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European Journal of Cancer 41 (2005) 1223–1236

European Journal of Cancer

www.ejconline.com

CpG island methylation and expression of tumour-associated genes in lung carcinoma

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Received 8 January 2005; accepted 3 February 2005
Available online 27 April 2005

Abstract

In this study, microarray analysis was used to identify tumour-related genes that were down regulated in lung carcinoma. The promoter sequences of the identified genes were analysed for methylation patterns. In lung cancer cell lines, CpG island methylation was frequently detected for *TIMP4* (64%), *SOX18* (73%), *EGF-like domain 7* (56%), *CD105* (71%), *SEMA2* (55%), *RASSF1A* (71%), *p16* (56%) *SLIT2* (100%) and *TIMP3* (29%). Methylation was however rarely observed in cell lines for *SLIT3* (18%) and *DLC1* (18%). In primary lung tumours, methylation of *TIMP4* (94%), *SOX18* (100%), *EGF-like domain 7* (100%), *CD105* (69%), *SEMA2* (93%), *DLC1* (61%), *RASSF1A* (44%), *p16* (47%), *SLIT2* (100%) and *TIMP3* (13%) was also detected. Methylation of several CpG islands was frequently found in normal lung tissue of cancer patients and this may have been attributed to epigenetic field defect and/or infiltrating tumour cells. Interestingly, inactivation of *RASSF1A* and *p16* correlated well with an extended smoking habit ($P = 0.02$), and exposure to asbestos ($P = 0.017$) or squamous cell carcinoma ($P = 0.011$), respectively. These results have identified genes whose aberrant promoter methylation could play a crucial role in the malignancy of lung carcinoma.

Keywords: Lung cancer; Methylation; Tumour-suppressor gene; Epigenetics

1. Introduction

Lung cancer is one of the leading causes of death in the United States and central Europe [1]. In the United States, the death rates from male lung cancers were decreasing in the 1990s reflecting reductions in tobacco use. Sadly however, an increase in female lung cancers has recently been reported in countries from Eastern Europe.

Loss of heterozygosity (LOH) on chromosome arms 3p, 4p, 5q, 8p 9p, 13q and 17p are among the most frequent alterations in lung cancer [2–4]. A limited number

Abbreviations: RASSF1, Ras association domain family 1 gene; TIMP, tissue inhibitor of metalloproteinase; SOX18, sex determining region Y box 18; DLC1, deleted in liver cancer 1; CD105, CD105 antigen; SEMA2, semaphorin 2; EGFL7, epidermal growth factor-like domain 7; SLIT, slit (Drosophila) homologue; LOH, loss of heterozygosity; 5-Aza-CdR, 5-aza-2′-deoxycytidine; MSP, methylation-specific PCR; COBRA, combined bisulfite restriction analysis; TNM, Tumour-Node-Metastasis; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

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doi:10.1016/j.ejca.2005.02.020

of tumour-suppressor genes have been identified as potential targets for chromosomal deletion events in pulmonary tumours and includes *RB* (13q), *p53* (17p), *p16* CDK inhibitor (9p) and *RASSF1A* genes (3p21) [5,6]. Unlike LOH, it is becoming clear that transcriptional inactivation by hypermethylation of CpG islands containing gene promoter regions, is one of the main mechanisms of tumour-suppressor gene inactivation in cancers [7]. Several studies have demonstrated that the CpG islands in *RB*, *p16*, *VHL*, *RASSF1A*, *CDH1*, *GSTP1*, *FHIT*, *MLH1*, and *BRCA1* genes are frequently methylated in a variety of human cancers and are usually methylation-free in the corresponding normal tissue. CpG island promoters that are hypermethylated in lung cancer includes *p16*, *MGMT*, *SLIT2*, *TIMP3* and *RASSF1A* [6,8–11]. It is speculated that inactivation of tumour-suppressor genes due to hypermethylation may play an important role in carcinogenesis. In lung cancer, it appears that DNA methylation analysis could also prove to be a powerful tool for diagnosis and prognosis. For instance, hypermethylation of the *RASSF1A* gene has been correlated with impaired survival and earlier recurrence of lung cancer in patients [12–14]. In addition, hypermethylation of *RASSF1A*, *MGMT* and *p16* was detected from cells in the sputum of smokers and cancer patients, indicating that promoter methylation analysis may serve as a useful molecular marker for early detection of lung cancer [15,16].

In order to investigate novel cancer-associated genes, DNA microarray analysis was used to identify genes that were down regulated in lung cancer patients. Microarray analysis was used to detect groups of genes that were of broad and specific interest. The epigenetic inactivation of several previously identified tumour-related genes (*TIMP4*, *SOX18*, *EGF-like domain 7*, *CD105*, *SEMA2* and *SLIT3*) were also analysed as well as the methylation status of other well known cancer-associated genes including *RASSF1A*, *p16*, *TIMP3*, *DLC1* and *SLIT2*.

2. Materials and methods

2.1. Patients and samples

Tumour and normal lung tissue samples were obtained from 89 consecutive patients with non-small cell lung cancer (NSCLC), who had undergone pulmonary resection surgery between 1999 and 2001 (Table 1). Only patients with clear histological classification as NSCLC (adenocarcinoma, squamous cell carcinoma) and without neo-adjuvant chemo- or radiotherapy were admitted to the study. All patients gave a written consent and the local ethical committee of the Medical Faculty approved the study. Immediately following resection, tumour tissues and matched normal lung tissues were snap-frozen and stored in liquid nitrogen. Tumour histology and stages were classified according to the WHO-classification and the TNM staging system of the UICC, respectively.

Table 1
Demographic data of lung cancer patients

	n	%
No. of patients	89	
Mean age (year)	65.5	
Male/female ratio	71/18	
Smoking/non-smoking ratio	60/29	
Asbestos/non-asbestos ratio	16/89	
Surgical procedure		
PE/diagnostic thoracotomy	6	6.8
Segementresection	5	5.5
Lobectomy	64	72.0
Bilobectomy	2	2.2
Pneumonectomy	12	13.5
Histology		
Squamous cell carcinoma	49	55.0
Adenocarcinoma	40	45.0
TNM staging		
I	31	34.8
II	23	25.8
III	29	32.6
IV	6	6.8
Grading		
Well/moderately well differentiated	25	28.0
Poorly differentiated/undifferentiated	64	72.0
Residual tumour situation		
R0	75	84.2
R1	6	6.8
R2	8	9.0

2.2. RNA preparation

Total RNA was prepared from cryo-preserved normal lung or cancer sample tissue by acid phenol/chloroform extraction (TRIzol; Invitrogen, Karlsruhe, Germany) followed by purification with RNeasy Mini Kit (Qiagen; Hilden, Germany) according to manufacturer's instructions. RNA was quantified at 260 nm by a spectrophotometer and quality was assessed by visualization of 18S and 28S RNA bands after electrophoresis through agarose gels.

2.3. Microarray expression analysis

A total of 10 μg RNA from each sample was used to prepare biotinylated target cRNA as previously described [17–19]. A detailed protocol is available at www.affymetrix.com. Samples were hybridized to a custom expression monitoring DNA microarray (EOS-K) designed by *Eos Biotechnology*, *Inc.* using Affymetrix GeneChip technology [20] that contained nearly all expressed human genes in the public domain at the time of design (June 2001). Sequences included on the array were derived from human genomic, expressed mRNA and EST databases in

GenBank [21]. Consensus sequences representing human expressed sequences were generated using the Clustering and Alignment Tool software (DoubleTwist, Oakland, CA), and prediction of the expressed genome from the human genome sequence was done using *ab initio* exon prediction [22]. The 59000 probe sets on this microarray represent approximately 45000 mRNA and EST clusters and 6200 predicted exons. Data was used after gamma-distribution normalization.

2.4. Gene chip analysis

For gene expression analysis in native tumour samples and normal tissues, total RNA was extracted using Trizol (Gibco, Karlsruhe, Germany) and biotinylated cRNA was prepared by *in vitro* transcription after synthesis of double-stranded cDNA using standard protocols. After cRNA-fragmentation and hybridization with microarrays (EOS-K), signals were detected with streptavidin–phycorythrin. Signal enhancement was performed using biotinylated goat-anti-streptavidin antibodies. Arrays were washed and stained with the GeneChip Fluidics Station 400 and scanned with a GeneArray Scanner. Primary image analysis was performed with *Microarray Suite 5.0* and images were scaled to an average hybridization intensity of 200. In total 56 squamous cell carcinomas, 43 adenocarcinomas and 15 normal lung samples were analysed (Table 1). All expression values below 60 were set to 60. Calculation of fold-change was performed by dividing the mean expression level of a gene in tumour samples by the mean expression level of the same gene in the normal lung samples. To identify specific genes that were differentially expressed in normal lung tissue as compared to tumours, a criterion that marked differential gene expression at an approximate significance level (determined by Bonferoni method) of 8.0×10^{-7} using Student's *t*-test for down-regulated genes was used.

2.5. Cell lines and DNA preparation

Fifteen human lung cancer cell lines were obtained from American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen und Zellkulturon (DSMZ) and cultured in the recommended growth medium. These cell lines included small cell lung cancers (SCLC) (H64, H69, H82, H146, H446, H1688 and DMS53) and NSCLCs (H322, H358, A549, A427, DV90, HCC366, HCC15, SK-MES1). Normal human bronchial epithelial cells were obtained from Clonetics (Brussels, Belgium) and grown in BEGM. Genomic DNA was extracted from cultured cells and frozen tissues by standard phenol/chloroform procedures. *In vitro* methylated DNA was obtained by modification of genomic HeLa DNA with the CpG methylase *Sss*I (New England Biolabs; Beverly, MA) according to conditions specified by the enzyme manufacturer.

2.6. Re-expression and real time RT-PCR

The A427 cancer cell line was treated with 5-aza-2′-deoxycytidine (5-Aza-CdR; Sigma). 2×10^6 cells were grown for 4 days in the presence of different concentrations of 5-Aza-CdR (0, 5 and 10 μM). RNA was isolated using of Trizol-Reagent (Gibco BRL, Life Technologies). Control RNA of normal tissue was obtained from BD Bioscience Clontech (RNA Master Panels). Real time RT-PCR conditions and primers for *RASSF1A* was used as previously described [23,24]. RT-PCR for *TIMP4* was performed with the primers U460TMP4 (5′-TCG AGC CCT GGG AGG ACC TGT C) and L707TMP4 (5′-CTA GGG CTG AAC GAT GTC AAC AAA CTC C) at 65 °C with expected product size of 275 bp. The real time PCR was done in a Rotor-Gene 2000 (Corbett Research, Sydney, Australia) and amplification of the *GAPDH* transcript was carried out to verify the integrity of the RNA [24].

2.7. Bisulfite modification of DNA and methylation analysis

To investigate the methylation status of gene promoters, the corresponding CpG island was determined by CpGplot (http://www.ebi.ac.uk/emboss/cpgplot/) with following parameters: window 100, step 1, obs/exp 0.6, minPC 50 and length 100. Bisulfite specific primers were designed for the CpG island region by MethPrimer (http://www.urogene.org/methprimer/). Genomic DNA was isolated from lung tissue and cell lines and treated for bisulfite modification [6]. As a control, unmethylated bisulfite treated DNA isolated from human fibroblast was included. The bisulfite reaction and methylation analysis was repeated for all samples to confirm methylation status. Primer sequences and PCR conditions for combined bisulfite restriction analysis (COBRA) are listed in Table 2. The methylation status of *SOX18*, *CDC105*, *SEMA2*, *SLIT3*, *TIMP4*, *EGF-like domain 7*, *DLC1 and RASSF1A* was analysed using COBRA. Briefly, 100ng of bisulfite-treated DNA was amplified in 25 μl reaction buffer containing 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 10 pmol of each primer (Table 2) and Taq polymerase (InViTek GmBH, Berlin, Germany) at 95 °C for 30 s, Tm for 30 s, and 72 °C for 30 s for 20 cycles or 40 cycles if semi-nested PCR was not preformed. For several genes a semi-nested PCR was performed using internal primers with similar conditions as described for the preceding PCR amplification with 30 cycles (Table 2). For the restriction enzyme analysis of PCR products from bisulfite-treated DNA, 20–50 ng of the PCR products were digested with 10 units of *Taq* I and *Hpych4* IV (New England Biolabs; Beverly, MA) according to

Table 2
Primer sequences for combined bisulfite restriction analysis

Primer	Primer sequence	T_m (°C)	PCR products (bp)	Enzyme (products in bp)
TIMP4U1	5′-GGTTTTGGGAAGGAGTTTTGTTGG	58	314	TaqI (222, 38)
TIMP4L1	5′-AAACTCCTCCCTTTTCCTCTAAACTCCT	58	260	
TIMP4L2	5′-CCCCACAACCACCCCCTACTATAA			
SOX18U1	5′-GTTTTTGATTATTGAAATTTTTTGGAAGAA	56	354	TaqI (259, 61, 34)
SOX18L1	5′-CCATATCACAACCCCCTAAAAACC			
SOX18U2	5′-TTTGGTAATTTTGTTAATAGGTTTTGGG	56	187	(92, 61, 34)
EGFL7U1	5′-TGGGGTTAGATTTTGATGGTTTAGGG	57	281	TaqI (104, 71)
EGFL7L1	5′-CTATCCACAAAAACCTCCCTTACAACC	57	245	
EGFL7U2	5′-TTATTTTTGGGTAGTTTGTTTAGGTTTTTTT			
CD105U1	5′-TGGGGTTAGGATTGTTGTTGTTATTG	57	323	HpyCH4IV (62, 48, 69, 23, 30)
CD105L1	5′-ACCAACAACAAAACAACAACCAAAAA	57	232	
CD105U2	5′-GGGTTTTTGTGTTTATTTTTTTTGATTTTT			
SEM2U1	5′-TTGTTTTATAAGTGGTGGTTTGGTGGTAG	59	331	TaqI (108, 89)
SEM2L1	5′-ACCCAAACAAAACACATACACACAAACAC	59	197	
SEM2U2	5′-TTGTTGGTTGTTAGGGGGTTTTTTG			
SLIT3U1	5′-GTTGTTTTTAGAGGATTTTTTGGAAGAT	54	233	TaqI (173, 60)
SLIT3L1	5′-AAAACCTAAAAAAAAAAAACAATAAAAAAA			
SLIT3U2	5′-GTGTAAAAATTAAAAAAAAAAAAGTGTTAGG	54	130	TaqI (96, 34)
SLIT3L2	5′-AAAAAAAAAACCAAAAAAAAAAAAAAC			
DLCU1	5′-AGGTGGTGTGGGGATAGTAGGATT	55	329	TaqI (193, 136)
DLCL1	5′-AAATATTCCCAAACAATAAACTCTCCC			
RASSF1U1	5′-GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT	55	377	TaqI (90, 81, 34)
RASSF1L1	5′-ACCCTCTTCCTCTAACACAATAAAACTAACC	55	205	
RASSF1L2	5′-CCCCACAATCCCTACACCCAAAT			

manufacturer's protocol and analysed on 2% (w/v) Tris–borate EDTA agarose gels. Additionally the methylation status of the CpG island of *RASSF1A*, *p16*, *DLC1*, *TIMP3* and *SLIT2* was investigated by methylation specific PCR (MSP). Published primer sequences and conditions were used [8,23,25] and MSP products were analysed on 2% (w/v) Tris–borate EDTA agarose gels.

2.8. Statistical analysis

Statistical analysis was carried out using SPSS 11.5. Categorical variables were plotted in contingency tables and evaluated using Pearson's Chi square analysis and Fisher's exact test for differences between groups. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Transcriptional silencing of tumour-associated genes in lung carcinoma

Differential microarray analysis of 89 lung cancer patients was performed using Affymetrix GeneChip Technology. The probe sets represented 45 000 mRNA and EST clusters and 6200 predicted exons. We detected

Table 3
Down regulated candidate genes in lung carcinoma patients

Gene[a]	Location	Function	CpG island[b] (bp)	Location of CpG island[c]	Normal/tumour[d] (*P*-value)
TIMP4 (U76456)	3p25	Tissue inhibitor of metalloproteinase 4	154	+368 to +521	1.01 (0.96)
SOX18 (AB033888)	20q13.3	HMG-box transcription factor	1780	−303 to +1477	1.19 (4.3E − 12)
EGFL7 (AA448958)	9q34.3	NOTCH4-like	209	+48 to +256	1.40 (2.6E − 12)
CD105 (AI672727)	9q33-34	TGF-ß receptor associated	194	−212 to −19	1.50 (2.6E − 15)
SEMA2 (AB029496)	3p21.31	Neuronal development	310	+183 to +492	1.77 (4.7E − 12)
SLIT3 (AB017169)	5q35	Ligand of ROBO receptor	817	−584 to +232	3.05 (6.9E − 17)
DLC1 (AF035119)	8p22	RhoGAP	908	−694 to +213	2.5 (5.4E − 17)
RASSF1A (AF132675)	3p21.3	Ras association domain family 1	636	−198 to +437	Not available
p16INK4α (U38945)	9p21	Inhibitor of cyclin dependent kinase	977	− 449 to +527	0.41 (0.0025)
SLIT2 (AA022569)	4p15.2	Ligand of ROBO receptor	297	−82 to +214	2.10 (4.6E − 19)
TIMP3 (U33114)	22q12.1	Tissue inhibitor of metalloproteinase 3	780	−617 to +163	1.75 (9.7E − 14)

[a] Genbank accession number is indicated.
[b] CpG island was determined by CpGplot.
[c] The location of the CpG island is indicated relative to the translational start site (ATG).
[d] Expression of matching normal lung *versus* tumour sample in 89 patients.

388 EST, which were down regulated in tumours *versus* normal matching lung tissue (data not shown). Most of these EST represented unknown clones and therefore no association to cancer development could be evaluated. However, the promoter regions of 50 gene candidates were evaluated for the presence of CpG island promoters by *in silicio* analysis (CpGplot) and several genes were selected for further study (Table 3). The epigenetic inactivation of *RASSF1A*, *p16*, *SLIT2* and *TIMP3* have been previously reported in lung cancer [6,8,10]. Results presented here show that *SOX18*, *EGF-like domain 7*, *CD105*, *SEMA2*, *SLIT3*, *DLC1*, *SLIT2* and *TIMP3* are significantly down regulated in lung tumours (Table 3). For the *RASSF1A* isoform no microarray data was available and surprisingly *p16* was up regulated in the tumour samples.

3.2. Epigenetic silencing of *TIMP4* and *RASSF1A* in lung cancer

As aberrant methylation of *TIMP3* promoter is frequently reported in tumours and *TIMP4* inhibits the growth of Wilm's and breast tumour [26,27], we considered *TIMP4* as a potential gene candidate. The expression of *TIMP4* and *RASSF1A* was evaluated by real-time PCR (Fig. 1(a)). Transcription of *TIMP4* was detected in all analysed normal tissues including lung samples. Interestingly, in primary lung tumours and several lung cancer cell lines (e.g., A427, H322, DMS53 and H64) the mRNA levels of *TIMP4* were highly reduced (Fig. 1(a)). However, the transcription was restored when A427 cells were treated with 5 and 10 μM of the DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (5-Aza-CdR) and suggested that DNA methylation is involved in the transcriptional repression of *TIMP4* in lung cancer (Fig. 1(a), inset). Similarly, we analysed the expression of *RASSF1A* (Fig. 1(b)). In normal lung, high levels of *RASSF1A* transcript were found, whereas in several primary tumours and lung cancer cell lines, a reduced expression was detected. As observed for *TIMP4*, when A427 cells were treated with 5-Aza-CdR, *RASSF1A* expression was induced (Fig. 1(b), inset). In addition, the methylation of *TIMP4* CpG island was investigated using combined bisulfite restriction analysis (COBRA). In cell lines (H322, H358, H1688 and A427) with silent *TIMP4*, the promoter region was methylated which was also confirmed by sequencing (Fig. 2). This methylation was prevented by treatment with 5-Aza-CdR in A427 and was absent in normal bronchial epithelial cells (Fig. 2).

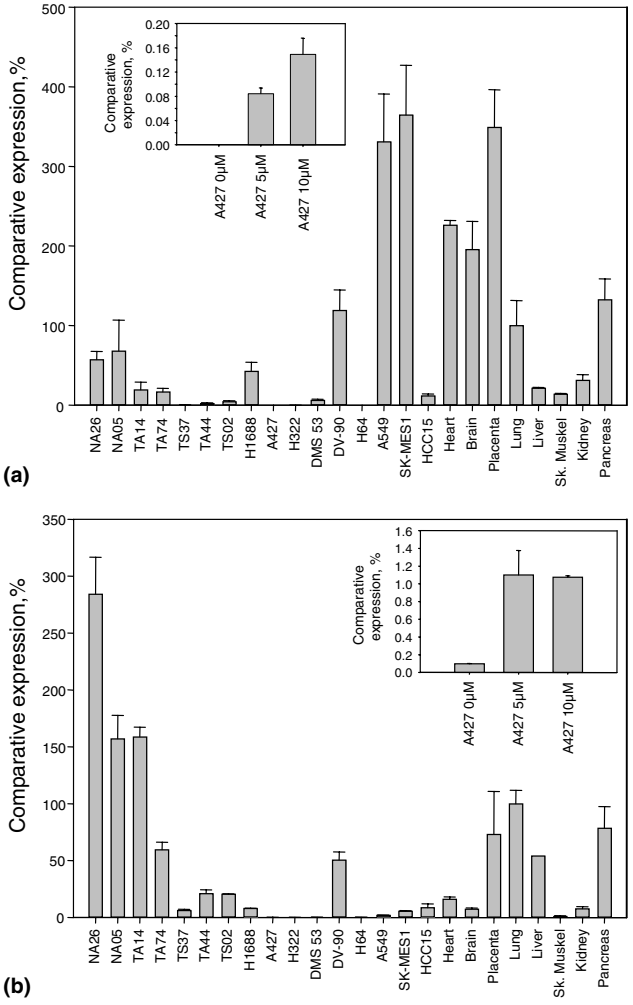

Fig. 1. Real time analysis of *TIMP4* and *RASSF1A* expression in lung cancer. (a) Expression of *TIMP4* was analysed in seven primary lung tissues (N: normal tissue matching, TA: tumour adenocarcinoma and TS: tumour squamous cell carcinoma), in nine lung cancer cell lines (H1688, A427, H322, DMS53, DV90, H64, A549, SK-MES1 and HCC15) and in eight normal tissues by real-time RT-PCR. Inset: The cell line A427 was treated with 5-Aza-CdR (0, 5 and 10 μM) for 4 days and expression was analysed. All experiments were repeated at least three times (standard derivation is indicated) and adjusted for *GAPDH* expression. Real time data were plotted on graphs (lung = 100%). (b) Expression of *RASSF1A* in lung cancer and normal samples was analysed by real time RT-PCR. As for *TIMP4* data was plotted on graphs (lung = 100%). Inset: effect of 5-Aza-CdR (0, 5 and 10 μM) on *RASSF1A* expression in A427 cells.

3.3. Methylation profile of candidate and tumour-suppressor genes in lung cancers

In 15 lung cancer cell lines, including seven SCLCs, two bronchio-alveolar carcinomas, four adenocarcinomas and two squamous cell carcinomas, the methylation status of the gene promoters was evaluated by COBRA and a representative selection is shown in Fig. 3. COBRA was also used to investigate aberrant methylation in 18 primary lung carcinomas (nine adenocarcinomas and nine squamous cell carcinomas) and eight matching normal lung tissues, a selection of which are illustrated in Fig. 4. In COBRA, PCR fragments obtained from bisulfite-modified DNA were further digested with an enzyme that has CpG in its consensus sequence [28]. All results of the methylation analysis are summarised

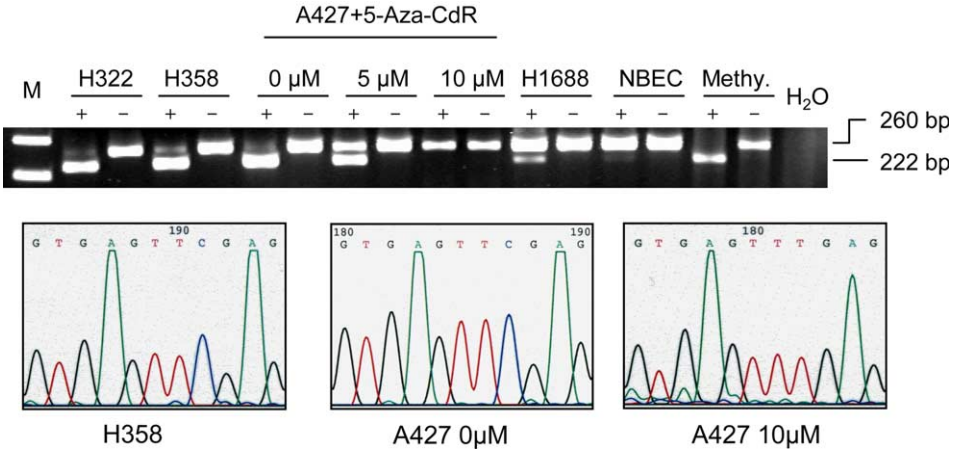

Fig. 2. Methylation analysis of the *TIMP4* CpG island. In lung cancer cell lines (H322, H358, A427 and H1688), the methylation of *TIMP4* was analysed by COBRA and sequencing. NBEC (normal bronchial epithelial cells) and *in vitro* methylated DNA (Methy.) were used as controls. The cell line A427 was treated with 5-Aza-CdR (0, 5 and 10 μM) for 4 days and methylation was analysed by COBRA and sequencing.

in Tables 4 and 5. The methylation of *TIMP4* was detected in nine out of 14 (64%) lung cancer cell lines, in 17 out of 18 (94%) of primary lung carcinoma and in seven out of eight (88%) normal tissues. Aberrant methylation of *SOX18* was found in eight out of 11 (73%) of lung cancer cell lines. *SOX18* was frequently methylated in all analysed primary lung tissues including tumours (100%) and normal samples (100%). In five out of nine cancer cell lines methylation of *EGFLD7* promoter was observed. Methylation of *EGFLD7* was also found in 14 out of 14 (100%) primary tumours and in three out of four (75%) normal samples. The promoter of the *CD105* gene, that codes for a proliferation-associated protein and is expressed in angiogenic endothelial cells, was aberrantly methylated in five out of seven (71%) of cancer cell lines, weakly methylated in 11 out of 16 (69%) of lung tumours and 80% of normal lung tissue. Similarly, weak methylation of *SEMA2* was detected in most tumour samples, normal tissues and cancer cell lines (13/14 = 93%, 6/8 = 75% and 6/11 = 55%, respectively). *DLC1* was only methylated in two out of 11 (18%) cell lines. In primary lung tissue, methylation of *DLC1* was investigated by methylation specific PCR (MSP) (selection in Fig. 4). In 11 out of 18 (61%) lung tumours and in two out of eight (25%) matching normal lung, hypermethylation was observed (Table 5). Additionally, methylation of two human homologues of the Drosophila *Slit* gene, *SLIT2* and *SLIT3*, was investigated by MSP and COBRA, respectively. *SLIT2* was intensely methylated (100%) in cell lines, lung tumours and normal lung (Table 4, Fig. 5). In contrast, *SLIT3* methylation was detected less frequently in cancer cell lines (18%) and absent in primary tumours (Fig. 4, Table 5). Hypermethylation of *RASSF1A* was detected in 10 out of 14 (71%) cancer cell lines and in seven out of 16 (44%) primary tumours, but not found in any normal lung sample. In lung cancer case TS37, hypermethylation of *RASSF1A* was associated with a reduction in transcript (Fig. 1(b)). Methylation of *p16* was found in five out of nine (56%) cancer cell lines and in eight out of 17 (47%) primary lung tumours (selection shown in Fig. 5, Table 5). Aberrant methylation of *TIMP3* was less frequent and detected in two out of seven (29%) cancer cell lines and two out of 15 (13%) lung tumours (Table 5).

3.4. Promoter methylation in normal lung samples

In this study, we detected high methylation frequency for several genes (*TIMP4*, *SOX18*, *EGFL7*, *CD105*, *SEMA2* and *SLIT2*) in normal lung tissue isolated from cancer patients (Table 5). There are several reasons for the methylation present in histologically normal samples such as infiltrating tumour cells, epigenetic field defect, imprinting or tissue specific methylation. As a control, to see if methylation could be derived from incomplete bisulfite reaction, an unmodified negative DNA control (Mock) originating from human fibroblast was included (Figs. 4 and 5). The bisulfite reaction and methylation analysis was repeated for all samples. The mock-treated DNA was not amplified and showed that primers generated for deaminated DNA sequences did not anneal to unmodified DNA. To investigate the epigenetic status of normal cells, we analysed the methylation frequencies of all genes in normal human bronchial epithelial cells (NBEC) and normal human fibroblast (HF) (Table 4 and Fig. 5). Most genes were completely unmethylated in both normal cell lines, however methylation of *EGFL7* was frequently detected in both. Moreover, low methylation was also detected for *SOX18*, *CD105* and *SEMA2* in HF or NBEC (Fig. 5). Methylation of *TIMP4*, *SLIT3*, *DLC1*, *RASSF1A*, *p16*, *SLIT2* and *TIMP3* was not detected in neither of the normal cell lines (Table 4).

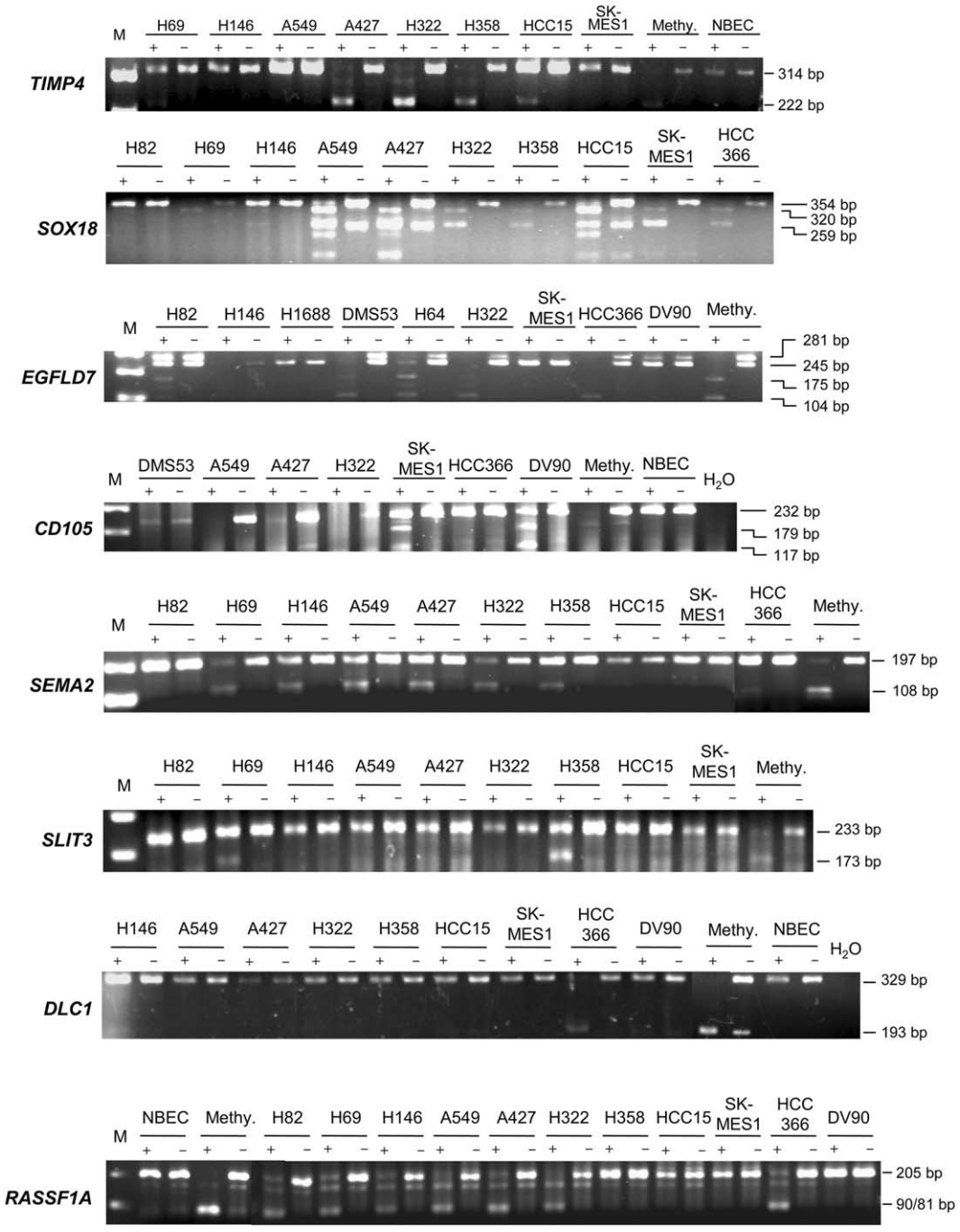

Fig. 3. Methylation analysis of cancer-related genes in lung cancer cell lines. CpG island of *TIMP4*, S*0X18*, *EGFLD7*, *CD105*, *SEMA2*, *SLIT3*, *DLC1* and *RASSF1A* were analysed by COBRA in lung cancer cell lines. PCR products after ± restriction digestion with enzymes *Taq*I and *Hpych4*IV were resolved on 2% (w/v) TBE gels together with a 100 bp DNA ladder (M). Length of PCR products and restriction fragments are indicated. NBEC (normal bronchial epithelial cells) and *in vitro* methylated DNA (Methy.) were used as controls.

3.5. Clinical significance of promoter methylation in lung cancer

The methylation status of down regulated genes was correlated with distinct clinico-pathological characteristics (Table 6). For several genes the methylation frequency in normal tissue was similar to tumours or only of low frequency (*TIMP3*). No correlation to lung cancer pathogenesis can be reasonably assumed in those cases. In contrast, methylation of *RASSF1A*, *p16* and *DLC1* were detected mainly in tumour samples (Table 5). Interestingly, the patients with methylated *RASSF1A* were long time smokers ($P = 0.02$) and were generally younger than the patients with unmethylated *RASSF1A* (Table 6). An association with smoking habit was not detected

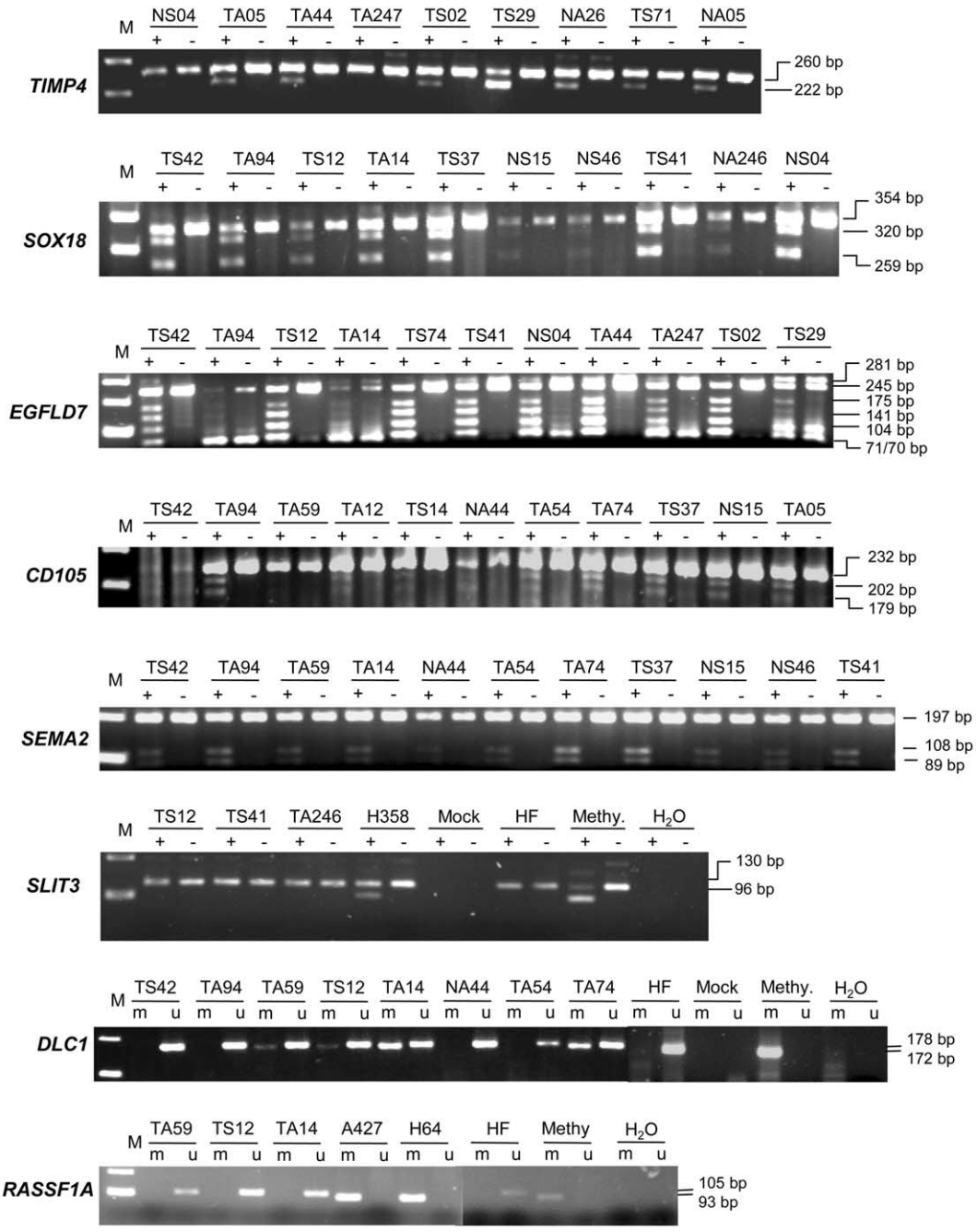

Fig. 4. Analysis of methylation in primary lung cancer specimen. CpG island of *TIMP4*, S*0X18*, *EGFLD7*, *CD105*, *SEMA2* and *SLIT3* were analysed by COBRA in primary lung cancer tissues (TA: tumour adenocarcinoma; TS: tumour squamous cell carcinoma and N: normal tissue matching). PCR products after restriction digestion with (+) or without (−) enzyme were resolved on 2% (w/v) TBE gels together with a 100-bp DNA ladder (M). To analyse the methylation of *DLC1* and *RASSF1A*, methylation-specific (m) and unmethylation-specific (u) primers were used for MSP. Mock-bisulfite treated DNA (Mock) and bisulfite treated DNA isolated of human fibroblast (HF), normal bronchial epithelial cells (NBEC) and *in vitro* methylated DNA (Methy.) were used as controls.

for *p16* or *DLC1*. However, methylation of *p16* was detected in all four patients who were exposed to asbestos ($P = 0.017$). Methylation of p16 and *DLC1* was significantly also associated with squamous cell carcinoma ($P = 0.011$) and in patients with distant metastasis, respectively.

4. Discussion

Epigenetic inactivation of tumour-suppressor genes plays a crucial role in the pathogenesis of tumours, including lung cancer. In our study, we detected methylation of several cancer-related genes that were down reg-

Table 4
Methylation-profile of candidate genes and TSGs in lung cancer

	Gene / Samples	*TIMP4*	*SOX18*	*EGFL 7*	*CD105*	*SEMA2*	*SLIT3*	*DLC1*	*RASSF1A*	*p16*	*SLIT2*	*TIMP3*
SCLC	H64		na		na	na	na	na		U	na	M
	H69			na	na			U		na	na	na
	H82		U	U	na	U	U	U		M	na	na
	H146		U		na		U	U		na	na	M
	H446		na	na	na	na	na	na		na	na	na
	H1688		na	U	na	na		na		M/U	na	na
	DMS53		na		U	na	na	na	na	U	na	na
BA	H322						U	U		U	M/U	U
	H358			na	na					M	na	U
Adenoca	A549	U		na			U	U		na	na	na
	A427			na			U	U		na	M	U
	DV-90	U		U		U	U	U	U	na	na	na
	HCC366	na			U	U	na			M	na	na
SC	HCC15			na	na	U	U	U	U	U	M	U
	SK-MES1	U		U		U	U	U	U	M/U	na	U
HF		U			U		U	U	U	U	U	U
NBEC		U	na			U	U	U	U	U	U	U
Primary adenocarcinoma	TA05			na			U	M/U	M/U	U	M	U
	NA05		na	U			U	M/U	U	U	M	U
	TA14						U	M/U	U	U	M	na
	TA44						U	U	U	M/U	M/U	U
	NA44		na	na			U	U	U	U	na	U
	TA54		na	na			U	U	U	U	M/U	U
	TA59		na	na	na		U	M/U	U	U	M/U	M/U
	TA74		na				U	M/U	U	U	M/U	U
	TA94						U	U	M/U	na	na	U
	TA246		na			na	U	U	na	U	M/U	U
	NA246			na	na	U	U	U	U	U	M/U	U
	TA247	U			U		U	M/U	M/U	U	M/U	M/U
	NA26						U	M/U	U	na	M/U	na
Prim. squamous cell carcinoma	TS02				U		U	M/U	U	M/U	M/U	na
	TS03		na		U	na	na	U	na	U	M/U	U
	TS12					na	U	M/U	U	M/U	M	U
	TS29				U	na	U	U	M/U	M/U	M/U	U
	TS37			na			U	M	M/U	U	M/U	U
	TS41				na		U	M/U	U	M/U	M/U	na
	TS42				U		U	U	M/U	M/U	na	U
	TS68					U	U	M/U	U	M/U	M/U	U
	NS68		na		U	U	U	U	na	U	M/U	na
	TS71						U	M/U	M/U	M/U	M/U	U
	NS04	U			na		U	U	U	U	M/U	U
	NS15			na			U	U	U	M/U	M	na
	NS46			na	na		na	U	U	U	M	na

BA: bronchio-alveolar carcinoma; SC: lung squamous cell carcinoma; HF: human fibroblast; TA: tumour adenocarcinoma and TS: tumour squamous cell carcinoma; N: normal matching tissue; U: unmethylated by COBRA or MSP; light gray box: weak methylation by COBRA (10–40%); dark gray box: strongly methylated by COBRA (50–90%); black box: completely methylated by COBRA; M: methylated by MSP, M/U partially methylated by MSP; na, not analysed.

ulated in microarray assays. Several genes (*TIMP4*, *SOX18*, *EGF-like domain 7*, *CD105*, *SEMA2* and *SLIT3)* have not been previously described for promoter methylation and could represent novel targets for hypermethylation in cancer. In this report we have shown for the first time that *TIMP4* is frequently methylated in lung cancer. Down regulation of *TIMP4* has been reported to contribute to the tumourogenic and invasive potential of tumour cells [26]. Aberrant methylation of *TIMP3* has also been reported for several

Table 5
Summary of methylation analysis in lung carcinomas

Gene	Methy. in lung cancer cell lines	Methy. in lung tumours	Methy. in matching normal lung
TIMP4	64% (9/14)	94% (17/18)	88% (7/8)
SOX18	73% (8/11)	100% (13/13)	100% (5/5)
EGFL7	56% (5/9)	100% (14/14)	75% (3/4)
CD105	71% (5/7)	69% (11/16)	80% (4/5)
SEMA2	55% (6/11)	93% (13/14)	75% (6/8)
SLIT3	18% (2/11)	0% (0/17)	0% (0/7)
DLC1	18% (2/11)	61% (11/18)	25% (2/8)
RASSF1A	71% (10/14)	44% (7/16)	0% (0/7)
p16	56% (5/9)	47% (8/17)	14% (1/7)
SLIT2	100% (3/3)	100% (16/16)	100% (7/7)
TIMP3	29% (2/7)	13% (2/15)	0% (4/4)

tumour entities including lung cancer [10,11]. Here, we detected *TIMP3* methylation in 13% of lung carcinomas and was not seen in normal tissue. *TIMP4* hypermethylation was found in 94% of lung cancer cases. However, methylation was also present in the normal matching lung samples but was not found in normal lung epithelial cells. *TIMP4* methylation may represent an epigenetic field defect, which is frequently found in resected bronchial margins of lung carcinomas [29]. The *TIMP4* gene has been located to chromosome 3p25 and as allelic loss of 3p is frequently observed in lung cancer [3,4], down regulation of *TIMP4* may occur by LOH. Interestingly, *TIMP4* has been implicated in the inhibition of tumour growth and metastasis [27] and it could be that its inactivation is involved in the pathogenesis of lung cancer.

The *DLC1* gene located on chromosome 8p22 was identified by its high deletion frequency in liver cancer and reports have shown the inhibitory effects of *DLC1* on the proliferation of hepatoma cell lines [30]. Also, epigenetic inactivation of *DLC1* was detected in gastric cancer and hepatocellular carcinoma [25,31]. The DLC1 protein shares homologies to RhoGAP that acts as a negative regulator of Rho proteins by stimulating its intrinsic GTPase activity. In our report, we detected significant down regulation of *DLC1* and moreover frequent aberrant methylation was found in primary lung tumours and cancer cell lines. These findings suggest that *DLC1* may also serve as tumour-suppressor gene in lung cancer. The role of *DLC1* as tumour-suppressor is supported by the frequent LOH of 8p21-22 in lung cancer [4].

The *SLIT* genes are a family of extracellular matrix secreted ligands of the repulsive guidance receptor ROB and recently hypermethylation of *SLIT2* has been reported for several tumour entities including lung cancer [8]. In this study, down regulation of *SLIT2* from microarray analysis was also observed and could be attributed to its epigenetic inactivation. However, high methylation frequencies in both tumour and normal lung samples were recorded. The methylation observed in normal samples may have been attributed to infiltrating cancer cells or represented an epigenetic field defect. In addition, results from our microarray analysis showed significant down regulation of *SLIT3*. Aberrant methylation of *SLIT3* was not found in primary tumours and suggests that an alternative mechanism may be responsible for its inactivation in lung cancer such as loss of 5q35 heterozygosity [2].

The SOX proteins belong to the HMG box super family of transcription factors involved in a wide range of processes including blood vessel development [32]. It is suggested that SOX7 represses Wnt/ß-catenin signaling and involved in tumourogenesis [33]. SOX18 is closely related to SOX7 and mutations in SOX18 result in cardio-vascular dysfunction [32] and may be involved in inhibition of angiogenesis. The microarray data from this work showed a reduction in *SOX18* expression as well as hypermethylation of *SOX18* CpG islands in all tumours. It appears that methylation of *SOX18* may be involved in its repression.

CD105 is a proliferation-associated and hypoxia inducible protein that is important for angiogenesis [34]. Elevated levels detected in patients with cancer have been positively correlated with metastasis and proliferation [35,36]. Our analysis showed slight methylation of the *CD105* promoter in several samples including lung cancer cell lines. Since the CpG island of its promoter is only 200 bp long, the weak methylation in most probes may represent a spreading of methylation, which is abundant in exons in normal cells.

In lung cancer, allelic loss of chromosome 3p21.3 is frequently detected [4,37]. We analysed the epigenetic inactivation of two genes in this segment: *Semaphorin 2* (*SEMA2*) and *RASSF1A*. The Semaphorin family encodes for secreted and membrane associated proteins that play important roles in the immune system and may affect organogenesis, vascularization and angiogenesis [38]. Semaphorins may also be associated with potential oncogenic properties. Interestingly, a decrease in *SEMA2* expression and hypermethylation of *SEMA2* in lung cancer cells was observed. Thus, epigenetic

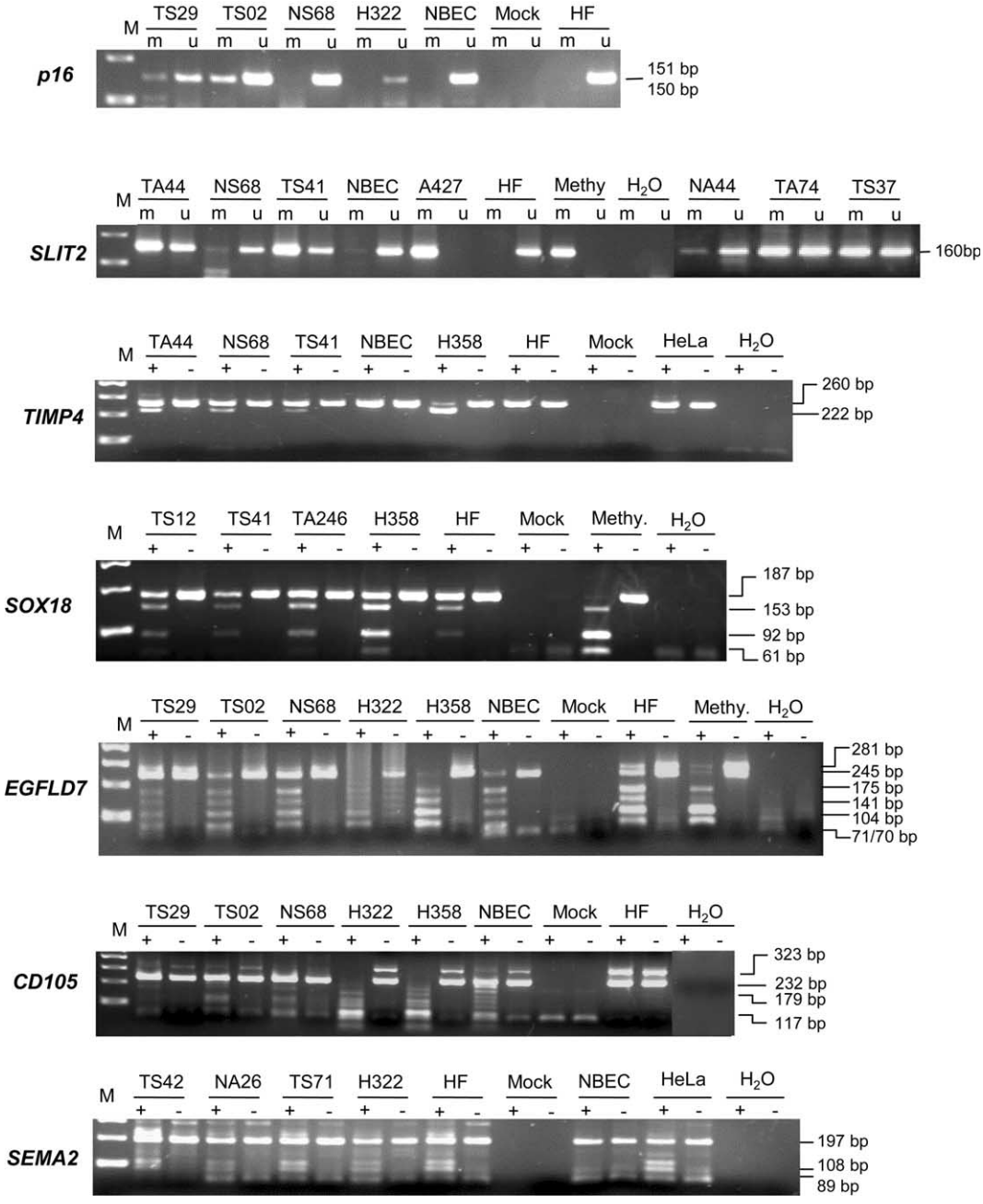

Fig. 5. Analysis of methylation in lung cancer specimen and controls. To analyse the methylation of *p16* and *SLIT2*, methylation-specific (m) and unmethylation-specific (u) primers were used for MSP. The methylation status of *TIMP4*, S0*X18*, *EGFLD7*, *CD105*, and *SEMA2* was investigated as described in Fig. 4. To verify the bisulfite analysis, we repeated the bisulfite reaction and included mock-bisulfite treated DNA (Mock) and bisulfite treated DNA (HF) isolated of human fibroblast. NBEC (normal bronchial epithelial cells), lung cancer cell lines (H358, H322 and A427) and samples from patients (TA, TS, NS and NA) were included in the analysis.

silencing of *SEMA2* may represent the second hit for its inactivation in lung cancer. Epigenetic inactivation of *RASSF1A* is frequently detected in lung cancer including SCLC [6,9,12]. The observed methylation frequency of 44% in lung tumours is similar to studies in NSCLC [6,12]. Like other published reports, our data showed that aberrant methylation of *RASSF1A* is associated with exposure to smoke and correlated to long term smoking habit [13,39]. In agreement, hypermethylation of *RASSF1A* has been detected from cells in the sputum and bronchioloalveolar lavages of smokers [16]. Moreover, inactivation of *RASSF1A* is also correlated with advanced tumour stage and an impaired survival of lung cancer patients [12–14]. Hypermethylation analysis of *RASSF1A* may serve as a diagnostic and prognostic marker for lung cancer.

Table 6
Correlation of methylation and clinicopathological characteristics

	DLC1 methylated, $N = 13$	*DLC1* unmethylated, $N = 11$	*RASSF1A* methylated, $N = 7$	*RASSF1A* unmethylated, $N = 15$	*p16* methylated, $N = 9$	*p16* unmethylated, $N = 13$
Mean age ± SD	61 ± 10	64 ± 7	59 ± 8	63 ± 9	65 ± 6	60 ± 10
Male	10 (78%)	10 (91%)	7 (100%)	11 (73%)	9 (100%)	10 (77%)
Years of smoking ± SD	24 ± 15	25 ± 13	33 ± 11[a]	19 ± 13[a]	24 ± 12	26 ± 15
Exposure to asbestos ($n = 4$)	1	3	2	2	4[b]	0[b]
Squamous cell ca	6	6	4	7	8[c]	4
Adenocarcinoma	7	5	3	8	1[c]	9
T1	3	3	2	4	1	4
T2	6	6	2	8	4	6
T3	3	1	2	2	2	2
T4	1	1	1	1	–	1
I	5	3	1	7	4	4
II	4	3	5	1	3	4
III	2	5	1	5	2	3
IV	2	–	–	2	–	2
G1	–	–	–	–	–	–
G2	2	2	1	3	1	3
G3	11	9	6	12	8	10
MO	11	11	7	13	9	11
M1	2	–	–	2	–	2
N0	9	3	3	9	5	7
N1	1	3	3	–	2	2
N2	3	4	1	5	1	4
N3	–	1	–	1	1	–

T1–T4; primary tumour; I–IV; TMN staging; G1–G3; grading; M0–M1; distant metastasis; N0–N3; regional lymph nodes.
[a] $P = 0.02$ (95% CI 2.4–26.8; *T*-test).
[b] $P = 0.017$ (Fisher exact test).
[c] $P = 0.011$ (Fisher exact test).

Hypermethylation of the cell cycle inhibitor *p16* is frequently observed in lung cancer [10,11]. We also report frequent hypermethylation of *p16*. The methylation detected in the matching normal cells might be attributed to tumour cells infiltration in these probes or to age related methylation of tumour-related genes [40]. Interestingly, in all lung cancer patients, which were exposed to asbestos aberrant methylation of *p16* was detected. It is reported that aberrant promoter methylation of *p16* is frequently detected in sputum of smokers and patients exposed to radon [15]. Additionally, we found inactivation of *p16* was significantly associated with squamous cell carcinoma.

Methylation of *TIMP4*, *SOX18*, *EGF-like domain 7*, *CD105*, *SEMA2* and *SLIT2* was frequently detected in histologically normal tissue originating from cancer patients. This methylation was not due to incomplete bisulfite modification and may be attributed to field epigenetic field defects, infiltrating tumour cells or related to methylation reported in aging [29,41]. To clarify this issue, we analysed the methylation of these genes in NBEC and HF cells. Interestingly, no methylation of *TIMP4* or *SLIT2* was detected in these apparently normal cells. Most likely the methylation of both genes in normal tissue from cancer patients represented infiltrating tumour cells or field defect. It has been reported that histologically negative bronchial margins of resected NSCLC exhibit frequent hypermethylation changes in multiple genes [29]. This aberrant methylation is also present in the primary tumour and may represent a field defect of preneoplastic changes that occurs early in carcinogenesis. In contrast, methylation of *SOX18*, *EGFL7*, *CD105* and *SEMA2* was found in normal cell lines and therefore may represent a tissue specific methylation of these genes. It is unlikely that the methylation of these genes is due to imprinting. However, *SOX18* is located at 20q13.3 and in this segment, the gene responsible for a form of pseudohypoparathyroidism is paternally imprinted [42] and it will be interesting to analyse if *SOX18* is expressed hereditarily.

In summary, we provide new evidence that down regulation and aberrant methylation of several novel genes are involved in the pathogenesis of cancer. The methylation differences in cancer cell lines were more pronounced than in primary tumours. This may be attributed to establishment of aberrant methylation pattern in the propagation of cell lines [43]. The evaluation of the epigenetic status of silenced genes from microarray studies may represent an interesting strategy to identify new aberrant methylated candidate genes in lung cancer. However, it must be noted that for several genes, transcriptional silencing in lung cancer is

unnecessarily associated with aberrant methylation of CpG islands. In our study, we describe the inactivation of *SLIT3*, *SOX18*, *EGF-like Domain 7*, *CD105*, *SEMA2*, *DLC1*, and *TIMP4*, which may represent novel targets with recessive oncogenic properties in the development of lung cancer. These findings warrant the investigation of these tumour-related genes in growth assays.

Conflict of interest statement

None declared.

Acknowledgements

We are grateful to Prof. Heidi Foth of the Institute for Environmental Toxicology, University Halle for the possibility to perform real-time PCR. We thank Christel Trümpler for technical assistance. This work was supported by BMBF (FKZ 01ZZ0104), Land Sachsen-Anhalt and DFG (DA 552-1) Grants to Reinhard Dammann and DFG (SFB 610 TPB1) and Deutsche Krebshilfe (70-2787-Bu3) Grants to Stefan Burdach.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2005.02.020.

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