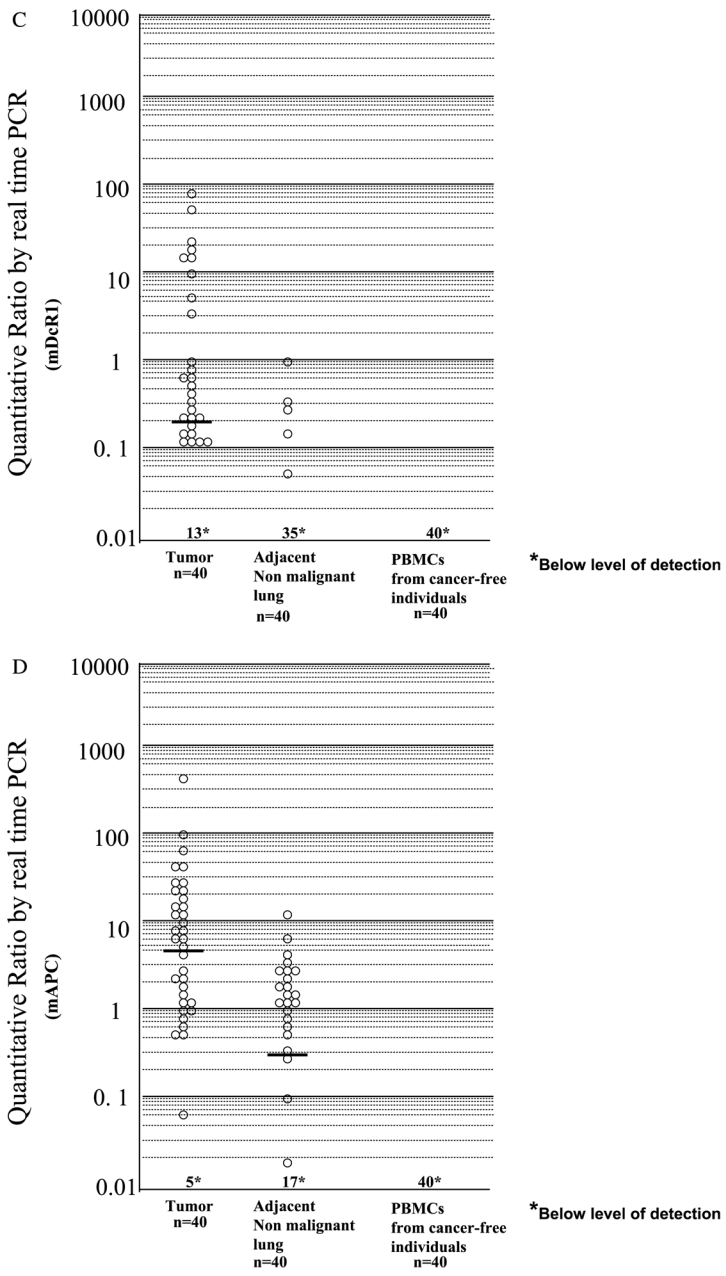


Fig. 1.

Fluorescence curves generated from real time PCR reactions for methylated 3-OST-2 (A), methylated *RASSF1A* (B) and *MYOD1*(C). The data represent analysis of the 20 normal tumor pairs from our study. A (*3-OST-2*) and B (*RASSF1A*), the samples with the lowest Ct value (curves on the far left for both genes, Ct 35.64 for *3-OST-2* and Ct 32.18 for *RASSF1A*) represent positive controls (SS1DNA) Ct values for of all the adjacent non-malignant lung samples ranged from 42 to 50 (Ct value of 50 means below the threshold) both in *3-OST-2* and *RASSF1A* groups. The Ct values for methylation positive tumors were always below 42 both in *3-OST-2* and *RASSF1A* groups. The range of Ct values for *MYOD1* of different tumor and normal lungs was much narrower, 31.18–37.88 (Ct value for *MYOD* of positive control was 36.47). Standard curves are shown for methylated *3-OST-2* (D) and methylated *RASSF1A* (E). The standard curves demonstrate a linear relationship between fluorescence intensity (log quantity) and cycle number (Ct cycle) over more than three logs.





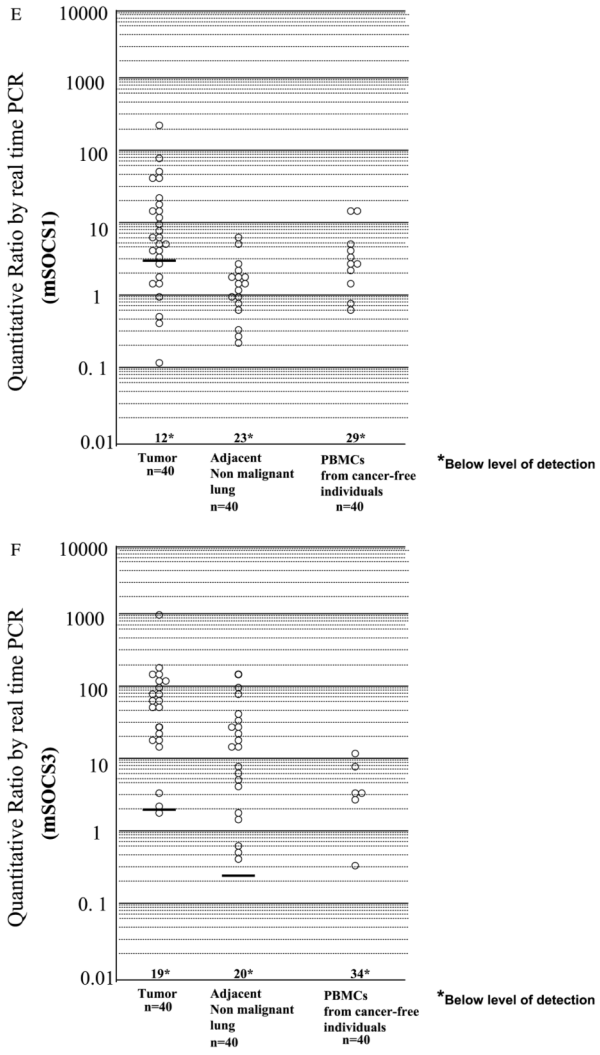


Fig. 2.

Methylation levels of m*3-OST-2* (A), m*RASSF1A* (B), m*DcR1* (C), m*APC* (D), m*SOCS1* (E) and m*SOCS3* (F) in NSCLC (tumor), adjacent non-malignant lung and PBMCs from cancer-free individuals. Methylation levels were quantitated by semiquantitative real time PCR. Real time analysis was performed as described in Section 2. Quantitative ratio is defined as the ratio of the fluorescence emission intensity values for the PCR products of the biomarker gene to those of PCR products of *MYOD1* multiplied by 100. The ratio is a measure for the relative level of methylation in an individual sample. Because values are expressed on a log scale, completely negative values are expressed as values of 0.01. The solid horizontal bar represents the median value for each group. No solid horizontal bar was shown when the median value for a group is zero.

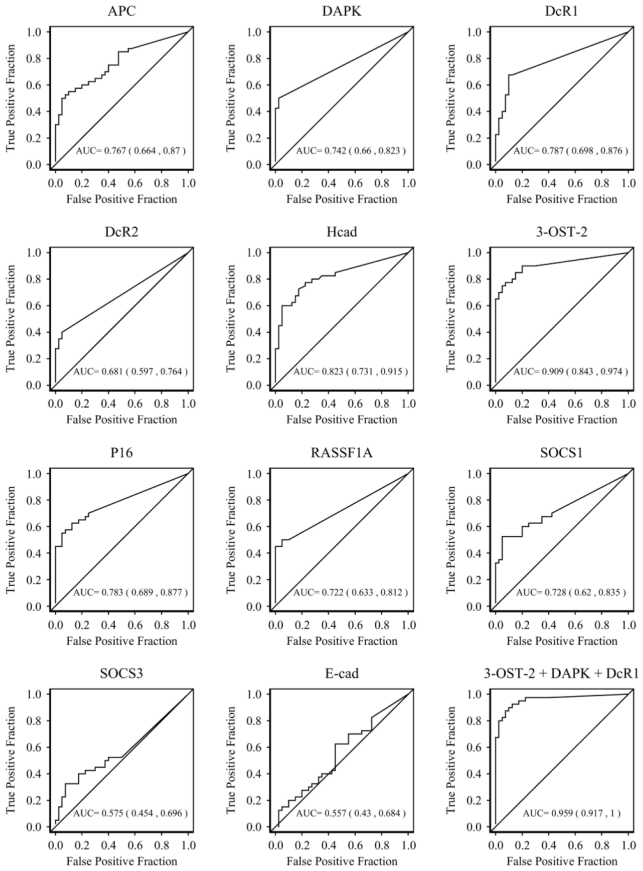


Fig. 3.

ROC curves for each of the 11 genes (methylated form) at separating cancer from the adjacent non-malignant lung. ROC curves are plots of the true positive rate (vertical axis) against the false positive rate (horizontal axis) for the different possible cutoff points of a diagnostic test. The closer the curve follows the left-hand border and then the top border of the ROC space, the more accurate the test.

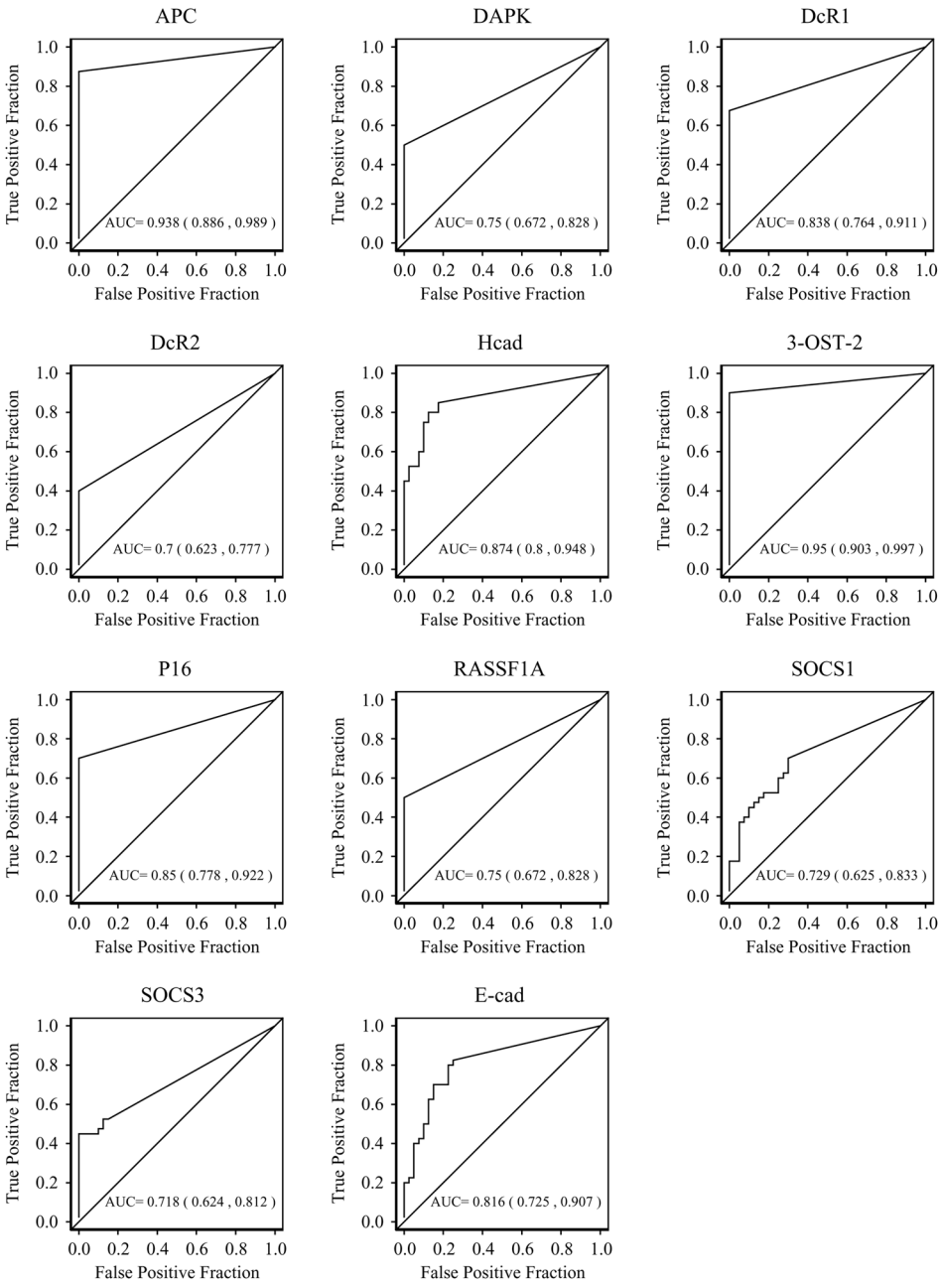


Fig. 4.

ROC curves for each of the 11 genes (methylated form) at separating cancer from PBMCs derived from healthy individuals with family history of cancer. The description of ROC curves as in Fig. 3 and in Section 2.

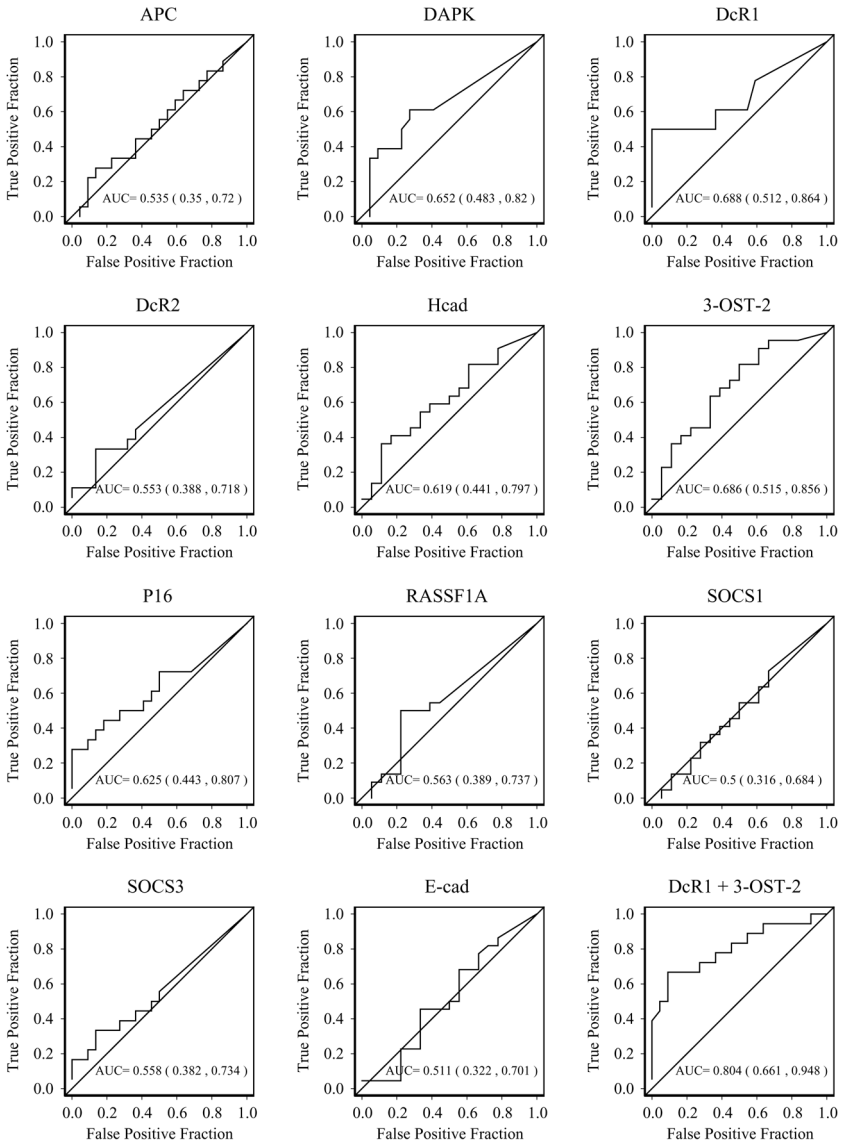


Fig. 5.

ROC curves for each of the 11 genes (methylated form) at separating adenocarcinoma from squamous cell carcinoma. The description of ROC curves as in Fig. 3 and in Section 2.

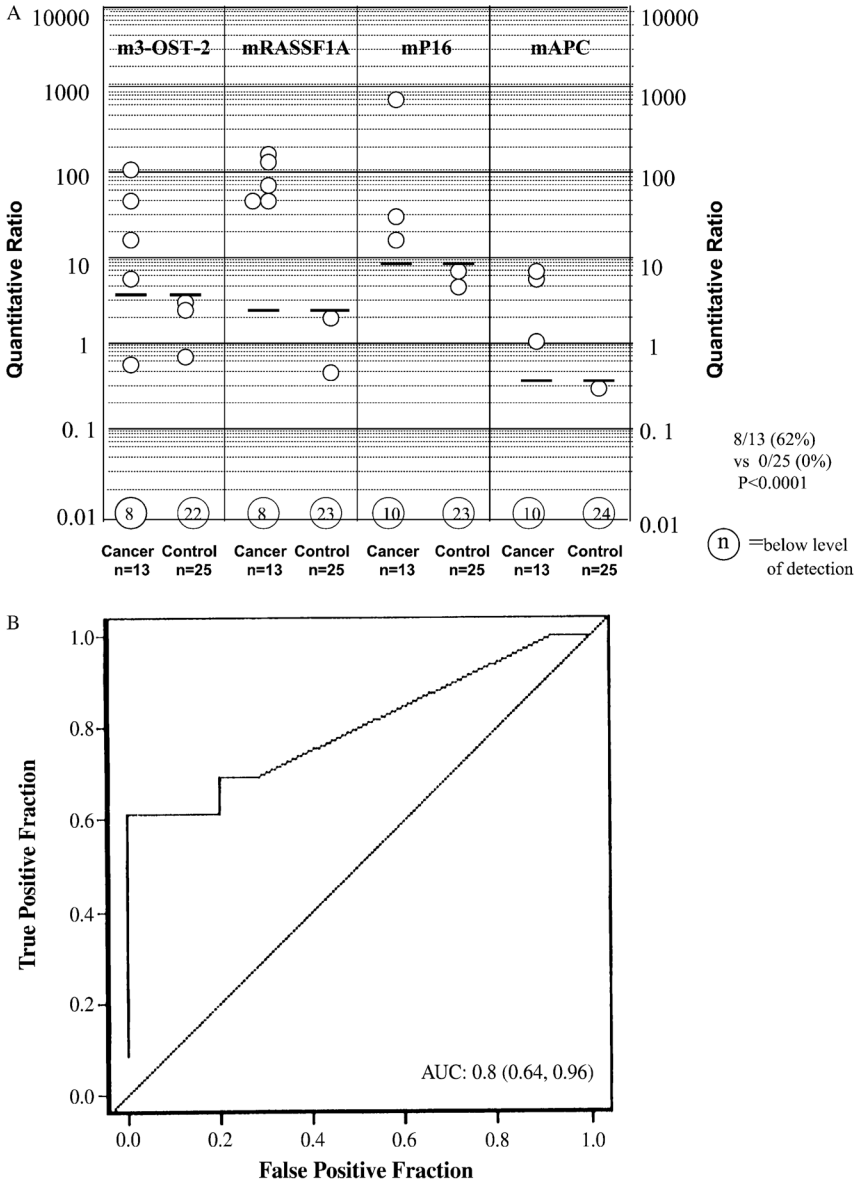


Fig. 6.

(A) Methylation levels of m*3-OST-2*, m*RASSF1A*, mp16, m*APC* in sputum from NSCLC patients and in sputa from patients without malignancy. Methylation levels were quantitated by semiquantitative real time PCR. Real time analysis was performed as described in Section 2. Quantitative ratio is defined as the ratio of the fluorescence emission intensity values for the PCR products of the biomarker gene to those of PCR products of *MYOD1* multiplied by 100. The ratio is a measure for the relative level of methylation in an individual sample. Because values are expressed on a log scale, completely negative values are expressed as values of 0.01. The solid horizontal bar represents the cutoff ratios established based on the highest QR values in control groups. The large open circles with numbers indicates the number of samples below level of detection. The statistical comparison between the methylation frequencies (combined for the four genes) of sputum specimens from cancer patients and those from normal controls was done using Fisher's exact test. (B). ROC curves to evaluate for the ability of the combination of *m3-OST-2.*

mRASSF1A, mP16 and *mAPC* in separating sputa of patients with lung cancer from sputa of patients without lung cancer. ROC curves are plots of the true positive rate (vertical axis) against the false positive rate (horizontal axis) for the different possible cutoff points of a diagnostic test. The closer the curve follows the left-hand border and then the top border of the ROC space, the more accurate the test.

Table 1

Summary data of genes analysed for methylation with quantitative PCR

Gene	Gene name	Chromosomal location	Known function	Refs.
3OST2	Heparan sulfate (glucosamine) 3-*O*-sulfotransferase	6p12	Heparan sulfate biosynthesis signal transduction involved in multiple physiological pathways	[15,16]
DcR1	Decoy receptor 1	p22	Apoptosis	[18,19]
DcR2	Decoy receptor 2	8p22	Apoptosis	[18,19]
RASSF1A	Ras association domain family protein1a	3p21	Growth signals	[17]
P16	Cyclin-dependent kinase inhibitor 2A	9p21	Cell cycle	[20]
APC	Adenomatous polyposis coli gene	5q21	Adhesion	[21]
DAPK	Death-associated protein kinase	9q34	Apoptosis	[22]
SOCS1	Suppressor of cytokine signaling-1	16q13	Suppression of cytokine signaling	[23]
SOCS3	Suppressor of cytokine signaling-3	17q25	Suppression of cytokine signaling	[23]
H-CAD	H-Cadherin	16q24	Adhesion	[25]
E-CAD	E-Cadherin	16q22	Adhesion	[25]

Table 2

Clinico-pathological information and quantitative methylation data for sputum samples

Sputum sample no.	Age	Gender	Diagnosis	Stage	m3-OST-2	mRASSF1A	mP16	mAPC
CWDNA10	71	Male	No lung cancer	NA	0	0	0	0
CWDNA11	68	Female	No lung cancer	NA	0	0	0	0
CWDNA14	80	Male	No lung cancer	NA	0	0	0	0
CWDNA15	54	Female	No lung cancer	NA	0	0	3.99	0.28
CWDNA16	63	Female	No lung cancer	NA	3.21	0.42	0	0
CWDNA17	78	Male	No lung cancer	NA	0	0	0	0
CWDNA20	64	Male	No lung cancer	NA	0	0	0	0
CWDNA22	71	Male	No lung cancer	NA	0	0	7.67	0
CWDNA23	54	Female	No lung cancer	NA	0	0	0	0
CWDNA26	79	Male	No lung cancer	NA	0	0	0	0
CWDNA27	66	Male	No lung cancer	NA	0	1.12	0	0
CWDNA29	52	Male	No lung cancer	NA	0	0	0	0
CWDNA33	54	Male	No lung cancer	NA	0	0	0	0
CWDNA35	63	Female	No lung cancer	NA	0	0	0	0
CWDNA36	80	Male	No lung cancer	NA	0	0	0	0
CWDNA37	69	Male	No lung cancer	NA	0	0	0	0
CWDNA39	54	Male	No lung cancer	NA	0	0	0	0
CWDNA24	45	Female	Breast carcinoma	NA	0	0	0	0
CWDNA2	77	Male	No lung cancer	NA	0	0	0	0
CWDNA6	50	Female	No lung cancer	NA	0.85	0	0	0
CWDNA8	61	Female	No lung cancer	NA	0	0	0	0
CWDNA12	73	Male	Prior lung cancer*	ND	0	0	0	0
CWDNA18	68	Male	Prior lung cancer*	IA	0	0	0	0
CWDNA30	73	Male	Prior lung cancer*	IB	2.98	0	0	0
CWDNA1	58	Male	Prior lung cancer*	IIIA	0	0	0	0
CWDNA3	81	Male	Squamous cell carcinoma	IIB	0	0	512.86	0
CWDNA4	71	Male	Adenocarcinoma	IV	0	159.59	0	5.4
CWDNA5	79	Male	Squamous cell carcinoma	IIIA	33.11	125.6	0	0.32
CWDNA7	55	Male	Adenocarcinoma	IV	100	0	0	0

Sputum sample no.	Age	Gender	Diagnosis	Stage	m3-OST-2	mRASSF1A	mP16	mAPC
CWDNA9	68	Male	Squamous cell carcinoma	IIIA	0	0	0	0
CWDNA13	83	Male	Adenocarcinoma	IV	0	0	0	0
CWDNA19	78	Male	Squamous cell carcinoma	IIIA	0	50.84	29.32	4.86
CWDNA21	52	female	Adenocarcinoma	IA	0	0	0	0
CWDNA25	77	Male	Squamous cell carcinoma	IIIB	0	0	0	0
CWDNA31	39	Male	Adenocarcinoma	IIB	4.62	36.73	14.45	0.91
CWDNA32	68	Male	Squamous cell carcinoma	IA	13.49	0	0	0
CWDNA38	65	Male	Squamous cell carcinoma	ND	0	35.48	0	0
CWDNA40	73	Male	Squamous cell carcinoma	IV	0.42	0	0	0

All the controls were free of lung cancer at the time the sputum was collected. Four contol patients (*) had lung cancer >1 year before the sputum was collected. Control patient CW24 had breast cancer at the time the sputum was collected. NA, not applicable; ND, information not available.

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Table 3

Primers and TaqMan probes for real time MSP assays

Gene symbol	Forward primer	TaqMan probe	Reverse primer
m3OST2	TCGGCGTACGTAAGAGTTTGG	AGCGTTC-GAGTCGTTCGGTTGTTCG	ATCTCCCGATCCTAAACGA-TAAAA
mDcR1	GGAGCGTTTTTTATCGTTAGGGA	CGGTCGTTTGATGGTCGAGG-TAGGGT	CCCGACGCCGTCCTAAAT
mDcR2	GGGATAAAGCGTTTCGATCG	CGAGCGTTCGAGTAGGGCGT-TATTTAGG	TCTAATTCCCGACGCTATCCTAA
mRASSF1A	GCGTTGAAGTCGGGGTTC	ACAAACGCGAACCGAAC-GAAACCA	CCCGTACTTCGCTAACTT-TAAACG
mP16	CGCAACCGCCGAACG	CGCGATCCGCCCCACCCT	TTTTTTCGTTAGTATCGCAG-GAAGA
mAPC	GAACCAAAAACGCTCCCCAT	CCCGTCGAAAACCCGCCC-GATTA	TTATATGTCGGTTACGTGCGTT-TATAT
mDAPK	GGATAGTCGGATCGAGT-TAACGTC	TCGGTAATTCGTAGCGG-TAGGGTTTGG	CCCTCCCAAACGCCGA
mSOCS1	GCGAGAGTTTCGATTGTTTTTTC	AGTTGTTGGAGTAT-TACGTGGCGGCG	CCCCAACATACGACGCG
mSOCS3	TCGCGTTTTTTTTTTCGTAGTTT	TCGGGATGCGGTAGCGGTCG	CGCGACCTCCGCACA
mH-CAD	GGGATGTTATTTTCGCGGG	CGCGGTTAGTAGGGCGGGT-TAGGG	CCTtCCTAACGCTCCCTCGT
mE-CAD	AATTTTAGGTTAGAGGGT-TATCGCGT	CGCCCACCCGACCTCGCAT	TCCCCAAAACGAAACTAACGAC
MYOD1	CCAACTCCAAATCCCCTCTCTAT	TCCCTTCCTATTCCTAAATC-CAACCTAAATACCTCC	TGATTAATTTAGATTGGGTTTA-GAGAAGGA

Table 4

Median quantitative ratios for methylation of genes in NSCLC and adjacent non-malignant tissue

Methylated gene	Median quantitative ratio for NSCLC (*n*=40)	Median quantitative ratio for adjacent non-malignant lung (*n*=40)	Median quantitative ratio for PBMCs from cancer-free individuals (*n*=40)	% Samples +ve for methylation in NSCLC	Cutoff value
3-OST-2	14.19(0–975)	0(0–4.1)	0(0–0)	28/40(70%)	4.1
RASSF1A	0.69(0–883.10)	0(0–4.013)	0(0–0)	18/40(45%)	4.013
P16	2.00(0–753.36)	0(0–2.90)	0(0–0)	18/40(45%)	2.9
DcR1	0.19(0–72.44)	0(0–0.96)	0(0–0)	9/40(23%)	0.96
DcR2	0(0–481.94)	0(0–4.37)	0(0–0)	11/40(28%)	4.37
APC	4.1(0–331.13)	0.29(0–13.95)	0(0–0)	12/40(30%)	13.95
DAPK	0.10(0–4.70)	0(0–0.22)	0(0–0)	17/40(43%)	0.22
SOCS-1	2.72(0–111.39)	0(0–6.3)	0(0–16)	13/40(33%)	6.3
H-CAD	2.92(0–148.94)	0(0–7.59)	0(0–3.6)	11/40(28%)	7.58
SOCS-3	1.88(0–912.04)	0.21(1–186.2)	0(0–11.73)	2/40(5%)	186.2
E-CAD	3.98(0–467.7)	2.56(0–39.8)	0(0–18.84)	5/40(12%)	39.8

Cutoff value=the highest QR value in adjacent non-malignant lung. For SOCS3 and E-CAD cutoff value is too high and thus no meaningful conclusion can be drawn about % samples+ve for methylation in NSCLC.

Table 5

Relation between QR of methylated 3-OST-2 and tumor stage

Stage I	Stage II	Stage III	Stage IV	Stages II–IV
1.36	5.74	7.9	68.39	5.74
18.26	97.72	316.23	114.81	97.72
4	758	15.49		758
3.1	18.62	975		18.62
4.37	12.88	219.7		12.88
1.89	45.1	501.2		45.1
0	37.16	35.48		37.16
1.7	776.28	38.9		776.28
0	93.33	169.8		93.33
7.32	0	5.37		0
1.2	1			1
0.6	0			0
3.21				7.9
36.98				316.23
3.5				15.49
				975
				219.7
				501.2
				35.48
				38.9
				169.8
				5.37
				68.39
				114.81
Mean				
5.8	153.8	228.5		179.7

Mann–Whitney *U*-test $P<0.05$. Stage I vs. stage II $P=0.0315$, stage I vs. stage III $P=0.0002$, stage I vs. stages II–IV $P=0.0003$. For one NSCLC stage was not known.

Table 6

Relation between QR of methylated RASSF1A and tumor stage

Stage I	Stage II	Stage III	Stage IV	Stages II–IV
37.15	0	69.66	0	0
0	22.8	2	0	22.8
16.6	0	92.89		0
1.37	7.94	0		7.94
0	147.57	0		147.57
0	0	416.86		0
67.44	72.11	418.78		72.11
0	222.3	51.62		222.3
65.05	883.1	0		883.1
0	0	208.93		0
0	11.07			11.07
0	222.33			222.33
0				69.66
0				2
0				92.89
				0
				0
				416.86
				418.78
				51.62
				0
				208.93
				0
				0
Mean				
12.5	132	126		119

Mann–Whitney *U*-test $P<0.05$. Stage I vs. stage II $P=0.0588$, stage I vs. stage III $P=0.0319$, stage I vs. stage II–IV $P=0.0349$. For one NSCLC stage was not known.