RESEARCH PAPER

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Low expression of ITIH5 in adenocarcinoma of the lung is associated with unfavorable patients' outcome

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Abbreviations: EGFR, Epidermal growth factor receptor; ITIH, inter-α-trypsin inhibitor heavy chain.

Inter-α-trypsin inhibitor heavy chain 5 (ITIH5) is supposed to be involved in extracellular matrix stability and thus may play a key role in the inhibition of tumor progression. The current study is the first to analyze in depth ITIH5 expression and DNA methylation, as well as its potential clinical impact in non-small-cell lung carcinoma (NSCLC). We examined *ITIH5* mRNA expression in tumor and adjacent normal lung tissue specimens of NSCLC patients. In addition, methylation frequency of the *ITIH5* promoter was investigated using methylation-specific PCR and pyrosequencing. Significance of our data was validated by independent data sets from The Cancer Genome Atlas and the Kaplan-Meier Plotter platform. Furthermore, ITIH5 protein expression was evaluated by immunohistochemistry utilizing a tissue microarray with 385 distinct lung tissue samples. Based on our tissue collections, *ITIH5* mRNA expression was significantly decreased in NSCLC compared to normal lung tissue in line with an increased methylation frequency in lung cancer tissue. Independent TCGA data confirmed significant expression loss of *ITIH5* in lung cancer concordant with *ITIH5* promoter hypermethylation in NSCLC. Of interest, low *ITIH5* mRNA expression was particularly found in the magnoid and squamoid ADC expression subtype, concordant with an unfavorable patients' outcome in squamoid as well as tobacco smoking ADC patients. In conclusion, *ITIH5* may be a novel putative tumor suppressor gene in NSCLC with a potential molecular significance in the squamoid ADC subtype and further clinical impact for risk stratification of adenocarcinoma patients. In addition, *ITIH5* may serve as a novel biomarker for prognosis of tobacco smoking ADC patients.

Introduction

Lung cancer is the most common cause of global cancer-related mortality, leading to over a million deaths each year.[1,2] Due to this high mortality rate, a thorough understanding of tumor biological and molecular processes is mandatory to enable individual prognosis and novel targeted therapies.

To date, targeted therapies for non-small-cell lung cancer (NSCLC), the most common bronchial tumor, have dramatically improved treatment for patients whose tumors harbor driver mutations or rearrangements resulting in constitutively active mutant signaling proteins, most commonly occurring in oncogenes such as *EGFR*, ALK, *HER2*, *BRAF*, *PIK3CA*, *AKT1*, *ROS1*, and *MAP2K1*.[3] Therapy involving tyrosine kinase inhibitors tailored to the genetic background of individual tumors can lead to improved clinical benefit compared to cytotoxic chemotherapy. However, up to 25% of NSCLC patients harbor clinically challenging *KRAS* mutations, which are currently not treatable by kinase inhibitors, or in other potentially clinical relevant oncogenic drivers (33%), resulting in conventional chemotherapy treatment.[3,4]

Within the last year, microarray expression profiling of adenocarcinoma of the lung, the most frequent histological type of NSCLC, revealed intrinsic molecular subtypes encompassing diverse functional pathways and patients' outcomes.[5-11] Using microarray data analysis, Hayes et al. originally defined 3 ADC gene expression subtypes, namely bronchioid, magnoid, and squamoid, dependent on transcriptomic similarities with histologically defined bronchioalveolar, large-cell, and squamous-cell carcinoma.[6] Transcriptional expression profiles co-occur with distinct mutations and alterations in patient tumors.[6,7] For instance, bronchioid ADC are generally of lower grade, have a higher proportion

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of *EGFR* mutations, occur predominantly in women and never-smokers, and have favorable overall survival.[6,7] In contrast, magnoid and squamoid ADC harbor more *KRAS* mutations, occur more often in men and smokers, and have poorer overall survival.[6,7] However, the clinical impact of these expression signatures is currently not well understood, thus limiting progress in targeted therapy development in ADC patients.[7,12]

Deciphering of novel biomarkers affecting molecular pathways within the distinct expression signatures of ADC is a critical challenge for efficient stratification of patients who will benefit from a particular therapy regimen improving patients' survival. In this context, the previously identified inter-α-trypsin inhibitor heavy chain 5 (ITIH5) could play a valuable biological and clinical role. Loss of ITIH5 expression in mammary, colon, and bladder tumors, due to frequent hypermethylation of the *ITIH5* promoter, was shown to be associated with malignant progression and unfavorable patients' outcome, indicating a putative tumor suppressor function in breast,[13] colon,[14] and bladder cancer.[15] Furthermore, the ITI heavy chains effectively stabilize the ECM.[16] and have been shown to suppress processes such as tumor invasion.[17] and metastasis,[18] while the biological relevance of ITIH5 in NSCLC has not been investigated yet.

The present study is the first to show that expression of ITIH5 is clearly deregulated in NSCLC, providing evidence for a potential role as tumor suppressor particularly in adenocarcinoma of the lung. Moreover, an unfavorable patients' outcome in squamoid as well as tobacco smoking ADC patients, due to the epigenetic silencing of the *ITIH5* gene promoter, point toward a possible clinical impact of ITIH5 in adenocarcinoma of the lung.

Results

ITIH5 expression is lost in non-small-cell lung carcinoma

In a recent study, we showed that *ITIH5* promoter hypermethylation is the molecular cause for *ITIH5* gene silencing in breast,[13] colon,[14] and bladder cancer,[15] which was associated with unfavorable patients' outcome. To investigate the biological relevance of ITIH5 in lung cancer we initially analyzed mRNA expression in 10 NSCLC tumor and 11 normal lung tissues by real-time PCR. We verified a significant ($P < 0.0001$) loss of *ITIH5* mRNA expression in tumor (median expression level: 0.0965) when compared to normal lung tissues (median expression level: 0.9003) (**Fig. 1A**). Available matched normal and tumor tissue pairs from the same patients (n = 3) confirmed expression loss in tumor compared to adjacent normal lung tissue.

To assess the significance of our data, we analyzed *ITIH5* gene expression in a large dataset of an independent study. Using data of The Cancer Genome Atlas (TCGA) we verified a prevalent loss of *ITIH5* gene expression in the major histological NSCLC subtypes, including ADC and SCC, when compared to adjacent normal lung tissue specimen (**Fig. 1B and C**). We found abundant *ITIH5* mRNA expression in ADC (median expression level: 216) compared to SCC (median expression level: 94) ($P < 0.0001$) (**Fig. 1C**). Of interest, stratifying ADC patients by expression subtypes illustrated a significant decrease of *ITIH5* mRNA expression, particularly in magnoid (median expression level: 136) and squamoid (median expression level: 178), contrary to bronchioid ADC (median FC: 370) ($P < 0.0001$) (**Fig. 1D** and **Table 1**). In addition, we could demonstrate a significant ($P < 0.05$) association of high *ITIH5* mRNA expression concerning female and lower stage ADC patients (**Table 1**), while there were no significant correlations with any clinicopathological parameter in SCC patients.

In concordance to the mRNA expression data, immunohistochemistry analysis based on a well-established polyclonal anti-ITIH5 antibody, confirmed abundant ITIH5 expression in epithelial cells of healthy respiratory tissue (**Fig. 2A**). As expected from the RNA expression pattern, NSCLC tumor cells showed decreased ITIH5 protein staining (**Fig. 2B-D**) or almost complete loss of ITIH5 protein (**Fig. 2E and F**). An association of ITIH5 protein expression with clinical data of the tissue microarray can be found in **Table S1**.

Decreased ITIH5 expression is caused by promoter hypermethylation, particularly in adenocarcinoma of the lung

Next, to prove if promoter methylation could be responsible for ITIH5 expression loss in lung cancer, we analyzed a set of 13 ADC and 14 SCC tissue samples by qualitative methylation-specific PCR (MSP). Methylation frequency of the *ITIH5* gene promoter was 50% in SCC (i.e., 7 of 14 samples were methylated) and 54% in ADC (i.e., 7 of 13 samples were methylated). Considering the highest methylation value (6%) in normal lung tissue as the cut-off value, quantitative pyrosequencing in our independent cryoconserved tissue collection revealed a methylation frequency of 66% in ADC (i.e., 10 of 15 samples were methylated) as well as SCC (i.e., 4 of 6 samples were methylated) (**Fig. 3A**). Median methylation was significantly ($P < 0.05$) increased in ADC and SCC tissue samples compared to adjacent normal lung tissue, while ADC patients showed a higher median methylation (18%) in contrast to SCC patients (8.5%).

Based on the TCGA data set, CpG sites within the *ITIH5* promoter that are closely located to the transcription start were commonly found methylated in primary lung cancer samples (**Fig. 3B**). In line with the data from our cryoconserved tissue collection, median methylation frequency of the *ITIH5* gene promoter in the TCGA data were increased in ADC (including all ADC-specific expression subtypes) compared to SCC (**Fig. 3C**). To further examine the relation between *ITIH5* mRNA expression loss and DNA-methylation, we performed a spearman correlation analysis between *ITIH5* mRNA expression and methylation. This analysis revealed a higher inverse association between *ITIH5* mRNA expression and DNA methylation in ADC (spearman r: −0.4053, $P < 0.0001$) (**Fig. 3D**) compared to SCC (spearman r: −0.2778, $P < 0.0001$).

A functional association between *ITIH5* promoter methylation and *ITIH5* gene silencing was further supported by *in vitro* demethylation experiments using 2 different lung cancer cell lines lacking endogenous *ITIH5* mRNA expression (SK-MES-1 (human squamous cell carcinoma) and SK-LU-1 (human adenocarcinoma). Real-time PCR analyses showed a clear re-expression

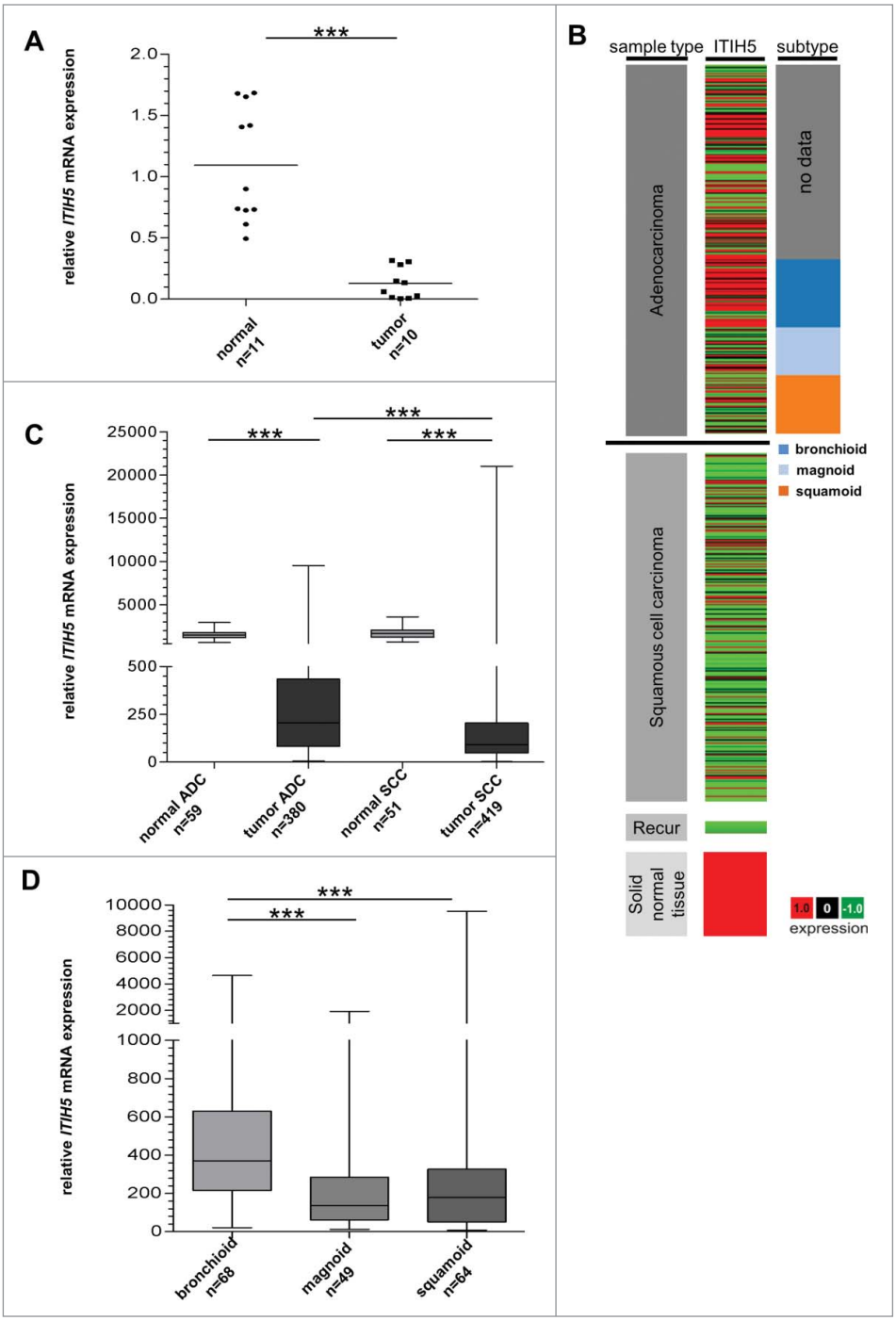

Figure 1. *ITIH5* gene expression is lost in NSCLC cancer tissue. (**A**) *ITIH5* mRNA expression is strongly decreased in NSCLC compared with normal lung tissue. Box plot analysis illustrates reduced *ITIH5* mRNA expression in tumor tissue with a median expression level of 0.1402 compared to normal lung tissue (median expression level: 0.9003). Horizontal lines: grouped medians. ***$P < 0.0001$. (**B**) Tumor samples (based on TCGA Illumina HiSeq mRNA expression platform) are divided in adenocarcinoma (n = 380), squamous cell carcinoma (n = 419), recurrent ("Recur") tumors (n = 2), and normal tissue samples (n = 110) (left panel). The middle panel illustrates relative values of *ITIH5* mRNA expression: red (high expression) and green (low expression). Additionally, adenocarcinoma are stratified by expression subtype: bronchioid (n = 68), magnoid (n = 49), and squamoid (n = 64) (right panel). (**C**) Box plot analysis of *ITIH5* mRNA expression in adenocarcinoma and squamous cell carcinoma compared to adjacent normal lung tissue. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak, and minimum. ***$P < 0.0001$. (**D**) Box plot analysis illustrates decreased expression of the *ITIH5* gene in magnoid as well as squamoid adenocarcinoma. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak, and minimum. ***$P < 0.0001$.

of *ITIH5* after 5-aza-2′-deoxycytidine (DAC) and trichostatin A (TSA) treatment in both cell lines compared to untreated cells (**Fig. 4A**), while methylation pattern in SK-MES-1 remained stable in contrast to SK-LU-1 cells (**Fig. 4B**).

Abundant ITIH5 expression predicts favorable outcome in patients diagnosed with squamoid ADC, with a pronounced clinical impact in tobacco smokers

To reveal whether *ITIH5* mRNA expression has an impact on patients' survival, a descriptive data analysis was performed with overall survival (OS) and recurrence free survival (RFS) data of the TCGA and the Kaplan-Meier Plotter (KMP) platform. RFS and OS data of the TCGA platform were compared between invasive ADC showing abundant *ITIH5* mRNA expression (median expression ≥204) and SCC (median expression ≥90) by univariate statistics (**Table 2**). Concerning both ADC and SCC patients, KMP analysis revealed no prognostic impact of a strong *ITIH5* mRNA expression concerning longer RFS or OS. However, with respect to a low *ITIH5* mRNA expression in magnoid and squamoid ADC, we performed stratified univariate analysis within the expression subgroups. Interestingly, a favorable RFS could be demonstrated in the subgroup of squamoid ADC with abundant *ITIH5* expression. Squamoid ADC patients with higher *ITIH5* mRNA expression had an estimated median RFS of 2.96 years [95% confidence interval (CI): 1.36 – 4.57], compared to 1.86 years (95% CI: 1.07 – 2.65) in patients with decreased *ITIH5* expression ($P = 0.027$) (**Fig. 5**). Next, we calculated a multivariate Cox regression model, including all factors potentially influencing the RFS in ADC, but statistical

Table 1. Clinicopathological parameters in relation to *ITIH5* mRNA expression in ADC of the lung using the TCGA data platform

Variable		*ITIH5* mRNA expression			
Clinicopathological factors	**n[a]**	**low≤ 204[b]**	**high> 204[b]**	***P*-value[c]**	**r-value[d]**
Age at diagnosis (median: 65 years)					
≤ 65 years	185	99	86	0.128	0.080
> 65 years	178	81	97		
gender					
Female	205	91	114	0.018	0.121
Male	175	99	76		
Tumor size					
pT1–2	331	164	167	0.649	−0.022
pT3–4	47	25	22		
Lymph node status					
pN0	242	116	126	0.304	−0.053
pN1–3	129	71	58		
Tumor stage					
Stage I	209	94	115	0.026	−0.114
Stage II-IV	170	96	74		
Expression subtype					
Bronchioid	68	15	53	<0.001	−0.297
Magnoid	49	35	14		
Squamoid	64	36	28		
KRAS mutation status					
No mutation	127	64	63	0.236	0.088
mutation	54	22	32		
EGFR mutation status					
No mutation	158	77	81	0.392	0.064
Mutation	23	9	14		
ITIH5 methylation					
Low (≤47.4%)	162	64	99	0.001	−0.187
High (>47.4%)	170	98	71		

Significant *P*-values marked in bold face.
[a]Only patients with primary, invasive adenocarcinoma of the lung were included.
[b]Median *ITIH5* mRNA expression values.
[c]Fisher's exact test at a 2-sided significance level of 0.05.
[d]Pearson correlation coefficient.

independency was missed. Of clinical relevance, evaluating data of the KMP platform, strong *ITIH5* mRNA expression indicated a favorable outcome in tobacco smoking ADC patients (smoker at some point) (**Fig. S1**): Smoker patients with low *ITIH5* expression had a worse OS compared to smoker patients showing high *ITIH5* expression. In contrast, ITIH5 expression revealed no influence on patients' survival within the group of non-smoking adenocarcinoma patients.

To give a first insight into the close association of *ITIH5* mRNA expression regarding the smoking-related mechanism of ADC patients, we re-analyzed a published transcriptomic microarray analysis consisting of 58 tissue samples of adenocarcinoma and 49 paired noninvolved lung tissue from current (n = 40), former (n = 36), and never smokers (n = 31).[19] Again, array-based class comparison analysis revealed a significant ($P < 0.0001$) downregulation of *ITIH5* mRNA expression in ADC cancer specimen compared to adjacent normal tissue samples (FC: 2.25). Besides, we identified more than 962 genes that are predominately downregulated in current compared to never smokers and 986 genes in former compared to never smokers, including *ITIH5*. Therewith, *ITIH5* loss seems to be part of a common gene signature typical for the tobacco-smoking patients. A part of this signature, including *ITH5*, is shown as heatmap in **Figure S2**.

Discussion

Today, several lines of evidence suggest a potential role of ITIH5, a member of the ITIH family, in tumor biology, particularly in the prevention of tumor development and progression.[13,20,21] We previously showed that loss of ITIH5 expression in breast,[13] bladder,[15] and colon cancer,[14] caused by aberrant promoter hypermethylation, is associated with unfavorable prognosis. However, molecular relevance of ITIH5 in lung cancer remains elusive. The current study is the first to analyze in depth ITIH5 expression and DNA methylation, as well as its potential clinical impact toward NSCLC.

Initially, we verified, by both real-time PCR and immunohistochemistry, that ITIH5 was downregulated in human NSCLC tissue, suggesting that ITIH5 expression is lost in the course of tumor progression. To prove the accuracy of our results, we further analyzed independent *ITIH5* mRNA expression data of the TCGA platform in NSCLC samples. In line,

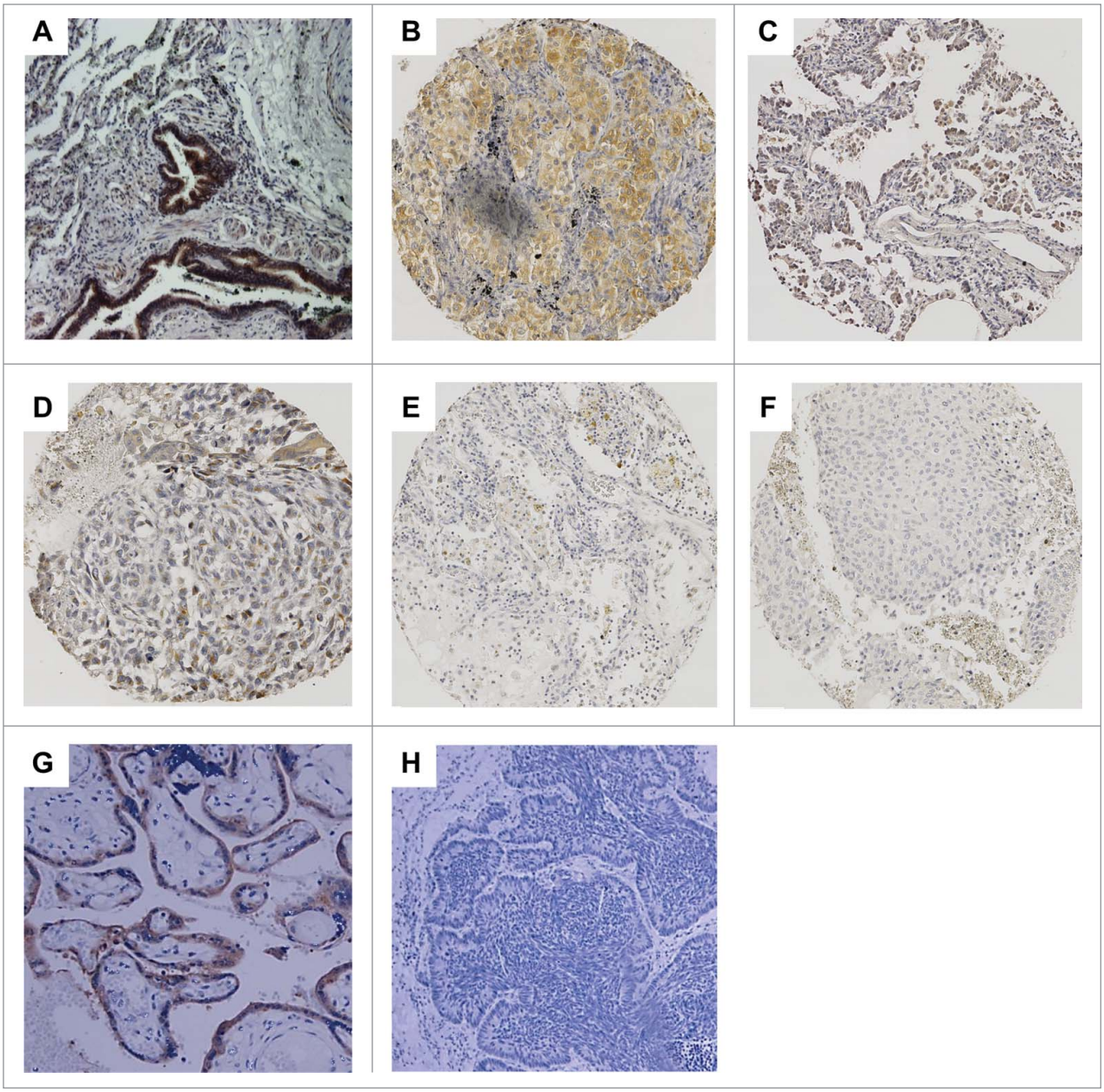

Figure 2. Loss of ITIH5 protein expression in human lung cancer. (**A**) Strong ITIH5 expression in epithelial cells of normal respiratory tissue. (**B–D**) Moderate ITIH5 immunoreactivity in adenocarcinoma cells (**B and C**) and squamous cell carcinoma (**D**). (**E**) and (**F**) Very low staining in squamous cell carcinoma. (**G**) Strong ITIH5 protein expression in placenta tissue that served as positive control for ITIH5 staining. **(H)** Negative control of adenocarcinoma lung tissue. The application of primary antibody was omitted. Magnifications: (**A, G, and H**): × 200; (**B, C, D, E, and F**): × 100.

TCGA data analyses revealed a significantly decreased *ITIH5* mRNA expression in NSCLC compared to normal lung tissue. Furthermore, TCGA data illustrated a significantly higher *ITIH5* mRNA expression in ADC compared to SCC of the lung. To analyze the molecular cause of downregulation in ADC and SCC, we investigated the epigenetic configuration of the *ITIH5* gene promoter, as it is known that the *ITIH5* promoter sequence contains distinct CpG islands. Based on our cryoconserved tissue collection analyzed with quantitative pyrosequencing, we demonstrated a higher median methylation level in ADC compared to SCC tumor specimen. Moreover, hypermethylation of distinct CpG dinucleotides within the *ITIH5* gene promoter discriminate significantly between lung cancer and normal tissue specimen. In line, TCGA data analyses confirmed the results of our independent cryoconserved tissue cohort by indicating a higher methylation frequency in ADC (median methylation: 47%) compared to SCC (median methylation: 20%). Concerning this data, one may hypothesize that *ITIH5* promoter methylation is the leading molecular mechanism regulating *ITIH5* gene expression in ADC, while in SCC further mechanisms may exist, resulting in low *ITIH5* gene expression in unmethylated SCC. Indeed, *in vitro*

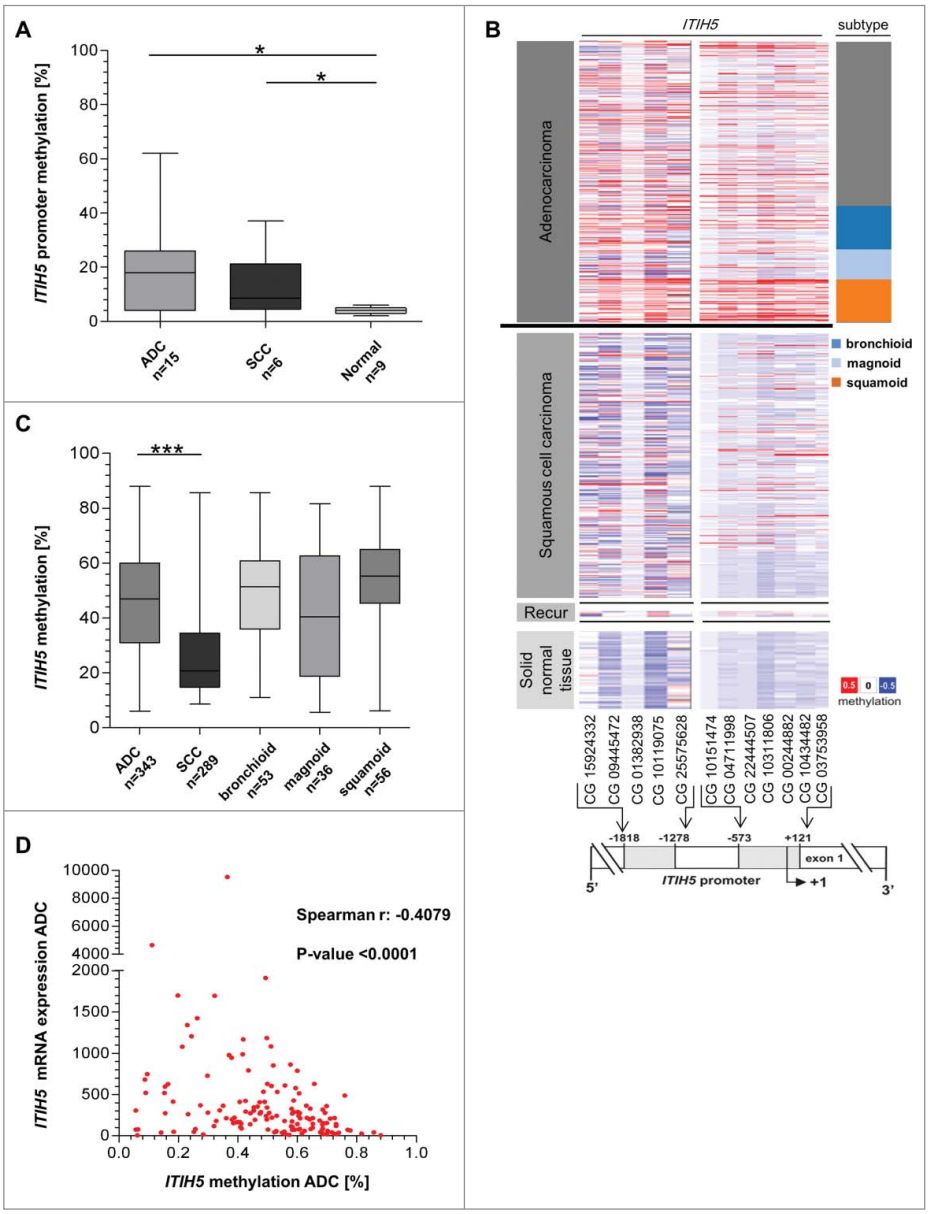

Figure 3. Expression of *ITIH5* mRNA correlates with epigenetic inactivation particularly in adenocarcinoma. (**A**) Box plot diagram illustrates *ITIH5* promoter hypermethylation between adenocarcinoma (ADC) (n = 15), squamous cell carcinoma (SCC) (n = 6), and normal lung (n = 9) specimens, based on quantitative pyrosequencing analysis. (**B**) DNA hypermethylation (Illumina HumanMethylation450 platform) of the *ITIH5* promoter analyzed in lung cancer samples from the TCGA data portal. Tumor samples are divided in adenocarcinoma (n = 329), squamous cell carcinoma (n = 241), recurrent ("Recur") tumors (n = 2), and normal tissue samples (n = 88) (left panel). The middle panel illustrates relative values of *ITIH5* DNA hypermethylation for each CpG duplet: red (high methylation), white (mean methylation), and blue (low methylation). The relative positions of 12 analyzed CpG duplets (−1818 bp to +121 bp; 5′ to 3′) are indicated within a schematic map of the human *ITIH5* promoter region. +1: *ITIH5* transcription start site. The right panel shows the adenocarcinoma expression type bronchioid (n = 53), magnoid (n = 36), and squamoid (n = 56). (**C**) Box plot analysis demonstrates a significantly higher *ITIH5* methylation level in all adenocarcinoma expression subtypes of the TCGA data portal compared to squamous cell carcinoma. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum, $^{*}P < 0.05$, $^{***}P < 0.001$. (**D**) Scatter plot illustrates the association between expression (Illumina HiSeq mRNA expression platform) and DNA methylation status (Illumina HumanMethylation450 platform) of *ITIH5* in 149 primary adenocarcinoma samples based on available TCGA data. Spearman correlation coefficient: r = −0.4053, $P < 0.0001$.

demethylation of the human ADC cell line SK-LU-1 and the human SCC cell line SK-MES-1 revealed restoration of *ITIH5* mRNA expression in both cell lines, but *ITH5* promoter DNA methylation in SK-MES-1 cells remained stable. Additionally, we demonstrated a pronounced inverse correlation (r = −0.4079) of *ITIH5* methylation and mRNA expression in ADC compared to SCC (r = −0.2778), strengthening promoter hypermethylation as the molecular cause of the *ITIH5* loss in adenocarcinoma of the lung.

The predominant association of *ITIH5* gene expression and promoter DNA methylation in ADC prompted us to take a deeper look into the putative impact of ITIH5 within the complex histological group of lung adenocarcinoma. In 2006, Hayes et al.[6] described 3 major intrinsic molecular subtypes in ADC of the lung showing distinct gene expression patterns associated with significant morphologic and molecular heterogeneity.[5,8-11] Clinical evaluation of these potentially intrinsic subtypes of ADC confers new opportunities for therapeutic strategies in the management of lung cancer patients, as it has successfully been demonstrated for the intrinsic breast cancer subtypes.[22,23] Recently, new classification systems and developments at the molecular level using microarray expression profiling of ADC patients revealed further intrinsic molecular subtypes encompassing diverse functional pathways and patients' outcomes.[24] Several studies confirmed an unfavorable prognosis of ADC patients with squamoid compared to those with bronchioid gene expression pattern.[6] Contrary to the bronchioid subtype, a decreased *ITIH5* mRNA expression was observed in magnoid and squamoid ADC. The low *ITH5* mRNA expression in magnoid and squamoid ADC, in line with the different clinical characteristics of distinct expression subtypes, prompted us to perform a stratified univariate survival analysis in bronchioid compared to magnoid and squamoid ADC. Of clinical interest, we revealed a linkage of abundant *ITIH5* mRNA expression with favorable RFS concerning the squamoid subtype. Because of its epigenetic mediated silencing, *ITIH5* may be a potential novel

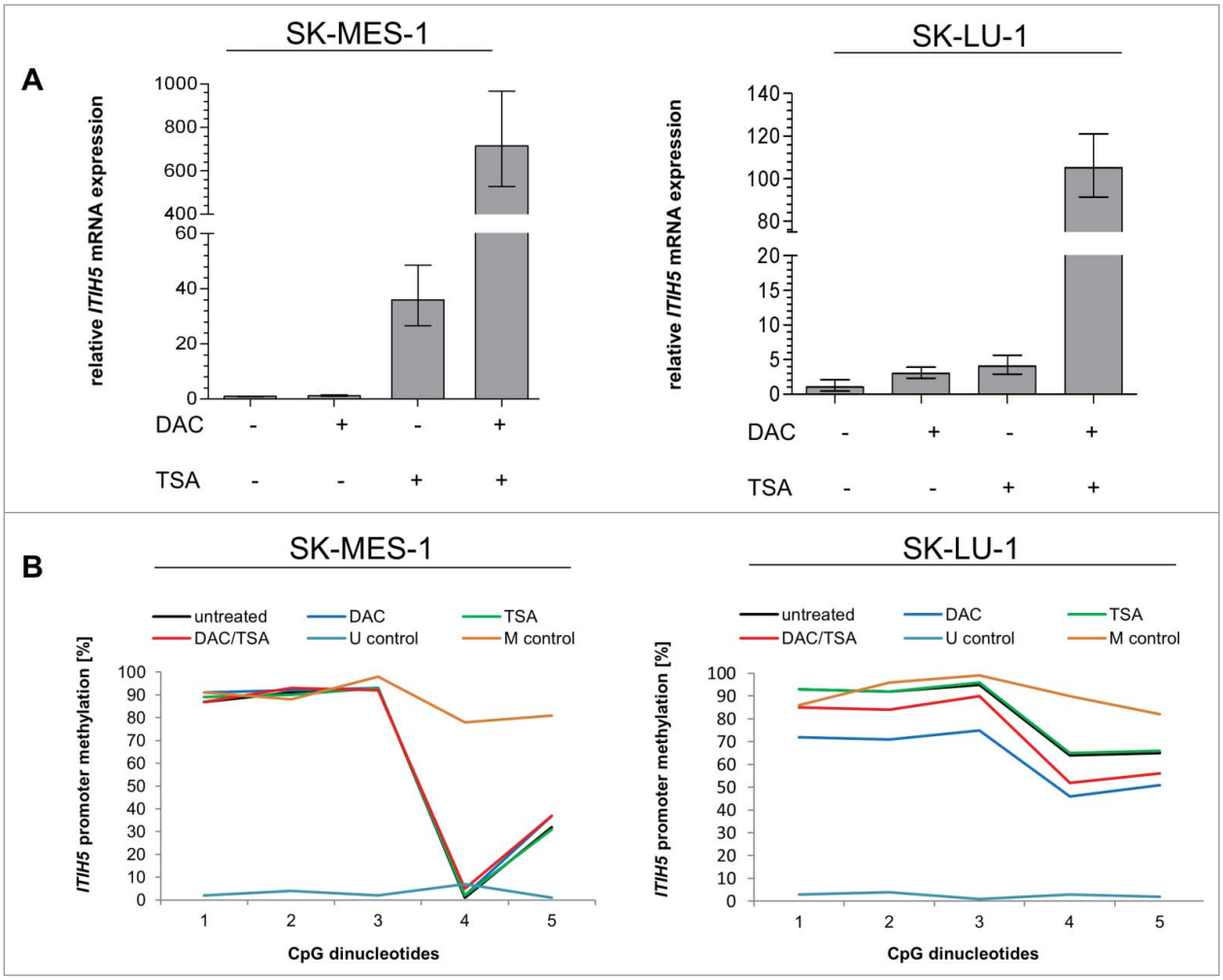

Figure 4. *ITIH5* re-expression after *in vitro* demethylation. (**A**) Semi quantitative real-time PCR showing *ITIH5* mRNA expression without and after treatment with 5-aza-2′-deoxycytidine (DAC) and Trichostatin A (TSA) in human SK-MES-1 and SK-LU-1 cells. Gain of *ITIH5* mRNA expression is indicated as fold-change relative to each baseline expression. Expression of *GAPDH* served as a control for equal starting amounts of cDNA. Relative Y-axis scaling is related to non-treated SK-MES-1 and SK-LU-1 cells (set to one). SEM was derived from triplicate experiments. (**B**) Quantitative *ITIH5* promoter methylation analysis of 5 CpG sites of SK-MES-1 and SK-LU-1 cells. Pyrogram of non-treated as well as DAC and TSA treated cells. Unmethylated and *in vitro* methylated DNA (Qiagen, Hilden, Germany) served as control.

tumor suppressor gene in ADC, particularly in the challenging clinical subgroups of squamoid and magnoid ADC, displaying an impact on tumor development.

Additionally, a subscribed molecular feature of squamoid adenocarcinoma of the lung is frequent *KRAS* mutation, while, in contrast, bronchioid ADC harbor mainly *EGFR* driver mutations.[7] Interestingly, KMP data revealed a clear impact of abundant *ITIH5* mRNA expression in tobacco-smoking patients, often showing multiple mutations, including a *KRAS* driver mutation,[25,26] compared to non-smoking patients, who are known to have more frequent *EGFR* mutations. Hence, there is still a lack of suitable prognostic biomarkers for risk stratification in ADC patients showing *KRAS* mutation. Accordingly, all patients harboring *KRAS* mutation receive adjuvant chemotherapy, while the benefit is still controversial. In this context, ITIH5 may represent a novel prognostic biomarker with clinical utility in squamoid and *KRAS* mutated adenocarcinoma patients helping to estimate favorable patients' outcome. Of interest, based on *in silico* transcriptomic microarray analysis, we found a decreased *ITIH5* expression level related to a common gene signature in tobacco-smoking ADC patients, providing strong evidence that *ITIH5* loss is associated with smoking-related mechanism of ADC tumorigenesis. Nevertheless, the biological role of ITIH5 in the subtype- and smoking-related signaling pathway has to be unraveled in further studies.

In conclusion, these findings provide for the first time evidence that *ITIH5* could act as a tumor suppressor gene in normal lung tissue. In addition, *ITIH5* is potentially valuable as a prognostic biomarker for the clinically important group of patients with squamoid adenocarcinoma, whose disease management has to be adjusted to a personalized progression risk. Moreover, abundant *ITIH5* expression might be an improved novel biomarker concerning tobacco-smoking ADC patients, with prognostic significance for patient survival. Further investigation of

Table 2. Univariate analysis of clinicopathological factors regarding overall survival (OS) and recurrence-free (RFS) in ADC patients

Variable	OS			RFS		
Clinicopathological factors	**n[a]**	**Events**	***P*-value[b]**	**n[a]**	**Events**	***P*-value[b]**
Gender						
Female	113	28	0.168	113	27	0.715
Male	131	34		131	43	
Tumor size						
pT1–2	214	52	0.004	214	59	0.024
pT3–4	28	10		28	10	
Lymph node status						
pN0	157	26	0.037	157	35	0.027
pN1–3	80	35		80	4	
Tumor stage						
Stage I	133	19	0.001	133	29	0.030
Stage II-IV	110	43		110	41	
Expression subtype						
Bronchioid	33	10	0.642	33	10	0.014
Magnoid	20	8		20	11	
Squamoid	37	15		37	15	
KRAS mutation status						
No mutation	66	26	0.605	66	26	0.067
Mutation	24	7		24	10	
EGFR mutation status						
No mutation	78	26	0.064	78	30	0.557
Mutation	12	7		12	6	
ITIH5 expression						
Low (≤204)	108	30	0.934	108	39	0.113
High (>204)	124	28		124	29	
ITIH5 methylation						
Low (≤47.4%)	112	29	0.403	112	38	0.018
High (>47.4%)	108	26		108	26	

[a]Only patients with primary, invasive adenocarcinoma of the lung were included.
[b]Fisher's exact test at a 2-sided significance level of 0.05.

the contribution of ITIH5 to lung cancer progression concerning the potentially biological relevance in the ADC-specific intrinsic expression subtypes displaying distinct clinical characteristics may help to understand underlying pathways in more detail, finally helping to improve disease management.

Material and Methods

FFPE and cryoconserved patient samples

DNA methylation was analyzed using formalin-fixed, paraffin-embedded (FFPE) tissue collection, including ADC (n = 13) and SCC (n = 14) of the lung. In addition, an independent cryoconserved tissue collection for *ITIH5* mRNA expression and DNA methylation analysis from patients with primary NSCLC, including ADC (n = 17) and SCC (n = 6), as well as adjacent normal tissue (n = 11), were obtained from the RWTH centralized biomaterial bank (RWTH cBMB; http://www.cbmb.rwth-aachen.de). All patients gave informed consent for retention and analysis of their tissue for research purposes (local ethical review board of the medical faculty of the RWTH Aachen, ref no. EK-206/09). Hematoxylin and eosin-stained sections were prepared for assessment of the percentage of tumor cells, only samples with >70% tumor cells were selected. An overview of the clinical characteristics of the patients is summarized in **Tables S2 and S3**.

In silico patient samples

Data from primary NSCLC tissues, including ADC and SCC of the lung, recurrent lung cancer tissues, and solid normal lung tissues were used from The Cancer Genome Atlas (TCGA), comprising patients data of 2 independent platforms: Illumina Infinium DNA methylation [HumanMethylation450 array and IlluminaHiSeq mRNA expression (ADC, n = 398; SCC, n = 419)]. An overview of the clinical characteristics of the patients is summarized in **Table S4**. In addition, data of the KMP portal was used to analyze a possible prognostic influence of abundant *ITIH5* mRNA expression in ADC patients.[27]

Cell lines and reagents

The human adenocarcinoma cell line SK-LU-1 and the human squamous carcinoma cell line SK-MES-1 were obtained, tested, and authenticated from Cell Lines Service (Eppelheim, Germany) and were resuscitated before using in experiments. Used cell lines were regularly tested for mycoplasma infection using the PCR-based Venor® GeM Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany).

Nucleic acid extraction and reverse transcription PCR

Genomic DNA from FFPE and cryoconserved NSCLC as well as normal lung tissue samples was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. By using TRIzol reagent (Invitrogen, Darmstadt, Germany) total cellular RNA from cell culture and tissue specimen was prepared. cDNA was synthesized using the reverse transcription system (Promega, Madison, WI) as previously described.[13]

Semiquantitative real-time PCR

cDNAs were amplified by semiquantitative real-time PCR using SYBR-Green PCR mix (Bio-Rad Laboratories, Munich, Germany) performed in an iCycler IQ5 (Bio-Rad Laboratories). Gene expression was quantified by the comparative C_T method, normalizing C_T-values to the housekeeping gene *GAPDH*, and calculating relative expression values.[28] All primers used spanned at least one intron, and were described earlier.[15] To ensure experiment accuracy, all reactions were performed in triplicate.

DNA bisulfite modification

The extracted tissue DNA was bisulfite-converted using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA), as previously described.[13]

AZA/TSA treatment

A demethylation treatment of the lung cancer cell lines SK-LU-1 and SK-MES-1 was performed as previously described.[29]

Pyrosequencing

Pyrosequencing analysis of a distinct *ITIH5* promoter region was performed by using the PyroMark PCR Kit (Qiagen) for initial fragment amplification. Afterwards, the PyroMark96 ID

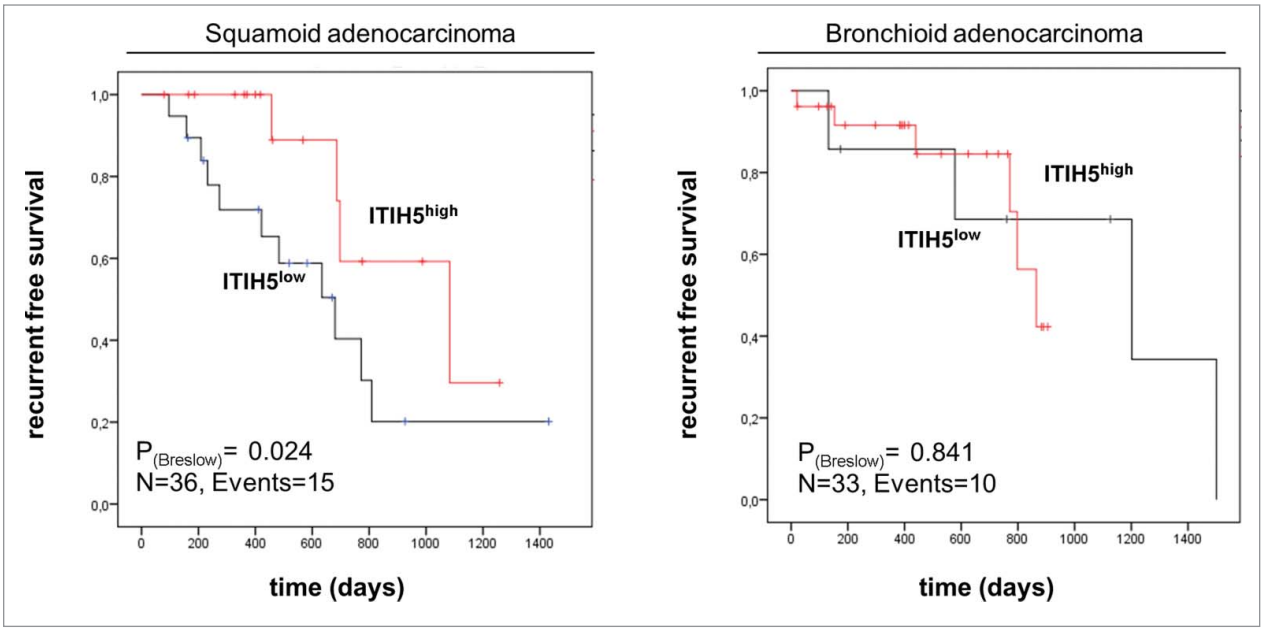

Figure 5. Univariate survival analysis of *ITIH5* mRNA expression according to the Kaplan–Meier Plotter (KMP) method reveals a favorable RFS in patients with squamoid adenocarcinoma. KMP analysis illustrating a prognostic value for *ITIH5* in the clinically challenging group of patients with squamoid adenocarcinoma of the lung (which is not demonstrable in bronchioid adenocarcinoma patients). Red line: abundant *ITIH5* expression (median $\geq$ 204); black line: weaker *ITIH5* expression (median $<$ 204). Vertical lines: censored cases.

device and the PyroGoldSQA Reagent Kit (Qiagen) were implemented as previously described.[30] The *ITIH5* assays were designed by using the Pyromark Assay Design Software (Qiagen). Primers were described previously.[15]

ITIH5 immunohistochemistry

A lung cancer-specific tissue microarray (TMA) was kindly provided by Prof. I. Petersen (Institute of Pathology, University Hospital Jena) previously published by his working group.[31] The TMA was constructed containing 380 lung cancer samples including SCC (n = 194), ADC (n = 83), non-small-cell lung cancer not otherwise specified (n = 47), lung cancer metastases (n = 29), neuroendocrine tumors (n = 10), large cell lung cancer (n = 7), and small-cell lung cancer (n = 10). Patients were operated between the years 1999 and 2002. Immunohistochemical staining was performed as previously described.[15] with slight modifications. TMA slides were incubated with a polyclonal ITIH5 rabbit anti-human antibody (1:50) (Atlas Antibodies, Stockholm, Sweden). FFPE sections of non-cancerous placenta tissue served as positive control.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (SPSS, Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). The non-parametric Mann-Whitney U-test was used in order to compare *ITIH5* mRNA expression between tumor and normal lung tissue, as well as ADC and SCC tumor specimen. Differences were considered statistically significant if the 2 sided *P*-values were equal or below 5% ($\leq$0.05).

ITIH5 mRNA expression and DNA methylation pattern in human NSCLC samples and adjacent normal lung tissue were assessed using 2 independent own tissue collections, as well as an independent public data set (TCGA). Correlation of the *ITIH5* mRNA expression (TCGA Illumina sequencing platform) and *ITIH5* methylation data (TCGA HM450 platform) was performed by calculating a Spearman correlation coefficient. Recurrence-free survival (RFS) was measured from surgery until local or distant relapse and was censored for patients alive without evidence of relapse at the last follow-up. Multivariate Cox-regression analysis was carried out to test for an independent prognostic value of *ITIH5* mRNA expression. Selection of the prognostic factors to be included in the multivariate model was based on statistical significance in univariate Breslow tests.

Re-analysis of gene expression using HG-U133A Affymetrix array to compare tobacco-smoking to never-smoking lung cancer patients.[19] was performed using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team version 4.4.0 – Stable. In order to significantly identify genes differentially expressed between 2 classes the *class comparison* evaluation was used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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