Quantitative detection of *TUSC3* promoter methylation - a potential biomarker for prognosis in lung cancer

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Received March 14, 2016; Accepted June 17, 2016

DOI: 10.3892/ol.2016.4927

Abstract. Aberrant promoter methylation of tumor relevant genes frequently occurs in early steps of carcinogenesis and during tumor progression. Epigenetic alterations could be used as potential biomarkers for early detection and for prediction of prognosis and therapy response in lung cancer. The present study quantitatively analyzed the methylation status of known and potential gatekeeper and tumor suppressor genes [O-6-methylguanine-DNA methyltransferase (*MGMT*), Ras association domain family member 1A (*RASSF1A*), Ras protein activator like 1 (*RASAL1*), programmed cell death 4 (*PDCD4*), metastasis suppressor 1 (*MTSS1*) and tumor suppressor candidate 3 (*TUSC3*)] in 42 lung cancers and in corresponding non-malignant bronchus and lung tissue using bisulfite-conversion independent methylation-quantification of endonuclease-resistant DNA (MethyQESD). Methylation status was associated with clinical and pathological parameters. No methylation was found in the promoter regions of *PDCD4* and *MTSS1* of either compartment. *MGMT*, *RASSF1A* and *RASAL1* showed sporadic (up to 26.2%) promoter methylation. The promoter of *TUSC3*, however, was frequently methylated in the tumor (59.5%), benign bronchus (67.9%) and alveolar lung (31.0%) tissues from each tumor patient. The methylation status of *TUSC3* was significantly associated with smaller tumor size (P=0.008) and a longer overall survival (P=0.013). Pooled blood DNA of healthy individuals did not show any methylation of either gene. Therefore, methylation of *TUSC3* shows prognostic and pathobiological relevance in lung cancer. Furthermore, quantitative detection of *TUSC3* promoter methylation appears to be a promising tool for early detection and prediction of prognosis in lung cancer. However, additional studies are required to confirm this finding.

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Key words: tumor suppressor candidate 3, lung cancer, prognosis, survival, quantitative methylation analysis

Introduction

Of all types of cancer, lung cancer is the malignancy with the greatest incidence and associated mortality, worldwide (GLOBOCAN 2012, v1.0). The poor chances of survival for lung cancer patients are often due to a late diagnosis (1). A variety of genetic and epigenetic factors contributing to the development of lung cancer have been discovered, yet a valid marker set for the early detection of disease has not been established. Methylation of promoter regions is a known cause for transcriptional repression (2) and can, therefore, contribute to carcinogenesis and tumor progression (2). This epigenetic mechanism of gene silencing has already been described in lung cancer for several genes, including cyclin-dependent kinase inhibitor 2A, O-6-methylguanine-DNA methyltransferase (*MGMT*), Ras association domain family member 1A (*RASSF1A*), retinoic acid receptor β (3-5), mutL homolog 1 (6), fragile histidine triad, death associated protein kinase, runt related transcription factor 3, TIMP metallopeptidase inhibitor 3 (4) or adenomatous polyposis coli (5). Certain studies even report promoter methylation in the bronchial lavage (7) or blood samples (8) of lung cancer patients, whereas others elucidate the increasing risk of developing this tumor with the accumulation of this epigenetic change in sputum (9). In future, the knowledge of the methylation status of tumor related genes could be helpful for the identification of persons at risk, the early detection of lung cancer and for the prediction of prognosis and therapeutic success. Certain studies suspect an association between promoter methylation and smoking habits (10) or exposure to environmental and industrial factors, such as smoky coal emission or chromate exposure (6,11).

To identify potential marker genes in lung cancer and in possible precursor lesions, the methylation status of *MGMT*, *RASSF1A*, Ras protein activator like 1 (*RASAL1*), programmed cell death 4 (*PDCD4*), metastasis suppressor 1 (*MTSS1*) and

tumor suppressor candidate 3 (*TUSC3*) in lung tumors and corresponding non-malignant bronchus and lung tissue were quantitatively assessed using methylation-quantification of endonuclease-resistant DNA (MethyQESD). MethyQESD is a reliable bisulfite-conversion independent quantitative methylation sensitive polymerase chain reaction (qPCR) method. These six genes were chosen as they all appear to be relevant in tumor development and progression.

The inactivation and, therefore, loss of expression of *MGMT* and *RASSF1A* in lung cancer has been described previously (3-6,8,12-17). The reduced expression of *RASAL1* was observed in several malignant tumors, including brain, head and neck, bladder, breast, colorectal, hepatocellular and thyroid carcinoma (18-21), and appears to be associated with neoplastic progression (19). In addition, certain studies provide evidence of an association between promoter *RASAL1* methylation and loss of expression (18-21), while treatment with the DNA methyltransferase inhibitor 5'-azacitidine restored the expression of *RASAL1* (18). *PDCD4* is also suspected to act as tumor suppressor gene (22). Reduction or loss of expression has also been observed in lung cancer (23,24), and may be associated with tumor progression and a poorer prognosis (23). The impact of *MTSS1* on malignant tumors is not yet well defined. Certain studies provide evidence that *MTSS1* promotes tumor initiation and progression in early stages (25), whereas *MTSS1* appears to have a tumor suppressive effect in advanced stages and in metastasis (25-27). Furthermore, the reduction and loss of expression of *MTSS1* has been observed in malignancies (26,27). Other studies contradict this finding, and found increased levels of *MTSS1* in aggressive and metastatic tumors and tumor cell lines (28). However, in lung cancer cell lines, *MTSS1* was downregulated compared with benign human bronchial epithelial cell lines (29). Finally, in a number of studies, genetic and epigenetic changes and a loss of expression of *TUSC3* have been detected in several malignancies, including prostate, ovarian, colorectal, larynx and pharynx carcinoma and acute lymphoblastic leukemia (30-34). In addition, *TUSC3* involvement has already been observed in non-small cell lung cancer (NSCLC) downregulation (24) and promoter methylation (35). Therefore, it is likely that *TUSC3* functions as a tumor suppressor gene.

Materials and methods

Sample collection. In total, 42 patients who had been diagnosed with primary lung cancer at the University Hospital of Regensburg (Regensburg, Germany) were selected for the present study. In particular, 42 primary lung tumors (17 adenocarcinomas, 20 squamous carcinomas, 3 large cell carcinomas and 2 neuroendocrine carcinomas) as well as corresponding normal lung tissue (n=42) and non-malignant bronchus tissues (n=28 for *MGMT* and *TUSC3*, n=29 for *RASSF1A*, n=27 for *RASAL1* and *PDCD4* and n=24 for *MTSS1*) were retrospectively analyzed. Archival tissue samples were obtained from the Institute of Pathology at the University Hospital of Regensburg, of which the Institutional Review Board approved the study in January 1997. All lung cancer patients underwent surgical resection between January 2000 and November 2002 at the Department of Thoracic Surgery, University Hospital of Regensburg and all histological data were provided by the Department of Pathology. Tumor staging was performed according to the 7th edition of the TNM Classification of Malignant Tumours. The age of the patients at diagnosis ranged between 38 and 77 years (mean, 59 years). In total, 33 patients were males (78.6%) and 9 were females (21.4%). Two patients received neoadjuvant therapy. Clinical and histopathological data are given in Table I.

DNA extraction. Formalin-fixed paraffin-embedded 4-μm thick slides were deparaffinized after incubation at 70˚C for 30 min in xylene, and then rehydrated in ethanol (graded series, 100, 96 and 70%) and deionized water. Subsequently, the slides were stained with 0.01% methylene blue. Microdissections of the bronchus, lung and tumor tissues were performed with a stereo microscope (magnification, x40). DNA was isolated using the MagNA Pure LC DNA-Isolation Kit II (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's recommendations, and quantified photometrically. Samples with low DNA content were concentrated with Amicon® Ultra 0.5 ml Centrifugal Filters (Merck Millipore, Darmstadt, Germany).

Quantitative methylation analysis. Methylation analysis was performed using MethyQESD (36), a combination of methylation-sensitive digestion and qPCR. A methylation specific quantification digestion (MQD) containing 40 units (U) of the methylation-sensitive endonuclease, Hin6I (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and a calibrator digestion (CalD) containing the methylation-independent endonucleases, XBaI (20 U) and DraI (20 U) (Fermentas; Thermo Fisher Scientific, Inc.), were set up for each sample. Each digestion, with a total reaction volume of 20 μl, was performed using 5 μl DNA (minimum concentration, 20 ng/μl) and 2 μl of 10x Buffer Tango™ (Fermentas; Thermo Fisher Scientific, Inc.). Cell-line DNA served as a positive control (SW48 for *MGMT*, *RASSF1A*, *MTSS1* and *TUSC3*; HT29 for *RASAL1*; none for *PDCD4*) whereas pooled blood DNA from healthy individuals and a sample containing no DNA were used as negative controls. Following incubation at 37˚C overnight, the reaction was stopped at 70˚C for 20 min. qPCR was performed using the LightCycler 1.0 (Roche Diagnostics) for *MGMT*, *RASSF1A*, *RASAL1*, *PDCD4* and *MTSS1* and the LightCycler® 480 (Roche Diagnostics) for *TUSC3*. The total reaction volume contained 10 μl QuantiTect® SYBR® Green PCR Kit (Qiagen GmbH, Hilden, Germany), 0.5 μM Primer and 3 μl digested DNA. The primers sequences were as follows: *MGMT* forward: 5'-CCCGGATATGCTGGGACAG-3'; *MGMT* reverse: 5'-CCCAGACACTCACCAAGTCG-3'; *RASSF1A* forward: 5'-GCTGGGCGCGCTGGGAAG-3'; *RASSF1A* reverse: 5'-CAGGGACCAGCTGCCGTGT-3'; *RASAL1* forward: 5'-CTCCAGACGCCTCGGCAAGAG-3'; *RASAL1* reverse: 5'-AGCGCCCGTCCGGACTCTAC-3'; *PDCD4* forward: 5'-CCAGTCCCAGGAGCCACAT-3'; *PDCD4* reverse: 5'-GAGGAAAAGGGAGAGGAGTGA-3'; *MTSS1* forward: 5'-GAGCCCAGCCAGAGCGAGC-3'; *MTSS1* reverse: 5'-CGGCGTCCGGATCTGTTGCT-3'; *TUSC3* forward: 5'-TACCGCGCGTGGAGGAGACA-3'; *TUSC3* reverse: 5'-GTGGGCAGGTACCGCAGCC-3'. After an initial denaturation of 15 min at 95˚C 45 cycles of amplification followed: Denaturation at 95˚C for 10 sec (*MGMT*,

Table I. Clinicopathological characteristics of 42 non-small cell lung cancer patients.

Characteristics	No. of patients (%)
Total	42 (100.0)
Gender	
Male	33 (78.6)
Female	9 (21.4)
Age at diagnosis, years	
≤60	23 (54.8)
>60	19 (45.2)
Survival	
Yes	13 (31.0)
No	28 (66.7)
Unknown	1 (2.4)
Smoking status	
Smoker	37 (88.1)
Non-smoker	5 (11.9)
Histology	
Adenocarcinoma	17 (40.5)
Squamous carcinoma	20 (47.6)
Large cell carcinoma	3 (7.1)
Neuroendocrine carcinoma	2 (4.8)
T category	
T1a	8 (19.0)
T1b	2 (4.8)
T2a	16 (38.1)
T2b	8 (19.0)
T3	3 (7.1)
T4	5 (11.9)
N category	
N0	28 (66.7)
N1	11 (26.2)
N2	3 (7.1)
M category	
M0	38 (90.5)
M1	3 (7.1)
Unknown	1 (2.4)
Grading	
G2	24 (57.1)
G3	18 (42.9)
Resection boundaries	
R0	38 (90.5)
R1	3 (7.1)
R2	1 (2.4)
Stage	
I	19 (45.2)
II	10 (23.8)
III	9 (21.4)
IV	3 (7.1)
Unknown	1 (2.4)

T, tumor; N, node; M, metastasis.

RASSF1A, *PDCD4*) or 15 sec (*RASAL1*, *MTSS1*, *TUSC3)* and annealing at 60 ˚C (MGMT, PDCD4, TUSC3), 65 ˚C (RASSF1A) and 66 ˚C (RASAL1, MTSS1) for 17 sec (MGMT, RASSF1A, PDCD4), 20 sec (MTSS1) and 34 sec (TUSC3). For *RASAL1* a two-step PCR was performed at 66˚C for 20 sec. For the melting point analysis, PCR products were heated from 55 to 98˚C with an increase of 0.2˚C/sec (*MGMT*, *RASSF1A*, *RASAL1*, *PDCD4*, *MTSS1*) or 0.11˚C/sec (*TUSC3*). Fluorescence was measured continuously. Methylation was quantified according to the formula: Methylation (%) = $E^{(C_tCalD - C_tMQD)}$ x100. (C_t = C_t value; E = PCR efficiency). For calculation of the Ct-Value LightCycler Software Version 3.5 (Roche Diagnostics) (*MGMT, RASSF1A, RASAL1, PDCD4* and *MTSS1*) and LightCycler® 480 Software 1.5.0 (Roche Diagnostics) (TUSC3) were used. PCR-efficiency was obtained by standard curves for MQD and CalD with the dilution levels 1:4, 1:16, 1:64, 1:256, 1:1024, 1:4096 and 1:16384, respectively. LightCycler Software ver. 3.5 and LightCycler® 480 Software ver. 1.5.0 was used to calculate PCR efficiencies, which were 1.97 for *MGMT*, *RASSF1A*, *RASAL1*, *PDCD4* and *MTSS1* and 1.94 for *TUSC3*. The cut-off value for positive methylation was >4%.

Statistical analysis. The association between two variables was analyzed using Fisher's exact test (two-sided). Survival was estimated according to Kaplan-Meier, and comparisons between differences in survival were performed with the log-rank test. $P<0.05$ was assumed to indicate a statistically significant difference.

Results

Methylation frequencies. The promoter methylation frequencies of *MGMT*, *RASSF1A*, *RASAL1*, *PDCD4*, *MTSS1* and *TUSC3* in lung tumor and corresponding non-malignant bronchus and lung tissues were quantitatively assessed, with a cut-off value of >4 per cent for positive methylation applied (Table II). No methylation was identified for *PDCD4* and *MTSS1* in any tissue types, and only sporadically for *RASAL1* [bronchus, 0.0% (0/27); lung, 2.4% (1/42); tumor, 4.8% (2/42)]. *MGMT* showed methylation in 7.1% of benign bronchus (2/28) and tumor (3/42) samples, as well as in 2.4% (1/42) of non-malignant lung tissue. Simultaneous methylation in two tissue types occurred only in one case (tumor and lung). *RASSF1A* was scarcely methylated in bronchial tissue (3.4%; 1/29), not at all in normal lung (0.0%; 0/42) and in 26.2% of tumor tissues (11/42). However, in the one case of bronchus methylation, no other tissue type was affected. The highest methylation frequencies were detected for *TUSC3* in all three tissue types: Bronchus, 67.9% (19/28); lung tissue, 31.0% (13/42); and tumor tissue, 59.5% (25/42). Notably, 5 cases demonstrated *TUSC3* methylation in all three tissue types, 8 cases demonstrated *TUSC3* methylation in the bronchus and tumor tissues, 3 cases in the lung and tumor tissues, and 9 cases demonstrated *TUSC3* methylation in the tumor tissues only. Of the 3 cases with methylation in the lung and tumor and from 6/9 cases with tumor methylation, bronchial material was not available for examination. In addition, 12 patients showed methylation of >1 gene in the three tissue types. *MGMT*, *RASSF1A* and *TUSC3* were methylated more often in the bronchus compared

Table II. Frequencies of methylation in lung cancer and corresponding non-malignant lung tissues from the same patients.

	Bronchus tissue		Lung tissue		Tumor tissue	
Analyzed gene	% patients	n/n_{total}	% patients	n/n_{total}	% patients	n/n_{total}
MGMT	7.1	2/28	2.4	1/42	7.1	3/42
RASSF1A	3.4	1/29	0.0	0/42	26.2	11/42
RASAL1	0.0	0/27	2.4	1/42	4.8	2/42
PDCD4	0.0	0/27	0.0	0/42	0.0	0/42
MTSS1	0.0	0/24	0.0	0/42	0.0	0/42
TUSC3	67.9	19/28	31.0	13/42	59.5	25/42

MGMT, O-6-methylguanine-DNA methyltransferase; *RASSF1A*, Ras association domain family member 1A; *RASAL1*, Ras protein activator like 1; *PDCD4*, programmed cell death 4; *MTSS1*, metastasis suppressor 1; *TUSC3*, tumor suppressor candidate 3.

with in the lung tissue: *MGMT,* 7.1% (2/28) vs. 2.4% (1/42); *RASSF1A,* 3.4% (1/29) vs. 0.0% (0/42); and *TUSC3,* 67.9% (10/28) vs. 31.0% (13/42). No methylation of any gene was detected in pooled blood DNA of healthy individuals.

Survival and clinicopathological parameters. There was no significant association between survival time and gender (P=0.864), smoking habits (P=0.322), tumor histology (P=0.788) and grading (P=0.301). However, patients diagnosed with lung cancer that were older than 60 years of age lived significantly longer (P=0.034) compared with patients that were younger than 60 years of age at the time of diagnosis. Furthermore, the association between survival time and detailed T-(tumor; detailed: Stadium subdivided in a and b), N (node)-, and M (metastasis)-stadium (P=0.019; P=0.000; P=0.000), tumor stage (P=0.000) and R-classification (P=0.017) reached statistical significance.

Promoter methylation and clinicopathological parameters. No statistically significant associations between promoter methylation and gender, age at diagnosis, smoking habits, lymph node and distant metastasis, grading, R-classification and tumor histology were observed for *MGMT, RASSF1A, RASAL1, PDCD4, MTSS1* and *TUSC3* and bronchus, lung and tumor tissue. However, tumor promoter methylation of *TUSC3* significantly associated with R0-resection (R0 vs. R1 and R2, P=0.021) and smaller tumor size (T1a, T1b and T2a vs. T2b, T3 and T4; P=0.008; Table III). The latter effect, however, disappeared when dismissing the subdivision of T-Stadiums in a and b (T1 and T2 vs. T3 and T4, P=0.235). TUSC3 was also significantly more often methylated in bronchus tissues in lower tumor stages (stage I and II vs stage III and IV, P=0.035). In addition, a slight tendency for higher methylation frequencies of *TUSC3* in bronchus (10 of 17 vs.9 of 11 patients) and lesser in tumor (13 of 23 vs. 12 of 19 patients) can be observed in patients >60 years at time of diagnosis.

Promoter methylation and survival. Patients with promoter methylation of *TUSC3* in tumor tissues lived significantly longer compared with those without this epigenetic modification (P=0.039; Fisher's exact test). The Kaplan-Meier curve is shown in Fig. 1 (P=0.013; log-rank test).

Discussion

In the present study, the promoter methylation status of *MGMT*, *RASSF1A*, *RASAL1*, *PDCD4*, *MTSS1* and *TUSC3* was quantitatively examined using a highly reliable methylation-sensitive qPCR, avoiding bisulfite-conversion.

In the patients studied, *MGMT* methylation was less frequently observed in lung tumor tissues compared with other studies, which describe methylation frequencies of 10.0-77.8% (3,4,6,8,12,16,17). In contrast to the present study, the majority of other studies did not analyze normal tissue; therefore, statements concerning the use of *MGMT* methylation in the early detection of lung cancer by analyzing non-malignant samples cannot be reasonably made. However, two reports (3,12) with normal tissues had greater methylation frequencies (~20%) compared with the 7.1% *MGMT* methylation found in bronchial or 2.4% found in lung specimens in the present study, which are similar to the 3% methylation reported by Safar *et al* (16).

The findings of low *RASSF1A* promoter methylation levels are in accordance with other reports, which describe methylation frequencies of 0.0-6.0% in normal tissues (5,13,16,37,38), 12.8% methylation in benign lung tissues (3) and 15.0-45.0% in NSCLC (3-5,7,8,13-16,37,38).

Differences in *MGMT* and *RASSF1A* methylation frequencies between the results of the present study and the results of previous studies may be due to different analysis methods. In contrast to the MethyQESD technique (36), previous studies used methods such as bisulfite-conversion, which bears the risk of incomplete conversion of unmethylated cytosines (39), leading to an overestimation of methylation. Furthermore, the use of methylation specific primers without enough discrimination capacity between methylated and unmethylated DNA can also result in false positive methylation results (39). In addition, cut off values are not mentioned (12), or set at 0% (8), or methylation is analyzed only qualitatively (3,4,6,14,15,17). Certain studies employed a nested PCR (3,6), which improves sensitivity but can lower specificity and reproducibility (40). This effect is further demonstrated by the experiments of Lee *et al* (38), which show that *MGMT* methylation differed depending on whether methylation specific PCR (qualitative analysis) or pyrosequencing (quantitative analysis) was used. Therefore, the comparability of methylation studies is limited.

Table III. Number of patients with positive methylation status and clinicopathological data.

	MGMT			*RASSF1A*			*RASAL1*			*TUSC3*		
Tissue	B	L	T	B	L	T	B	L	T	B	L	T
Gender												
Female	2/22	1/33	3/33	1/23	0/33	8/33	0/21	1/33	2/33	17/22	12/33	20/33
Male	0/6	0/9	0/9	0/6	0/9	0/9	0/6	0/9	0/9	2/6	1/9	5/9
Age												
≤60	2/18	1/23	2/23	0/18	0/23	6/23	0/17	1/23	2/23	10/17	7/23	13/23
>60	0/10	0/19	1/19	1/11	0/19	5/19	0/10	0/19	0/19	9/11	6/19	12/19
Survival												
Yes	1/8	0/13	1/13	0/9	0/13	2/13	0/8	0/13	0/13	7/9	6/13	11/13[a]
No	1/20	1/28	2/28	1/20	0/28	9/28	0/19	1/28	2/28	12/19	7/28	13/28[a]
Smoking												
Yes	2/26	1/37	3/37	1/27	0/37	9/37	0/27	1/37	2/37	19/26	11/37	22/37
No	0/2	0/5	0/5	0/2	0/5	2/5	0/2	0/5	0/5	0/2	2/5	3/5
T category												
T1a	0/2	1/8	1/8	0/3	0/8	2/8	0/2	1/8	1/8	3/3	2/8	6/8[b]
T1b	0/2	0/2	0/2	0/2	0/2	1/2	0/2	0/2	0/2	0/2	2/2	0/2[b]
T2a	1/10	0/16	1/16	1/10	0/16	3/16	0/10	0/16	0/16	8/10	5/16	14/16[b]
T2b	0/7	0/8	1/8	0/7	0/8	4/8	0/6	0/8	1/8	5/6	3/8	2/8[b]
T3	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/3	0/3	1/3	0/3	1/3[b]
T4	1/4	0/5	0/5	0/4	0/5	0/5	0/4	0/5	0/5	2/4	1/5	2/5[b]
N category												
N0	2/18	0/28	1/28	0/19	0/28	7/28	0/17	0/28	1/28	14/18	9/28	18/28
N1	0/8	1/11	2/11	1/8	0/11	3/11	0/8	1/11	1/11	4/8	2/11	5/11
N2	0/2	0/3	0/3	0/2	0/3	1/3	0/2	0/3	0/3	1/2	2/3	2/3
M category												
M0	2/26	1/38	3/38	1/27	0/38	10/38	0/25	1/38	2/38	19/26	11/38	23/38
M1	0/2	0/3	0/3	0/2	0/3	1/3	0/2	0/3	0/3	0/2	2/3	1/3
Grading												
G1	1/15	1/24	1/24	0/16	0/24	6/24	0/15	1/24	2/24	11/16	7/24	15/24
G2	1/13	0/18	2/18	1/13	0/18	5/18	0/12	0/18	0/18	8/12	6/18	10/18

Table III. Continued.

	MGMT			*RASSF1A*			*RASAL1*			*TUSC3*		
Tissue	B	L	T	B	L	T	B	L	T	B	L	T
Resection boundaries												
R0	2/26	1/38	2/38	1/26	0/38	11/38	0/25	1/38	2/38	18/26	11/38	25/38[c]
R1	0/1	0/3	1/3	0/1	0/3	0/3	0/1	0/3	0/3	1/1	1/3	0/3[c]
R2	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1[c]
Histology												
AC	1/11	0/17	0/17	0/11	0/17	6/17	0/11	0/17	0/17	6/11	3/17	12/17
SCC	0/13	1/20	2/20	1/14	0/20	4/20	0/12	1/20	2/20	10/13	9/20	11/20
LCA	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	2/3	1/3	1/3
Carc	0/1	0/2	1/2	0/1	0/2	1/2	0/1	0/2	0/2	1/1	0/2	1/2
Stage												
I	1/11	0/19	1/19	0/12	0/19	4/19	0/11	0/19	0/19	9/12[d]	6/19	15/19
II	0/7	1/10	2/10	1/7	0/10	4/10	0/6	1/10	2/10	6/6[d]	3/10	4/10
III	1/8	0/9	0/9	0/8	0/9	2/9	0/8	0/9	0/9	4/8[d]	2/9	4/9
IV	0/2	0/3	0/3	0/2	0/3	1/3	0/2	0/3	0/3	0/2[d]	2/3	1/3

Data are expressed as n/n_{total}. Groups with significant P-values from multiple comparison analysis: [a]P=0.039, [b]P=0.005, [c]P=0.021 and [d]P=0.041. *MGMT*, O-6-methylguanine-DNA methyltransferase; *RASSF1A*, Ras association domain family member 1; *RASAL1*, RAS protein activator like 1; *TUSC3*, tumor suppressor candidate 3; B, bronchus; L, lung; T, tumor; T/N/M category, tumor/node/metastasis category; AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; Carc, carcinoid.

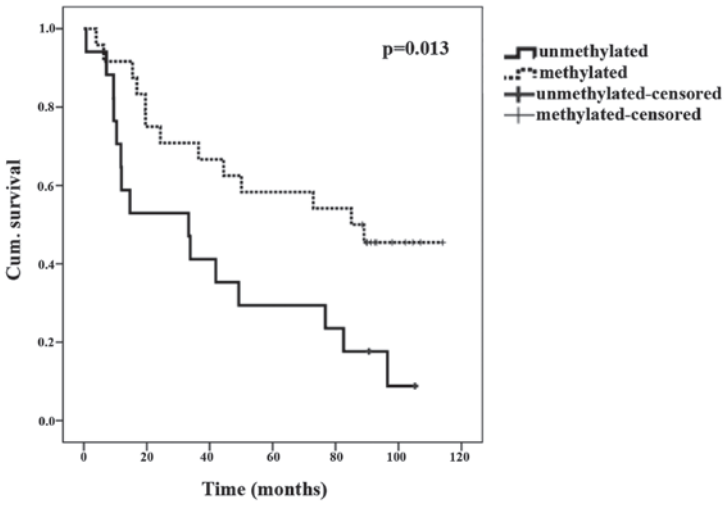

Figure 1. Kaplan-Meier overall survival curve. Dotted line, tumor patients with *TUSC3* methylation; solid line, patients without *TUSC3* methylation. *TUSC3*, tumor supressor candidate 3.

In the present study, only low levels or no methylation of *RASAL1* (tumor tissue, 4.8%; lung, 2.4%; bronchus, 0.0%) were observed, and consequently no association with clinicopathological parameters was found. To the best of our knowledge, only two other studies have dealt with methylation of *RASAL1* in lung carcinoma: Jin *et al* (18) observed methylation in 2/4 lung cancer cell lines; whereas Calvisi *et al* (20) reported a methylation frequency of 16.7% (5/30) in primary tumors, but did not describe the characteristics of the patient population nor the histology of the tumors analyzed. This is especially important as *RASAL1* shows tumor and tissue-specific expression (19) with increased expression levels in endocrine organs (19), which raises the question of whether *RASAL1,* in general, is a cancer relevant gene in lung adenocarcinoma.

In the present study, no methylation of *PDCD4* or *MTSS1* was found in any of the 3 tissue types. Promoter methylation of *PDCD4* and *MTSS1* genes has been previously examined in other tumors (41,42), but not yet in lung cancer. According to the current results, factors other than methylation could be involved in the regulation of the expression of *MTSS1*, such as binding of DNA (cytosine-5-)-methyltransferase 3β to the *MTSS1* 5' region (43,44), and *PDCD4*, such as transcription growth factor β (22), zinc finger protein 148 and histone modifications (44).

In contrast to the other analyzed genes, *TUSC3* showed frequent methylation in all three tissue types: Bronchus, 67.9% (19/28); lung, 31.0% (13/42); and tumor, 59.5%, (25/42). Thus, *TUSC3* promoter methylation could be an early event during bronchial tumor development, and the detection of *TUSC3* methylation could be beneficial for the early detection of lung cancer. Regarding prognostic aspects, the present study shows that patients with *TUSC3* methylation in tumor tissues lived significantly longer compared with patients without this epigenetic modification (Fig. 1). In addition, patients that were diagnosed with lung cancer when they were older than 60 years of age lived significantly longer (P=0.034) compared with patients diagnosed when they were younger than 60 years of age, and showed slightly more methylation in bronchus and tumor tissues, although without statistical significance. This finding could be due to an association between longer survival and *TUSC3* methylation. Tumor methylation was significantly associated with R0-resection and smaller tumor size as T-stadiums were subdivided into a and b. Although longer survival is an evident result, the potential causality between *TUSC3* methylation and longer survival remains to be elucidated.

In certain aspects, the present study contrasts with previous studies, which found an association between *TUSC3* methylation and a poorer prognosis or advanced tumor stage in other tumor types (31,33). Furthermore, the first evidence of a loss of *TUSC3* expression was found in metastatic prostate cancer (30), which implies that the loss of *TUSC3* expression may be associated with progressed disease in this tumor type. Contrarily, *TUSC3* can be reasonably assumed to have a non-oncogenic function due to a defect in *TUSC3* that was previously described to cause non-syndromic autosomal mental retardation without tumor formation in affected patients (45).

Finally, the methylation of *TUSC3* may potentially occur due to collateral damage along with other gene methylation events in the 8p22 chromosome region (46). Consequently, its loss could be without direct consequence for tumorigenesis. Previous studies showed that *TUSC3* methylation was not beneficial for tumor growth in one cell culture experiment (32), whereas in another experiment, it was (31).

In conclusion, the present study identified little or no promoter methylation of *MGMT*, *RASSF1A*, *RASAL1*, *PDCD4* and *MTSS1* in bronchial, lung and lung cancer tissues, but the relatively frequent methylation of *TUSC3* in the same tissues. The fact that *TUSC3* methylation was found to be associated with a longer survival time contradicts the hypothesis that *TUSC3* has a tumor suppressor function and underlines that *TUSC3* methylation has a prognostic value in lung cancer patients. In addition, the methylation of *TUSC3*, particularly in combination with other markers, may be useful for the early detection of lung cancer, as *TUSC3* was frequently observed in the tumor and benign bronchus and lung tissues of lung cancer patients, but not in pooled blood DNA of healthy individuals. Additional studies are required to clarify the functional role of *TUSC3* methylation in lung cancer. Prospective studies are

recommended to be undertaken to further evaluate *TUSC3* methylation as a prognostic biomarker and its usefulness for the early detection of disease in lung cancer patients.

Acknowledgements

The present study was supported by the Wilhelm-Sander-Foundation (grant no., 2000.127.1). The authors would like to thank Mrs Irene Schardt, Mrs Beate Reil, Mrs Sigi Appel and Mrs Jutta Förster for excellent technical assistance and Dr Corinna Lang-Schwarz for tissue sampling.

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