Expression of Snail in upper urinary tract urothelial carcinoma: Prognostic significance and implications for tumor invasion

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Running title: Prognostic significance of Snail expression in upper urinary tract urothelial carcinoma

Key words: Snail, E-cadherin, epithelial-mesenchymal transition (EMT), upper urinary tract urothelial carcinoma, MMP2, MMP9, Vimentin

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Translational Relevance

Upper urinary tract urothelial carcinoma (UTUC) is a relatively rare occurrence. There are only a few molecular markers that predict the prognosis. Therefore, the investigation of molecular markers that predict the prognosis of UTUC is considered to be a high priority. Snail, which contributes to epithelial-mesenchymal transition (EMT), has been documented in cancer progression, but not clear yet in UTUC. We therefore addressed the expression and biological significance of Snail in UTUC. Strong Snail staining was observed in many of the nucleus of invasive UTUC. Multivariate analysis revealed that elevated Snail expression was an independent prognostic predictor of recurrence-free survival and cancer-specific survival. Targeting of Snail mRNA expression in UMUC-3 cells with Snail-specific siRNA resulted in decreased invasion activity, accompanied by mesenchymal marker and MMPs reduction. Snail-induced EMT represents a clinically relevant mechanism of UTUC invasion and an attractive target for the treatment.
Abstract

Purpose: There are few molecular markers known to predict upper urinary tract urothelial carcinomas (UTUC) prognosis. Snail, which contributes to epithelial-mesenchymal transition (EMT), has been documented in cancer progression, but not clear yet in UTUC. We therefore addressed the expression and biological significance of Snail in UTUC.

Experimental Design: To elucidate the biologic significance of Snail in UTUC, we examined the immunohistochemical expression of snail in UTUC and analyzed its clinical significance in 150 patients with UTUC. Biological effects of Snail in EMT and invasion were evaluated by using small interfering RNA (siRNA) specific for Snail in UC cell lines and the Matrigel invasion assay.

Results: Nuclear Snail staining was very weak in superficial UTUC. In contrast, strong Snail staining was observed in many of the nucleus of invasive UTUC. Snail expression was significantly higher in the high tumor stage, high grade, and in tumors showing lymphovascular invasion (LVI). Multivariate Cox regression analysis revealed that elevated Snail expression was a significant and an independent prognostic predictor of recurrence-free survival and cancer-specific survival. Patients with positive LVI and high Snail expression showed the worse outcome. Targeting of Snail mRNA expression in UMUC-3 cells with Snail-specific siRNA down-regulated the mRNA expression of Snail, Vimentin, MMP2 and MMP9. Furthermore, the cells with siRNA for Snail showed decreased invasion activity in comparison to the cells transfected with a non-targeting siRNA.

Conclusion: Snail-induced EMT represents a clinically relevant mechanism of UTUC progression and an attractive target for the treatment of patients with UTUC.
Introduction

Upper urinary tract urothelial carcinoma (UTUC) accounts for approximately 10% of all renal tumors and for approximately 5% of all urothelial carcinomas(1). UCUC is a relatively rare tumor and there are few molecular markers known to predict the disease prognosis (2-3). Radical nephroureterectomy (RNU) with excision of the bladder cuff is the standard of procedure for UTUC. Lymphovascular invasion (LVI) has been recently reported to be an independent predictor in a large, multicenter, international, retrospective data set of patients treated with RNU(4). Despite the clinical importance, little is known about the molecular mechanism of LVI and the subsequent establishment of markers for predicted LVI, which may contribute to improvement of patients with urothelial carcinoma (UC). Therefore, it is important to examine the factors playing a significant role in LVI with patient with UTUC.

Epithelial to mesenchymal transition (EMT) is a process that was initially observed during embryonic development in which cells lose epithelial characteristics and gain migratory and invasive properties of mesenchymal cells. According to several reports, EMT is a key process in cancer development and progression (5-6). Recent study has indicated that EMT may contribute to chemoresistance(7-8). Tumor cells transform from a non-invasive to an invasive phenotype through a series of metastatic steps, whereby epithelial cells lose polarity and invade the lymphovasculature.

Recent research has documented the role of Snail in EMT(5). Snail is a member of a superfamily of zinc-finger transcription factors, which was first identified in Drosophila melanogaster(9). Snail mediates events in the mesoderm, neuroectoderm, and development od other organs in the embryo(10). Snail is considered a key regulator of EMT and thereby of tumor progression and metastasis(11). Snail represses the transcription of E-cadherin(12-14) by binding to E-box elements found in the proximal E-cadherin promoter(5), thereby triggering a complete EMT with acquisition of invasive properties. In squamous cell carcinoma and hepatocarcinoma, Snail expression has been associated with MMP expression
(11, 15-18) and invasiveness. The expression of Snail in breast carcinoma is associated with tumor recurrence and poor prognosis (19-23). Despite the importance of Snail in EMT and cancer progression, its expression and underlying molecular mechanisms of action have not been well characterized in UTUC.

In this study, we evaluate Snail expression to determine its prognostic significance in patients with UTUC. Furthermore, in order to elucidate the molecular mechanisms involved in the invasion activity, we analyze Snail function using small interfering RNA (siRNA) and Matrigel invasion assay.
Materials and Methods

Patient Selections

Surgical specimens from 150 patients who had been surgically treated for UTUC at Keio University Hospital from 1990 to 2007 were employed. The median follow up was 54 months and the mean patient age was 66.1 years (range, 36 to 89 years). The patients did not receive any chemotherapy or radiation therapy before surgery. The patients with distant metastasis at the time of diagnosis and incomplete clinical data were excluded from this study. Nephroureterectomy with removal of the bladder cuff was the most common procedure (N=147). Partial ureterectomy was performed in 3 patients. Regional lymphadenectomy was generally performed in patients with suspicious lymph nodes on preoperative axial imaging or with adenopathies detected during intraoperative examination. Extended lymphadenectomy was not routinely performed. The patients were followed postoperatively with urinary cytology every 3 months for 2 years and every 6 months thereafter. Computed tomography as well as cystoscopy and magnetic resonance imaging (MRI) were performed every 6 months for 5 years and annually thereafter.

Tissue samples

All the specimens were fixed in 10% formalin and embedded in paraffin, and all slides were re-reviewed by genitourinary pathologists. Tumors were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer TNM classification. Tumor grading was assessed according to the 1998 WHO/International Society of Urologic Pathology consensus classification(24). LVI was defined as the presence of tumor cells within an endothelium-lined space without underlying muscular walls (table 1).

Immunohistochemistry

Sections (4μm) of formalin-fixed and paraffin-embedded material were analyzed. The sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. After antigen retrieval with citric acid (pH6.0), endogenous peroxidase activity was blocked.
with 1% hydrogen peroxide for 30 min followed by washing with distilled water. To bind nonspecific antigens, the sections were incubated with 5% skim milk for 15 min. The sections were incubated with either an anti-E-cadherin mouse monoclonal antibody (1:100 dilution, DAKO JAPAN, Tokyo) at room temperature for 1h or a anti-Snail rabbit polyclonal antibody (1:800 dilution, Abcam, Cambridge, U.K) at room temperature for 1h. Both antibodies were detected using the avidin-biotin complex peroxidase method. Color was developed with 3,3’-diaminobenzamine tetrahydrochloride in 50 mM Tris-HCl (pH7.5) containing 0.005% hydrogen peroxide. The sections were counterstained with hematoxylin.

Evaluation of immunostaining

To evaluate Snail staining, cancer cells with positive staining in the nucleus were counted in at least 10 representative fields, and the mean percentage of positive cancer cells and staining intensity, which was stratified from 0 to 3 (0, no staining; 1 slight staining; 2, medium staining; 3, strong staining) were estimated. Expression of Snail was assessed using a semiquantitative scoring system. Histoscore (Snail score) was calculated by applying the formula: Mean percentage × intensity (range 0 to 300). E-cadherin expression in the tumor cells was scored as the average proportion of detectable immunoreactions in 10 representative fields (range 0% to 100%) for each tumor. The immunoreactivity for Snail and E-cadherin was analyzed independently by two uropathologists.

Cell culture

Three urothelial cancer cell lines (UMUC-3, T24, 5637) were routinely maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% FBS at 37°C in a humidified 5% CO₂ atmosphere. UMUC-3, T24, 5637 were obtained from the American Type Culture Collection.

Cell extracts and Western blot analysis

Whole-cell extracts were obtained using RIPA buffer (50 mM, Tris-HCL pH 7.5, 150 mM NaCl, 1%NP-40, 0.5% deoxycholate, 0.1%SDS) containing protease inhibitors. For Western blot analysis, 20μg of nuclear protein for Snail and 50μg of total protein for
E-cadherin, Vimentin from each sample were loaded on 12.5% SDS-polyacrylamide gels. Immunoblotting was also done according to a standard method. Proteins were transferred onto a polyvinylidene difluoride membrane in blocking solution (5% nonfat dry milk in TBS containing 0.1% Tween 20). The primary antibodies for Snail and E-cadherin were the same we used for immunohistochemistry. The primary antibody for Vimentin (1:200 dilution, Dako), MMP2 (1:400 dilution, Abcam) or MMP9 (1:400 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) were used for the study. After washing, the membranes were incubated for 1.5 hr at room temperature linked with peroxidase secondary antibody (DAKO, Carpinteria, CA), and signals were detected using RAS3000 Image Analysis System (Fuji Film, Tokyo).

**Small-interference RNA for Snail**

Snail expression was transiently down-regulated using the following predesigned duplex siRNA directed against Snail (si-SnailA and B) and non targeting control siRNA (Ambion negative control siRNA): The sense sequences of siRNA for Snail were as follows: si-SnailA: 5’-AGGCCUUCAACUGCAAAUAdTdT-3’: si-SnailB: 5’-AGGCCUUCAAACUGGAAAUAAdTdT-3’. UMUC-3 cells were transiently transfected with 10 nmol of si-SnailA, B or control siRNA using Fugene (Roche, Indianapolis, IN). After 12h, siRNA was removed by replacing the culture medium with fresh RPMI 1640 containing 10% fetal bovine serum and cells were cultured for additional 36h. A mock-transfection control was prepared using the transfection reagent only.

**Real-time PCR**

Total RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany), and the quantity and quality were evaluated by spectrophotometry. Reverse transcription of RNA to cDNA was done using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative gene expression was done for Snail (Hs00195591_m1), E-cadherin(Hs01023894_m1), Vimentin(Hs00185584_4m1), MMP-2(Hs00234422_m1), MMP9(Hs00234579_m1), and β-actin (Hs99999903_m1) with gene-specific probes (Applied...
Biosystems) using Taqman Universal PCR Master Mix and the 7500 Fast Real-time PCR system (Applied Biosystems). The cycling conditions were 50°C for 10 minutes, 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The data were then quantified using the comparative C_t method for relative gene expression compared to β-actin as endogenous control.

**In vitro cell invasion assay**

Invasion of tumor cells was assessed by counting the number of cells that migrated through transwell inserts with a polyethylene terephthalate membrane (8μm pore size) coated with a uniform layer of BD Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA), according to the protocol recommended by the manufacturer. Briefly, cells (5×10^4) suspended in 1% FBS 0.5mL RPMI 1640 were seeded in the upper chamber, whereas the lower chamber was loaded with 0.5 mL of 10% FBS medium. After 24 h at 37°C in a well-humidified incubator, the cells that remained inside the inserts were removed, and those that invaded the reverse side of the inserts were rinsed and counterstained using a Diff-Quik stain kit (Sysmex Co., Hyogo, Japan). The cells that had migrated through the membranes were counted in six randomly chosen visual fields at ×100 magnification.

**Cell viability assay**

UMUC-3 cells were plated in 96-well plates, allowed to attach for 24 h, and then treated with different concentrations of cis-dichlorodiammineplatinum (CDDP). At the end of the incubation period, WST reagents were added to each well and incubated for 1 hr. Cell viability was estimated by colorimetry, reading the color intensity in a plate reader at 570 nm. WST regent was purchased from Takara Bio Inc. (Kyoto, Japan). CDDP was purchased from Sigma.

**Statistical analysis**

The association between Snail and E-cadherin, and clinicopathological features was assessed using chi-square test. Differences between Snail expression score and clinical
variables were analyzed using Mann-Whitney’s U-test. Cancer specific survival (CSS) and recurrence free survival (RFS) curves were calculated by the Kaplan-Meier methods. We compared survival rates according to certain clinicopathological variables using log-rank test, including patient age, gender, tumor location, pathological tumor stage (pT), grade, LVI, E-cadherin expression, and Snail expression. We used the Cox proportional hazards regression analysis to assess the prognostic indicators including age, gender, tumor location, pT, grade, LVI, E-cadherin expression, and Snail expression for survival. The level of statistical significance was set at p<0.05. These analyses were performed with SPSS version 16.0 statistical software package (SPSS Corporation, Chicago, IL).
Results

Snail expression in UTUC

All the specimens were fixed in 10% formalin and embedded in paraffin, and all slides were re-reviewed by genitourinary pathologists. To elucidate the biologic significance of Snail in UTUC, we examined the immunohistochemical expression of snail in UTUC (Fig.1). Nuclear Snail staining was very weak in superficial UTUC (Fig.1A). On the other hand, in the invasive UTUC, strong Snail staining was observed in many of the nucleus of cancer cells (Fig.1B). As it has been reported that Snail represses the transcription of E-cadherin, we also investigated the relationship between Snail and E-cadherin expression. E-cadherin staining was very strong in superficial UTUC in the cytoplasm of many cancer cells (Fig.1C). On the other hand, in the invasive UTUC, weak E-cadherin staining was observed (Fig.1D).

Association between Snail expression and clinico-pathological features

Table 1 showed the clinic-pathological features in overall 150 UTUC patients. We analyzed the association between Snail expression and the clinico-pathological features of 150 UTUC samples. We set the cut-off point at 100 based on the frequent distribution of Snail score and the median score. Among the features, the level of Snail expression was significantly higher in UTUC with LVI (p<0.001), high grade (p<0.001), and higher pathological stage (p<0.001) (Table 1). A weak or loss of E-cadherin expression was significantly associated with higher pathologic tumor stage (p<0.001), high grade (p=0.001) and LVI (p<0.001) (Table 1).

Prognostic significance of Snail expression in UTUC patients

To examine the prognostic value of Snail and E-cadherin expression, uni- and multivariate Cox regression analyses were performed (Table 2). Univariate analysis revealed that pathological stage, tumor grade, LVI, and Snail score, were associated with RFS and CSS. E-cadherin expression was associated only with RFS. Multivariate Cox regression analysis showed that Snail expression and LVI were independent prognostic indicators of RFS and
CSS. Kaplan-Meier curves also demonstrated significant differences in RFS (p<0.001, Fig.2A) and CSS (p<0.001, Fig.2B) in regard to Snail expression. The 5-year RFS and CSS rates were 45.0% and 51.2%, respectively, for patients with higher Snail expression, compared to 81.8% and 96.4% for patients with lower Snail expression. Kaplan Meier curves demonstrated no significant differences between higher and lower level of E-cadherin for RFS (p=0.12) and CSS (p=0.09).

Survival of patients with different profiles of LVI and Snail score

We distributed the patients into four different groups according to LVI and Snail expression (Fig.2C). Patients with LVI and high Snail expression showed the worse outcome among the 4 groups. Whereas, patients without LVI and low Snail expression had a longer survival. There were significant differences in the five-year CSS rate between patients with LVI / high Snail expression (37.5%) and patients with LVI / low Snail expression (80.8%) (p=0.011); as well as between patients without LVI / low Snail expression (91.6%) and patients without LVI / high Snail expression (72.1%) (p=0.018).

Prognostic significance of Snail expression in UTUC patients with pathological stage T3 or higher

In patients with pathological T3 or higher (N=68), multivariate analyses demonstrated that LVI and Snail expression were independently associated with RFS and CSS (Fig.2D). The 5-year RFS and CSS rates in patients with high Snail expression were 45.0% and 51.2%, respectively, compared to 81.8% and 96.4% in patients with low Snail expression (p=0.001 for RFS; Fig. 2E, and p=0.001 for CSS; Fig.2D).

Expression of Snail, E-cadherin, Vimentin and invasive activity in human urothelial carcinoma cell lines

The expression of Snail, E-cadherin and Vimentin was analyzed at the protein level by Western blot in three urothelial carcinoma cell lines (5637, T24, UMUC-3) (Fig.3A). Snail in the nucleus was expressed with different intensities in all cancer cell lines. UMUC-3 cells
showed a strong expression of Snail accompanied by relatively weak E-cadherin and strong Vimentin expression. The expression of Snail, E-cadherin, and Vimentin was also analyzed at the mRNA level by Real-time PCR in these cell lines (5637, T24, UMUC-3) (Fig.3A). Real-time PCR demonstrated that the mRNA expression was correlated with the protein level in these cell lines. The Matrigel invasion assay for 24 h revealed that UMUC-3 cells, which showed strong Snail and weak E-cadherin expression, significantly more invasive (40.6 ± 1.6, p=0.001, Fig.3B) than other cells; 5637: 7.6 ± 0.6, T24: 13.3 ± 1.6 (Fig.3B).

**Effect of siRNA for Snail in UMUC-3 cells.**

To investigate the effects of Snail knock-down, we transfected UMUC-3 cells with si-Snail-A or B. Real-time PCR demonstrated that si-Snail-A markedly reduced the level of Snail mRNA expression by 91.5% and 91.1% in comparison to mock or cells treated with the control siRNA (control), respectively (Fig.3C). Similarly, si-Snail-B significantly reduced the level of Snail mRNA expression by 88.4% and 87.8%, compared with mock or control (Fig. 3C). Western blot analysis showed a significantly reduced level of Snail expression in comparison to mock or control group at a protein level (Fig.3D). si-Snail-A down-regulated the mRNA expression of Vimentin by 90.6% (p<0.01, Fig.3C) and up-regulated the mRNA expression of E-cadherin by 2680% (p<0.01, Fig.3C), compared with control. Similarly, si-Snail-B down-regulated the mRNA expression of Vimentin by 58.4% (p<0.05, Fig.3C) and up-regulated the mRNA expression of E-cadherin by 1914% (p<0.01, Fig.3C). This knock-down effect on E-cadherin and Vimentin was confirmed by western blot at a protein level (Fig.3D).

**Effect of Snail knock-down on cancer cell invasion in vitro**

Matrigel invasion assay revealed that UMUC-3 cells transfected with si-Snail-A and si-Snail-B exhibited decreased invasion activity by 83.5% and 77.6% in comparison to control, respectively (Fig.4A). Furthermore, si-Snail-A down-regulated the mRNA expression of MMP-2 and MMP-9 by 95.1% (p<0.01) and 46.4% (p<0.05, Fig.4B), respectively.
Similarly, si-Snail-B down-regulated the mRNA expression of MMP-2 and MMP-9 by 86.2% (p<0.05, Fig.4B) and 43.6% (p<0.05, Fig.4B), respectively.

**Effect of Snail knock-down on the sensitivity to CDDP**

We performed additional experiments to examine the effect of Snail knock-down on the sensitivity of UMUC-3 cells to cis-dichlorodiammineplatinum (CDDP). CDDP is commonly used conventional chemotherapeutic agent for the treatment of advanced bladder cancer. We did not observe significant enhanced effect of si-Snail on the UMUC-3 cells under CDDP treatment (Fig.4D).
Discussion

In the current study, we retrospectively analyzed the impact of nuclear-localised Snail protein expression by immunohistochemistry in a series of UTUC from our single center experience. Our results indicated that Snail expression, which was closely related to tumor grade and stage and LVI, was prognostic indicator. To the best of our knowledge, this is the first study examining the expression pattern of Snail in case matched many UTUC specimens.

Prognostic significance of Snail expression and EMT in UTUC

Recently, histological evidence of Snail expression in human tissue samples has also been reported in gastric cancer (25), hepatocellular carcinoma (26) and ovarian cancer (27) and is expected to serve as a novel prognostic indicator. In this study, we analyzed the nuclear-localized Snail, indicated as active form as transcriptional factor. We found significant correlation between Snail expression and LVI (p<0.001), grade (p<0.001), pathological tumor stage (p<0.001). Additionally, we demonstrated that higher Snail expression was significantly associated with a lower CSS and was an independent prognostic factor. These results indicate that the role of Snail as a regulator of EMT in UTUC might be of special importance for its progression and the establishment of metastasis.

We analyzed the association between Snail expression and the clinico-pathological features of bladder tumor (BT) samples obtained from total cystectomy. The level of Snail expression was significantly associated with a high grade or higher pathological stage and the presence of lymph node involvement. Kaplan-Meier curves demonstrated significant differences in RFS and CSS in regard to Snail expression. These results indicated that the contribution of Snail to clinic-pathological behavior tended to be similar between UTUC and invasive BT (data not shown).

Association between Snail expression and LVI in UTUC

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited. Copyright © 2010 American Association for Cancer Research
Recently, we have reported that LVI was an independent predictor for UTUC in large, multicenter, international, retrospective data set of patients treated with RNU(4). This study indicated that LVI was an independent predictor of clinical outcomes in non metastatic patients who underwent RNU for UTUC. Assessment of LVI may help identify patients who could benefit from multimodal therapy after RNU. We addressed the question of whether there exists an effective molecular target in UTUC, specially focusing on patients with LVI or high stage. Then, combining LVI and Snail expression, proven to be independent predictors in the current study, we examined whether there was a specific combination of Snail expression and LVI in UTUC that was of prognostic value. As shown in Fig 2A, among patients with negative LVI, there was statistically significant difference regarding Snail expressions. The patients without LVI and higher Snail expression showed a worse prognosis than those without LVI and low Snail expression. Furthermore, to examine the significance of Snail in high stage UTUC, we analyzed RFS and CSS in high stage patients. High Snail expression was significantly associated with low RFS and CSS rates. These observations indicate that Snail-associated EMT is closely associated with the mechanism of UTUC progression not only just before UC cells invasion but also after UC cells progression.

**Function of Snail in UC**

We tranfected cells with siRNA targeting Snail mRNA expression in UMUC-3 cells and performed an in vitro invasion assay. The specific siRNA for Snail down-regulated its expression at the mRNA and protein level accompanied by down-regulation of Vimentin and up-regulation of E-cadherin. Under the culture condition used in the present study, Snail-specific siRNA inhibited the Matrigel invasion by UMUC-3 cells, accompanied by reduction of both MMP2 and MMP9. As an EMT mediator, Snail controls the expression of MMPs that contribute to the phenotypic changes associated with invasion through the induction of MMP-1, MMP-2, MMP7, MMP9, and MT1-MMP, in carcinoma cell lines (15-18, Research.
Our results confirmed that Snail knock down in UMUC-3 cells led to significantly but differently downregulated mRNA expression of MMP-2 and MMP-9, accompanied by decreased invasive properties. The mechanism of MMPs regulation by Snail is thought to be more complicated. We speculate Snail may indirectly regulate MMP2 and MMP9 transcription differently. Snail-induced EMT may be an important mechanism as a trigger of the multi-step processes involved in urothelial carcinoma invasion and metastasis accompanied by MMP2 and MMP9 expression. Taking these results into consideration, we propose a simplified schema to understand the involvement of Snail and EMT in UC (Fig. 4D).

**Molecular control of EMT in cancer progression**

Accumulating evidences have suggested the contribution of EMT to therapeutic resistance (8, 29-33). In bladder cancer cells, Sayan et al. founded ZEB2, one of EMT master regulators, contributed to ultraviolet-induced and cisplatin-induced apoptosis (33). We did not observe significant enhanced effect of si-Snail on the UMUC-3 cells under CDDP treatment. This may be due to the differences in the character and origin of the cancer cells or anti-cancer agent. Further investigations will be needed to elucidate the association between Snail and chemotherapeutic resistance in UC. Recently, some exciting reports have suggested that microRNA plays an important role in EMT regulation (7, 34-35). The miR-200 family binds directly to the mRNAs encoding Zeb-1 or Zeb-2, promoting their degradation and blocking translation (7).

In conclusion, our study demonstrated that Snail, which proved to be a significant prognostic factor, may be an attractive target for the treatment of patients with UTUC. Snail-induced EMT may be an important mechanism of cancer progression and a potential target of treatment.
Acknowledgement

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References


10. Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell


**Figure Legends**

Figure 1

Immunostaining for Snail in pTa, high grade and LVI negative superficial UTUC (A), and pT3b, high grade, LVI positive invasive UTUC (B) Immunostaining for E-cadherin in the same case of A(C), and B (D). Note the Strong nuclear staining in invasive UC. Bar indicated 0.5 mm.

Figure 2

The Kaplan-Meier analysis of the Recurrence-free survival (A) and Cancer-specific survival (B) of the patients after surgery for UTUC according to the Snail expression.

(C) The Kaplan-Meier curves of the Cancer-specific survival of the patients after surgery for UTUC according to the Snail expression and LVI, which are stratified according to risk factors.

(D) The Kaplan-Meier curves of the Cancer-specific survival of the patients with pathological stage T3 or higher according to the Snail expression.

Figure 3

(A) The expression of Snail, E-cadherin, Vimentin was analyzed at a protein level by Western blot in three urothelial carcinoma cell lines (5637, T24, UMUC-3). Snail expression in the nuclear extract was detected with different intensities. The expression of Snail, E-cadherin, Vimentin was also analyzed at a mRNA level by Real-time PCR. Expression level of the protein and mRNA showed matched expression.

(B) The cells that invaded through the BD MatrigelTM Basement Membrane Matrix were stained with Diff-Quik. The Matrigel invasion assay for 24 h revealed that UMUC-3 cells significantly strong invasive activity. Bars, mean ± SE (n=6); *, p < 0.01. Two siRNA for Snail down-regulated the mRNA (C) and protein (D) expression of Snail, accompanied by
reduced Vimentin and increase E-cadherin in UMUC-3 cells at a mRNA level and at a protein level. §, §§, #, ψψ; p< 0.01, ## p< 0.05.

Figure 4
(A) The cells that invaded through the BD Matrigel™ Basement Membrane Matrix were stained. The number of cells that invaded through was significantly reduced the cells transfected with siRNA for Snail in comparison to those with mock or non-targeting siRNA. Bars indicate mean ± SE (n=6); #, ## p < 0.01
Two siRNA for Snail down-regulated the mRNA expression of MMP-2 and MMP-9 (B), in comparison to the cells with mock and control siRNA. Bars indicate the mean ± SE.
(C) Effect of Snail knock-down on the sensitivity to CDDP in UMUC-3 cells.
(D) Simplified schematic representation of how the Snail would be involved in the development and progression, including several points at which Snail and invasion can be associated in UC.
Table 1 Correlation of clinicopathological parameters and Snail or E-cadherin expression

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<th>p-value</th>
<th>E-cadherin score (mean ± SD)</th>
<th>p-value</th>
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<tr>
<td><strong>Grade</strong></td>
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<tr>
<td>low</td>
<td>30</td>
<td>49.4 ± 71.8</td>
<td>&lt; 0.001</td>
<td>48.4 ± 35.0</td>
<td>&lt; 0.001</td>
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<tr>
<td>high</td>
<td>120</td>
<td>90.0 ± 99.3</td>
<td>&lt; 0.001</td>
<td>26.3 ± 30.4</td>
<td>&lt; 0.001</td>
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<tr>
<td><strong>Lymph node status</strong></td>
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<tr>
<td>pNx or pN0</td>
<td>139</td>
<td>80.6 ± 95.9</td>
<td></td>
<td>31.2 ± 32.9</td>
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<tr>
<td>pN1,2</td>
<td>11</td>
<td>98.5 ± 94.8</td>
<td>0.174</td>
<td>22.4 ± 25.0</td>
<td>0.684</td>
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<tr>
<td><strong>LVI</strong></td>
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<tr>
<td>Negative</td>
<td>90</td>
<td>55.4 ± 75.8</td>
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<td>38.7 ± 34.3</td>
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<td>60</td>
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<td>18.7 ± 25.2</td>
<td>&lt; 0.001</td>
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<td>Multivariate Analysis</td>
<td>Cancer-Specific Survival</td>
<td>Multivariate Analysis</td>
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<td>LVI</td>
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<td>2.556 (1.175-5.561)</td>
<td>0.018</td>
<td>2.745 (1.107-6.807)</td>
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<td>E-cadherin</td>
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<tr>
<td>Snail</td>
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<td>2.413 (1.204-4.837)</td>
<td>0.013</td>
<td>2.407 (1.094-5.297)</td>
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</table>
Expression of Snail in upper urinary tract urothelial carcinoma: Prognostic significance and implications for tumor invasion

Takeo Kosaka, Eiji Kikuchi, Shuji Mikami, et al.

Clin Cancer Res  Published OnlineFirst October 14, 2010.

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