Epigenetic inactivation of ITIH5 promotes bladder cancer progression and predicts early relapse of pT1 high-grade urothelial tumours

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Inter-α-trypsin inhibitor heavy chain 5 (ITIH5) has been associated with tumour suppression in various cancers. However, its putative role in bladder cancer is completely unknown. Therefore, we initiated a study analysing ITIH5 expression as well as its prognostic and functional impact on human urothelial cancers (UCs). Expression analysis showed a clear down-regulation of ITIH5 mRNA in 61% (n = 45) of UCs, especially in muscle-invasive tumours (P < 0.001). ITIH5 loss in UCs was further evident on protein level (65.5%, n = 55) as detected by immunohistochemistry. DNA methylation analysis demonstrated tumour-specific ITIH5 promoter methylation in 50% of papillary non-invasive pTa (n = 30) and 68% of invasive (n = 28) UCs. Aberrant ITIH5 promoter methylation in bladder tumours was tightly linked (P < 0.001) with loss of ITIH5 mRNA expression, which was furthermore functionally confirmed by demethylation analysis in cell lines. Pyrosequencing analysis revealed that ITIH5 promoter hypermethylation was closely associated with progressive bladder cancers. Subsequently, a large cohort (n = 120) of clinically challenging pT1 high-grade UC was analysed for ITIH5 expression. Of clinical significance, we found an association between loss of ITIH5 expression and unfavourable prognosis of UC patients without distant metastasis at first diagnosis (recurrence-free survival; hazard ratio: 4.35, CI, confidence interval; CIS, carcinoma in situ; FC, fold change; FFPE, formalin fixed paraffin embedded; HA, hyaluronic acid; IRS, immunoreactive scoring system; ITIH5, inter-α-trypsin inhibitor heavy chain 5; MSP, methylation-specific PCR; NU, normal urothelium; PBS, phosphate-buffered saline; RFS, recurrence-free survival; TSA, trichostatin A, UC, urothelial cancer.

These authors contributed equally to this work.

Introduction

Accumulating evidence suggests that inter-α-trypsin inhibitors (ITIs), which comprise a family of serine proteases (1), play a crucial role in human carcinogenesis. These secreted protein complexes consist of two different proteins: a light chain (bikunin) and homologous heavy chains (ITIH 1–5) (2). Beyond that, ITI heavy chains also have biological functions independently of bikunin (3) and were originally referred to as serum-derived hyaluronic acid-associated protein (SHAP) (4,5). Owing to the covalent linkage with hyaluronic acid (HA), the ITI heavy chains are thought to generate ‘cable-like HA structures’, leading to a stabilization of the extracellular matrix (6,7), and thus might influence the enormous activity spectrum of HA (8). In tumorigenesis, ITIH deregulation has been associated with malignant progression (1,9–11). For instance, Paris et al (12) demonstrated that over-expression of ITI heavy chain 1 and 3 in the human lung cancer cell line H460M notably reduced the number of induced metastases in mice. The recently characterized new ITI family member inter-α-trypsin inhibitor heavy chain 5 (ITIH5) was shown to be down-regulated during breast cancer development (13), which predicted unfavourable recurrence-free and overall survival (14). ITIH5 loss in breast cancer was furthermore associated with regional lymph node invasion as well as distant metastasis (15). ITIH5 gene silenceing in breast tumours is triggered by aberrant promoter hypermethylation (14) that has been recently suggested as a putative biomarker for early breast cancer detection (16). Beyond that, absence of ITIH5 has been associated with thyroid cancer development (17), and an expression array study by Lu et al (2010) (18) indicates ITIH5 mRNA dysregulation in urothelial cancer (UC).

Apart from the latter, nothing is known about a putative role of ITIH5 in bladder carcinogenesis. With >386 000 new cases in 2008 and estimated 150 000 deaths, UC is the most frequent urogenital malignant tumour concerning both sexes worldwide (19). Most of these tumours (~80%) are low grade and non-invasive at first diagnosis (20). However, 20% of bladder tumours exhibit muscle infiltration (pT ≥ 2) (20) and cause a mortality rate of at least 50% within 2 years of diagnosis (21,22). In contrast, the overall prognosis of non-muscle-invasive bladder cancer is favourable (23) but different subtypes of non-muscle-invasive bladder cancer predict a heterogeneous clinical outcome. Low-grade pTa tumours progress in only 5%, whereas pT1 high-grade UCs are associated with a progression rate between 20 and 40% (24,25). Shahin et al. (26) further revealed that 30% of patients with pT1G3 tumour die due to metastatic disease, whereas another 30% of pT1G3 patients never display recurrence after transurethral resection of the bladder.

Current prognostic factors, helping to define high risk for recurrence or progression in pT1 UCs, refer to a scoring system based on clinico-pathological characteristics such as early recurrence or multifocality of tumours (27). Due to the lack of further prognostic and predictive biomarkers (28), disease management and hence, the selection of an adequate therapy is difficult and often insufficient (29). Therewith, deciphering of novel factors affecting invasive bladder cancer pathways is an important prerequisite for the development of therapeutic strategies. For this reason, we present in this study for the first time a comprehensive analysis of ITIH5 expression in human bladder cancer with a special focus on its prognostic and functional role in the subgroup of early invasive (pT1) high-grade tumours.

Material and methods

Patient samples

Formalin-fixed paraffin-embedded (FFPE) primary bladder cancer samples were obtained from the pathology archives/departments of urology of RWTH Aachen University/LMU Munich (cohort I; Supplementary Table 1, available at Carcinogenesis Online) and University of Regensburg/Erlangen (cohort II; Supplementary Table 2, available at Carcinogenesis Online). Haematoxylin and eosin (H&E)-stained sections were prepared for estimating the percentage of tumour cells by a pathologist. For DNA methylation and RNA expression experiments, only micro-dissected cancer and normal
samples of cohort I were used. Patients gave informed consent and the retrospective study was approved by the local Ethics Committee (EK 122/04, 173/06 and 206/09).

**Cell line and reagents**

The human bladder cancer cell lines UROtsa, RT112 and J82 were originally obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were resuscitated before using in experiments. The cell lines HCV29 and K47 were a gift from Dr Alexander Buchner (LMU München, Germany). All cell lines were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco) and were regularly tested for mycoplasma infection using the PCR-based Venor® GeM Mycoplasma Detection Kit (Minerva Biolabs, Berlin, Germany).

**Generation of anti-ITIH5 C-terminal antibody**

Polyclonal antiserum against the human ITIH5 protein was engineered at Pineda Company (Berlin, Germany) by immunizing rabbits with a synthesized peptide corresponding to amino acids NH$_2$-CYLASHFPTDGMTLGRGMSREL-COOH. Afterwards, the antibody was isolated, affinity purified and tested to ensure specificity.

**Immunohistochemistry**

Immunohistochemical analysis was carried out according to the manufacturer’s instructions (DAKO 5001; DAKO, Glostrup, Denmark). FFPE sections (3 µm) were incubated for 60 min with a polyclonal ITIH5 rabbit anti-human antibody (1:100) (Pineda Company). FFPE sections of non-cancerous placenta tissue served as positive control (14). ITIH5 protein staining was quantified by a pathologist using an adapted immunoreactive scoring system (IRS) according to Remmele and Stegner (30).

**Western blotting**

Whole-cell lysates were homogenized and sonicated in an appropriate volume of 1x NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA) supplemented with 5% dithiothreitol and heat denatured (5 min 95°C). They were loaded on 4–12% gradient gels (NuPAGE; Invitrogen) for protein separation and then transferred onto 0.2 µm nitrocellulose membranes (Whatman, Dassel, Germany) (1 h, 100 V) for immunodetection. Blots were blocked in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) and 5% non-fat dry milk (Merck, Darmstadt, Germany) overnight at 4°C. Blocked blots were probed with anti-β-actin (A5316, 1:5000; Sigma–Aldrich, Deisenheim, Germany) or anti-ITIH5 (C-term, 1:2000; Pineda) in blocking solution buffer, washed [0.01% (vol/vol) Tween-20/PBS] and incubated with rabbit anti-mouse (1:8000; Dako) or goat anti-rabbit (1:8000; Dako) secondary peroxidase-conjugated antibody. After washing [0.01% (vol/vol) Tween-20/PBS], antibody detection was accomplished with Pierce ECL Western blotting Substrate (Thermo Scientific).

**Nucleic acid extraction and reverse transcription–PCR**

Genomic DNA from FFPE bladder samples was extracted by using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. Total RNA from in vitro cultured cells and FFPE samples was isolated by using TRIzol reagent (Invitrogen). Afterwards, cDNA was synthesized using the reverse transcription system (Promega, Madison, WI) as described previously (14).

**Bisulphite modification and methylation-specific PCR**

Bisulphite treatment of DNA and methylation-specific PCR (MSP) analysis were performed as described previously (14).

**Pyrosequencing**

Pyrosequencing analysis of a distinct ITIH5 promoter region was performed by using the PyroMark PCR Kit (Quagen) for initial fragment amplification. Afterwards, the PyroMark96 ID device and the PyroGoldSQA reagent Kit (Quagen) were implemented as described previously (31). The ITIH5 assays were designed by using the PyroMark Assay Design Software (Quagen); Primers are listed in Supplementary Table 3, available at Carcinogenesis Online.

**Semi-quantitative real-time PCR**

cDNAs were amplified by semi-quantitative real-time PCR using SYBR-Green PCR mix (Bio-Rad Laboratories, Munich, Germany). PCRs were performed in an iCycler IQ5 (Bio-Rad Laboratories) and quantified as described previously (32). All primers used spanned at least one intron and are listed in Supplementary Table 4, available at Carcinogenesis Online.

**In vitro demethylation**

**In vitro** demethylation experiments were carried out as recently performed (31).

**Transfection of human RT112 cells**

The ITIH5-pBK-CMV expression vector, containing the full-length human ITIH5 cDNA derived from normal breast tissue, was described recently (14). RT112 cancer cells were transiently or stably transfected with either the pBK-CMV (empty vector) or the ITIH5-pBK-CMV construct using Fugene 6 according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). To provide independent stable clones, 48 h after transfection, RT112 cells were single cell cloned by limiting dilution and grown in media containing 0.8 mg/ml G418 for 2 weeks ensuring genomic cDNA integration. Isolated clones were expanded to 75 cm$^2$ flasks and were afterwards characterized by both real-time PCR and western blotting for expression of ITIH5. In case of the transient in vitro model, 72 h after transfection functional assays were performed.

**Migration assay**

In vitro motility was assessed by performing a monolayer scratch wound assay as recently described (33).

**Colonization formation assay**

Two-dimensional colony formation assays were performed as previously specified (32).

**Statistical analysis**

Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). Differences were considered statistically significant if the two-sided P values were ≤0.05. The non-parametric Mann–Whitney U test was used in order to compare two groups. In case of more than two generated groups, the Kruskal–Wallis test was used. Descriptive Fisher’s exact tests and two-sided log-rank tests were performed in order to correlate clinico-pathological parameters with ITIH5 promoter methylation or protein expression, respectively. 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 expression. Selection of the prognostic factors to be included in the multivariate model was based on statistical significance in univariate log-rank tests.

**Results**

**ITIH5 expression is lost in human bladder cancer**

Semi-quantitative ITIH5 mRNA expression analysis of 45 bladder cancer tissues (UCs) containing papillary non-invasive (pTa) low- and high-grade, flat non-invasive carcinoma in situ (CIS) and invasive bladder tumour samples (pT2–4) showed a median down-regulation of 61% when compared with ITIH5 mRNA expression of normal urothelium (NU) (n = 15) (Figure 1A). For cohort characteristics of analysed samples, see Supplementary Table 1, available at Carcinogenesis Online. A comparison between UC stages/subtypes and tumour grades revealed a highly significant (P < 0.001) loss of ITIH5 mRNA expression in the invasive UC stage [fold change (FC): 6.3; Figure 1B] as well as in high-grade UC (UC-hg: papillary pTa-hg, CIS and invasive UC) (FC: 7.7; Figure 1C). The close association between loss of ITIH5 mRNA expression and high-grade UC was confirmed by using a Fisher’s exact test (P < 0.05; Supplementary Table 5, available at Carcinogenesis Online). Besides that, a correlation of ITIH5 mRNA expression with both gender and bladder cancer subtypes was observed (Supplementary Table 5, available at Carcinogenesis Online). Using patient-matched specimens including NU, CIS and invasive tumour tissues, a clear decrease of ITIH5 expression in the course of tumour progression could be detected (Figure 1D).

Next, we aimed to verify loss of ITIH5 expression on the protein level by performing immunohistochemistry analysis. ITIH5 protein staining was quantified according to an adapted immunoreactive score (IRS) developed by Remmele and Stegner (1987). In line with the mRNA data, we found abundant cytoplasmic ITIH5 protein expression in normal urothelial cells (Figure 2A and B). In parts of the urothelium, ITIH5 protein staining was predominantly detected in differentiated superficial urothelial cells (see arrows in Figure 2C). In contrast, bladder tumours showed decreased ITIH5 protein staining (Figure 2D–F) or almost complete loss of ITIH5 protein (Figure 2G and H).
**ITIH5 in human bladder cancer**

After spotting a clear down-regulation of ITIH5 expression in human bladder cancer tissues, we analysed whether ITIH5 promoter methylation could be responsible for ITIH5 expression loss in human bladder cancer cells. The analysed promoter region upstream of the transcription start site included the MSP primer-binding sites and the potential regulatory sequences described recently (14) (Figure 3A). The human bladder cancer cell lines RT112 and J82 and also the immortalized normal bladder cell line UROtsa revealed a methylated ITIH5 promoter (Figure 3B). We quantified the methylation level of nine CpG sites within >200 bp of the ITIH5 promoter by using pyrosequencing technique. Consistent with our MSP results, the normal bladder cell line UROtsa as well as the malignant bladder cell lines (RT112, KK47 and J82) showed high methylation levels (Figure 3C). Besides, the cell line HCV29, derived from tumour-associated NU, showed also ITIH5 promoter methylation. Quantitatively, the median methylation value of all nine CpG dinucleotides was 59.1% (HCV29), 56.1% (UROtsa), 63.4% (RT112), 62.7% (KK47) and 54.7% (J82) and significantly correlated with a nearly complete loss (3000-fold reduction, \(P < 0.001\); Figure 3D) of ITIH5 mRNA expression in these five bladder cell lines (Figure 3D).

The functional association between ITIH5 promoter methylation and ITIH5 gene silencing was further supported by *in vitro* demethylation experiments (Supplementary Figure 2, available at Carcinogenesis Online). After demethylation treatment of bladder tumour cell lines (RT112, KK47 and J82), MSP analysis indicated decreased ITIH5 promoter hypermethylation (Supplementary Figure 2A, available at Carcinogenesis Online). This finding was quantified by using pyrosequencing. A representative pyrogram of RT112 cells, before and after 5-aza-2'-deoxycytidine and trichostatin A (TSA) treatment, is shown in Supplementary Figure 2B, available at Carcinogenesis Online. The median reduction rate of ITIH5 promoter methylation level in RT112 tumour cells was significantly \(P < 0.001\) reduced by AFC: 77.8% (Supplementary Figure 2C, available at Carcinogenesis Online). The median reduction rate of ITIH5 promoter methylation level was 35.5 and 69.6% in KK47 and J82 cell, respectively (Supplementary Figure 2C, available at Carcinogenesis Online). In concordance with this effective demethylation of the ITIH5 promoter, ITIH5 mRNA expression was clearly restored after 5-aza-2'-deoxycytidine and TSA treatment (Supplementary Figure 2D, available at Carcinogenesis Online). The up-regulation of ITIH5 mRNA expression was 540-fold, 1160-fold and 2252-fold in RT112, KK47 and J82 cancer cells, respectively. Of interest, in RT112 cells, 5-aza-2'-deoxycytidine treatment without TSA supplement already triggered ITIH5 mRNA re-expression. Contrary to that a sole TSA treatment did not suffice to re-express ITIH5 mRNA in all three cell lines, indicating that ITIH5 promoter DNA methylation plays a major epigenetic role in modulation of ITIH5 expression.

**ITIH5 promoter hypermethylation correlates with both ITIH5 mRNA down-regulation in human urothelial tumours and higher progressive tumour stages**

Subsequently, we analysed human bladder cancer samples (\(n = 58\) for ITIH5 promoter methylation by using MSP (Figure 4A) and found a methylation frequency of 59% (34/58) in this cohort. A significant association of ITIH5 promoter methylation with clinicopathological characteristics such as tumour size or grade was not observed (Supplementary Table 6, available at Carcinogenesis Online). According to the bladder cancer stages, 15 out of 30 (50%) non-invasive tumours (pTa) and 19 out of 28 (68%) invasive (pT > 1) bladder tumours exhibited a methylated ITIH5 promoter. All analysed normal bladder tissues (\(n = 15\)) did not show ITIH5 promoter methylation, corroborating a tumour-associated aberrant mechanism in bladder carcinogenesis.
Fig. 2. Loss of ITIH5 protein expression in human UC. (A and B) Very strong ITIH5 immunoreactivity in epithelial cells of NU (intensity: 3+) and faint staining of fibroblasts (arrow). (C) Strong ITIH5 protein expression in epithelial cells of NU (intensity: 2+) with enhancement in terminally differentiated superficial urothelial cells (arrows) (intensity: 3+). (D and E) Moderate ITIH5 immunoreactivity in cells of papillary non-invasive pTa tumours (intensity: 2+). (F) Low staining in invasive tumour cells (intensity: 1+). (G and H) Very low staining in muscle-invasive tumour cells (intensity: 0). Scale bar: 50 µm. (I) Box plot analysis demonstrating significant loss of ITIH5 protein expression in all bladder tumours \( (n=55) \) compared with control tissue \( (n=8) \). Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum; *\( P < 0.05 \), ***\( P < 0.001 \). pTa: papillary non-invasive UC; invasive: invasive UC (pT2-4). (J and K) Negative controls of NU. The application of primary antibody was omitted. Scale bar: 50 µm. (L) Strong ITIH5 protein expression in FFPE section of placenta tissue that served as positive control for ITIH5 staining. Scale bar: 100 µm.
To address the question whether *ITIH5* promoter methylation contributes to *ITIH5* expression loss in primary bladder cancer, we correlated methylation and mRNA expression data of those cancer patient samples for which both data sets were available (n = 36). These included 16 unmethylated and 20 methylated bladder cancer samples. Ten unmethylated normal bladder tissues served as control and their median expression rate was set to 1 (Figure 4B). Compared with these normal bladder tissues, unmethylated UCs showed an almost equal median *ITIH5* expression rate of 1.18 independently of the tumour grade (Figure 4C). In contrast, methylated tumour tissues exhibited
**Fig. 4.** *ITIH5* promoter hypermethylation in primary human bladder is associated with loss of *ITIH5* mRNA expression and bladder cancer progression cancer. (A) Representative MSP results of the *ITIH5* promoter methylation status in four papillary non-invasive tumours (pTa) as well as four invasive bladder cancer (UC) samples in comparison to four normal tissue specimens. Bands labelled with U and M reflect unmethylated and methylated DNA, respectively. Bisulphite-converted unmethylated genomic (U-co) and polymethylated genomic (M-co) DNA were used as positive controls. NTC: non-template control. (B) Relative *ITIH5* mRNA expression levels of NU and bladder cancer samples arranged in relation to their *ITIH5* promoter methylation status. U: unmethylated normal or tumour specimens; M: methylated tumours. Error bars: ±standard error of margin. (C) FC of *ITIH5* down-regulation according to hypermethylated *ITIH5* promoter status. U: unmethylated normal (NU) or tumour (UC) specimens; M: methylated tumours (UC). Horizontal lines: grouped medians. Boxes: 25–75% quartiles, Vertical lines: range, peak and minimum; ***P < 0.001. (D) Pyrogram of patient-matched normal (n = 3), CIS (n = 3) and invasive tumour (n = 3) specimens is shown. Methylation levels for each of the pyrosequenced CpG dinucleotides (10–14) within the *ITIH5* promoter region, representing the mean methylation frequency of three independent patients. (E) Box plot demonstrating significant increase of *ITIH5* promoter methylation frequency during bladder tumorigenesis. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum; ns: not significant, *P < 0.05. (F) Mean methylation frequency for each pyrosequenced CpG duplet (10–14) of NU (n = 3), pTa-lg (n = 9), pTa-hg (n = 8) and invasive (n = 9) bladder cancer samples is shown as pyrogram. (G) Box plot demonstrating significant increase of *ITIH5* promoter methylation frequency in association to bladder cancer progression. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum; ns: not significant, ***P < 0.001. pTa: papillary non-invasive UC; CIS: flat non-invasive UC; invasive: invasive UC (pT2-4); pTa-lg: papillary non-invasive pTa low-grade bladder cancer; pTa-hg: papillary non-invasive pTa high-grade bladder cancer.
a significant (P < 0.001) loss of ITIH5 mRNA expression (median expression: 0.16, ΔFC: 7.4). This correlation between loss of ITIH5 mRNA expression and ITIH5 promoter methylation was statistically supported by using a Fisher’s exact test (P = 0.001; Supplementary Table 6, available at Carcinogenesis Online), suggesting that aberrant ITIH5 methylation presents a major mechanism for ITIH5 gene silencing in primary bladder cancer.

Given that ITIH5 loss was tightly linked with ITIH5 promoter methylation in both bladder cancer cell lines and primary bladder tumours, we aimed to determine whether this epigenetic modification is correlated with bladder cancer progression. To carefully address this question, we quantified ITIH5 methylation frequency in matched patient samples (n = 3), where NU, CIS and solid invasive tumour tissue each was available. Analysing individual CpG sites of the ITIH5 promoter by pyrosequencing (Figure 4D) revealed that ITIH5 methylation frequency is significantly (P < 0.05) increased with bladder cancer progression in these matched samples (Figure 4E). The mean ITIH5 methylation level of tumour-adjacent normal tissue was 7.7% (SD ±3.8%). In contrast to that, median ITIH5 methylation frequency was increased in CIS and tumour tissue by 2.3-fold (mean ITIH5 methylation: 17.2%, SD 2.7%) and 3.7-fold (mean ITIH5 methylation: 20.8%, SD 8.5%, P < 0.05), respectively.

Parallel to that, we analysed in more detail the correlation between ITIH5 methylation level and the two principal bladder cancer pathways (i.e. papillary versus invasive) by pyrosequencing. We focused on pTa low-grade (pTa-lg; n = 9) and pTa high-grade tumour (pTa-hg; n = 8) samples in comparison to muscle-invasive tumours (n = 9). Normal tissues (n = 3) served as control (median ITIH5 methylation: 5%). Compared with these healthy samples, we detected a constant increase in ITIH5 methylation level associated with the progressive tumour stage and grade (Figure 4F and G). pTa low-grade tumours exhibited a mean ITIH5 methylation of 13.4% (SD 4.5%), whereas it was clearly increased to 20.8% (SD 9.9%) in pTa high-grade tumours. However, muscle-invasive tumour samples exhibited a mean ITIH5 promoter methylation level of 38.2% (SD 12.5%). Taken together, aberrant ITIH5 promoter methylation was existent in both early and late stages of the papillary-invasive bladder cancer pathway but increased in invasive tumours up to ~40%.

**ITIH5 loss is associated with early relapse in the clinically challenging group of patients with pT1 high-grade UC**

Owing to the increased ITIH5 promoter methylation found in pTa high-grade tumours, we decided to take a closer look on ITIH5 protein expression in 120 papillary high-grade UC samples (for cohort characteristics of analysed samples, see Supplementary Table 2, available at Carcinogenesis Online) and its impact on patients’ outcome. Altogether, 98 out of 120 (81.7%) pT1 high-grade tumours showed an ITIH5 protein staining of IRS ≥4. The median ITIH5 IRS was 4 (average IRS: 6.6, SD ±1.5, range: 2–8), and in solely 18.3% (22/120) of pT1 bladder cancer specimens, a clear ITIH5 staining (IRS ≥ 6) was detected. According to that, high ITIH5 (IRS = 6–8) and low ITIH5 expression (IRS = 0–4) was dichotomized.

No associations were found between our ITIH5 immunohistochemistry results and the clinico-pathological characteristics of this cohort (Supplementary Table 7, available at Carcinogenesis Online). By univariate Kaplan–Meier analysis, we found that patients with high ITIH5 expression tend (P = 0.16) to have longer RFS [mean RFS: 144.9 months ± 17.2; 95% confidence interval (CI): 111.3–178.5] when compared with pT1 bladder tumours with low ITIH5 expression (mean RFS: 100.3 months ± 10.0; 95% CI: 80.6–119.9) (Figure 5A). After subgrouping our cohort, we found that ITIH5 expression has a significant (P < 0.05) prognostic impact in metastasis-free (pM0) patients (Figure 5B and Supplementary Table 8, available at Carcinogenesis Online). No significant correlation in patients with distant metastasis was observed (Figure 5C and Supplementary Table 8, available at Carcinogenesis Online). Non-metastasized patients with low ITIH5 expression had a poorer RFS (mean RFS: 96.9 months ± 11.1; 95% CI: 75.2–118.7) compared with non-metastasized patients showing high ITIH5 expression (mean RFS: 165.7 months ± 15.3; 95% CI: 135.6–195.7). High ITIH5 expression, therefore, indicated favourable outcome. The calculated Cox regression model, including all factors potentially relevant to influence RFS, also confirmed loss of ITIH5 expression to be a putative independent marker for early recurrence (Supplementary Table 9, available at Carcinogenesis Online). Bladder cancer patients with low ITIH5 expression have an estimated 4.3-fold increased risk for tumour relapse compared with patients with high ITIH5 expression (multivariate hazard ratio: 4.348, 95% CI: 0.05–0.99, P < 0.05).

**ITIH5 re-expression reduces tumour cell migration and colony growth of RT112 bladder cancer cells**

With respect to the prognostic impact of ITIH5 loss in papillary high-grade tumours, we hypothesise a functional involvement of ITIH5 in tumour progression associated mechanisms in bladder cancer. In order to address this hypothesis functionally, we restored ITIH5 expression in the papillary bladder cancer cell line RT112 by transient as well as by stable transfection with a full-length ITIH5 cDNA pBK-CMV expression vector (RT112-ITIH5 clones) or the empty vector alone (RT112 mock clones). Ectopic ITIH5 expression in transient and stable RT112 clones was confirmed by real-time PCR and western blotting (Figure 1A and Supplementary Figure 3, available at Carcinogenesis Online).

![Fig. 5. ITIH5 protein expression predicts longer RFS in the clinically important group of pT1 high-grade bladder cancer patients. Univariate Kaplan–Meier survival curves displaying RFS of patients with high ITIH5 (IRS = 6–8) expression (black line) in relation to low ITIH5 (IRS = 0–4) expression (grey line) in (A) all, (B) non-metastasized (pM negative) or (C) metastasized (pM positive) UC patients. Vertical lines: censored cases.](https://example.com/f5.png)
Subsequently, we used these *in vitro* tumour models to analyse the effect of ITIH5 re-expression on tumour cell behaviour. Although no significant influence on the proliferation rate of RT112 cancer cells was observed (data not shown), tumour cell migration was clearly impaired by ITIH5 in both the transient and the stable model (Figure 6B and 6C). Based on a monolayer wound healing assay, we observed that transiently ITIH5 re-expressing RT112 cells (RT112-ITIH5*\text{transient}\*) migrated much slower compared with mock-transfected RT112 control clones (RT112-mock*\text{transient}\*) (Figure 6B). These effects were strikingly confirmed using the stable ITIH5 gain-of-function

![Figure 6](image-url)

**Fig. 6.** ITIH5 re-expression suppresses migration and colony formation of papillary RT112 bladder cancer cells. (A) Transient ITIH5 expression in transfected RT112 tumour cells (72 h after transfection). Upper graph: Relative ITIH5 mRNA expression of transiently transfected (mock or ITIH5) RT112 cells is shown. Column: mean of triplicate determinations. Error bars: ± standard error of margin. Bottom: western blot analysis of the ITIH5 protein expression in mock-transfected and ITIH5-transfected RT112 cells. (B) Cell migration of transiently transfected RT112 cells was analysed by using a monolayer scratch assay. Comparison of migration of the control cell set (RT112-mock*\text{transient}\*) and the ITIH5 set (RT112-ITIH5*\text{transient}\*) over 3 days. Vertical lines: standard deviation of triplicates. Cell-free area on day 0 was set as 100% and used for standardization. (C) Cell migration based on stably transfected independent RT112 mock and ITIH5 clones. Comparison of migration of a control cell set (*n* = 5) and RT112-ITIH5 clones (*n* = 4) over 5 days. Vertical lines: standard deviation of triplicates. Cell-free area on day 0 was set as 100% and used for standardization. Δ*day 2*: differences of cell-free areas on day 2. (D) Left graph: detailed comparison of wound closure of each clone after 48 h. ***P < 0.001. Right images: wound documentation of a magnified area by phase contrast microscopy 48 h after scratching. Scale bar = 100 µm. White star: completely colonized (i.e. closed) wound area. Arrows: scratch direction. White border: cell-free wound area. (E) 2D colony formation assay. Representative 6-well plates containing RT112-WT, RT112-mock*\text{stable}\* (#1, #5, #10, #12) or RT112-ITIH5*\text{stable}\* clones (#9, #16, #17, #18) are shown 2 weeks after cell seeding. (F) Densitometrical evaluation of colony growth after 2 weeks. Box plot graph presents averages of colony growth of triplicate experiments for each clone. Horizontal lines: grouped medians. Boxes: 25–75% quartiles. Vertical lines: range, peak and minimum, ***P < 0.001.
RT112 cell model. After 2 days in culture, RT112-mockable clones as well as parental RT112 wild-type cells had repopulated almost the entire wound (on average 97.8%), whereas RT112-ITIH5able clone migration into the scratch was significantly (P < 0.001) inhibited (Figure 6C). At this time point, these ITIH5-expressing cells covered solely on average 44.6% of the wounded area, i.e. 60.1% (#16), 52.6% (#17), 37.6% (#9) and 27.9% (#18). Micrographs of magnified wound areas 48 h after scratching are shown in Figure 6D.

Beyond that, we studied the impact of ITIH5 expression on colony formation, which reflects, to some extent, tumour spreading capability. After 2 weeks, ITIH5 re-expressing clones clearly demonstrated impaired colony formation capability compared with the mock control clones (Figure 6E). Densitometric evaluation determined a highly significant retardation of ITIH5-expressing cells in colony forming by 88.6% (P < 0.001) (Figure 6F). Compared with controls whose median colony growth was defined as 100%, the median colony growth of ITIH5-expressing RT112 clones was 11.4%.

Discussion
ITIH5 is a novel member of the ITI heavy chain family (13,34) that is assumed to have similar extracellular matrix-stabilizing functions as described previously for other ITI heavy chain members (3). ITIH-mediated cross-linking of HA has been implicated in tumour suppression (3), arguing that loss of ITIH5 could also foster tumour development or progression. Indeed, accumulating studies suggested involvement of ITIH5 in human tumorigenesis of various entities like breast cancer (14), thyroid cancer (17), acute myelogenous leukaemia (35) and squamous cell carcinoma of the tongue (36). Also ITIH5 dysregulation in bladder cancer has been described recently, but solely on mRNA level (18). The current study is the first to analyse in depth ITIH5 expression and regulation in human bladder cancer as well as its potential clinical and functional impact towards this important cancer disease.

In normal bladder tissue, we clearly assigned strong ITIH5 expression to the urothelium verified both by real-time PCR and immunohistochemistry. Histologically, ITIH5 protein was particularly localized in the cytoplasm of superficial cells but was also substantially present in less differentiated cells of the urothelium. Only minimal ITIH5 expression was observed in stromal cells. This expression pattern might argue for a distinct ITIH5 function in NU whose extracellular matrix protein composition is known to provide crucial structural support for the bladder (37). Bearing in mind that a main structural scaffold factor, namely HA, is thought to have an important role in bladder homeostasis and injury response (37), it is likely that ITIH5 may be involved in these processes potentially affecting also tumour development.

In line with this notion, ITIH5 expression was notably downregulated in the course of bladder cancer development. ITIH5 loss occurred already in CIS, but the most abundant lack of ITIH5 expression was identified in muscle-invasive UC, as well as in papillary high-grade tumours. These findings suggest a putative tumour suppressive role of ITIH5, and the loss of ITIH5 may be essential for the malignant progression of bladder cancer as proposed for breast cancer by Veeck et al. (2008) (14).

Addressing the molecular cause for ITIH5 loss, we focused on DNA methylation as it is known that the ITIH5 promoter sequence contains distinct CpG islands and has been found to be methylated in human breast cancer (14,16). Indeed, tumour-specific ITIH5 promoter hypermethylation, a major mechanism to silence tumour suppressor genes (38), was evident in bladder cancer as well. In vitro cultured bladder cancer cell lines such as RT112 and J82 as well as UC tissue samples exhibited aberrant ITIH5 promoter methylation. Of importance, in both, there was a remarkable correlation between ITIH5 promoter methylation and ITIH5 expression, which may be indicative of an important biological function in these tumours. So far, a tumour-specific loss of ITIH5 clearly associated with promoter hypermethylation was only shown for breast cancer (14). Hence, our study gives further indication that this epigenetic mechanism might be a general means to silence the ITIH5 gene in human cancer types.

Beyond that, we revealed a linkage of ITIH5 methylation with clinico-pathological characteristics of UC. Referring to the subgroup, half of patients with papillary non-invasive (pTa) low-grade bladder tumours showed aberrant ITIH5 promoter methylation but a closer look by using pyrosequencing analysis clearly demonstrated that solely a marginal percentage of tumour areas seemed affected. In turn, papillary non-invasive (pTa) high-grade tumours exhibited a strikingly increased ITIH5 promoter methylation level. Furthermore, accumulated abundance of ITIH5 promoter methylation was detected in muscle-invasive UC. Two-thirds of patients with muscle-invasive bladder cancer showed a methylated ITIH5 promoter, and also a high proportion of tumour cells definitely carried this epimutation, suggesting that ITIH5 loss might provoke invasive bladder cancer pathways.

Genetically instable invasive bladder cancer is known to cause frequent metastasis (21), however, bladder cancer progression is a major challenge in the field of papillary non-invasive (pTa) high-grade and early invasive tumours (pT1). Although low-grade papillary non-invasive tumours often do not progress (23), pTa and pT1 high-grade tumours have a progression rate up to 40% within 5 years (24,25). By now, the disease management of patients with pT1 high-grade tumours is still poor due to lack of prognostic and predictive markers (28), and consequently one-third of these patients die in consequence of metastatic disease (26). With respect to that, we examined whether ITIH5 expression has an impact on the clinical outcome and focused on a patient cohort comprising pT1 high-grade UCs. The univariate analysis of all patients showed that ITIH5 expression tends to results in reduced patients’ RFS. As ITIH5 loss might mediate initial bladder cancer progression, we took next a closer look to cancer patients who did not show metastatic spread. Interestingly, in this clinically important patient group, ITIH5 protein expression showed a clear prognostic value: ITIH5 loss notably increased the risk for early tumour relapse and consequently the likelihood for progression of these tumours. Thus, ITIH5 might represent a novel prognostic biomarker helping to stratify high risk of recurrence or progression in pT1 tumours. Future studies will be necessary to validate the precise biomarker potential of ITIH5 in high-grade bladder cancer.

Further analysis of the functional impact of ITIH5 expression on papillary bladder cancer cells supported our hypothesis that ITIH5 might suppress bladder cancer progression. In fact, transient as well as stable ITIH5 re-expression in RT112 cancer cells, originally derived from a papillary G3 tumour (39), caused an inhibition of bladder tumour cell motility in vitro. This inhibition of cell migration supposed a decrease in colony spreading. Indeed, ITIH5 expression clearly mediates suppression of colony growth, whereas the proliferation rate of these cells was not altered. These in vitro findings imply that ITIH5 particularly mediates suppression of those cellular mechanisms, which have been defined as hallmarks for invasive cancer cells (40).

In conclusion, we provide for the first time evidence that ITIH5 down-regulation by aberrant ITIH5 promoter methylation during bladder cancer development may promote bladder cancer progression. Moreover, our results suggest a putative usability of ITIH5 as prognostic biomarker for the clinically challenging group of patients with papillary non-invasive (pTa) high-grade or pT1 bladder cancer whose disease management must be urgently adapted to the (individual) progression risk. Hence, further investigation of the contribution of ITIH5 to bladder cancer invasion and metastasis may help to approach this challenge, finally advancing more favourable patient outcome.

Supplementary material
Supplementary Tables 1–9 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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