Expression profile of urothelial transcription factors in bladder biopsies with interstitial cystitis

（間質性膀胱炎生検における膀胱粘膜転写因子の発現プロファイル）

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Abstract

Objectives

Interstitial cystitis (IC) is considered to manifest Hunner's lesion or glomerulation in the bladder based on NIDDK criteria, but the pathology remains elusive. While Hunner's lesion display an inflammatory gene signature, intrinsic abnormalities of the urothelium *per se* are largely unknown. Tissue specific master transcription factors (TFs) play pivotal roles in human development and disease, and we sought to characterize IC pathology based on the expression profile of urothelial master TFs.

Methods

To identify candidate urothelial master TFs, we used bladder carcinoma cell lines which are derived from the urothelial stem cells but display two contrasting characteristics: epithelial or mesenchymal. Gene expression was measured with quantitative RT-PCR. From the initial screening of 170 TFs (human homologs of Drosophila segmentation genes and known master TFs from a database), 28 TFs were selected. Subsequently we purified mRNA from bladder biopsies of IC patients and measured gene expression levels of known urothelial marker genes and candidate master TFs. Multivariate expression data were analyzed with SPSS software.

Results

Factor analysis decomposed the expression profile into four axes: Principal Axis (PA) 1 included retinoic acid (RA) receptors and 17 candidate master TFs. PA2 included *KRT5* and five candidates. PA3 included transcription factor *TP63* and two candidates. PA4 included *SHH* and two candidates. Principal component (PC) analysis segregated biopsies from Hunner's lesion in the PC1(RA)/PC2(SOX13)/PC3(TP63) space.

Conclusions

We developed a method to characterize IC biopsies based on the urothelial master TF signature and Hunner's lesion might be defined as 'decreased progenitors and higher regeneration'.

Keywords

Painful Bladder Syndrome, Principal Component Analysis, Retinoic Acid Receptor alpha, TP63, Urothelial Master Transcription Factors

Introduction

Interstitial cystitis (IC) is diagnosed by three requisites: symptoms (hypersensitivity, urinary frequency, bladder discomfort and pain), cystoscopic findings (Hunner's lesion as 'ulcerative' or bladder glomerulation after hydrodistension as 'non-ulcerative'), and exclusion of confusable diseases. Hunner's lesion is observed as a circumscript, reddish mucosal spot with small vessels radiating towards a central scar, with a fibrin deposit or coagulum attached to this area. This site ruptures with increasing bladder distension, with petechial oozing of bladder from the lesion and the mucosal margins in a waterfall manner.[1,2] Inflammatory collapse of umbrella cells is a major burden but the pathology of IC remains elusive.[3-6] Currently there is no curative or satisfactory treatment for IC. Therefore, elucidation of IC pathology is critical to improve diagnosis and radical treatment.[1,7] Recently, genome scientists have been keen to characterize master transcription factors (TFs) for human development and disease.[8-10] Master TFs control a battery of target genes critical for cellular identities and are themselves under feedforward regulation by their own super enhancers.[8-10] Thus, identification of master TFs represents a novel molecular approach to understand human development and diseases. In this study, we sought to characterize IC pathology based on gene expression profiles of urothelial master TFs. First, we screened candidate master TFs to understand

the crucial transcription cascades in patients' urothelium. Second, we investigated expression profiles of known urothelial marker genes and candidate master TFs in bladder biopsies of IC patients. Multivariate analysis decomposed four principal factors that reflect urothelial development and regeneration. We found that biopsies from Hunner's lesion display a characteristic 'decreased progenitors and higher retinoic acid signaling'.

Materials and Methods

Patients and biospecimens

This study was approved by the Institutional Review Board of our university hospital (ID: 27023), on the basis of the ethical guidelines. Patients who were diagnosed as having interstitial cystitis (IC) at our university hospital agreed to donate their bladder biopsies for research purposes.

Punch biopsy protocol

The sampling (punch biopsy) was performed endoscopically from the Hunner's lesion, trigonal region, and other parts of the bladder before the bladder hydrodistension. The size of specimens is around 1-2 mm. If the patient did not have a Hunner's lesion, the

urothelium with or without glomerulation during the bladder hydrodistension was sampled. However, punch biopsy from the bladder dome was avoided due to the high risk of bladder rupture.

Cell culture

We used bladder carcinoma cell lines which did or did not retain urothelial characteristics as initial screening material. BOY-12E (RCB2627), JMSU1 (RCB2227), T24 (RCB0431), and 5637 (RCB1191) cell lines were purchased from RIKEN Bio-resource Center (Tsukuba, Japan). HuB6 (named after Human Bladder) and HuB15 transitional carcinoma cell lines were established in our university hospital and described elsewhere.[11] We classified these cell lines into either epithelial (E) or mesenchymal (M) type, according to E-cadherin (a pan-epithelial marker) synthesis (Supplementary Figure S1A). 5637, HuB6 and HuB15 synthesized abundant E-cadherin and were grouped into E-type, while BOY-12E, JMSU1 and T24 were grouped into M-type (Supplementary Figure S1A). E-type cell lines concomitantly synthesized transcription factor TP63, a master regulator of stratified epithelium (Supplementary Figure S1A).[12,13] In general, bladder carcinoma is a tumor derived from urothelial stem cells[14] and several TFs in bladder diseases had been investigated in previous study, such

as c-Jun and E2F-1.[15,16] Importantly, bladder carcinoma frequently display mesenchymal phenotype through epithelial to mesenchymal transition (EMT). For example, a well-established cell line T24 is known for mesenchymal characteristics through EMT.[17] We hypothesized that the two contrasting behaviors (i.e. E- or M-type) could be exploited for screening of candidate urothelial master TFs (see below). The four purchased cell lines were cultured under supplier-recommended conditions (for details, visit the website http://cell.brc.riken.jp/en/). HuB6 and HuB15 were cultured using Dulbecco's Modified Eagle Medium (Wako, Osaka, Japan) supplemented with 5% Fetal Bovine Serum (Gibco, Waltham, MA, USA).

Protein extraction and immunoblotting

Bladder cancer cell lines were fixed with 10% trichloroacetic acid (Wako) and whole-cell proteins were dissolved with solubilizing reagent (7 M urea, 2 M thiourea, 2% Triton X-100, Wako). The entangled genomic DNA was sheared by sonication for 20 min. The protein concentration was measured using Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Procedures for immunoblotting have been described elsewhere.[18] Primary antibodies used in this study were as follows: E-cadherin antibody (#610181) was purchased from BD Transduction Laboratories (Franklin Lakes, NJ,

USA). TP63 (#13109), SHH (#2207) and GAPDH (#5174) were from Cell Signaling Technologies (Danvers, MA, USA). Representative data are shown in Supplementary Figure S1A; experiments were repeated at least twice at different passage points.

Strategy for master transcription factor screening

The strategy to identify candidate urothelial master transcription factors (TFs) is as follows: Master transcription factors are known to have several characteristics including: 1) controlling a battery of target genes critical for lineage determination and cellular identities, 2) controlling its own gene expression through auto-feedback super enhancers, and as a result, 3) maintaining exceptionally high expression levels of their own coding genes.[8] We exploited the third characteristic in our screening criteria because of its accessibility: When the mRNA expression level of its coding gene was higher in E-type than in M-type cell lines, the TF was defined as a candidate (Supplementary Figure S1B). Retinoic acid is a known chemical to induce urothelial cell differentiation[19] and we added all-trans-retinoic acid (ATRA, Wako) treated E-type cells to our screening materials. We used different concentrations and durations of ATRA because each cell line has different IC50 against ATRA (data not shown). The condition of ATRA treatment was as follows: 5637 (20μM, four days), HuB6 (5μM, five

days) and HuB15 (2.5μM, six days). We investigated three groups of TFs for the screening (Supplementary Figure S1B). The first group (85 genes) belong to evolutionary conserved homologues of Drosophila segmentation genes (Caudal, Bicoid, Hunchback, Krüppel, Tailless, Even skipped, Hairy, Odd skipped, Paired, Runt, Fushitarazu, Sloppy paired, Odd paired and Engrailed). The second group (33 genes) belong to master TFs identified from bladder tissue (UCSD_Bladder).[9] The third group (52 genes) belong to master TFs from biopsies of colon crypts or cultured mammary epithelial cells (HMEC).[9] We prioritize the 170 genes because know master TFs are developmental regulators evolutionally conserved. From the initial screening of 170 candidate transcription factors (TFs), 28 TFs were selected based on the following criteria: mRNA expression of each coding gene was higher in the epithelial (E) type than in the mesenchymal (M) type of bladder carcinoma cell lines (Supplementary Figures S1 and S2). *TP63* and *FOXA1* served as positive controls (Supplementary Figure S2).

Quantitative reverse transcription polymerase chain reaction

Total RNA was purified using RNAiso Plus (Takara, Osaka, Japan). Reverse transcription was performed using PrimeScript™ with gDNA Eraser (Takara).

Quantification of the copy number of each mRNA was performed using QuantStudio3™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) and the Taqman® method. Data were calculated using the delta-cycle of threshold (ΔCT) method. We used the ΔCT parameter for statistical analysis because mRNA expression follows a log-normal distribution, whereas ΔCT follows normal distribution. Each gene expression is represented as log scale in Table S3 and Table S4, where CT value was normalized by either *18S rRNA* or *CDH1* (E-cadherin). A gene-specific fluorescent probe was designed using a web-based program (https://qpcr.probefinder.com/organism.jsp). TableS1 shows the primer sequences and corresponding fluorescent probes. Gene specific Taqman® probes were purchased from Life Technologies, (Waltham, MA, USA, for Cat#, see TableS2). For PCR, Thunderbird® Probe qPCR mix (Toyobo, Osaka, Japan) was used and the PCR conditions consisted of 40 cycles of two steps (95°C for 15 s and 60°C for 50 s).

Statistical analysis

Comparison between the two groups (M type cells versus E type cells) and statistical differences in mean values (ΔCT) were determined by one-tailed *t* test (p-value < 0.1) using Excel software (Microsoft, Redmond, WA, USA). Statistical differences between

the two groups (IC specimens with Hunner's lesion vs without Hunner's lesion) were determined by two-tailed *t* test (p-value < 0.05) using Excel. Multivariate analyses for IC specimens were performed using SPSS software (IBM, Armonk, NY, USA). We used 'principal axis factoring' as an extraction method and 'promax with Kaiser normalization' as a rotation method (Supplementary DataS1). Initial extraction decreased the dimension down to six but we found that the fifth and sixth axes were less relevant due to smaller eigenvalues (Supplementary DataS1) and lower correlation with other factors. On the other hand, we performed 'principal component analysis' with an extraction method 'promax with Kaiser normalization'. E-type cell lines were added to the analysis to estimate the distance between urothelial dysplasia and IC specimens. 27 variables were chosen to segregate specimens between Hunner's lesion and 'apparently normal' (Supplementary DataS2).

Results

mRNA expression of the 28 candidate TFs as well as known urothelial marker genes were measured in IC specimens sampled endoscopically (For all the Log_2 expression data, see Supplementary TableS3 and TableS4). IC biopsies included various clinical status, including ulcerative type biopsies (Hunner's lesion or 'apparently normal'

urothelium counterpart from the same patient) and non-ulcerative type biopsies (urothelium with or without glomerulation during hydrodistension) (Supplementary TableS3 and TableS4). Initially, we noted that E-cadherin expression (encoded by *CDH1* gene) had a huge variation among different IC specimens (Figure 1A, Log_2 expression range: from 5.29 to 14.96, 815-fold expression difference). Endoscopic handling could have resulted in significant variation of epithelial/stromal ratio and we presumed that the contaminated stroma (i.e. submucosal tissue and inflammatory cells) significantly affected data interpretation. Therefore, we changed the internal control of RT-PCR from *18s ribosomal RNA* to *CDH1* (Figure 1A), thereby exclusively focusing on urothelial status. *UPK1A* expression (uroplakin 1A, a known marker of urothelial cells) was lower in IC biopsies from Hunner's lesion (ulcer positive) compared to urothelium from both ulcerative and non-ulcerative patients (collectively defined as ulcer negative) (Figure 1B). Moreover, expression of other urothelial marker genes, *TP63* (Figure 2A), *SHH* (Figure 2B), or *FOXA1* (data not shown, p=0.035) was lower in Hunner's lesion. Conversely, retinoic acid receptor *RARA* expression was higher in ulcer positive compared to ulcer negative urothelium (Figure 2C). Among 28 candidate TFs, expression of *EVX1* (Figure 3A), *OVOL1* (Figure 3A), *EHF* (Figure 3A), *ELF3* (data not shown, p=0.046), or *GRHL2* (data not shown, p=0.033) was lower in Hunner's

lesion. Conversely, expression of vitamin D receptor *VDR* (Figure 3B), *ELF4* (Figure 3B), *IRF1* (Figure 3B), *ETS2* (data not shown, p=0.026), *NR4A2* (data not shown, p=0.012), or *ZBTB7B* (data not shown, p=0.019) was higher in ulcer positive compared to ulcer negative urothelium. In order to grasp the entire profile, Pearson correlations among expression levels of known urothelial markers or candidate TFs are described in Supplementary TableS5. We noted that several factors are associated with each other and the multivariate expression data could be decomposed to fewer number of dimensions. Factor analysis reduced the expression complexity of known urothelial markers and the candidate TFs and we chose four axes for convenience of classification (Figure 4 and Supplementary DataS1). From the known markers, retinoic acid receptors *RARA* and *RXRA* were classified into the first axis (Figure 4A, Principal Axis: PA1), and the second axis (PA2) was related to the urothelial basal cell marker, *KRT5* (Figure 4A). Other known markers, *KRT7, UPK1B,* and *UPK3A* were classified into PA2 but these three were inversely correlated with *KRT5* (Figure 4B). The third axis (PA3) included four markers, *TP63, KRT20, UPK1A,* and *UPK3B* (Figure 4A), which are closely associated with each other (Figure 4B). The fourth axis (PA4) included *SHH* and *FOXA2* (Figure 4A). Most candidate TFs were classified according to these criteria but some were under-detected (*KLF3, KLF15, NR5A2* and *FOS*) in IC biopsies, and *EHF*

and *CEBPG* were not classified into the four PAs, due to poor correlation with other factors (Figure 4A). Finally, we sought to characterize IC biopsies according to these multivariate expression profiles (Figure 5). We added the expression dataset of E-type cell lines (initial screening materials) and performed a principal component (PC) analysis (Supplementary DataS2). We drew a scatter plot as shown in Figure 5 according to three principal components. Although there was no remarkable segregation between ulcer statuses (black circles: Hunner's lesion, white circles: 'apparently normal' urothelium counterparts from ulcerative type patients and urothelium from non-ulcerative type patients), biopsies from Hunner's lesion (black) tended to be located in particular PC areas (Figure 5, see discussion).

Discussion

According to NIDDK criteria, IC patients have either Hunner's lesion (ulcerative type) or glomerulation after hydrodistension (non-ulcerative type) but the boundary between them is sometimes obscure.[1] Importantly, the visible ulcer status does not necessarily reflect pain severity and relapse rate.[20] In contrast, 'apparently normal' urothelial counterpart of ulcerative IC patients could be 'pre-malignant', making the prognostic prediction even more difficult. Therefore, it is critical to define the molecular pathology

of intractable disease.

Several groups have reported comprehensive gene expression analyses for IC specimens using microarray.[21-23] Overall, Hunner's lesion display an inflammatory signature but the intrinsic abnormalities in urothelial homeostasis remain unclear. Erickson et al. revealed that patient-derived urothelial cells display normal differentiation capacity *in vitro*.[24] Nevertheless, inflammatory cells and urothelium mutually interact and the intrinsic alteration of urothelium *in situ* remains a pertinent question. In this study, we purified mRNA from bladder biopsies sampled endoscopically from IC patients and measured known urothelial marker gene expression levels and newly identified candidate urothelial master TFs. However, our attempt was hindered by the huge variability in *CDH1* (E-cadherin) expression (Figure 1A). Practically, it is difficult to keep the ratio of epithelial to mesenchymal tissues in the same range during the biopsy procedure (see Methods). Mesenchymal tissue including inflammatory cells could contribute to 'mesenchymal noise' against authentic 'urothelial signal'. To troubleshoot this problem, we switched the normalization gene (internal control) from *18S rRNA* to *CDH1* (Figure 1A). Although the validity of this normalization should be scrutinized, more genes displayed altered expression in Hunner's lesion under E-cadherin normalization (Figures 1-3), suggesting that the improved detection could be a

consequence of the modified methodology. Moreover, the multivariate data were well-correlated with each other (Supplementary TableS3), which prompted further analysis.

Bladder urothelium is organized into three layers, basal, intermediate and superficial layers.[19,25] The superficial layer is covered by umbrella cells, which are terminally differentiated cells specialized for mechanical extension (also termed as transition epithelium). During tissue injury and regeneration, urothelial progenitor cells inside the intermediate layer actively proliferate and replenish the umbrella cells.[19] We decomposed multivariate expression data into four principal axes (PAs) and found that the four PAs reflect the known urothelial biology (Figure 4). PA1 included retinoic acid receptor *RARA* and *RXRA*. Retinoic acid (RA) is a critical signal for urothelial development and regeneration.[19] Mice treated with cyclophosphamide (CPP) display rapid peel-off of urothelium, which regenerate after the damage. RA signal is activated during such experimental conditions.[19] *RARA* expression was higher in Hunner's lesion (Figure 2C), indicating that the urothelium could be undergoing regeneration. PA2 included *KRT5*, *KRT7, UPK1B,* and *UPK3A* but *KRT5* displayed an inverse correlation with the other three (Supplementary DataS1). KRT5 is a marker of the urothelial basal layer, while KRT7, UPK1B, and UPK3A are markers for the intermediate or superficial

layers.[19,25] We interpreted that PA2 reflects the layer localization inside the urothelium.[25] PA3 included *TP63, KRT20, UPK1A,* and *UPK3B*, whereas PA4 included *SHH* and *FOXA2* (Supplementary DataS1). TP63, SHH, and FOXA2 are endodermal markers and critical regulators for urothelial development.[25] TP63 is a master TF in stratified epithelium[12] and is localized at the basal and intermediate layers of the urothelium.[19,25] Lineage tracing analysis revealed that TP63 starts its expression in the urothelial stem cells.[26] In contrast, SHH (Sonic Hedgehog) is an evolutionally conserved developmental regulator. Developing urothelium and bladder stroma interact each other via SHH and WNT signals.[27] *Shh*-expressing cells play an important role in urothelial progenitors during regeneration in CPP-treated mice.[19,27]

We subsequently chose the 27 gene expression profile to segregate the Hunner's lesion from ulcer negative specimens in principal component analysis (Figure5). We noted that the specimens from Hunner's lesion tended to be plotted in PC1(RA)^{high}, PC2(SOX13)^{high} and PC3(TP63)^{low} (Figure5). Based on the interpretation of PA1 (RA) and PA3 (TP63) described above, the pathology of Hunner's lesion could be represented as having 'augmented regeneration' and 'decreased progenitors'. Urothelial regeneration should be within the physiological range if the progenitor cell replenishment and RA-induced differentiation are coupled with each other. In terms of

urothelial biology, Hunner's lesion could be explained as 'a disrupted homeostasis due to overloaded regeneration'. In future, we would like to obtain further evidence to strengthen our hypothesis.

Finally, we did not use the urothelial biopsies from healthy volunteers. To date, non-tumor counterpart tissue from bladder carcinoma patients have been used for 'normal urothelial control' but we used E-type cancer cell lines for the initial screening. Such cell lines retain physiological urothelial characters (see methods) and gene expression profile is close to IC specimens to some extent (Figure5C). In the future, we would like to overcome such technical issue by using cultured urothelial cells differentiated from iPS cells.

In conclusion, candidate urothelial master TFs could serve as novel diagnostic markers and potentially explain the molecular pathology of IC.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure legends

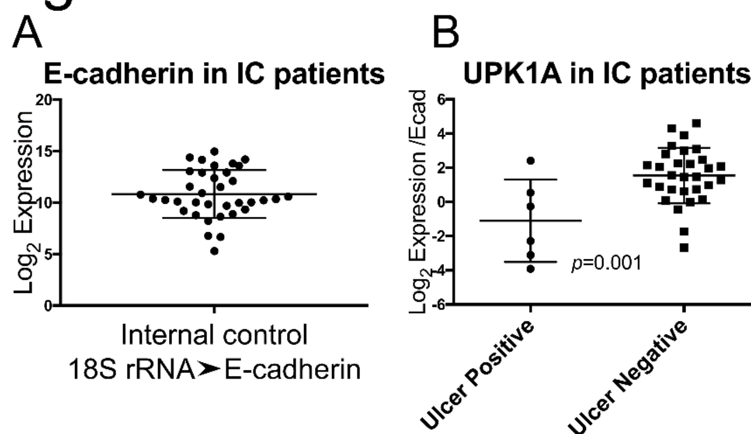


Figure 1

mRNA expression in interstitial cystitis (IC) biopsies. A. There is a huge variation in E-cadherin gene expression (*CDH1*) in IC specimens (Log_2 expression range: from 5.29 to 14.96, 815-fold expression difference). To minimize the variance in epithelial/stromal ratio, we switched the internal control gene from *18S rRNA* to *CDH1* (a gene encoding E-cadherin) for normalization of other genes of interest. B. mRNA expression of *UPK1A* gene (a known urothelial marker) was lower in Hunner's lesion (ulcer positive) compared to the urothelium from both ulcerative ('apparently normal' counterpart) and non-ulcerative IC patients (collectively defined as ulcer negative).

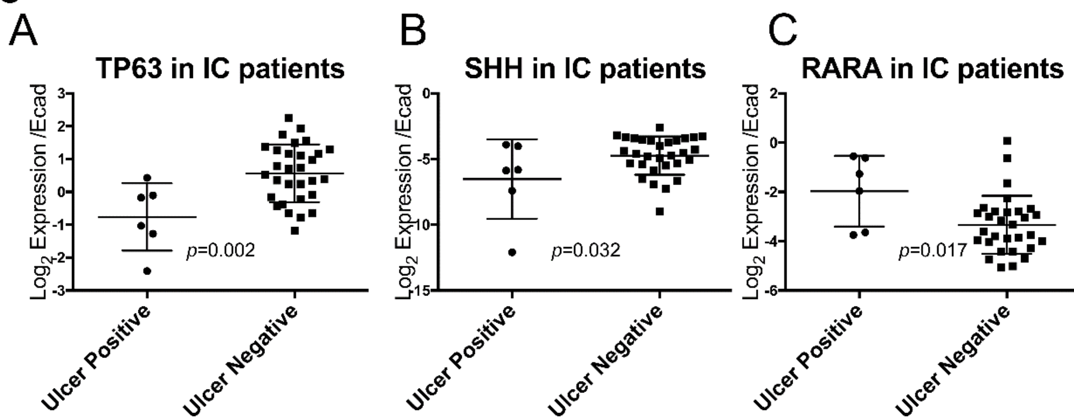


Figure 2

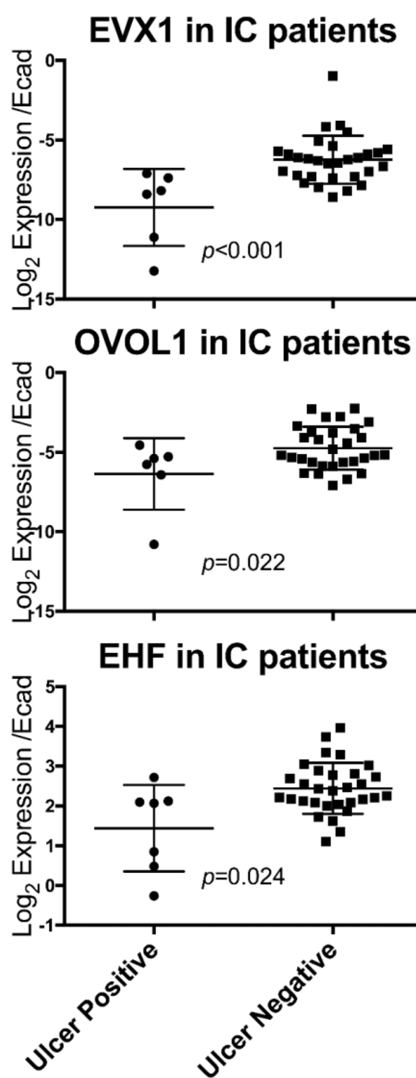
mRNA expression of known urothelial marker genes in IC biopsies. A and B. mRNA expression of *TP63* and *SHH* genes was lower in ulcer positive compared to ulcer negative IC biopsies. C. mRNA expression of *RARA* gene was higher in ulcer positive

compared to ulcer negative IC biopsies.

Figure 3

A

Lower in Hunner's lesion



B

Higher in Hunner's lesion

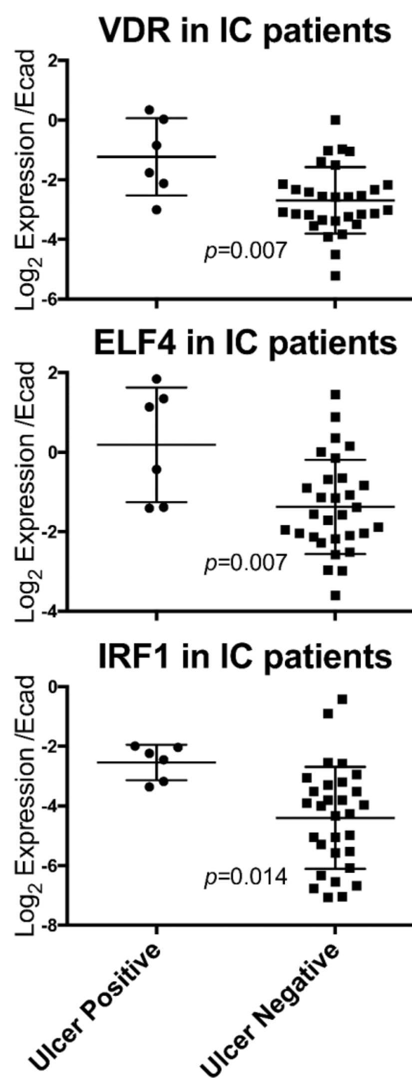


Figure 3

mRNA expression of candidate urothelial master TFs in IC biopsies. A. mRNA

expression of *EVX1, OVOL1,* and *EHF* genes was lower in ulcer positive compared to ulcer negative IC biopsies. B. mRNA expression of *VDR, ELF4,* and *IRF1* genes was higher in ulcer positive compared to ulcer negative IC biopsies.

Figure 4

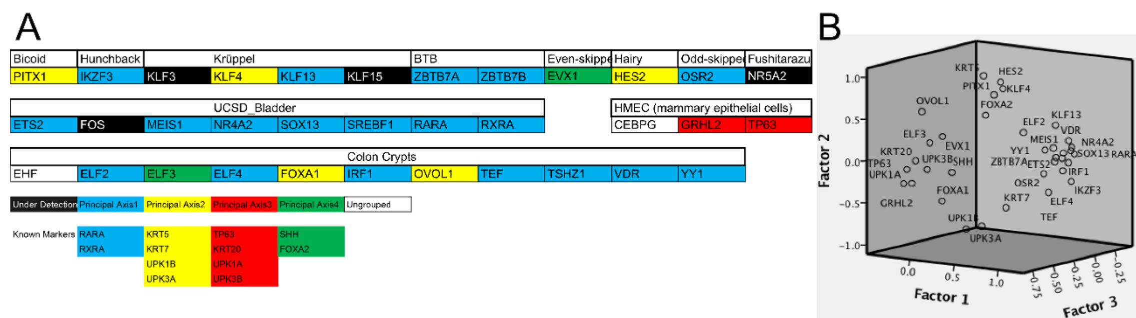


Figure 4

Candidate master TFs were classified based on expression profile in IC biopsies. A. Factor analysis classified candidate TFs into four categories (Principal Axes 1-4), with EHF and CEBPG as ungrouped exceptions. B. Three-dimensional factor plot in rotated factor space. Each factor represents a ‘Principal Axis’ shown in left.

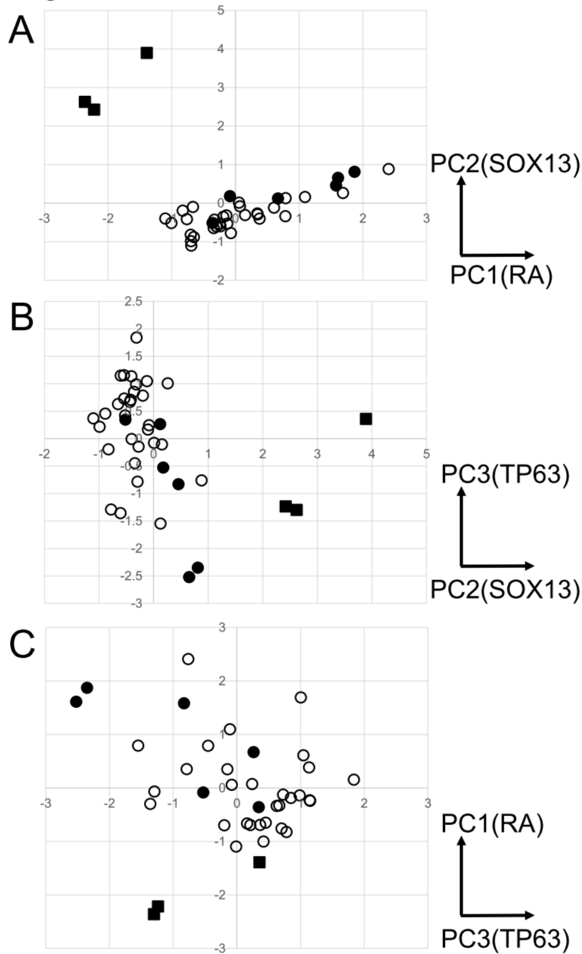


Figure 5

Expression profile of each IC biopsy was represented in a scatter plot in the Principal Components' plane. A. PC1 (*RARA* as a representative gene) and PC2 (*SOX13* as a representative gene). B. PC2 and PC3 (*TP63* as a representative gene). C. PC3 and PC1 Filled circles: biopsies from Hunner's lesion, open circles: 'apparently normal' counterpart of Hunner's lesion from ulcerative patients and urothelium from non-ulcerative patients. Filled squares: E-type cell lines (HuB6, HuB15 and 5637) PC: Principal Component.

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