Methylation profiling in non-small cell lung cancer: Clinical implications

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Abstract. The aim of this study was to identify a panel of methylation markers that distinguish non-small cell lung cancers (NSCLCs) from normal lung tissues. We also studied the relation of the methylation profile to clinicopathological factors in NSCLC. We collected a series of 46 NSCLC samples and their corresponding control tissues and analyzed them to determine gene methylation status using the Illumina GoldenGate Methylation bead array, which screens up to 1505 CpG sites from 803 different genes. We found that 120 CpG sites, corresponding to 88 genes were hypermethylated in tumor samples and only 17 CpG sites (16 genes) were hypomethylated when compared with controls. Clustering analysis of these 104 genes discriminates almost perfectly between tumors and normal samples. Global hypermethylation was significantly associated with a worse prognosis in stage IIIA NSCLC patients (P=0.012). Moreover, hypermethylation of the *CALCA* and *MMP-2* genes were statistically associated to a poor clinical evolution of patients, independently of TNM tumor stage (P=0.06, RR=2.64; P=0.04, RR=2.96, respectively). However, hypermethylation of *RASSF1* turned out to be a protective variable (P=0.02; RR=0.53). In conclusion, our results could be useful for establishing a gene methylation pattern for the detection and prognosis of NSCLC.

Introduction

Lung cancer is one of the most common cancer malignancies worldwide. According to the World Health Organization, it kills over one million people each year and it is the leading cause of cancer death in men and second leading cause in women. Clinically, lung cancer is divided into two subtypes, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is the most aggressive subtype, and accounts for 10-15% of all cases. The remaining 85-90% of cases is classified as NSCLC, which is further subdivided into four histological categories; adenocarcinoma (AD), squamous cell carcinoma (SQ), large cell carcinoma (LC) and 'others' (1). Lung cancer is unique among human solid cancers in that a single environmental factor, tobacco smoke, is believed to promote sequential changes in target cells that lead to carcinogenesis. Recently, progress has been made in elucidating changes involved in the transformation of a normal bronchial epithelium to frank malignancy (2). However, there is not a routine screening method that enables early detection, which is a key factor for decreasing the mortality rate of this cancer. Imaging and cytology analyses have been employed for early detection, but none have been demonstrated to reduce mortality. Thus, the quest for specific molecular markers has become so important in lung cancer.

DNA methylation has emerged as a highly promising biomarker and is being widely studied in every type of cancer. CpG (cytosine-guanine) islands are short regions of 0.5-4 kb rich in CpG content found in almost half of all genes and are frequently promoter-associated (1,2). The addition of a methyl group at the 5-position of the cytosine in a CpG dinucleotide is a normal process within cells in the mammalian genome and hypermethylation of CpG islands in promoter regions is a frequent mechanism for gene inactivation. Changes in the pattern of methylation in these regions have been widely described in cancer (3). Although global hypomethylation has been shown in tumors (4), hypermethylation of specific genes is the most frequent and well characterized epigenetic change (5). Therefore, the use of methylation markers to classify different types of cancer and predict patient survival is an interesting field of research.

Disruption of gene expression caused by methylation plays an important role in pathogenesis of NSCLC and it may be relevant as a detection or outcome prediction tool. Several studies have identified aberrant methylation events in different genes in NSCLC (6,7). Our group considers informative the

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study of the methylation status in a large number of genes, in order to establish a panel of relevant genes which are hyper or hypomethylated in a set of NSCLC patients.

Materials and methods

Patients and tissue samples. Forty-six primary NSCLCs and their corresponding control tissue samples were obtained from patients who underwent surgery between 2000 and 2005, at San Carlos Hospital in Madrid, Spain. Informed consent was obtained from patients prior to investigation and the project was approved by the Ethics Committee of San Carlos Hospital. Samples were snap-frozen in liquid nitrogen at the time of surgery and stored at -80˚C until use. No patient had received chemotherapy or radiation therapy before diagnosis. Cryostat-sectioned, H&E-stained samples from each tumor block were examined microscopically, to confirm the presence of ≥80% tumor cells by two independent pathologists. Paired normal tissues, used as controls, were obtained away from the margin of the tumor and microscopically confirmed. Forty-three patients were male and three female and the average age was 64.97 years (range 40-82). Tumors were staged pathologically using the tumor node metastasis (TNM) system (8) and consisted of 20 stage I, 4 stage II and 22 stage IIIA. According to the World Health Organization criteria, 28 tumors were squamous cell carcinomas (SCC) and 18 were adenocarcinomas (AC). The histological classification of tumors was established according to previous criteria (9). Thus, 11 tumors were well differentiated, 24 moderately, and 11 poorly differentiated. The follow-up period for patients was 5 years. The overall median survival of the series was 22.02 months (range 1-60).

DNA methylation profiling. Genomic DNA from fresh frozen tissue was isolated as described by Blin and Stafford (10). DNA methylation profiling using Illumina GoldenGate Methylation bead arrays (Illumina, San Diego, CA) was carried out at the Human Genotyping Unit-CEGEN from the Spanish National Cancer Research Centre (CNIO), Madrid, according to manufacturer's protocol. This array interrogated 1505 CpG loci associated with 807 cancer-related genes. GoldenGate array methylation data are publicly available on the Gene Expression Omnibus archive, accession GSE22290.

Statistical analysis. Illumina BeadStudio Methylation Software was used for data analysis. The methylation level at each CpG site (β-value) was defined as the ratio of the fluorescent signal for methylated (Cy5) allele to the sum of methylated and unmethylated (Cy3) alleles [β=(max(Cy5, 0))/(|Cy3|+|Cy5|+100)]. β-value is derived from the 30 replicate methylation measurements and ranged from 0 (completely unmethylated) to 1 (completely methylated). At each locus for each sample the detection P-value was used to determine sample performance. To ensure adequate sample quality, only samples having >75% loci with a detection P<0.05 were included for analysis. GoldenGate assay has nine controls which help to monitor each step of the process. Background normalization was carried out for the methylation analysis. The background value is derived by averaging the signals of built-in negative control bead types. Outliers are removed using the median absolute deviation method. The average signal of the negative controls is subtracted from the probe signals as done by Bibikova *et al* (11). The expected signal for unexpressed targets is equal to zero.

Gene data set was applied to complete-linkage hierarchical clustering analysis using the Spearman Rank correlation method in Cluster v3.0 and the resulting expression map was visualized with JavaTreeview v1.1.5r2. Unsupervised clustering was used to characterize methylation patterns, as performed in previous studies (12,13).

Clinical correlations were established using software SPSS (PASW Statistics 17). HRs for risk of death were estimated from a multivariate Cox proportional hazards model, with adjustments for age, sex, smoking status, tumor grade, tumor stage, and histology. Overall survival in relation to methylation status was evaluated by Kaplan-Meier survival curves and log-rank tests. A p-value <0.05 denoted significance.

Validation of microarray by methylation-specific PCR. Methylation status of specific genes was examined by methylation-specific PCR (MSP) (14) after bisulfite treatment. Genomic DNA from samples was subjected to bisulfite modification using the Epitect Bisulfite kit from Qiagen according to manufacturer's instructions. The bisulfite-converted genomic DNA was amplified by PCR using two sets of specific MSP-primers recognizing methylated or unmethylated DNA respectively. PCR conditions and primers for *RASSF1* were described by Wang *et al* (15) and for *p16* by Gonzalez-Quevedo *et al* (16).

Results

Methylation cluster analysis. DNA from 46 NSCLCs and its 46 control samples were subjected to a study using Illumina GoldenGate bead array to characterize the epigenetic profile. This panel interrogates 1505 CpG loci selected from 807 cancer-related genes where 28.6% contain one CpG site per gene, 57.3% contain two CpG sites, and 14.1% have three or more sites. 1044 CpG sites are located within CpG islands, and 461 are located outside the CpG islands. After analyzing the array, one non-tumor sample was taken out from the study, because its signal was too low to obtain reliable results.

Array methylation data were analyzed with unsupervised hierarchical clustering using Spearman Rank correlation for the 1505 CpG sites. As shown in Fig. 1, this cluster analysis helps to distinguish clearly NSCLC samples from non-malignant tissues. Just four paired matched samples (numbers 61, 100, 132 and 133) are not properly classified within its clusters.

In order to determine if a sample shows hypermethylation at one locus, methylation level (β average value) in tumor sample was compared with methylation level at its corresponding non-tumor control. When methylation level at tumor sample was at least 50% higher than methylation level at the corresponding non-tumor control, this sample was classified as 'hypermethylated' at that locus. When β-value at tumor sample was at least 50% lower than β-value at non-tumor control, this sample was classified as 'hypomethylated'. Following these criteria, we found that 120 CpG sites, corresponding to 88 genes, were hypermethylated in tumor samples and only 17 CpG sites (16 genes) were hypomethylated when compared with its control (Table I). Remarkably, not all of the CpG sites analyzed with the microarray are located in a CpG island. Only those CpG sites located at a CpG island are relevant for the expression of

Figure 1. Unsupervised hierarchical clustering of average β-values in tumor and non-tumor samples, based on Manhattan distance. In the heatmap each column represents a sample (45 tumors, blue, and 45 non-tumors, purple) and each row represents a CpG locus (1505 in 807 genes). Average β close to zero, green, means unmethylated and close to one means methylated, red.

the corresponding gene. In Table I those CpG sites that are not located at CpG islands are shown in bold. Using the KEGG database (www.genome.jp/kegg/), we identified that most of these genes are involved in proliferation, differentiation or apoptosis pathways. There are genes from the Wnt pathway (*FRZB*, *WNT2* and *SFRP1*) and transcription factors (*EYA4*, *HOXA5*, *HOXA9*, *HOXA11*, *HOXB2*, *HOXB13*, *IRF5*, *IRF7*, *ISL-1* and *MYOD1*, for example). Adhesion and extracellular matrix molecules also showed an altered methylation pattern (*CDH13, DCC, OPCML COL18A1, MMP-2, SPARC, TIMP-2*, and *SPP1*). Intriguingly, eight genes, *ASCL2, HTR1B, AGTR1, NTSR1, GABRB3, SLIT2, P2RX7* and *GALR1*, are involved in signaling pathways related to neuroactive ligand-receptor interaction or axon guidance.

Validation of the microarray by methylation specific PCR (MSP). We validated two CpG sites that showed elevated methylation in NSCLC samples according to Golden Gate microarray using MSP (14). MSP is the most widely used technique to check the methylation status of individual genes. Specific primers to methylated or unmethylated *p16* and *RASSF1* were used to amplify DNA samples in separate reactions. We selected randomly 24 patients and checked the methylation status in both tumor and

Table I. Genes with altered methylation profile.[a]

Hypermethylated genes in NSCLCs						Hypomethylated genes in NSCLCs	
Gene symbol	GeneBank reference	Gene symbol	GeneBank reference	Gene symbol	GeneBank reference	Gene symbol	GeneBank reference
ADCYAP1	NM_001117	***HLA-DOA***	NM_002119	*NPY*	NM_000905	***CASP8***	NM_001228
AGTR1	NM_000685	*HOXA5*	NM_019102	*NTRK3*	NM_002530	*CDH3*	NM_001793
ASCL2	NM_005170	*HOXA9*	NM_002142	*NTSR1*	NM_002531	***IL6***	NM_000600
ATP10A	NM_024490	*HOXA11*	NM_005523	*ONECUT2*	NM_004852	*MEST*	NM_002402
CALCA	NM_001033952	*HOXB2*	NM_002145	***OPCML***	NM_002545	*MLLT4*	NM_005936
CDH13	NM_001257	*HOXB13*	NM_006361	***p16***	NM_058195	*MUC1*	NM_002456
CFTR	NM_000492	*HS3ST2*	NM_006043	*P2RX7*	NM_177427	*NEO1*	NM_002499
CHFR	NM_018223	*HTR1B*	NM_000863	*PENK*	NM_006211	*NRAS*	NM_002524
CHGA	NM_001275	*IGF1R*	NM_000875	*PITX2*	NM_000325	***PTPN6***	NM_080548
COL18A1	NM_130444	*IGF2*	NM_000612	*PODXL*	NM_001018111	*RRAS*	NM_006270
DAB2	NM_001343	*IGF2AS*	NM_016412	***PTPRO***	NM_002848	*SFN*	NM_006142
DAB2IP	NM_032552	*IGFBP7*	NM_001553	***RARA***	NM_000964	***SPP1***	NM_000582
DBC1	NM_014618	*IGSF4*	NM_014333	*RASSF1*	NM_170712	***TNF***	NM_000594
DCC	NM_005215	*IHH*	NM_002181	*SCGB3A1*	NM_052863	***TNFSF10***	NM_003810
DLK1	NM_003836	*IL17RB*	NM_018725	*SEPT5*	NM_001009939	***VAMP8***	NM_003761
EPHA5	NM_182472	***IL18BP***	NM_005699	*SFRP1*	NM_003012	*ZMYND10*	NM_015896
EPO	NM_000799	*IRF5*	NM_032643	*SLC22A3*	NM_021977		
EYA4	NM_004100	*IRF7*	NM_004029	*SLC5A8*	NM_145913		
FES	NM_002005	*ISL1*	NM_002202	*SLIT2*	NM_004787		
FGF3	NM_005247	*JAK3*	NM_000215	*SOX1*	NM_005986		
FLT3	NM_004119	*MAF*	NM_005360	*SOX17*	NM_022454		
FLT4	NM_002020	***MFAP4***	NM_002404	*SPARC*	NM_003118		
FRZB	NM_001463	*MMP2*	NM_004530	*ST6GAL1*	NM_173216		
GABRB3	NM_021912	*MOS*	NM_005372	***STAT5A***	NM_003152		
GALR1	NM_001480	*MT1A*	NM_005946	*TAL1*	NM_003189		
GAS7	NM_003644	*MYCL2*	NC_000023	*TERT*	NM_198255		
GDF10	NM_004962	*MYOD1*	NM_002478	*TIMP2*	XM_941104		
GUCY2D	NM_000180	*NEFL*	NM_006158	*TPEF*	NM_016192		
HCK	NM_002110	*NPR2*	NM_003995	*WNT2*	NM_003391		
				WT1	NM_024424		

[a]Genes were considered as hypermethylated or hypomethylated after comparing methylation level in tumors to methylation level at the corresponding non-tumor controls.

normal samples. Data obtained from Golden Gate array for *p16* and *RASSF1* were confirmed in 85.7% and 95.8% of the patients (both tumor and normal samples) respectively.

Clinical correlations. Global hypermethylation (more than 20% of the CpG sites hypermethylated in one tumor sample) did not show statistical correlations with clinical and pathological parameters, such as TNM stage (P=0.778), size of tumor (P=0.479), lymph node dissemination (P=0.908) or histology (P=0.541). We then search for clinical and pathologic correlations with hypermethylation gene by gene. Statistical analyses of the hypermethylation of single genes did not show significant associations with TNM stage. However, differences in the frequencies of hypermethylation were seen between NSCLC histological subtypes. The frequencies of hypermethylation in twelve genes (*CALCA*, P=0.08, *HOXB2*, P=0.033, *IRF7*, P=0.013, *MOS*, P=0.039, *MT1A*, P=0.004, *MYCL2*, P=0.001, *p16*, P=0.023, *RARA*, P=0.027, *RASSF1*, P=0.048, *SEPT5*, P=0.033, *SOX17*, P=0.039, and *SPARC*, P=0.048) were significantly higher in squamous cell carcinomas (SCC) compared to adenocarcinomas. Some other genes, such as *HOXA5*, *PTPRO*, *SLC5A8*, and *ST6GAL1* showed a trend to be more methylated

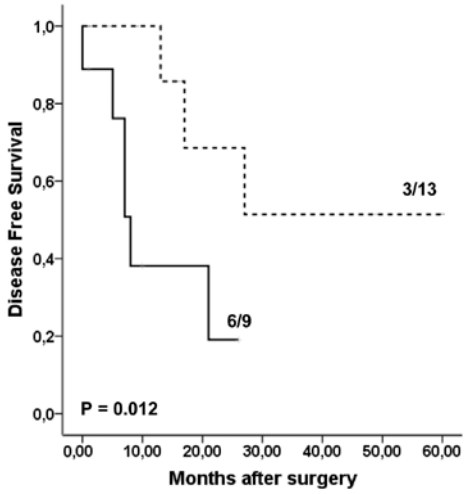

Figure 2. Kaplan-Meier survival curves for TNM stage IIIA NSCLCs showing global hypermethylation. Solid line represents patients with CpG hypermethylation and broken line represent patients without hypermethylation.

Table II. Cox multivariate analysis for TNM stage and *CALCA*, *MMP-2* and *RASSF1* methylation status in non-small cell lung tumors.

Variable	RR	95% Confidence Interval	P-value
TNM stage	1.822	1.040-3.192	0.036
CALCA hypermethylation	2.643	0.953-7.333	0.062
MMP-2 hypermethylation	2.956	1.067-8.190	0.037
RASSF1 hypermethylation	0.535	0.312-0.916	0.023

RR, relative risk.

in SCC compared to adenocarcinomas, but they did not reach statistic significance. Remarkably, no gene showed a trend to be more methylated in adenocarcinomas than in SCC. When we studied the frequencies of hypermethylation and compared them to differentiation grade, we found that well-differentiated tumors showed hypermethylation in *IRF5* (P=0.039) and *NPR2* (P=0.020).

Looking for prognostic factors, we found a tendency to a worse prognosis in those patients whose tumors showed global hypermethylation (P=0.065); 19 patients showed hypermethylation in at least 20% of the 1505 CpG sites studied. This trend toward a worse prognosis was statistically significant in TNM stage IIIA NSCLCs (P=0.012, Fig. 2). Hypermethylation of genes *CALCA* and *MMP-2* were statistically associated to a worse clinical evolution of patients (Fig. 3A and B, P=0.047 and P=0.020, respectively). However, hypermethylation of *RASSF1* turned out to be a protective variable in relation to patient prognosis (P=0.010, Fig. 3C). These results were independent of TNM tumor stage, as demonstrated by a Cox multivariate analysis (P=0.06, RR=2.64; P=0.04, RR=2.96; P=0.02; RR=0.53, respectively, shown in Table II).

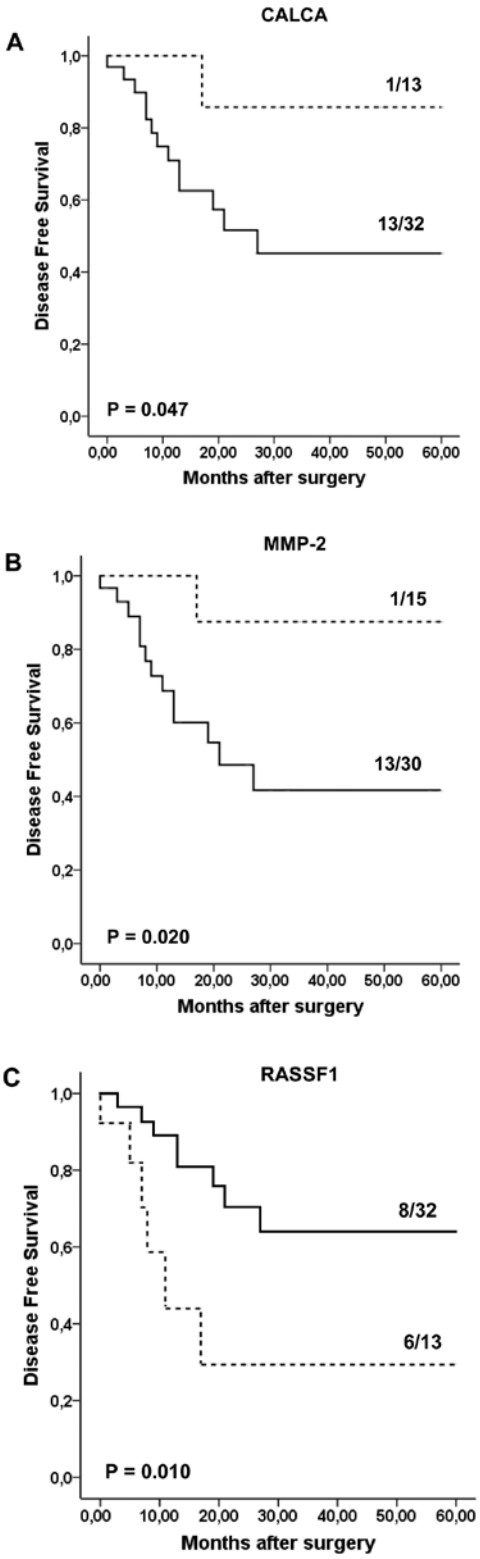

Figure 3. Kaplan-Meier survival curves for *CALCA* (A), *MMP-2* (B) and *RASSF1* (C) hypermethylation. Solid lines represent patients whose tumors were hypermethylated and broken lines represent patients without hypermethylation.

We then looked for clinical and pathologic correlations with gene hypomethylation. Global hypomethylation (more than 20% of the CpG sites hypomethylated) was not statistically associated to a different prognosis in NSCLCs (P=0.155). However,

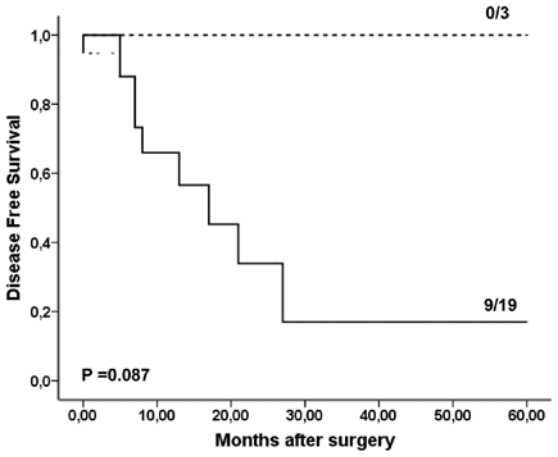

Figure 4. Kaplan-Meier survival curves for hypomethylation. Broken line represents patients with CpG hypomethylation and solid line represents patients without hypomethylation.

there was a clear trend toward a better prognosis in those patients considered as 'hypomethylated' when compared with non-hypomethylated in TNM stage IIIA NSCLCs (P=0.087, Fig. 4). Statistical analyses of the hypomethylation did not show significant associations with TNM stage or NSCLC histological subtypes. When we studied the frequencies of hypomethylation and compared them to differentiation grade, we found that undifferentiated tumors showed hypomethylation in *MLLT4*, *MUC1* and *SFN* (P=0.025, 0.035 and 0.031 respectively).

Discussion

The study of the epigenome and its relation to cancer progress is a field of growing interest. In this study, a series of 46 matched NSCLC case-control pairs we assessed the DNA methylation profile for 1505 CpG sites located at 807 cancer-related genes using Illumina GoldenGate bead array. We identified significant differences at 137 CpG sites in 104 independent genes. Of those 137 CpG sites, 120 (88 genes) were hypermethylated in tumor samples versus its corresponding controls and 17 (16 genes), were hypomethylated. In order to validate results obtained from Golden Gate array, we carried-out MSP in a set of patients for *RASSF1* and *p16*. Results were highly concordant with the methylation status determined by the microarray analysis. The overall validity of Illumina Golden-Gate method was already tested by Bibikova *et al* (11).

It is remarkable that 23 out of these 88 hypermethylated genes have been previously described as hypermethylated in lung cancer: *CALCA* (7), *CDH13* (17), *CHFR* (18), *DAB2IP* (19), *DBC1* (20), *GDF10* (21), *HOXA5* (22,23), *HOXA9* (24), *HOXB13* (25), *HTR1B* (26), *IGFBP7* (7), *IGSF4* (27), *IRF7* (28), *MMP-2* (29), *ONECUT2* (25), *OPCML* (30), *p16* (31,32), *PITX2* (33), *RASSF1* (34,35), *SCGB3A1* (36), *SFRP1* (37), *SLIT2* (38) and *SPARC* (31).

It is noteworthy that *RASSF1* is hypermethylated in our samples and this gene has been considered as a tumor suppressor gene (39). Its hypermethylation may cause an inactivation of the gene and it may impair its tumor suppressor gene function. Also remarkable is that our group had previously reported *p16* hypermethylation in a set of 98 NSCLCs. Thus, we found that 36.7% of samples without p16 expression showed *p16* promoter hypermethylation, this fact correlating to adverse patient prognosis (16). Furthermore, it is significant that we found hypermethylated *HOXA5*, *9* and *11* genes, all of them placed in a cluster at the chromosome 7p15.2. Epigenetic inactivation of this *HOXA* gene cluster has been described in breast and lung cancer (24,40). Likewise, *ASCL2, IGF2, IGF2AS* and genes are colocalized in chromosome 11p15.5.

Only one out of the sixteen hypomethylated genes has been previously found to suffer hypomethylation in lung cancer: *SFN* (41). *SFN*, also called 14-3-3 *sigma*, has been shown to be upregulated in lung cancer and it is thought to be involved in lung cancer tumorigenesis. Another remarkable result is the hypomethylation of *ZMYND10*, which has been shown to suffer frequent inactivation by promoter hypermethylation in lung cancers and is considered by some authors as a candidate tumor suppressor gene (42). Further, individualized studies on this gene are needed to clarify its role in cancer.

Unsupervised clustering of the DNA methylation profile of these 104 genes achieves excellent segregation of tumor from non-tumor samples. Thus, this panel may be useful as a screening method in diagnosis. It is important to stress that the use of DNA methylation profiling it is an advantage when comparing with the use of RNA gene expression profile, due to the different stability of RNA and DNA samples. Further studies will be necessary for defining in a more precise way a good panel of genes that could be useful for diagnosis and prognosis.

In the current study we found that methylation of a number of genes was significantly associated with histological type; squamous cell carcinomas was associated with hypermethylation of *CALCA*, *HOXB2*, *IRF7*, *MOS*, *MT1A*, *MYCL2*, *p16*, *RARA*, *RASSF1*, *SEPT5*, *SOX17* and *SPARC*. We did not find any gene more methylated in adenocarcinomas than in squamous cell carcinomas. However, Hawes *et al* (43) have recently found that adenocarcinoma was associated with increased methylation of *APC*, *CCND2*, *KCNH5*, and *RUNX*. Our panel of genes did not include *KCNH5* but the other three genes were included and we could not find any association between its hyper or hypomethylation and any clinical data.

Next, we studied prognostic correlations. First of all there was a clear tendency to a worse prognosis in those patients that showed global hypermethylation and this fact was statistically significant in stage IIIA NSCLCs. Thus, patients with TNM IIIA and hypermethylated tumors showed a clearly worse clinical evolution than those without this molecular event. Therefore, hypermethylation panel in TNM IIIA NSCLCs emerge as a useful molecular tool to predict patient prognosis. Furthermore, the event that we may consider as the 'opposite' of hypermethylation, global hypomethylation, showed clear trend towards a better prognosis in stage IIIA NSCLCs. Therefore, it seems that there is a kind of balance between hyper and hypomethylation that may have prognostic implications. Considering gene by gene methylation, *CALCA* and *MMP-2* were statistically associated to a worse clinical evolution of patients. However, hypermethylation of *RASSF1* turned out to be a protective variable in relation to patient prognosis.

CALCA (calcitonin-related polypeptide α) is an important regulator of bone calcium metabolism, and it has been found hypermethylated in oligodendrogliomas (44), lymphoblastic

leukemia (45), and cervical neoplasia (46). Its hypermethylation has also been found in NSCLC (7).

MMP-2 is a metalloproteinase that has been shown to be valuable as a prognostic factor in NSCLC (47). MMP-2 is considered as a molecular marker that predicts tumor recurrence and unfavorable outcome in NSCLC (48). Hypermethylation of its promoter has only been described in MCF-7 cells (49). In our set of patients we have found a correlation between *MMP-2* hypermethylation and a good prognosis. This strengthens its role as a negative prognostic factor.

RASSF1 is a gene located at 3p21, a region frequently deleted in human cancer (loss of heterozigosity) that encodes a protein that can bind with NORE1, the novel RAS effector. It is known that *RASSF1* blocks cell cycle progression through a suppression of the c-Jun-NH2-kinase pathway (50) and inhibits cyclin D1 accumulation (51). Altered expression of this gene has been associated with the pathogenesis of a variety of cancers, suggesting a tumor suppressor function of this gene. It is often disrupted by hypermethylation in lung cancer (52). Previously, Tomizawa *et al* (53) found that epigenetic inactivation of *RASSF1* plays an important role in the progression of lung adenocarcinoma, and that *RASSF1* hypermethylation correlated with adverse survival in a set of 110 patients with stage I lung adenocarcinoma. Endoh *et al* (54) showed that *RASSF1* methylation was associated with earlier recurrence. However, Choi *et al* (55) did not consider this gene as a good prognostic marker in a study with 116 cases of NSCLC, although they found that stage I NSCLC patients with *RASSF1* inactivation have a more adverse overall survival rate than stage I NSCLC patients without *RASSF1* inactivation. We agree with Buckingham *et al* (35), that epigenetic *RASSF1* inactivation may be a good prognostic factor. Our results are supported by the fact that *RASSF1* inactivation is related to cell division defects and induces premature *APC* activation, thereby resulting in acceleration of mitotic cyclin degradation and mitotic progression as well as induction of mitotic abnormalities (56).

In conclusion, in this study we have established a panel of 104 genes whose methylation status is altered in NSCLCs when compared to its corresponding non-tumor controls. These genes discriminate almost perfectly tumor samples from non-tumor samples. Moreover, global hypermethylation confers a poor prognosis to NSCLC patients, especially in patients affected by TNM stage IIIA tumors. We have found three genes whose hypermethylation may be a useful tool as a prognostic factor: *CALCA* and *MMP-2* hypermethylation confers poor clinical evolution and *RASSF1* hypermethylation confers a good prognosis to NSCLC patients.

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