Biology of Human Tumors

Identification of Differentially Expressed Long Noncoding RNAs in Bladder Cancer

Stefan Peter1, Edyta Borkowska1, Ross M. Drayton1, Callum P. Rakhit1, Aidan Noon1,2, Wei Chen3, and James WF Catto1

Abstract

Purpose: Loss of epigenetic gene regulation through altered long noncoding RNA (lncRNA) expression seems important in human cancer. LncRNAs have diagnostic and therapeutic potential, and offer insights into the biology disease, but little is known of their expression in urothelial cancer. Here, we identify differentially expressed lncRNAs with potential regulatory functions in urothelial cancer.

Experimental Design: The expression of 17,112 lncRNAs and 22,074 mRNAs was determined using microarrays in 83 normal and malignant urothelial (discovery) samples and selected RNAs with qPCR in 138 samples for validation. Significantly differentially expressed RNAs were identified and stratified according to tumor phenotype. siRNA knockdown, functional assays, and whole-genome transcriptomic profiling were used to identify potential roles of selected lncRNAs.

Results: We observed upregulation of many lncRNAs in urothelial cancer that was distinct to corresponding, more balanced changes for mRNAs. In general, lncRNA expression reflected disease phenotype. Focusing upon a promising candidate, we implicate upregulation of AB074278 in apoptosis avoidance and the maintenance of a proproliferative state in cancer through a potential interaction with EMP1, a tumor suppressor and a negative regulator of cell proliferation.

Conclusions: We report differential expression profiles for numerous lncRNA in urothelial cancer. We identify phenotype-specific expression and a potential mechanistic target to explain this observation. Further studies are required to validate lncRNAs as prognostic biomarkers in this disease.

Introduction

Bladder cancer (BC) is the fourth commonest male malignancy and one of the most expensive human cancers to manage (1, 2). The majority of tumors are urothelial carcinoma in histologic type. Clinicopathological data suggest that urothelial cancers are best stratified into two distinct phenotypes characterized by low- and high-grade cellular differentiation. Low-grade urothelial cancers frequently have mutations of FGFR3 and STAG2, partial deletion of chromosome 9 (3), and rarely progress to muscle invasion or metastases following endoscopic resection (4). In contrast, high-grade BCs are aggressive tumors that may present before or after the onset of muscle invasion (5). These poorly differentiated urothelial cancers have widespread chromosomal instability, multiple mutations, and are best characterized by deficiency of p53-mediated pathways (3). High-grade tumors share many molecular alterations regardless of stage and respond poorly to chemotherapy (6).

Although many reports detail genetic events in urothelial cancer, alterations of epigenetic gene regulation are also important in this disease (7). In general, epigenetic alterations reflect urothelial cancer disease phenotypes and associated genetic events (8, 9). For example, Wolff and colleagues reported that high-grade invasive urothelial cancers had widespread aberrant hypermethylation, whereas low-grade noninvasive tumors had regional hypomethylation (10). In general, epigenetic events reflect chromosomal changes with the disease and can be used as predictor of disease progression (11). With regard to miRNA, low-grade urothelial cancers are characterized by loss of expression of many species, including miR99a/100 that target FGFR3 (12, 13). In contrast, high-grade tumors have upregulation of many miRs, including miR21 that targets the p53 pathway (14) and miR129 (15).
Long noncoding RNA expression profiling

From each sample, 10 × 10 μm thick sections were microdissected to extract normal and malignant urothelial cells (>90% content). Total RNA was extracted using the mirVana kit (Ambion) according to manufacturer’s protocol (26), and measured using a 2100 Bioanalyzer (Agilent). The expression of long ncRNAs and protein coding mRNAs was determined using microarrays (NCode Human Non-coding RNA Microarrays, Invitrogen). Each sample was prepared according to Agilent’s one-color microarray protocol. Briefly, 200 ng RNA samples were mixed with spike in control RNA (Agilent), labeled with cyanine 3-CTP (Low Input Quick Amp Labeling Kit, one-color, Agilent), and hybridized to the microarray (Gene Expression Hybridization Kit, Agilent) according to the manufacturer’s protocol. After washing (Gene Expression Wash Buffer 1 and 2, Agilent), the microarray slides were scanned using the Agilent Microarray Scanner platform (High Resolution Microarray Scanner C) and raw probe fluorescence extracted. The microarray data are deposited with Gene Expression Omnibus (GSE55433). The NCode microarray contains duplicate probes to 17,112 ncRNAs and 22,074 mRNAs. The IncRNAs were identified by various strategies without annotation (27). To annotate the array, we converted all probes to hg 19,0 loci (using LiftOver, UCSC genome browser), matched to target gene, structure and the Gencode v7.0 annotation (22), and the nearest CpG island/differentially methylated region (28). For microarray validation, individual IncRNA expression was measured in triplicate using qRT-PCR. Total RNA was transcribed using random hexamer primers (Applied Biosystems) and the High Capacity Reverse Transcription Kit (Applied Biosystems) before diluting 10-fold in nuclease-free water. cdNA was mixed with SyberGreen MasterMix (Applied Biosystems) and PCR primers, and analyzed using the ABI 7900HTT real-time PCR system. cdNA expression was calculated using AC values normalized by subtraction of the mean of TEGT and HSP90AB1 expression (as reference genes) and fold change (FC = 2^ΔΔCt) calculated (29).

Protein coding potential score of ncRNAs

We investigated the protein coding potential of selected IncRNAs using geneID (30) and CPC against UniProt.
Reference Clusters (31). For both, negative scores suggest low protein coding potential.

siRNA knockdown of lncRNA expression

We selected representative lncRNAs for further analysis and modulated expression using custom siRNA. All experiments were performed in triplicate using hTERT immortalized NHU cells at 70% confluence transfected with siRNAs (synthesized with LifeTechnologies BLOCK-iT RNAi designer) specific to lncRNAs and controls (scrambled RNA sequence). Cells were seeded into 12-well dishes and incubated for 3 hours before transfection with 80 nmol/L RNAi using 2 mL Lipofectamine RNAiMAX (Invitrogen) per well. Transfection efficiency was determined 72 hours later by qRT-PCR (Applied Biosystems).

Growth analysis of siRNA modulated cells

The growth characteristics of siRNA-transfected cells were analyzed for cell-cycle regulation (propidium iodide flow cytometry), apoptosis (caspase-3 activation), and proliferation (MTT assay). For cell-cycle analysis, cells were harvested after 24, 48, and 72 hours of knockdown, centrifuged (3 minutes at 1,400 g) and washed in PBS. Cells (1 x 10^6) were resuspended in 1 mL PBS, mixed with 3 mL ice cold absolute ethanol, and fixed over night at 4°C before washing twice in PBS. Cells were then mixed with 5 µL RNase solution (2 mg/mL) and 300 µL propidium iodide solution (50 µg/mL), and incubated over night at 4°C. The next day, cells were analyzed on a FACS Calibur flow cytometry analyzer (Becton Dickinson). For apoptosis, we determined caspase-3 activity in 3 x 10^5 cells, 24 hours after transfection using the CaspGLOW Fluorescein Active Caspase-3 Staining Kit (MBL). Briefly, we resuspended the cells in 300 µL PBS, added 1 µL FITC-DEVD-FMK, and incubated them for 1 hour at 37°C. After pelleting the cells and washing them in washing buffer, they were resuspended in 300 µL wash buffer and analyzed on a FACS Calibur flow cytometry analyzer (Becton Dickinson). For proliferation, cells were seeded into 96-well plates following siRNA-mediated gene knockdown. The culture medium was replaced with 50 µL MTT-PBS solution (3 mg MTT/1 mL PBS) 24, 48, and 72 hours after knockdown, respectively. Cells were incubated at 37°C for 3 hours before 200 µl MTT dissolvent (DMSO) was added to each well. MTT absorbance was measured at 570 nm using a microplate reader.

Genetic consequences of lncRNA modulation

mRNA expression was determined in transfected cells using HG-U133 Plus 2.0 microarrays (Affymetrix, Cal.; ref. 32). Briefly, RNA was prepared with the Affymetrix protocol and annealed to an oligo-d(T) primer with a T7 polymerase-binding site. cDNA was generated using superscript II and E. coli DNA ligase and polymerase I, before the reaction was completed with T4 DNA polymerase and EDTA. Amplified cDNA was cleaned, biotin-labeled, and fragmented, before hybridizing to the microarray for 16 hours (45°C in a rotating oven at 60 rpm). After washing and staining, the arrays were scanned (GC3000 scanner) and data processed using Gene Chip Operating System software. mRNA expression was determined using

| Table 1. Details of patients and tissue samples analyzed in this study |
|-------------------------|-------------------------|-------------------------|-------------------------|
| Microarray cohort UCC   | Validation cohort UCC   |
| Low-grade NMI | High-grade NMI | Invasive | Normal urothelium | Low-grade NMI | High-grade NMI | Invasive | Normal urothelium |
| Total | 24 | 13 | 19 | 26 | 39 | 31 | 42 | 26 |
| Gender | | | | | | | | |
| Male | 13 | 11 | 15 | 6 | 23 | 25 | 29 | 26 |
| Female | 6 | 2 | 4 | 1 | 16 | 6 | 13 | |
| Age | | | | | | | | |
| Mean | 76.3 | 76.4 | 72.1 | 65.5 | 85 | 79 | 74 | 69.5 |
| Range | 48–82 | 54–84 | 46–89 | 59–82 | 54–100 | 65–95 | 41–92 | 61–88 |
| Stage | | | | | | | | |
| pTa | 24 | 2 | 0 | | 39 | 12 | 0 | |
| pTis | 0 | 2 | 0 | | 0 | 3 | 0 | |
| pT1 | 0 | 9 | 0 | | 0 | 16 | 0 | |
| pT2-4 | 0 | 0 | 19 | | 0 | 0 | 42 | |
| Progression | | | | | | | | |
| Yes | 2 | 2 | 11 | | 5 | 12 | 18 | |
| No | 22 | 11 | 8 | | 34 | 19 | 24 | |
| Follow-up (mo) | | | | | | | | |
| Mean | 31.8 | 31.6 | 14.1 | | 67 | 54.4 | 17.5 | |
Microarray Analysis Suite 5 (Affymetrix) and defined as expressed (perfect match probe set intensity greater than mismatch intensity) or absent (mismatch probe set intensity greater or equal to perfect match intensity). ANOVA analysis was then performed using Partek Genomic Suite 6.5 β and differentially expressed transcripts were defined as ≥2 relative fold change using a cutoff of \( P < 0.05 \). qRT-PCR was used to confirm the expression of individual mRNAs of interested in the second patient cohort.

**Statistical analysis**

Raw intensity values for each microarray probe were used to calculate IncRNA expression. The NCode microarray includes multiple probes for each target RNA. We excluded RNAs whose targeting probes were non-concordant (defined as signal ratio less than 0.5 or more than 2.0) in all the arrays. Normalization was achieved according to the 1-color default normalization of Agilent’s Gene Spring software (version 7), by dividing each raw intensity value by the median of the chip and the median expression of that RNA in all samples. Changes in IncRNA expression and statistical significance were calculated and illustrated using Volcano plots. Significant differences in expression between malignant versus normal urothelium, or between urothelial cancer phenotypes were defined using the Significance Analysis of Microarray software (33) as a t test \( P \) value of <0.05, a false discovery rate of <0.01, and an expression FC of ≥2. Hierarchical clustering was performed using Cluster 3.0 and visualized in Tree view (Eisen Lab 9). Correlation coefficients within SPSS (version 14.0, SPSS, Inc.). Area proportional to Venn diagrams were produced using BioVenn (34). IncRNA expression and tumor outcome were investigated using the log-rank test and plotted by the Kaplan–Meier method within SPSS. Tumor progression was defined as the presence of pathologic, radiologic, or clinical evidence of an increase in tumor stage and measured from the time of surgery to the time of proven event.

**Results**

**IncRNA expression in bladder cancer**

We investigated the expression of 17,112 IncRNAs and 22,074 mRNAs. The data are accessible through Gene Expression Omnibus Series accession number GSE55433. We filtered to 9,351 IncRNAs and 7,922 mRNAs, for which the microarray probe signals were concordant (Fig. 1A and Supplementary Table S1). Comparison between urothelial cancer and normal urothelium revealed 2,075 differentially expressed IncRNAs. In general, there was an increased expression of long ncRNAs in urothelial cancer [1,788 (86%) were upregulated (FC > 2) and 287 (14%) were downregulated (FC < 0.5)] when compared with normal urothelium. Fewer protein coding mRNAs (n = 1,410) were differentially expressed between the malignant and normal tissues, and their distribution was more balanced [836 (59%) upregulated and 574 (41%) downregulated in urothelial cancer] than for IncRNAs (t test \( P < 0.001 \)). We filtered the microarray transcripts to those in the Gencode v7 catalogue [ref (22); \( n = 3,885 \), Supplementary Table S2], of which 355 were IncRNAs differentially expressed in urothelial cancer. Although the majority were long intergenic ncRNA (IncRNA, \( n = 225 \)), we identified 130 IncRNAs located within protein coding genes (termed genic). These included RNAs coded in the sense [intronic (n = 20) and overlapping (n = 3)] and antisense [exonic (n = 50), intronic (n = 51), and overlapping (n = 6)] direction with respect to the mRNA gene (Supplementary Fig. S1). The proportion of aberrantly expressed IncRNAs in urothelial cancer, when compared with normal urothelium, did not vary with gene location (range from 15% for antisense exonic to 22% for overlapping antisense IncRNAs; Fig. 1B).

**Expression of IncRNAs with respect to tumor phenotype**

We compared RNA expression across urothelial cancer phenotypes (low-grade NM, high-grade NM, and invasive) with normal urothelium. Unsupervised hierarchical clustering revealed three different expression profiles fitting the disease-free, the low-grade, and the high-grade/invasive phenotypes (Fig. 1C). For all tumor phenotypes, we observed more up than downregulated IncRNAs, although the extent varied between groups (91% in invasive tumors, 61% in low-grade, and 53% in high-grade; Fig. 2A and Supplementary Fig. S2a). Accordingly, the magnitude of differential IncRNA expression varied between the invasive cohort (average fold change 2.57), and the low-grade and high-grade cohort (average fold change 1.75 and 1.73, respectively, Supplementary Fig. S2b). There was less difference in the magnitude of change in expression for mRNAs than IncRNAs (average fold change of 2.11 in invasive, 1.59 in low-grade, and 1.45 in high-grade, t test \( P < 0.01 \)). The majority of aberrantly expressed IncRNAs and mRNAs belonged to the invasive subset [1,800/2,034 IncRNAs (88%) and 1,050/1,410 mRNAs (74%)], followed by the low-grade cohort \([ n = 560 \) IncRNAs (28%) and \( n = 757 \) mRNAs (54%)\] and the high-grade cohort \([ n = 416 \) IncRNAs (20%) and \( n = 661 \) mRNAs (47%); Fig. 2B]. We defined a specific signature for each tumor phenotype by identifying RNAs significantly differentially expressed between each tumor type. Of these, 75% (1,356/1,800) of IncRNAs in the invasive phenotype, compared with 19% [109/560] of the low-grade cohort and only 3% [15/416] of the high-grade cohort were phenotype specific. Similarly for mRNAs, 45% in the invasive cohort, 17% and 4% of the low-grade and high-grade cohort, respectively, were phenotype specific. We identified \( n = 188 \) (9%) IncRNAs and \( n = 374 \) (26%) mRNAs that were altered in urothelial cancer regardless of tumor phenotype.

**IncRNA expression and tumor progression**

To identify IncRNAs with roles in urothelial carcinogenesis, we searched for those related to disease progression. We performed univariate log-rank analysis and identified 32/2,075 (1.5%) IncRNAs associated with tumor progression (Bonferroni adjusted log-rank \( P \) value < 0.05; Table 2, Fig. 3A). More of these tumor
progression-related lncRNAs were phenotype specific ($n=16$) than shared between all urothelial cancer ($n=6$). Eight were annotated within GENCODE. LncRNAs associated with tumor progression were balanced between up ($n=17$) and downregulation ($n=15$). Protein coding potential was calculated using two algorithms (Table 2; refs. 24, 25). The resultant scores were closely correlated (Pearson $r=0.81$) and suggested only one member was likely to be a misclassified mRNA (CR611332).

A role for AB074278 expression in cell proliferation and apoptosis

For functional validation, we investigated an aberrantly expressed lncRNA using siRNA in normal urothelial (hTERT-NHU) cells. We selected AB074278, as it was (i) associated with disease progression, (ii) upregulated in all urothelial cancer phenotypes (we were interested in lncRNAs generic to urothelial cancer not subtype specific), (iii) had low predicted protein-coding scores (thus likely to be an ncRNA), (iv) worse outcomes with high expression (thus a potential oncogenic role), (iv) also upregulated in urothelial cancer, and (v) appeared of particular interest as it was intronic (sense direction) to a protein coding host gene (sense to TANC2; as were most validated ncRNAs in GENCODE) also upregulated in urothelial cancer (thus potentially regulated by the lncRNA; ref. 35). siRNA Transfection in hTERT-NHU cells reduced expression by 80% after 24 hours and significantly increased cell apoptosis/death (Fig. 3B). Knockdown also significantly reduced proliferation (Fig. 3C) when compared with scrambled RNA controls. For comparison, we also knocked down two aberrantly expressed lncRNAs not associated with tumor progression (namely, G36639 and U50531). Proliferation was reduced for both these lncRNAs, but significantly less dramatically than for AB074278 (Supplementary Fig. S3).
mRNA interactions for AB074278: epithelial membrane protein 1

To explore interactions for AB074278, we compared changes in mRNA expression between siRNA knockdown and control NHU cells using HG-U133 Plus 2.0 microarrays in triplicate. We identified 471 mRNAs that were significantly differentially expressed (359 upregulated and 112 down regulated; Fig. 4A). As the cellular phenotype of cells with AB074278-siRNA is increased apoptosis and reduced proliferation, we selected the 87 of 471 mRNAs with roles in proliferation, cell death, and apoptosis (gene functions identified using DAVID Bioinformatics Resources, Vsn. 6.7; ref. 36; bold font in Supplementary Table S3). We identified 3 of 87 mRNAs (EMP1, CKS2, and PTGS2) correlated to AB074278 expression in the discovery NCode microarray dataset (Pearson \( r < -0.35 \) or \( >0.2, P < 0.001 \); Supplementary Table S4). We measured the expression of these three mRNAs and TANC2 (the host protein-coding gene for AB074278) in the validation cohort of 138 urothelial samples (Supplementary Fig. S4) to look for associations with disease phenotype. We identified that low expression of epithelial membrane protein 1 (EMP1) in urothelial cancer was associated with increased risk of progression and BC-specific mortality (Fig. 4B, 20% vs. 38% progression rate for tumors with high and low expression, respectively, log-rank \( P < 0.03 \)) and EMP1 was down regulated in urothelial cancer (Fig. 4C; ANOVA \( P = 0.005 \)), in contrast with the changes seen for AB07428 [worse outcomes with high expression (log-rank \( P < 0.05 \) and upregulation in urothelial cancer (ANOVA \( P = 0.02 \)]. To support a direct regulation, we also saw increased EMP1 expression (3.8 fold) following AB074278 knockdown in NHU-TERT cells (Fig. 4D; \( P < 0.01 \)). No significant changes were seen with respect to phenotype for TANC2, PTGS2, and CKS2.

Discussion

Technological improvements have revealed the importance of ncRNA in cellular function (20). Although the recent GENCODE annotated catalogue details an abundance and the distribution of many IncRNAs, little is known about their function. Here, we have performed the first large comprehensive screen of IncRNAs in urothelial cancer to identify those likely to play roles in the biology of this cancer. Putative IncRNAs were identified using an algorithm scoring characteristics of protein-coding genes, including open reading frame length, synonymous/nonsynonymous base substitution rates, and similarity to known proteins (27). We identified differential expression of many IncRNAs, without selection for genetic location, and found that the overall pattern of altered expression (mostly upregulation) was more extensive to that seen for protein coding mRNAs. Recently Wang and colleagues reported balanced changes in IncRNA expression in 12 urothelial cancer and preliminary data to suggest IncRNAs have malignant roles in mTOR and p53 signaling, and other cancer pathways (24). Although our observations do not suggest such a balanced alteration in expression, data in breast and neurologic cancers (23) and in human primary keratinocytes (35) support our findings. Given a lack of knowledge about IncRNAs and that our panel was designed computationally, we focused upon the minority (17%) of IncRNAs catalogued within GENCODE (22). This percentage compares with the 12% overlap for IncRNAs reported by Kapranov.
### Table 2. Detailed information on all lncRNA associated with tumor progression

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<th>Size [nt]</th>
<th>Exons</th>
<th>UCC expression(^a)</th>
<th>Expression ((n))(^b)</th>
<th>Progression rate(^b)</th>
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\(^a\)Urothelial cancer expression [phenotype: in which phenotype was abnormal expression seen (LG, low-grade NMI; HG, high-grade NMI; Inv, invasive urothelial cancer). FC, fold change in urothelial cancer vs. normal urothelium].

\(^b\)Expression (\(n\)): the number of cases with high or low expression and progression: the progression rates in these tumors, respectively.

\(^c\)Protein coding score calculated using GeneID and CPC (coding potential calculator). Negative values suggest a low protein coding risk.
identified many (regional epigenetic silencing) or direct interaction (43). To allow (e.g., through regional chromosomal instability or dysregulated expression), suggesting coordinated anatomical dysregulation (44% phenotype-specific expression vs. 26% common expression type specific vs. 9% were common to all urothelial cancer). This reflects (but varies in extent) changes in mRNA expression (71% of all aberrant expressed lncRNAs were phenotype specific, making the invasive phenotype distinguishable from the low- and high-grade tumors. This observation is in contrast with miRNA profiles within urothelial cancer, which often share differences between high-grade and invasive tumors (12), and suggests an exciting role to aid pathologic disease staging of high-grade tumors. Although our data require validation, we noted fewer progression events in our high-grade NMI tumors than typical (usually around 25%; ref. 5), suggesting that a chance enrichment for more indolent disease may have affected our comparisons. We focused upon lncRNAs that play potential roles in urothelial carcinogenesis, through selecting those associated with tumor progression. Most of those identified were phenotype specific, reflecting global trends within urothelial cancer, and have not been reported previously in cancer. We used loss-of-function studies for AB074278 in NHU cells to explore carcinogenic roles. This cell line has more intact cellular processes (e.g., epigenetic regulation) and fewer genetic events than most malignant cell lines, and so is better to model the subtle impact of epigenetic changes on gene expression (changes from genetic events, such as chromosomal loss/amplification, may dominate subtle modifications arising from epigenetic alterations). AB074278 was chosen because it was significantly upregulated in all tumor phenotypes (its overexpression was correlated (Pearson correlation $r > 0.5$ and $P < 0.05$). This relationship was strongest for genes within 3,000 base pairs (Supplementary Fig. S5a). There were 803 lncRNA/mRNA pairs (26%), where both members were significantly differentially expressed in urothelial cancer. Expression was directly correlated for the vast majority of these pairs (82%; Supplementary Fig. S5b), suggesting common transcriptional control or the epigenetic mediation of mRNA expression by nearby lncRNAs (22, 35), antisense RNAs (44), or promoter-associated lncRNAs (45).

The most altered transcripts were found in invasive urothelial cancer. This was especially true for upregulated lncRNAs (~64%), making the invasive phenotype distinguishable from the low- and high-grade tumors. The most altered transcripts were found in invasive urothelial cancer. This was especially true for upregulated lncRNAs (~64%), making the invasive phenotype distinguishable from the low- and high-grade tumors. This observation is in contrast with miRNA profiles within urothelial cancer, which often share differences between high-grade and invasive tumors (12), and suggests an exciting role to aid pathologic disease staging of high-grade tumors. Although our data require validation, we noted fewer progression events in our high-grade NMI tumors than typical (usually around 25%; ref. 5), suggesting that a chance enrichment for more indolent disease may have affected our comparisons. We focused upon lncRNAs that play potential roles in urothelial carcinogenesis, through selecting those associated with tumor progression. Most of those identified were phenotype specific, reflecting global trends within urothelial cancer, and have not been reported previously in cancer. We used loss-of-function studies for AB074278 in NHU cells to explore carcinogenic roles. This cell line has more intact cellular processes (e.g., epigenetic regulation) and fewer genetic events than most malignant cell lines, and so is better to model the subtle impact of epigenetic changes on gene expression (changes from genetic events, such as chromosomal loss/amplification, may dominate subtle modifications arising from epigenetic alterations). AB074278 was chosen because it was significantly upregulated in all tumor phenotypes (its overexpression in urothelial cancer samples could be confirmed by

![Figure 3. Tumor progression with respect to lncRNA expression. A. Tumor progression was analyzed with respect to low (dashed line) and high (solid line) lncRNA expression (dichotomized around median). Examples of aberrant up (AK127730, AK130230, and AB074278) and down (AF075063, BC015007, and AK122774) lncRNA regulation in urothelial cancer (plotted by the Kaplan-Meier method, log-rank P values shown). B, siRNA-mediated gene knockdown of the lncRNA AB074278 led to 84% reduction of its expression in hTERT NHU cells and caused cell death/apoptosis. Cell death was determined by propidium iodide flow cytometry and apoptosis by caspase-3 activation and flow cytometry. C, cell proliferation, measured by MTT assay, was also reduced following knockdown of AB074278.](attachment://figure3.png)
Cluster 3.0. Arrow identifies correlation similarity in expression of mRNA and corresponding changes in control scrambled siRNA (Scr RNA) knockdown and transfection with AB074278 after siRNA-mediated shown. D, expression of lncRNA measured by qRT-PCR. knockdown of AB074278 as in TANC2 AB074278 may regulate (by enhancing) the expression of supplementary Fig. S6), allowing for the speculation that lncRNA significantly following AB0724278 knockdown (Supplementary Materials S1–S3). These data suggest but do not confirm the exact relationship between AB074278 and EMP1, which now requires further investigation. In summary, we have identified many lncRNAs significantly altered in urothelial cancer and associated with disease progression and tumor subtypes. We specifically implicate AB074278 in apoptosis avoidance and cell progression, and has a low protein coding potential score. Furthermore, IncRNA AB074278 is intronic to TANC2, which was also upregulated in urothelial cancer, suggesting the potential for direct regulation. Although the expression of IncRNA AB074278 and TANC2 was not correlated in urothelial cancer cases, TANC2 expression did drop significantly following AB0724278 knockdown (Supplementary Fig. S6), allowing for the speculation that IncRNA AB074278 may regulate (by enhancing) the expression of TANC2 in cis (35). Little is known about the function of TANC2, although it is believed to play a role in embryonic development (46). Although these data are promising, we were keen to select mRNAs whose expression was abnormal in urothelial cancer and correlated to AB074278. Using two microarray screens, we identified a dynamic correlation between AB074278 and EMP1, and selected this gene due to its potential involvement in disease progression and its role in proliferation consistent with our observations. Our expression data of EMP1 matched its functional description in the literature (overexpression of EMP1 was found to inhibit the proliferation of EC9706 cells; ref. 47) and could explain the observed decrease in proliferation in transfected NHU-TERT cells. EMP1 is a putative tumor suppressor gene whose decreased expression is associated with advanced clinical stage and metastasis in oral squamous cell carcinoma (48) and reported to be directly involved in the inhibition of proliferation (47). To explore the link between AB074278 and EMP1, we compared the sequence of both RNAs and EMP1-associated genes (identified through Ingenuity Pathway Analysis, Ingenuity Inc. and EpiTect ChIP qPCR Primers by Transcription Factor search algorithm by SAbiosciences) using BLAT (UCSC, GRCh37/hg19) to investigate potential direct interactions. We found a 21nt stretch of identical sequence for AB074278 and SND1 (Staphylococcal nuclease domain-containing protein 1; Supplementary Table S5). SND1 is a transcription cofactor that regulates EMP1 through its interaction with EMP1’s transcription factor STAT5A (Supplementary Fig. S7 and Supplementary Materials S1–S3). These data suggest but do not confirm the exact relationship between AB074278 and EMP1, which now requires further investigation.
proliferation, potentially through regulating the expression of EMP1.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conceptualization and design: S. Peter, J.W.F. Catto
Development of methodology: S. Peter, R.M. Drayton, C.P. Rakhit, A.P. Noon, J.W.F. Catto
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Peter, E. Borkowska, C.P. Rakhit, A.P. Noon, W. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Peter, E. Borkowska, R.M. Drayton, C.P. Rakhit, J.W.F. Catto
Writing, review, and/or revision of the manuscript: S. Peter, J.W.F. Catto
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Peter
Study supervision: A.P. Noon, J.W.F. Catto

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References
29. Andersen CL, Jensen JL, Ornott TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based
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Stefan Peter, Edyta Borkowska, Ross M. Drayton, et al.


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