RESEARCH PAPER

Epigenetics 9:9, 1290–1301; September 2014; © 2014 Landes Bioscience

Epigenetic inactivation of the novel candidate tumor suppressor gene *ITIH5* in colon cancer predicts unfavorable overall survival in the CpG island methylator phenotype

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Keywords: ITIH5, tumor suppressor gene, DNA methylation, CpG island methylator phenotype, microsatellite instability, proximal colon

Abbreviations: AUC, area under the curve; cBMB, centralized biomaterial bank; CIMP, CpG island methylator phenotype; cfDNA, circulating-free DNA; C_T, cycle threshold; DNA, deoxyribonucleic acid; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *ITIH5*, Inter-alpha-trypsin inhibitor chain 5; MSI, microsatellite instability; qMSP, quantitative methylation-specific PCR; PCR, polymerase chain reaction; PMR, percentage of methylated reference; ROC, receiver operating characteristics

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 as well as its potential clinical and functional impact in colon cancer. Based on 30 tumor and 30 adjacent normal tissues we examined ITIH5 mRNA expression and promoter methylation, whose significance was further validated by independent data sets from The Cancer Genome Atlas (TCGA) platform. In addition, ITIH5 protein expression was evaluated using immunohistochemistry. ITIH5 mRNA expression loss was significantly associated ($P < 0.001$) with hypermethylation of the ITIH5 promoter in primary colon tumors. In addition, treatment of tumor cell lines with demethylating (DAC) and histone acetylating (TSA) agents induced ITIH5 expression. In line, independent TCGA data revealed a significant expression loss of ITIH5, particularly in the MSI-high and CIMP-positive phenotype concordant with an increased ITIH5 hypermethylation in CIMP-positive colon tumors ($P < 0.001$). In proximal, i.e., right-sided tumors, abundant ITIH5 expression was associated with longer overall survival (OS, $P = 0.049$) and the CIMP-positive ($P = 0.032$) subgroup. Functionally, ITIH5 re-expression mediated a reduced proliferation in HCT116 and CaCo2 cells. In conclusion, our results indicate that ITIH5 is a novel putative tumor suppressor gene in colon cancer with a potential impact in the CIMP-related pathway. ITIH5 may serve as a novel epigenetic-based diagnostic biomarker with further clinical impact for risk stratification of CIMP-positive colon cancer patients.

Introduction

Worldwide, over 1.2 million people are diagnosed with colorectal cancer (CRC) annually, and approximately one-half of CRC patients die from the disease.[1] Due to this high mortality rate, a thorough understanding of tumor biological and molecular processes in colon cancer is mandatory to enable individual prognosis and novel targeted therapies.

To date, current prognostic factors[2] with clear significance, helping to define high risk for progression or recurrence in CRC patients, refer mainly to the chromosomal instability pathway (CIN)[3] and the microsatellite instability pathway (MSI).[4] The latter is observed in approximately 15% of sporadic CRC[5-7] and in the majority of patients with hereditary non-polyposis colorectal cancer (HNPCC).[6,7] MSI reflects the presence of a defective mismatch repair (MMR) mechanism characterized by alterations of repetitive microsatellite nucleotide sequences throughout the genome.[8,9] Beyond germline mutations of the mismatch repair gene *MHL1*, acquired hypermethylation of the *MLH1* promoter is a known epigenetic mechanism that occurs mainly in sporadic colon cancer within the CpG island methylator phenotype (CIMP).[10,11] CIMP results in transcriptional silencing of specific tumor suppressor and DNA repair genes, including *MLH1*,[12,13] while the molecular mechanisms underlying CIMP is still elusive. However, CIMP-positive colon cancers appear to have a distinct molecular and clinical profile including proximal tumor location, high frequency of mutation of the proto-oncogene *BRAF,* low frequency of mutation of the proto-oncogene *KRAS,*

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Submitted: 06/16/2014; Revised: 07/16/2014; Accepted: 07/21/2014; Published Online: 08/04/2014
http://dx.doi.org/10.4161/epi.32089

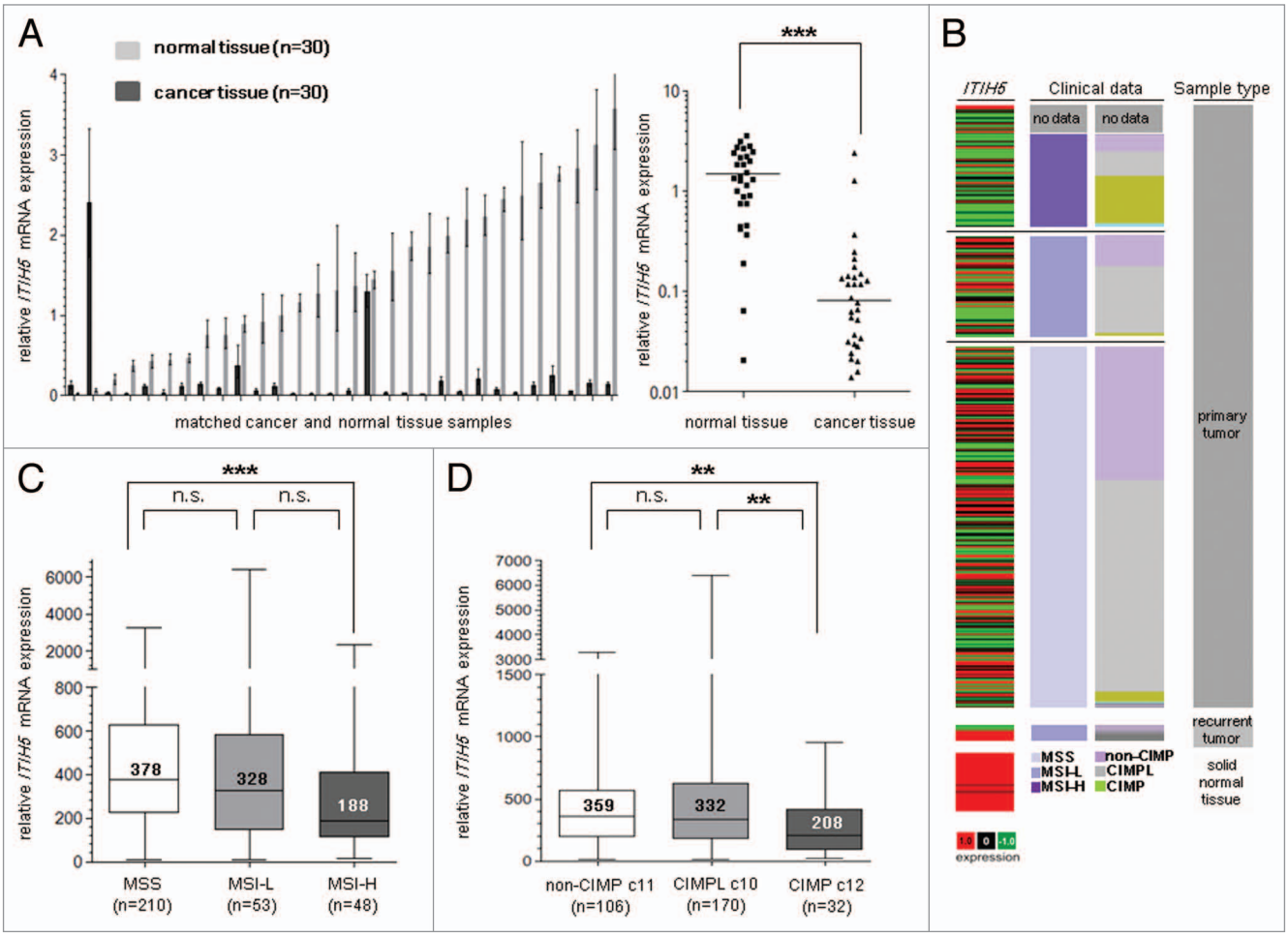

Figure 1. *ITIH5* gene expression is lost in colon tumor tissue. (**A**) *ITIH5* mRNA expression is strongly decreased in tumor tissue compared with matched normal tissue. Box plot analysis illustrates reduced *ITIH5* mRNA expression in tumor tissue with a median expression level of 0.0816 compared with normal tissue (median expression level: 1.332). (**B**) Tumor samples (based on TCGA IlluminaHiSeq mRNA expression platform) are stratified by the microsatellite subtype: MSS (n = 210), MSI-L (n = 53) and MSI-H (n = 48) (left panel) and CIMP subtype: non-CIMP (n = 106), CIMP-L (170) and CIMP-H (n = 32) (middle panel). The right panel shows sample type (dark gray: primary tumor (n = 326); light gray: recurrent tumor (n = 2); white: solid normal tissues (n = 100). (**C and D**) Box plot analysis of *ITIH5* expression in MSS, MSI-L and MSI-H as well as non-CIMP, CIMP-L and CIMP-H primary colon tumors. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum. ***$P < 0.0001$, ** $P < 0.001$, n.s.: not significant.

and poor differentiation.[12-16] Furthermore, CIMP-positive colon tumors have been reported to be associated with poor clinical outcomes.[17,18]

Deciphering of novel biomarkers affecting molecular pathways involved in the progression of CRC is a critical challenge for the extension of patients' survival. In this context, the previously identified inter-α-trypsin inhibitor heavy chain 5 (ITIH5) could play a valuable biological role.[19] Downregulation of ITIH5 in mammary as well as bladder tumors, due to a frequent hypermethylation of the *ITIH5* promoter, was shown to be associated with a higher proliferation rate and malignant progression indicating a putative tumor suppressor function in breast and bladder cancer.[20-22] Furthermore, the ITI heavy chains effectively stabilize the ECM[23] and have been shown to be involved in processes such as tumor invasion[24] and metastasis,[25] while the biological relevance of ITIH5 in CRC remains unknown.

By now, there has been no approach to investigate the molecular and clinical relevance of the putative tumor suppressor gene *ITIH5* in human colon cancer. In the present study, we show that the expression pattern of ITIH5 is clearly deregulated in colorectal adenocarcinoma, providing evidence for a potential role as a tumor suppressor gene in CRC. A worse overall survival in proximal colon tumors, due to an epigenetic silencing of the *ITIH5* gene promoter especially in the subgroup of CIMP-positive patients, reinforces a possible clinical impact of ITIH5 in colon cancer.

Results

ITIH5 expression is downregulated during colon cancer development

In a recent study, we showed *ITIH5* promoter hypermethylation as the molecular cause for *ITIH5* gene silencing in breast [26] and bladder cancer,[22] which was associated with poor prognosis particularly in lymph node negative

patients.[21] We could also demonstrate that *ITIH5* is hypermethylated in circulating free DNA (cfDNA) of colon cancer patients with a frequency of 21% (12 of 58 serum cfDNA specimen was methylated).[27] To assess the biological relevance of ITIH5 in colon cancer we initially analyzed mRNA expression in 30 tumor tissue samples and 30 adjacent normal tissues from the same patient by real-time PCR. We verified a significant ($P < 0.0001$) loss of *ITIH5* gene expression in colon tumors (median expression level: 0.0816) when compared with normal colon tissues (median expression level: 1.332) (**Fig. 1A**). To evaluate the significance of our data, we analyzed *ITIH5* gene expression in a large data set of an independent study.[28] Using data of The Cancer Genome Atlas (TCGA) we verified a prevalent loss of *ITIH5* gene expression in colon tumors when compared with normal colon tissues (**Fig. 1B**). Classifying the data set by clinicopathological characteristics (i.e., age at diagnosis, tumor stage, TNM classification, gender, anatomic subdivision, microsatellite status and CIMP status) we found a significant loss of *ITIH5* mRNA expression in MSI-high (median expression level: 188) compared with MSS tumors (median expression level: 378) ($P < 0.0001$) as well as in CIMP-high (median expression level: 208) in contrast to non-CIMP (median expression level: 359) ($P = 0.0018$) colon cancer tissue samples (**Fig. 1C and D**). There were no significant correlations of mRNA expression with the other mentioned clinicopathological characteristics (**Table S1**).

To give a first insight into the close association of *ITIH5* mRNA expression with MSI colon tumors we re-analyzed a published transcriptomic micro-array analysis consisting of 34 MSS and 19 MSI-H colon cancer specimens[29] with respect to a Gene Ontology (GO) based categorization. Array based class comparison analysis revealed a significant ($P = 0.001$) downregulation of *ITIH5* expression in MSI-H compared with MSS cancer specimen (FC: 1.8). Besides, we identified more than 1500 genes that are predominately downregulated in the MSI tumor subtype (**Table S2**). Therewith, *ITIH5* loss seems to be part of a common gene signature typical for the MSI phenotype. A part of this signature including *ITH5* is shown as heatmap in **Fig. S1**. Furthermore, over-represented gene annotations revealed a strong association with Cellular Component (CC) categories such as "histone methyltransferase complex" and "histone deacetylase complex" (**Table S3**) emphasizing the well-known epigenetic gene silencing in MSI tumors, particularly overlapping with the CpG island methylator phenotype (CIMP) pathway.[30,31]

However, based on immunohistochemistry analysis using a well-established polyclonal ITIH5 antibody,[22] we verified abundant ITIH5 protein expression in normal epithelial colon tissues (**Fig. 2A–C**). ITIH5 staining was predominantly found in the cytoplasm of goblet cell of healthy crypts (see arrows in **Fig. 2A and B**). A sporadic ITIH5 expression was further detected in stromal cells of the connective tissue (see arrow in **Figure 2C**). Contrary to this observation, tumor cells showed decreased ITIH5 protein staining (**Figs. 2D and E**) or almost complete loss of ITIH5 protein (**Figs. 2F and G**).

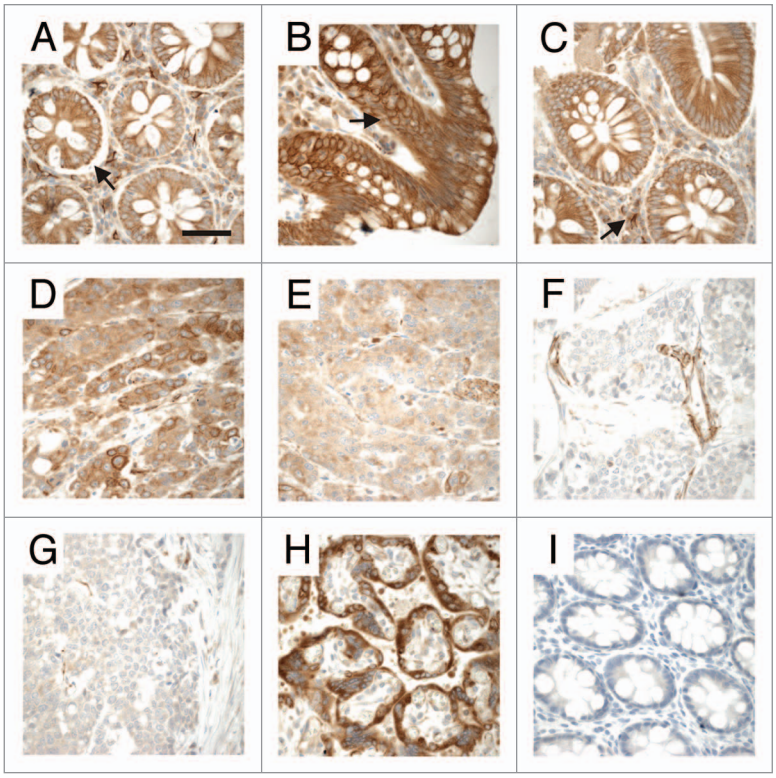

Figure 2. Loss of ITIH5 protein expression in human colon cancer. (**A–C**) Strong ITIH5 expression in epithelial cells of normal colon tissue, especially in goblet cells (arrows in **A and B**). Clear staining in stroma-associated cells (arrow in **C**). (**D and E**) Moderate and low ITIH5 immunoreactivity in cells of colon carcinoma. (**F and G**) Very low staining in progressed colon cancer cells. (**H**) Strong ITIH5 protein expression in FFPE section of placenta tissue that served as positive control for ITIH5 staining. (**I**) Negative control of normal colon tissue. The application of primary antibody was omitted. *Scale bar*: 50 μm.

***ITIH5* loss is caused by promoter hypermethylation in colon cancer**

Next, to prove if promoter methylation could be responsible for ITIH5 expression loss, like it is already suggested due to the transcriptomic micro-array analysis, we analyzed the set of 60 tissue samples by quantitative MSP. In line with the decreased mRNA level in tumor samples, methylation of the *ITIH5* promoter was significantly ($P < 0.0001$) increased in tumor tissue (median PMR value: 4.195%), compared with normal colon tissue (median PMR value: 0.269%) (**Fig. 3A**). Considering the highest PMR value (PMR: 6,467%) in normal colon tissue as cut-off value, methylation frequency in tumor tissue was 43% (i.e., 13 of 30 samples were methylated).

To address the question of whether *ITIH5* promoter methylation contributes to *ITIH5* expression loss in primary colon cancer, we compared methylation and mRNA expression data of both data sets. While *ITIH5* expression was relatively abundant in normal colon tissue in accordance with a low level of *ITIH5* promoter methylation, tumor tissues exhibited clearly increased methylation levels and low *ITIH5* mRNA expression (**Fig. 3B**).

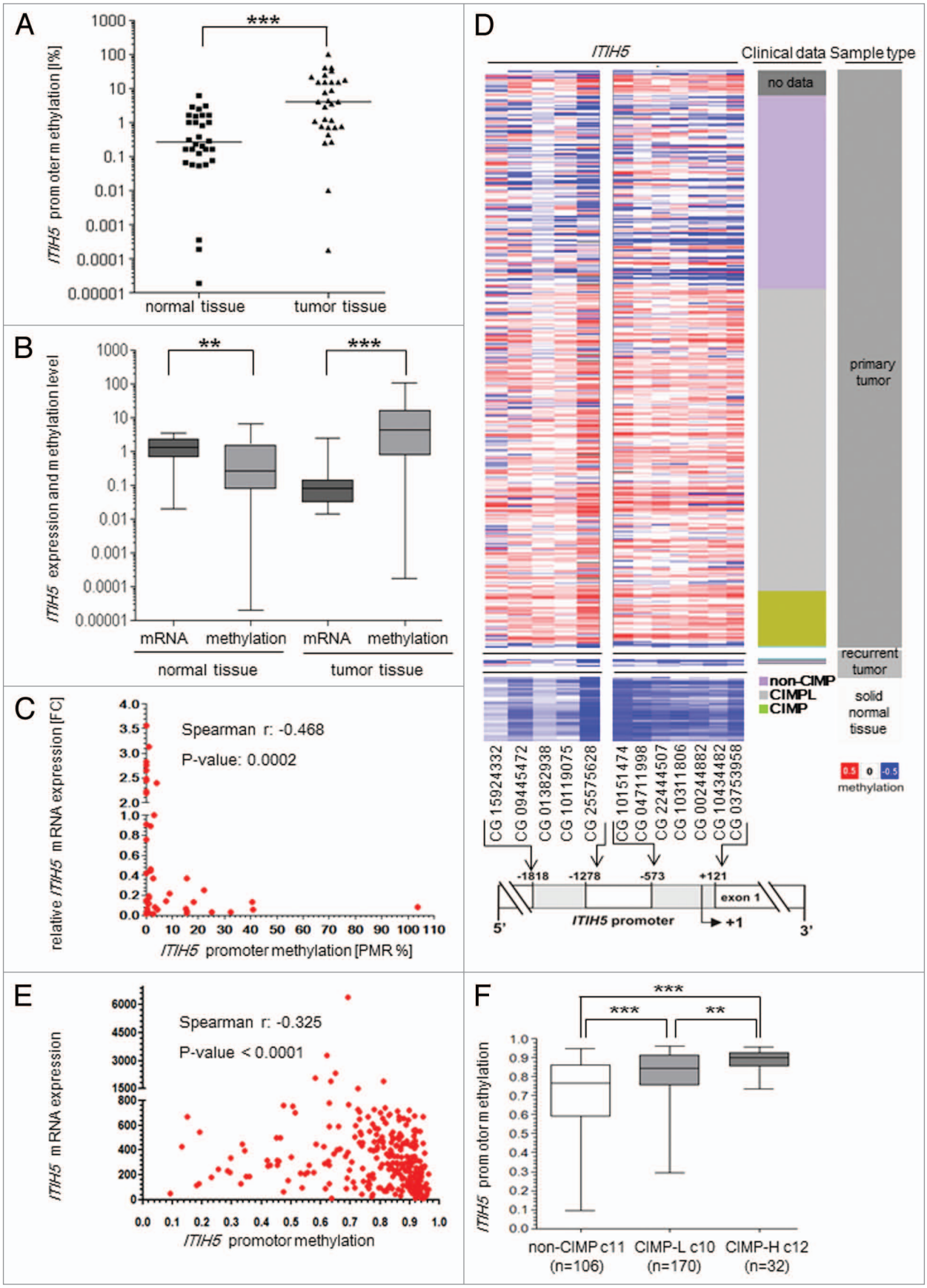

Figure 3. For figure legend see page 1294.

Figure 3 (see previous page). Expression of the *ITIH5* gene correlated with epigenetic inactivation. (**A**) Scatter plot illustrates significant *ITIH5* promoter hypermethylation between cancer and adjacent normal tissue specimen. Horizontal lines: grouped medians. ***$P < 0.001$. (**B**) Box plot analysis demonstrates significant low *ITIH5* methylation level in normal colon tissue compared with *ITIH5* RNA expression, while methylation of the ITIH5 promoter is significantly increased in tumor tissue accordant to a low *ITIH5* RNA expression. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum, **$P < 0.01$, ***$P < 0.001$. (**C**) Scatter plot shows the association between mRNA expression and DNA methylation status of the *ITIH5* gene in 60 colon tissue samples. Spearman correlation coefficient: -0.468, $P = 0.0002$. (**D**) DNA hypermethylation (Illumina HumanMethylation 450 platform) of the *ITIH5* promoter analyzed in colon cancer samples from the TCGA data portal. The left panel illustrates relative values of *ITIH5* DNA hypermethylation for each CG: 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 left panel shows the CIMP subtype (non-CIMP (n = 106), CIMP-L (170) and CIMP-H (n = 32). The right panel shows sample type (dark gray: primary tumor (n = 326); light gray: recurrent tumor (n = 2); white: solid normal tissues (n = 100). (**E**) Scatter plot illustrates the association between expression (IlluminaHiSeq mRNA expression platform) and DNA methylation status (Illumina HM450 platform) of *ITIH5* in 326 primary colon cancer samples based on available TCGA data. Spearman correlation coefficient: r = -0.325, $P < 0.0001$. (**F**) Box plot analysis demonstrates significant higher *ITIH5* methylation level in CIMP-L and CIMP-H tumors tissues of the TCGA data portal compared with non-CIMP cancer specimen. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum, **$P < 0.01$, ***$P < 0.001$.

To further examine the relation between *ITIH5* expression loss and methylation we performed a spearman correlation analysis between mRNA expression and methylation (**Fig. 3C**). This analysis revealed a highly significant inverse association between *ITIH5* mRNA expression and promoter DNA methylation (spearman r: –0.468, $P = 0.0002$) analyzing 60 tissue samples. Interestingly, *ITIH5* promoter methylation correlated significant ($P = 0.028$) with proximal colon tumors (**Table S4**).

Based on the TCGA data, CpG sites that are closely located to the transcription start site of the *ITIH5* promoter were also commonly found methylated in primary colon cancer samples (**Fig. 3D**). In line with our data, a negative correlation of *ITIH5* promoter methylation and *ITIH5* mRNA expression was seen in the TCGA cohort as well (Spearman r: –0.325, $P < 0.0001$) (**Fig. 3E**). Furthermore, an increased hypermethylation of the *ITIH5* promoter was revealed in CIMP-positive compared with non-CIMP colon tumors ($P < 0.0001$) (**Fig. 3F**), which is in line with the decreased *ITIH5* mRNA expression in CIMP-high colon tumors (see **Fig. 1D**).

A functional association between *ITIH5* promoter methylation and *ITIH5* gene silencing was further supported by in vitro demethylation experiment using three colon cancer cell lines (HCT116, SW480, and CaCo2) lacking endogenous *ITIH5* expression. Real-time PCR analyses showed a clear re-expression of *ITIH5* after 5-aza-2′-deoxycytidine (DAC) and trichostatin A (TSA) treatment in all cell lines compared with untreated cells (**Fig. 4**).

***ITIH5* mRNA expression predicts a longer overall survival in proximal colon cancer with a pronounced clinical impact in CIMP-positive tumors**

To reveal whether *ITIH5* 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 platform. RFS and OS were compared between invasive colorectal adenocarcinoma showing abundant *ITIH5* expression (median expression ≥ 329) and all other invasive tumors by univariate statistics. Concerning all colorectal cancer samples Kaplan-Meier analysis revealed no prognostic impact of a strong *ITIH5* expression with longer RFS or OS. However, with respect to the association of a low *ITIH5* mRNA expression in MSI- and CIMP-high tumors concordantly with an increased methylation frequency in CIMP-positive tumors, both known to occur predominantly in the proximal colon,[30,32,33] we divided the TCGA data cohort in the subgroups of proximal, i.e., right-sided (Cecum, Ascending Colon, Hepatic Flexure, and Transverse Colon) and distal, i.e., left-sided (Splenic Flexure, Descending Colon, Sigmoid Colon, Rectosigmoid Junction, Rectum) tumor specimen. Interestingly, a prognostic value of *ITIH5* concerning OS could be demonstrated in the proximal subgroup, while there was no prognostic relevance of *ITIH5* in distal colon cancers (**Table 1**). Patients with an abundant *ITIH5* expression in the right-sided tumors had an estimated mean OS of 7.65 y (95% confidence interval [CI]: 5.45 – 9.86) compared with 6.42 y (95% CI: 4.71–8.13) in patients with low *ITIH5* expression ($P = 0.049$) (**Fig. 5A**). Next, we calculated a multivariate Cox regression model, including all factors potentially influencing the OS time in right-sided colon cancer, but statistical independency ($P = 0.060$) was barely missed (**Table S5**). In a stratified univariate analysis, the prognostic value of *ITIH5* became even more pronounced in the subgroup of CIMP-positive proximal colon tumors (**Fig. 5B**) for OS. Particularly, a 2.3 y longer OS for CIMP-positive patients ($P = 0.032$) was demonstrated with high *ITIH5* expression compared with low *ITIH5* expression. Cox regression analysis supports an independent prognostic value for a lower risk of death for CIMP-positive tumors with high *ITIH5* mRNA expression (HR: 0.363, 95% CI: 0.147–0.893, $P = 0.027$) (**Table 2**). Furthermore, of clinical relevance, strong *ITIH5* expression in early stage tumors, i.e., lymph node-negative tumors, indicated a favorable outcome in proximal colon cancer patients (**Fig. S2**): Nodal-negative patients with low *ITIH5* expression had a worse OS (mean OS: 6.13 y ± 1.14; 95% CI: 3.89–8.37) compared with patients showing high *ITIH5* expression (mean OS: 8.76 y ± 1.32; 95% CI: 6.16–11.37). The calculated Cox regression model indicated *ITIH5* mRNA expression in this important patient group to be a putative independent marker for OS (**Table S6**).

***ITIH5* reveals an improved diagnostic and prognostic value compared with known biomarkers for classifying CIMP-positive tumors**

Next, to emphasize the clinical impact of *ITIH5* in CIMP-positive tumors, we analyzed the well-known Weisenberger et al. CIMP classification panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*)[13] in the TCGA data platform, with respect to the prognostic value of these biomarkers. *IGF2* and

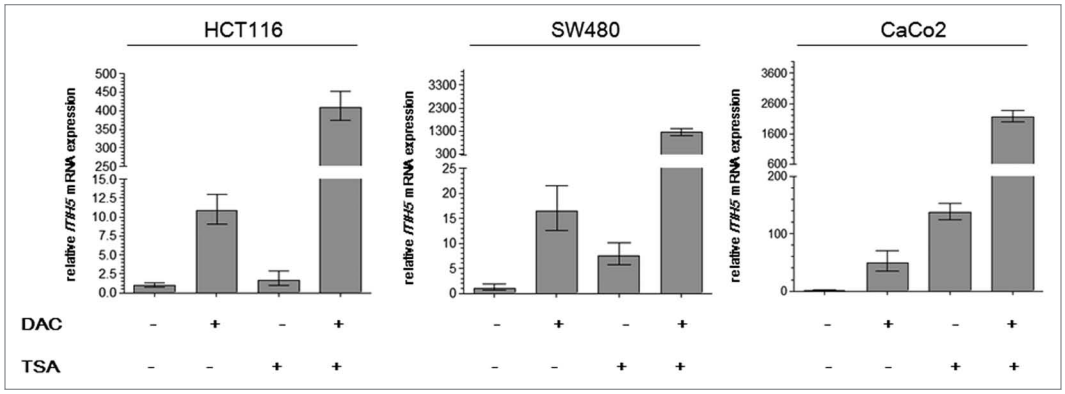

Figure 4. *ITIH5* re-expression after in vitro demethylation. Semiquantitative real-time PCR showing *ITIH5* mRNA expression without and after treatment with 1 mM 5-aza-2′-deoxycytidine (DAC) and 300 nM Trichostatin A (TSA). *ITIH5* mRNA expression is induced after demethylating and histone acetylating treatment. 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 HCT116, SW480 and CaCo2 cells (set to one). SEM was derived from triplicate experiments.

NEUROG1 data were not available in the TCGA cohort. Instead, we examined two of the most traditional CIMP markers, namely *CDKN2A* and *MLH1*, supplementary.

As expected, a strong median promoter hypermethylation of *CDKN2A* and *MLH1* correlated positively with CIMP-high tumors ($P < 0.01$). Pearson correlation was slightly higher for *CDKN2A* (r = 0.298, $P < 0.01$) and *MLH1* (r = 0.249, $P < 0.01$) compared with *ITIH5* methylation (r = 0.194, $P < 0.01$). In contrast, promoter methylation of *CACNA1G*, *RUNX3*, and *SOCS1* showed no statistical association with CIMP-positive tumors. Furthermore, ROC-analysis of promoter methylation indicated inappropriate AUC-values in CIMP-high tumors for *CACNA1G* (AUC: 0.605, 95% CI: 0.493–0.718), *RUNX3* (AUC: 0.505, 95% CI: 0.407–0.603) and *SOCS1* (AUC: 0.442, 95% CI: 0.322–0.562) in comparison to significant AUC-values for *ITIH5* (AUC: 0.723, 95% CI: 0.647–0.795), *CDKNA2* (AUC: 0.913, 95% CI: 0.858–0.967) and *MLH1* (AUC: 0.900, 95% CI: 0.828–0.973) ($P < 0.0001$) (**Fig. S3A**). Based on the ROC data, we would suggest proposing a novel CIMP-defining marker panel consisting of only three genes, namely *ITIH5*, *CDKN2A*, and *MLH1*. This panel identifies CIMP-high tumors with 87.5% sensitivity and 91.7% specificity as compared with 87.5% sensitivity and poorly specificity of 10% by the Weisenberger biomarkers *CACNA1G*, *RUNX3*, and *SOCS1* (**Fig. S3B**), hence highlighting *ITIH5* methylation as putative diagnostic biomarker for CIMP-classifying whose expression level might also usable for risk stratification of this patient group.

To shed light on the prognostic impact of *CACNA1G*, *RUNX3*, *SOCS1, CDKN2A*, and *MLH1, a* descriptive data analysis was performed. In contrast to *ITIH5* expression, data analysis revealed no prognostic relevance concerning *CACNA1G*, *RUNX3*, *SOCS1, CDKN2A*, and *MLH1* promoter methylation and expression for OS neither in all cases nor in the stratified subgroups of proximal and CIMP-positive tumors.

ITIH5 expression leads to decreased proliferation in HCT116 as well as CaCo2 colon cancer cells

An increased cell proliferation is a fundamental hallmark of cancer cells.[34] To study the impact of a forced ITIH5 expression on proliferation behavior in vitro, we transiently transfected two different colon cancer cell lines with the eukaryotic expression vector pBK-CMV. Concerning the different expression patterns of *ITIH5* in MSS and MSI tumors, we selected the MSI-H cell line HCT116 and in contrast the MSS cell line CaCo2. For a better verification of our data, we performed two independent transfections for each cell line. ITIH5 expression in the ITIH5-transfected cells and lack of expression in the mock-transfected cells had been verified by real-time PCR and western blotting (**Fig. 6A**).

Based on these transient in vitro models, a proliferation assay was performed using XTT assays (**Fig. 6B**). In CaCo2 cells, we observed that in a bulk of transiently transfected cells, i.e., a large part of non-transfected CaCo2 cells is present, ITIH5 re-expression led to a significant cell growth suppression by 13% ($P = 0.019$), compared with the mock-transfected cells 96h after plating (**Fig. 6B**, left graph). In line, tumor cell growth retardation of the ITIH5-transfected HCT116, compared with HCT116 mock cells, reached a 16.4% decreased proliferation rate ($P < 0.001$) in cells showing abundant ITIH5 protein expression (**Fig. 6B**, right graph).

Discussion

Today, several lines of evidence suggest a potential role of ITIH5, a novel member of the ITIH family, in tumor biology, particularly in tumor development and progression.[21,35] We previously showed that loss of ITIH5 expression in breast[21,27] and bladder[22] cancer, caused by aberrant promoter hypermethylation, is associated with unfavorable prognosis.[19,21,22] Furthermore, we demonstrated a moderate hypermethylation of the *ITIH5* promoter in circulating free DNA in the blood of colon cancer patients,[27] whereas the biological relevance of ITIH5 in colon cancer remains elusive. The current study is the first to analyze

Table 1. Univariate analysis of clinicopathological factors regarding overall survival (OS) in proximal colon cancer

Variable	*OS*		
	n[a]	Events	*P* value[b]
Clinicopathological factors:			
Tumor size			
pT0–2	28	2	0.206
pT3–4	119	27	
Lymph node status			
pN neg	89	12	**0.003**
pN1–3	58	17	
Tumor stage			
stage 1–2	125	20	**<0.001**
stage 3–4	15	8	
CIMP status			
Non-CIMP	23	5	0.806
CIMP	117	23	
Microsatellite status			
MSS	82	16	0.440
MSI	60	12	
ITIH5 (median expression)			
≤329	74	20	**0.049**
>329	74	9	

MSS, Microsatellite stable; MSI-L, Microsatellite instable low; MSI-H, Microsatellite instable high, CIMP-L, CpG island methylator phenotype low, CIMP-H, CpG island methylator phenotype high, ITIH, inter-a-trypsin inhibitor heavy chain. Significant *P* values marked in bold face. [a]Only patients with primary, proximal colon cancer were included. [b]Breslow test at the two-sided significance level of 0.05

in depth ITIH5 expression, as well as its potential clinical and functional impact toward colon cancer.

Initially, we verified by both real-time PCR and immunohistochemistry that ITIH5 was downregulated in human colon tumor 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[28] in colon cancer samples. In line, TCGA data analyses revealed a decreased *ITIH5* mRNA expression in colon cancer tissue compared with normal tissue. Interestingly, *ITIH5* expression was significantly lower in MSI-high and CIMP-high tumor specimen compared with MSS and non-CIMP tissue samples, respectively. To analyze the molecular cause of downregulation, we investigated the epigenetic configuration of the *ITIH5* gene promoter in primary colorectal carcinoma, as it is known that the *ITIH5* promoter sequence contains distinct CpG islands. In fact, *ITIH5* gene promoter was methylated in 43% of the analyzed colorectal tumor tissues, in line with a restoration of *ITIH5* expression through in vitro demethylation of the cell lines HCT116, CaCo2 and SW480. Again, TCGA data analyses confirmed our results by indicating a frequent hypermethylation in primary tumor tissue and, accordingly, demonstrated an inverse correlation (r = −0.392) of *ITIH5* methylation and mRNA expression indicating promoter hypermethylation as the molecular cause of the *ITIH5* loss in colon cancer.

A strong relationship between the MSI phenotype and promoter hypermethylation in colon cancer was demonstrated in numerous studies.[10,16,17,36] Some of these studies suggest that CpG island methylation precede the development of MSI tumors, e.g., beyond germline mutations the mismatch repair gene *MHL1* is known to become silenced by epigenetic modifications.[12,15] So far, *MHL1* methylation has been defined as a marker of a further pathway in colon cancer, namely the CpG island methylator phenotype (CIMP),[37] suggesting a strong overlap of MSI and CIMP pathways in colorectal carcinogenesis. Indeed, based on in silico transcriptomic micro-array analysis, we found clear indications of deregulated epigenetic pathways in sporadic MSI-high tumors. Of interest, a decreased *ITIH5* expression level was related to a common gene signature in MSI colon cancer, providing strong evidence that *ITIH5* loss is associated with the microsatellite unstable phenotype. However, *ITIH5* may be a potential novel tumor suppressor gene in colon cancer displaying an impact on tumor development in case of its epigenetic mediated silencing.

Importantly, concerning the distinct proximal anatomic subdivision of MSI-high and CIMP-positive colon cancers, we revealed a linkage of *ITIH5* expression with longer OS in proximal tumor specimens of the TCGA data portal. The potential clinical impact of abundant *ITIH5* mRNA expression in colon cancer was enforced by a pronounced OS of patients with proximal CIMP-positive colon cancer. Several studies confirmed an unfavorable prognosis of patients with CIMP-positive tumors compared with those with non-CIMP status.[17,18] In this context, a high *ITIH5* expression might be a novel independent biomarker for prognosis of patients with proximal CIMP-positive colon cancer. Nevertheless, the biological role of ITIH5 in the CIMP-related signaling pathway has to be unraveled in further studies. In agreement with our breast cancer related study, ITIH5 remains a significant prognostic factor in the clinically important subgroup of patients with node-negative proximal tumors as shown by univariate and multivariate analysis. Lymph node involvement is one of the most important predictors for disease recurrence in colon cancer[38] and, consequently, one-half of CRC patients die from the disease due to the high metastatic potential.[1] Hence, there is still a lack in suitable prognostic biomarker for risk stratification in early stage CRC patients.[39] Accordingly, all patients with node-positive tumors receive adjuvant therapy while the value of adjuvant therapy for node-negative cases is still controversial.[40,41] Therewith, ITIH5 may represent a novel biomarker of nodal disease spread with clinical utility in CRC helping to estimate favorable patients' outcome in lymph node negative tumors and prevent unnecessary chemotherapy. However, these findings have to be evaluated in an independent cohort with more cases.

Of importance, since the defined CIMP-positive classifying biomarkers *CACNA1G*, *RUNX3*, *SOCS1*, *MLH1*, and *CDKN2A*

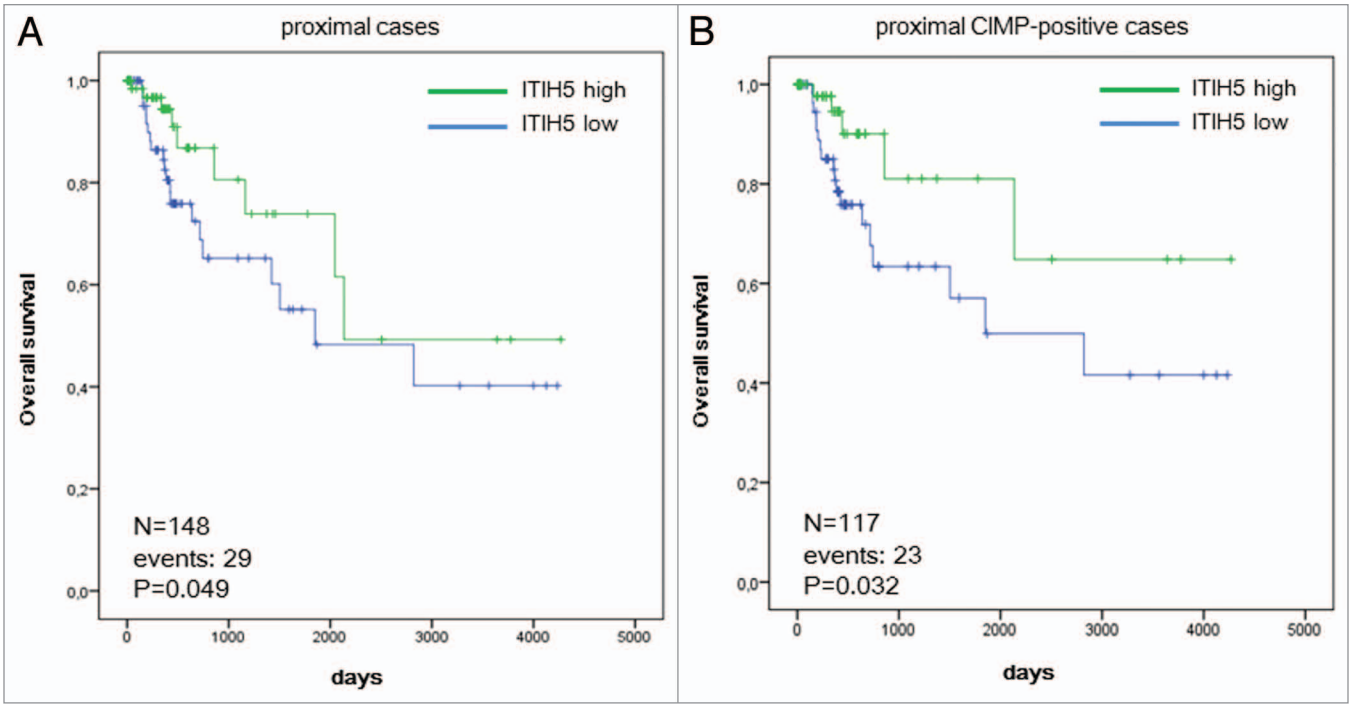

Figure 5. Univariate survival analysis of *ITIH5* mRNA expression according to the Kaplan–Meier (KM) method revealed a longer overall survival in patients with proximal colon cancer especially in CIMP-positive tumors. (**A**) The KM-analysis of all proximal cases is shown. (**B**) KM-Analysis illustrating a strong prognostic value of *ITIH5* in the clinically relevant group of patients with proximal CIMP-positive colon cancer, which is not demonstrable in non-CIMP patients. Green line: strong *ITIH5* expression (median ≥ 329); blue line: weaker *ITIH5* expression (median < 329). Vertical lines: censored cases.

[13], revealed no clinical relevance in the TCGA data cohort, the prognostic value of abundant *ITIH5* expression became even more important. In contrast, *ITIH5* exhibits a remarkable capability to detect methylation in CIMP-high tumors, i.e., *ITIH5* promoter methylation has a stronger predictive value (AUC value) for CIMP-high tumors than *CACNA1G*, *RUNX3*, and *SOCS1* promoter methylation. Indeed, defining a novel three-gene methylation biomarker panel for classifying CIMP-high tumors (*ITIH5*, *MLH1*, and *CDKN2A)* increased its specificity from 10% to 91.7%.

To give a first insight into the molecular function of ITIH5 in colon cancer, we evaluated the proliferation activity in the MSI-H cell line HCT116 and the MSS cell line CaCo2 by a functional XTT assay. Both cell lines showed a reduced proliferation rate in ITIH5-transfected cells as compared with mock-transfected control cells, emphasizing a putative tumor suppressive role in colon tumors in vitro.

In conclusion, these findings provide for the first time evidence that *ITIH5* acts as a tumor suppressor gene in normal colon tissue. To our knowledge, this is the first study so far demonstrating a putative tumor suppressive function of *ITIH5* in colon cancer. In addition, *ITIH5* is potentially valuable as a prognostic biomarker for the clinically important group of patients with proximal CIMP-positive and lymph node negative cancer whose disease management has to be adjusted to a personalized progression risk. With respect to the known defined CIMP-positive classifying biomarkers, *ITIH5* might be an improved novel biomarker for CIMP-high tumors with both functional significance to tumorigenesis and prognostic significance for patient survival. Further investigation of the contribution of ITIH5 to colon cancer progression concerning the potentially biological relevance in the epigenetic CIMP pathway may help to understand this pathway in more detail, finally helping to improve disease management.

Material and Methods

Cryoconserved patient samples

Tumor and adjacent normal tissue samples for methylation and mRNA expression analysis from 30 patients with primary CRC were obtained from the tumor bank of Euregional comprehensive Cancer Center Aachen (ECCA), now being part of 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). Tumor material was snap-frozen in liquid nitrogen directly after surgery. 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 **Table S7**.

In silico patients samples

Data from primary colon cancer tissues, recurrent tissues and solid normal tissues were used from The Cancer Genome Atlas

Table 2. Multivariate Cox regression analysis including all factors potentially influencing OS in CIMP-positive right-sided colon cancer

Variable		HR	*P* value	95%CI	
				lower	upper
***ITIH5* expression**					
ITIH5 low		1.000			
ITIH5 high		0.363	**0.027**	0.147	0.893
Lymph node status					
pN neg		1.000			
pN 1–3		1.258	0.646	0.472	3.351
Tumor stage					
stage 1–2		1.000			
stage 3–4		5.160	**0.003**	1.766	> 10

Significant *P* values are marked in bold face. HR, hazard ratio; CI, confidence interval.

(TCGA),[28] comprising patients data of two independent platforms: Illumina Infinium DNA methylation (HumanMethylation 450) and IlluminaHiSeq mRNA expression (n = 326). An overview of the clinical characteristics of the patients is summarized in **Table S8**. In addition, data from a transcriptomic micro-array analysis with 34 MSS and 19 MSI-high colon cancer specimens profiled on Affymetrix GeneChips (HG-U133 Plus 2.0)[29] was re-analyzed in this study.

Cell lines and reagents

The human colon cancer cell lines HCT116, CaCo2, and SW480 were obtained, tested, and authenticated from the American Type Culture Collection (ATCC) and were resuscitated before using in experiments. Medium was additionally supplemented with 1 mM sodium pyruvate and 10 mg/ml insulin. Used cell lines were regularly tested for mycoplasma infection using the PCR-based Venor® GeM Mycoplasma Detection Kit (Minerva Biolabs).

Nucleic acid extraction and reverse transcription PCR

Genomic DNA from cryoconserved colon tumors and adjacent normal tissue was isolated using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. By using TRIzol reagent (Invitrogen) total cellular RNA from cell culture and tissue specimen was prepared. cDNA was synthesized using the reverse transcription system (Promega) as previously described.[21]

Semiquantitative real-time PCR

cDNAs were amplified by semiquantitative real-time PCR using SYBR-Green PCR mix (Bio-Rad Laboratories) 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.[42] All primers used spanned at least one intron, and described earlier.[22] 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) as previously described.[21]

Quantitative methylation-specific polymerase chain reaction (qMSP)

Bisulfite-modified DNA used as template for fluorescence-based MSP, as previously described.[27] Amplification reactions were performed in triplicate in a volume of 25 μl containing 20 ng bisulfite-modified DNA. Primers and probes were designed specifically to amplify bisulfite-converted DNA for the *ITIH5* gene and the reference gene *GAPDH* to normalize for input DNA.[27] Amplifications were performed in an iCycler iQ5 (Bio-Rad). Each plate included positive controls for the methylated sequence (in vitro methylated human leukocyte DNA) and unmethylated sequence (human leukocyte DNA from a healthy donor), as well as multiple water blanks.

AZA/TSA treatment

A demethylating treatment of the colon cancer cell lines HCT116, CaCo2, and SW480 was performed as previously described.[43]

Transfection of HCT116 and CaCo2

For transient transfection experiments, HCT116 and CaCo2 cells were grown at 60–70% confluence and transfected with either the pBK-CMV (empty vector) or the ITIH5-pBK-CMV construct[19] using Fugene HD according to the manufacturer's instructions (Roche Diagnostics). After 72h, transfected cells were grown in media containing 1 mg/ml G418 for two weeks. Transfected cells were characterized by both real-time PCR and western blot for expression of ITIH5.

Western blotting

Protein lysates were analyzed from control cells (–ITIH5) and ITIH5-transfected (+ITIH5) cell lines under reducing (50 mM DTT) conditions in NuPAGE lysis buffer (Invitrogen). Approximately 20 mg of protein were separated in 4–12% Bis-Tris gels (Invitrogen) using MOPS-SDS running buffer and electroblotted onto nitrocellulose membranes. Membranes were incubated with primary antibody (polyclonal anti-ITIH5 200 ng/ml, Pineda Company) overnight at 4 °C. After washing with Tris-buffered saline Tween-20 (TBS-T), membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Chemicon International; 1:4000) for 1h at room temperature. Antibody detection was performed with the ECL

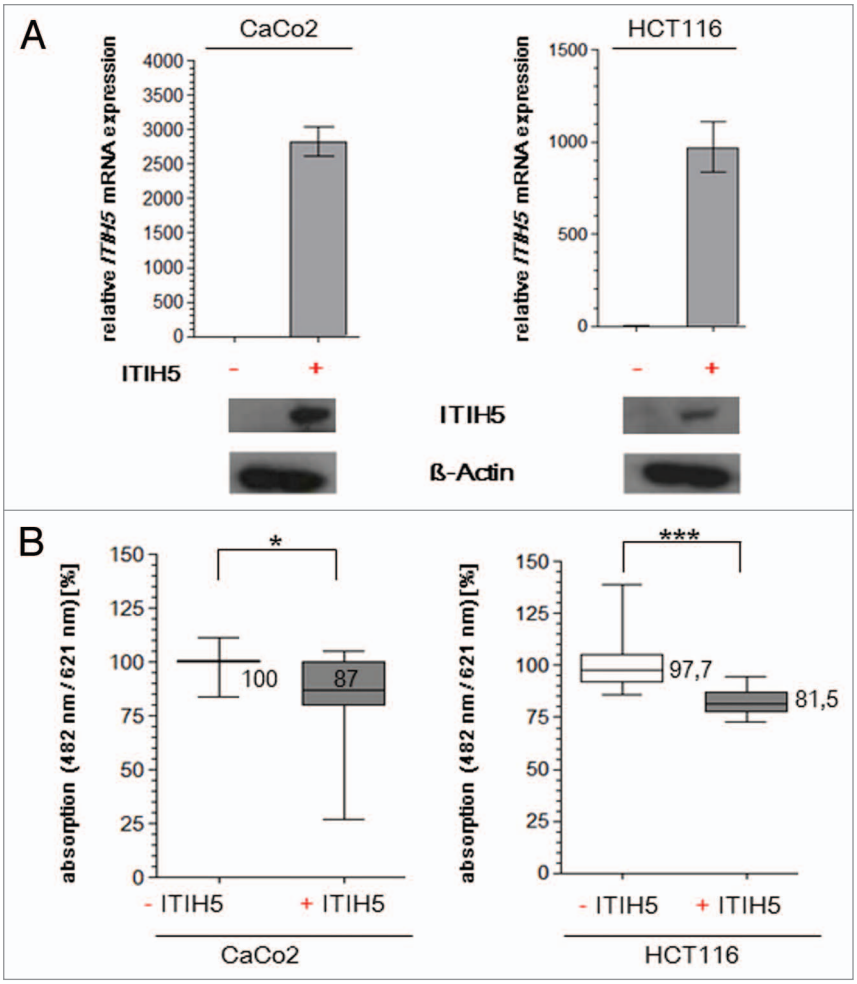

Figure 6. ITIH5 re-expression leads to a decreased proliferation in the ITIH5-deficient cells lines HCT116 and CaCo2. Two independent transfection experiments were performed for each cell line under the same conditions. (**A**) *ITIH5* mRNA re-expression in ITIH5 positive cells (+ITIH5) compared with mock cells (-ITIH5) in CaCo2 cells (left) and in HCT116 cells (right). Gain of ITIH5 mRNA expression is indicated as fold-change relative to each baseline expression of mock clones (set to one). Expression of GAPDH served as a control for equal starting amounts of cDNA. SEM was derived from triplicate experiments. Protein extracts were analyzed by western blot using indicated antibodies. To demonstrate equal protein loading, the membranes were re-probed with an antibody specific for β-actin. (**B**) Box plot analysis illustrates proliferation rate of CaCo2 (left) and HCT116 (right) cells after 96h measure. The baseline level at 24h for each clone was set to one. Proliferation of ITIH5 positive CaCo2 cells (+ITIH5) was 13% reduced compared with mock cells (–ITIH5). Proliferation of ITIH5 positive HCT116 cells (+ITIH5) was 16.4% reduced compared with mock cells (–ITIH5). Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum, $*P < 0.05$, $***P < 0.001$.

western blotting detection system (Amersham Life Science). Equal protein loading was monitored by using β-actin specific antibody.

ITIH5 immunohistochemistry

Immunohistochemical staining was performed as previously described[22] with slide modifications: FFPE sections were incubated with a polyclonal ITIH5 rabbit anti-human antibody (1:200) (Pineda Company). FFPE sections of non-cancerous placenta tissue served as positive control.

Proliferation assay

The XTT proliferation assay from Roche (Frankfurt, Germany) was used. Cells were plated and cultivated in flat 96-well plates (1×10^3 cells/well). To each well, 100 ml of growth medium and 50 µl of XTT reagent solution was added and the plate incubated for 4 h at 37 °C. Proliferation rate examined after 24h, 48h, 72h, and 96h of growth. The absorbance of the samples was measured at 450 nm.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 (SPSS) and GraphPad Prism 5.0 (GraphPad Software Inc.). The non-parametric Mann-Whitney U-test was used in order to compare *ITIH5* expression between tumor and normal colon tissue and ITIH5-positive and negative cells. Differences were considered statistically significant if the two sided *P* values were equal or below 5% (≤0.05).

Gene expression re-analysis of MSS and MSI-high colon cancer specimen[29] was performed using BRB-ArrayTools developed by Dr. Richard Simon and BRB-ArrayTools Development Team version 4.3.0 Beta. In order to significantly identify genes differentially expressed between two classes, the class comparison evaluation was used.

ITIH5 methylation status and expression in human colon cancer samples was assessed using an independent and public data set (TCGA). Correlation of the *ITIH5* expression (TCGA Illumina sequencing platform) and *ITIH5* methylation data (TCGA HM450 platform) was performed by calculating a Spearman correlation coefficient. Overall survival (OS) was measured from surgery until death and was censored for patients alive without evidence of death at the last follow-up. Multivariate Cox-regression analysis was performed to test for an independent prognostic value of *ITIH5* expression. Selection of the prognostic factors to be included in the multivariate model was based on statistical significance in univariate Breslow tests. Receiver-operating-characteristics (ROC) curves were calculated to evaluate the diagnostic performance of different marker combinations.[44]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Sonja von Serényi and Jennifer Wipperfürth for excellent technical assistance.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/32089

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