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Role of androgen receptor and associated lysine-demethylase coregulators, LSD1 and JMJD2A, in localized and advanced human bladder cancer

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Abstract

Bladder cancer is approximately three times more common in men as compared to women. We and others have previously investigated the contribution of androgens and the androgen receptor (AR) to bladder cancer. JMJD2A and LSD1 are recently discovered AR coregulator proteins that mediate AR-dependent transcription via recently described histone-lysine demethylation (KDM) mechanisms. We used immunohistochemistry to examine JMJD2A, LSD1 and AR expression in 72 radical cystectomy specimens, resulting in evaluation of 129 tissue samples (59 urothelial carcinoma, 70 benign). We tested levels of these proteins for statistical association with clinicopathologic variables and patient survival. Expression of these markers was also assessed in human bladder cancer cell lines. The effects of pharmacological inhibition of LSD1 on the proliferation of these bladder cancer cells was determined.

JMJD2A and AR levels were significantly lower in malignant *versus* benign urothelium, while increased LSD1 levels were observed in malignant urothelium relative to benign. A significant reduction in all three proteins occurred with cancer stage progression, including muscle invasion (JMJD2A/LSD1/AR), extravesical extension (JMJD2A/LSD1) and lymph node metastasis (JMJD2A/AR). Lower JMJD2A intensity correlated with additional poor prognostic features, including lymphovascular invasion, concomitant carcinoma *in situ* and tobacco usage, and predicted significantly worse overall survival. Pharmacological inhibition of LSD1 suppressed bladder cancer cell proliferation and androgen induced transcription. Our results support a novel role for the AR-KDM complex in bladder cancer initiation and progression, identify JMJD2A as a promising prognostic biomarker, and demonstrate targeting of the KDM activity as an effective potential approach for bladder cancer growth inhibition.

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BACKGROUND

Urothelial cell carcinoma of the bladder is the fourth most common solid tumor malignancy among men and ninth most common solid tumor malignancy among women in the U.S. [1]. Risk factors include smoking, male gender, advanced age, Caucasian race and prior pelvic irradiation [2,3]. While most tumors are superficial, recurrence after endoscopic resection with or without intravesical pharmacotherapy is frequent and carries a lifelong risk. Radical cystectomy achieves a durable cure, however relapse can be expected in approximately one third of patients, with worse outcomes associated with advanced tumor stage, lymphovascular invasion, lymph-node metastasis, concomitant carcinoma *in situ* and surgical margin involvement [4–7]. Worse outcomes also correlate with certain clinical traits, including tobacco use, female gender and preoperative hydronephrosis [2,8–10]. Relapse after definitive surgery is typically fatal, with current cytotoxic chemotherapies providing no definite survival benefit in the adjuvant or salvage setting. Thus an urgent need exists to identify key molecular signaling pathways governing bladder carcinogenesis. Such knowledge may improve patient outcomes by enabling development of better biomarkers for patient risk stratification and novel targeted therapies.

In this study we investigate the role of the androgen receptor (AR, NR3C4) signaling complex in human bladder carcinogenesis. Embryologically, the bladder derives from the urogenital sinus, where AR is expressed [11]. A role for AR signaling in bladder carcinogenesis is suggested epidemiologically by a male predominance (~3:1) in bladder cancer diagnoses in the U.S. independent of tobacco usage or occupational carcinogen exposure [12]. Interestingly, although males more frequently develop bladder cancer, they have a better prognosis than females, suggesting a complex role for androgens in bladder carcinogenesis [2,8,9]. In rodent models employing the T-antigen oncogene or the chemical carcinogen, N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), bladder tumors are induced preferentially in wild-type males relative to females or castrate males [13,14]. Formation of these tumors is increased by androgen supplementation and decreased by AR blockade or experimental downregulation, with androgens and AR exerting independent effects [13–17]. However, as neither BBN nor T-antigen plays a known role in human bladder carcinogenesis, the clinical relevance of these findings remains unknown.

In human tissue, current understanding of AR signaling in normal and malignant bladder urothelium is limited. Independent recent reports by Miyamoto *et al* and Boorjian *et al*. in human bladder cancer cell lines suggest a growth-promoting role for AR, consistent with the BBN and T-antigen rodent models [13,18]. In contrast, AR immunostaining in clinical bladder cancers indicate reduced AR levels with progression to muscularis propria (detrusor muscle) invasion [19,20]. Thus, while AR may be implicated in early events of bladder carcinogenesis, it may not be required for progression of disease, and the prognostic significance of AR loss during clinical bladder cancer progression remains unclear.

AR function is dependent upon the direct interaction of the AR with multiple coregulator proteins with diverse enzymatic actions that can serve to either activate (coactivators) or repress (corepressors) AR-mediated transcription [21]. Aberrant AR signaling could therefore arise from changes in the expression of AR coregulators, independent of levels of AR or endogenous androgen hormone. This phenomenon is well described in prostate cancer, where AR-coregulators have been implicated in prostate carcinogenesis and the progression to androgen-independent disease [22–25]. Epigenetic coregulators mediate AR transcription *via* chemical modifications of histone proteins [21,26]. Lysine-Specific Demethylase 1 (LSD1, also referred to as KDM1A/AOF2/BHC110) and members of the Jumoni-domain containing (JMJD2) coregulator family (JMJD2A, JMJD2C/GASC1; JMJD2D) are recently indentified nuclear receptor epigenetic coregulators that cooperatively

mediate sex steroid hormone-induced transcription by removal of methyl groups from specific histone lysine residues, causing chromatin reconfiguration [27–29]. Until recently, it was believed that histone lysine methylation was a biochemically irreversible state. However it is now established that in the presence of hormone-activated AR, the LSD1-JMJD2 complex cooperate to remove the repressive histone H3-lysine-9 (H3K9) tri-methylation mark that facilitates transcriptional activation [21,27,30]. JMJD2 family members are required to initiate the demethylation of tri-methylated lysine residues [30]. LSD1 completes the demethylation of mono- and di-methylated lysine residues via an FAD-monoamine oxidase mechanism [27,30]. LSD1 also regulates other transcription factors, including the estrogen receptor (ER) [29] and p53 [31] and can function as a transcriptional corepressor [32]. The recent description of their KDM-enzymatic activity has drawn considerable attention to JMJD2A and LSD1 as novel potential therapeutic targets for the reversal of aberrant epigenetic expression/silencing. Investigation of selective and non-selective agents are underway, the latter including the monoamine oxidase inhibitor (MAOI) class of antidepressants [27,33–38]. Novel inhibitors of the JMJD2-family of KDMs are also in development [35].

Consistent with their roles in sex steroid hormone receptor regulation, JMJD2A and LSD1 are both implicated clinically in the regulation of hormonally responsive malignancies [28,39,40]. Specifically, JMJD2A is expressed at higher levels during prostate tumorigenesis [28], while higher LSD1 levels are observed in advanced prostate and breast cancers, predicting recurrent disease in the former [39,40]. *In vitro*, JMJD2A and LSD1 expression drives AR-mediated prostate cancer cell growth, while siRNA-mediated downregulation or pharmacologic inhibition of LSD1 suppresses this effect [27,28]. However, whether these two proteins play a similar role in bladder carcinogenesis has yet to be studied.

In this study we tested the hypothesis that levels of the AR coregulators LSD1 and JMJD2A are altered in clinical bladder cancer. Protein expression of AR and these coregulators was measured by immunohistochemical staining in 129 bladder tissue specimens and assessed for statistical association with the presence of malignancy, adverse cancer histopathology, and multiple clinical variables, including known risk factors for bladder carcinogenesis and/ or bladder cancer-specific survival. Association between protein levels and patient outcomes after radical cystectomy, including recurrence and survival, was also investigated. Finally, RNA expression of *AR, LSD1* and *JMJD2A* was measured in four bladder cancer cell lines, and the effect of LSD1 pharmaco-inhibition on bladder cancer cell proliferation and androgen induced transcription was determined using MAOI drugs, specifically pargyline and tranylcypromine.

MATERIALS AND METHODS

Patients and tissue specimens

Institutional Review Board approval was acquired for all parts of this study. Seventy-two radical cystectomy specimens accessioned from May 2002 to December 2007 were retrieved from the surgical pathology archives of the Department of Pathology and Laboratory Medicine at New York-Presbyterian Hospital/Weill Cornell Medical College. Although all samples were acquired by cystectomy, the cohort reflects the clinical spectrum of localized to advanced bladder cancers. One hundred twenty-nine formalin-fixed, paraffin-embedded tissue samples, encompassing 59 urothelial cell carcinomas and 70 sections of benign urothelium, were subjected to immunohistochemistry for the purposes of this study. All patients had preoperatively diagnosed urothelial carcinoma of the bladder and underwent pelvic lymphadenectomy at the time of cystectomy. Thirteen patients had no detectable cancer at the time of surgery (stage pT0), and only benign urothelium was acquired in these cases. For 57 of the remaining 59 patients, both benign and malignant tissue samples were

obtained; the remaining two patients had large, advanced primary tumors such that only malignant tissue was available for immunohistochemistry. Clinicopathologic and survival data were collected for all patients in a prospectively updated cystectomy database. Clinical variables included age, gender race, body mass index, smoking history, preoperative hydronephrosis, pelvic radiation history and history of intravesical or neoadjuvant systemic chemotherapy. Pathologic variables included primary tumor stage, lymphovascualar invasion, perineural invasion, concomitant CIS, surgical margin positivity and lymph node metastasis. Tumor stage was defined according to the American Joint Committee on Cancer guidelines. Cancer recurrence after cystectomy was defined as radiographic suggestion of disease on postoperative CT scan or MRI obtained during routine clinical surveillance. Death certificates and/or autopsy reports were reviewed to determine whether cause of death was cancer-related or from another cause. Patient characteristics are summarized in Table 1.

Immunohistochemistry

Immunohistochemical staining was performed using the Leica BOND-MAX Autostainer and supplied reagents (Leica Microsystems), except where otherwise specified. Each antibody was evaluated individually on serial, 5-micron sections of representative whole tissue blocks from each cystectomy specimen. Sections were deparaffinized, and endogenous peroxidase was inactivated followed by Heat-Induced Epitope Retrieval (HIER)-1 (for LSD1 and JMJD2A antibodies) or HIER-2 (for AR antibody) according to the manufacturer's instructions (Leica Microsystems). Primary antibodies for LSD1 (1B2E5, #NB-100-1762, Novus Biologicals), JMJD2A (# NB100-57563, Novus Biologicals) and AR (AM256, BioGenex) were detected using the Leica Microsystems Refine Detection Kit. Negative controls were also performed by using normal serum from the same species and at the same concentration as the primary antibody. Benign and malignant prostate tissue was used as a positive control, and negative control staining without primary antibody was also performed. All stained tissue sections were then evaluated by a urologic pathologist (BDR), and separate scores were given to each sample based on: a) tissue positivity, *i.e.*, the percentage of tissue staining positive (0–100%), and b) the intensity of positively staining cells (0– none, 1– weak, 2– moderate, and 3– strong). Only nuclear staining was considered positive, and a minimum of 100 tumor nuclei were evaluated for each case.

Bladder cancer cell lines and culture conditions

Four bladder cancer cell lines (HTB1, HTB3, HTB5 and HT1376) were purchased from the American Type Culture Collection (Rockville, MD). The HTB1 (J82), HTB5 (TCCSUP), and HT1376 cell lines are derived from high-grade, invasive transitional cell carcinomas, while HTB3 (SCaBER) cell line originated from an invasive squamous cell carcinoma of the bladder. HTB5 expresses AR, while HTB1 is reported to be AR-negative [13,18]. Bladder cancer cells were cultured as described (ATCC) and maintained at 37°C, 10% CO_2 in MEM supplemented with 100 µg/mL streptomycin, 100 units/mL penicillin, and 10% FCS. Cell lines were verified according to ATCC guidelines (Technical Bulletin 8).

RNA extraction and RT-PCR

Total cellular RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). RNA-extraction experiments were repeated in triplicate. First-strand cDNA was synthesized from 1 µg of total RNA by reverse transcription with Superscript II (Invitrogen) or qScript (Quanta) reverse transcriptase at 42°C for 60 minutes. PCR amplification of cDNA for *AR* (GeneID: 376), *LSD1*(GeneID: 23028), and *JMJD2A*(GeneID: 9682), was performed using iQ Supermix (BioRad) and transcript-specific oligonucleotide PCR primers. Primer sequences spanned intron-exon boundaries, preventing amplification of any contaminating genomic DNA. PCR amplification was internally controlled using primers for *GAPDH* to confirm the integrity of cDNA. Primers and annealing temperatures were as follows:

GAPDH – F:agccacatcgctcagacac, R:gaggcattgctgatgatcttg, 63°C; *LSD1* - F: ggaagaaaatgaaagtgagcctgaag, R:cttctagcaaccggttaaactcttgc, 59°C; *JMJD2A*- F: gtggtcttcattacctgctttcgg, R:ggaccaaactggagacagtcctgg, 59°C; *AR* - F: caacccagaagctgacagtgtcac, R: ccatctggtcgtccacgtgta. Negative control PCR using reverse osmosis–grade water in the place of template were included in all experiments. PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. The identity of the cDNA product was confirmed by comparison of the PCR-amplified DNA to the predicted fragment size and DNA sequencing. PCR experiments were repeated on three independent RNA preparations.

Real time PCR was performed using Perfecta SYBR Green Super Mix for iQ (Quanta Bioscience) in a BioRad MyIQ real time system. The oligonucleotides employed were F: tggaggtcttggtcaagcatacag, R-caaggctgttagagagagataattgga for *NEP* (GeneID: 4311), and *HPRT* (GeneID: 3251) was as described previously [41]. The threshold cycle (Ct) was determined by the MyIQ software (BioRad). The Ct values showed a linear correlation with relative cDNA input for both the *NEP* and *HPRT* genes. Reaction conditions were template denaturation at 95°C for three minutes followed by 45 cycles of 94°C, 10s; primer annealing at 60°C or 55°C for *NEP* and *HPRT* respectively; extension at 72°C for 30s and fluorescence was read after each cycle at 80°C. Real time analysis was performed in triplicate on two independent cell treatments. Negative control PCRs using water in the place as template were incorporated into each experiment. Expression of *NEP* was normalized by *HPRT* and compared to the levels detected in the vehicle treated control cells using the ΔΔCt method [42].

Functional analysis of the effects of pargyline and tranylcypromine on bladder cancer cells

Growth analyses were performed on bladder cancer cell lines plated at 1×10^6 cells/well in six-well culture dishes. Dose response comparison of the effects on cellular proliferation of 3 mM and 1 mM concentrations of the non-selective monoamine oxidase/LSD1 inhibitors, pargyline and tranylcypromine (Sigma-Aldrich) were performed on HTB-1, HTB-3, HTB-5 and HT-1376 bladder cancer cell lines. Cells were treated with drug for 48 hours and counted using a Coulter Counter (Beckman). Each treatment condition represents five data points accrued over two separate experiments.

The effects of tranylcypromine on androgen induced transcription were measured in cultured HTB1 and HTB5 bladder cancer cells. Tranylcypromine (50M) is an effective irreversible inhibitor of LSD1 enzymatic activity [43]. HTB1 and HTB-5 bladder cells were treated for 48 hours with vehicle control or tranylcypromine (50μM). Following this 48 hour treatment, androgen regulated transcription was stimulated in cells by treatment with the synthetic androgen, R1881, (1nM) for three hours. In addition cells treated with tranylcypromine for 48 hours but not stimulated with R1881 were used as controls. Quantitative PCR was performed in triplicate on two independent experiments, starting from drug treatment. Relative expression of *NEP* normalized to *HPRT* in bladder cancer cell lines was determined using RT-qPCR with vehicle control treated cells set as the calibrator sample.

Statistical analysis

Fisher's Exact Test was used to test for statistical association between AR, JMJD2A and LSD1 immunostaining scores and the clinicopathologic variables shown in Table 1. Separate analyses were performed for tissue positivity and intensity scores. Tissue positivity was tested as a categorical variable using a 4-tier scale (0–10%, 11–40%, 41–70%, 71– 100%). Immunostaining intensity score was tested using the 0–3 scale described above. Patients with final pathology of urothelial carcinoma *in situ* (pTis) were censored from

analyses testing for association with concomitant CIS. Staining scores were tested for association with each other using a two-tailed Spearman Rank Correlation Test. Spearman coefficients >0.8 were considered to represent strong correlations, while coefficients between 0.4 and 0.8 were considered to have slight correlation. The Kaplan-Meier method was used to generate survival curves stratified by immunostaining scores for each protein. Univariate testing of immunostaining scores and each clinicopathologic variable shown in Table 1 for association with disease recurrence, cancer-specific mortality or overall mortality (yes/no) was performed using a Fisher's Exact Test. Multivariate analysis using exact logistic regression was used to determine the ability of immunostaining scores to predict overall mortality after adjusting for other clinicopathologic variables demonstrating significant association on univariate analysis. For *in vitro* growth assays an ANOVA test were utilized to determine statistical significance between mean cell counts. RT-qPCR data were for analyzed for statistical significance using student's t test. Statistical analyses were conducted using StataSE 10® (StataCorp College Station, TX, USA) or Prism4 (GraphPad). In all cases, p≤ 0.05 was considered significant.

RESULTS

JMJD2A/LSD1/AR immunostaining: association with cancer histopathology

Significantly lower levels of JMJD2A immunostaining were detected in malignant *versus* benign urothelium, based on both %-tissue positivity and intensity scores (Table 2, Figures 1, 2). For example, >10% tissue positivity was observed in the vast majority (90%) of benign specimens compared to around only one third of malignant specimens (Figures 1, 2). Among JMJD2A positive cases, staining intensity was typically low or moderate (1–2+). However, patients with 2+ or 3+ staining in benign urothelium generally had only 0+ or 1+ staining in matching cancer specimens (11/14, 79%). By both %-tissue positivity and intensity scoring, lower JMJD2A levels correlated significantly with the acquisition of an invasive phenotype, including detrusor muscle invasion (Table 2, Figures 1, 2). Lower JMJD2A intensity but not %-tissue positivity also correlated significantly with the presence of extravesical tumor extension (Table 2, Figures 1, 2). This association was most pronounced for pT4 disease, with complete JMJD2A loss being present in 64% of these specimens compared to only 8% of all lower staged cancers (0% pTa, 0% pTis, 0% pT1, 20% pT2, 18% pT3). As detailed in Table 2, reduced JMJD2A levels were also significantly associated with other adverse histopathologic descriptors, including lymphovascular invasion (JMJD2A intensity), concomitant carcinoma *in situ* (JMJD2A %-tissue positivity), lymph node positivity (JMJD2A %-tissue positivity) and lymph node stage (JMJD2A %-tissue positivity). There was also a trend towards association between JMJD2A intensity and perineural invasion, but this finding did not reach statistical significance (Table 2).

In contrast to JMJD2A, LSD1 stained with at least >10% tissue positivity in nearly all (96%) bladder specimens, with no differences observed between malignant and benign urothelium (Table 2, Figures 1, 2). Greater than 70% tissue positivity was detected in the majority of these cases. However, intensity of staining tended to be higher among malignant specimens (p=0.096) (Table 2, Figures 1, 2). This finding reached statistical significance when comparing only those patients with both benign and cancer specimens available (N=57, p=0.05). In contrast, LSD1 intensity decreased significantly with acquisition of the invasive phenotype, including detrusor muscle invasion and extravesical extension (Table 2, Figures 1, 2). LSD1 levels were not significantly associated with other adverse histopathologic parameters, including lymphovascular invasion, perineural invasion and concomitant carcinoma *in situ*, although we observed a nonsignificant trend towards association of lower LSD1 intensity with the presence of lymph node metastasis (Table 2).

As with JMJD2A, significantly lower AR staining intensity and %-tissue positivity were observed in malignant urothelium relative to benign urothelium (Table 2, Figures 1, 2). For example, >10% tissue positivity was found in two thirds of benign specimens compared to less than one quarter of cancer specimens, and total absence of AR was observed in roughly half of all cancer specimens compared to just 16% of benign specimens. As with JMJD2A and LSD1, lower AR %-tissue positivity correlated significantly with the acquisition of an invasive phenotype, including detrusor muscle invasion, and also correlated significantly with the presence of lymph node metastasis (Table 2, Figures 1, 2). A trend towards association of AR %-tissue positivity with extravesical extension did not reach statistical significance (Table 2, Figures 1, 2). No association was detected between AR levels and other adverse histopathologic descriptors, including lymphovascular invasion, perineural invasion and concomitant carcinoma *in situ* (Table 2).

JMJD2A/LSD1/AR immunostaining: association with clinical risk factors

Immunostaining levels of AR, JMJD2A and LSD1 were tested for association with several clinical variables, including known risk factors for bladder cancer diagnosis or cancer-specific mortality (Table 2). Lower JMJ2DA staining intensity was found to be strongly associated with active patient smoking (p=0.001). There was also a nonsignificant trend towards association of JMJD2A staining intensity with preoperative hydronephrosis (p=0.065) and female gender (p=0.062), as 30% of female patients had JMJD2A-negative tumors compared to just 9% of males. AR %-tissue positivity was significantly associated with preoperative intravesical chemotherapy (p=0.009), and there was a nonsignificant trend towards association between LSD1 %-tissue positivity and neoadjuvant systemic chemotherapy (p=0.09). No association was detected between JMJD2A/LSD1/AR levels and patient age, race, body mass index or pelvic radiation history (Table 2).

JMJD2A/LSD1/AR immunostaining: association with patient survival

Immunostaining scores for AR, JMJD2A and LSD1 were tested alongside other clinicopathologic variables for association with bladder cancer recurrence, cancer-specific mortality and overall mortality by univariate analysis. Disease recurrence was predicted by tumor stage (p>0.001) and lymph node stage/positivity/density (p=0.003/0.005/0.006), while cancer-specific mortality was predicted by tumor stage (p=0.007), lymph node stage/positivity/density (p=0.02/0.04/0.01) and lymphovascular invasion (p=0.01). AR, JMJD2A or LSD1 levels were not associated disease recurrence or cancer-specific survival (Table 2). In contrast, lower JMJD2A staining intensity was significantly associated with overall mortality (p=0.033), as was tumor stage (p<0.001), lymph node stage/positivity/density (p=0.001/0.007/0.001), lymphovascular invasion (p<0.001), neoadjuvant chemotherapy (p=0.041) and active tobacco usage (p=0.012), but not levels of LSD1 or AR (Table 2). We performed a multivariate analysis to determine whether the association of JMJD2A intensity with overall mortality was independent of other clinicopathologic variables, however no variable carrying significance on univariate analysis reached significance on multivariate analysis likely due to the limited number of death events (data not shown). Kaplan-Meier curves for overall survival were generated for each of the three AR-complex proteins and are displayed in Figure 3. While 100% of patients with moderate or high (2–3+) JMJD2A intensity demonstrated durable overall survival, patients with JMJD2A negative (0+) tumors experienced had accelerated death, with a 20% survival rate at just 2 years after cystectomy. Patients with low (1+) JMJD2A staining intensity had intermediate survival outcomes, with a >75% survival rate at 2 years after cystectomy.

JMJD2A/LSD1 immunostaining: association with AR expression

Levels of JMJD2A and LSD1 immunostaining were tested for association with levels of AR and each other using a Spearman correlation test (Table 3). Weak, but statistically

significant correlations were observed between LSD1 tissue positivity and AR tissue positivity and intensity, and between JMJD2A intensity and AR intensity (Table 3). LSD1 tissue positivity also weakly but significantly correlated with JMJD2 tissue positivity and intensity (Table 3).

Bladder cancer cell growth response to LSD1 inhibition

Levels of *AR, JMJD2A* and *LSD1* mRNA transcripts were assessed by RT-PCR in four human bladder cancer cell lines derived from advanced stage tumors. Consistent with previous reports, the *AR* transcript was detected at low levels in the HTB-5 and HTB-3 cell lines and was not detected in the HTB-1 and HT1376 cell lines (Figure 4A) [13,18]. In contrast, mRNA expression of AR coregulators, *JMJD2A* and *LSD1*, were detectable in all four cell lines (Figure 4A). Each cell line was treated with the non-specific LSD1 inhibitors pargyline and tranylcypromine (1 mM and 3 mM) to determine the dose response effects on proliferation (Figure 4C-F). Both drugs showed dose-dependent suppression of cancer cell proliferation in the AR-positive cell lines, HTB3 and HTB5. In the AR-negative cell lines, HTB1 and HT1376, higher dose (3 mM) pargyline similarly inhibited cell proliferation, whereas the lower dose (1 mM) had no significant effect. Identical results were observed for tranylcypromine in the AR-negative cell line, HT1376. (Figure 4C-F)

Effect of tranylcypromine on AR-regulated gene expression in human bladder cancer cells

We utilized RT-PCR to identify a representative AR-regulated test gene expressed in bladder cancer cells. This analysis confirmed expression of the androgen receptor regulated neutral endopeptidase (*NEP)* gene [44] in both HTB-1(AR negative) and HTB-5 (AR positive) bladder cell lines (data not shown). We next examined the effects of tranylcypromine on androgen induced transcription in cultured HTB-1 and HTB-5 bladder cancer cells. R1881 induced a statistically significant increase in *NEP* expression in AR-positive HTB-5 cells, but not in HTB-1 AR-negative bladder cells (Figure 4F). Tranylcypromine effected a modest repression of basal *NEP* expression in HTB-5 cells and blocked R1881 induced expression of *NEP* in HTB-5 cells (Figure 4F). Tranylcypromine and R1881 had no detectable effect on *NEP* expression in HTB-1 cells (Figure 4F).

DISCUSSION

While recent studies implicate AR signaling in bladder carcinogenesis [13], the underlying mechanisms are unknown. AR coregulators represent a enzymatically diverse set of proteins whose direct interaction with AR is required for assembly of a functional transcriptional complex [21]. Alteration in coregulator levels and/or activity was shown to dysregulate epigenetic transcriptional regulation independent of AR and circulating androgen levels [22]. Indeed there is mounting evidence for key roles of AR coregulators in the development of androgen-independent disease [22–25]. These findings highlight the dependence of AR transcriptional activity on AR-coregulator complex composition and underscore the signaling complexity that can result from differences in coregulator expression levels.

The current study investigated the role of recently identified AR coregulators JMJD2A and LSD1, in human bladder cancer. JMJD2A and LSD1 employ a recently described lysine demethylase mechanism to induce chromatin structural alterations required for AR-mediated transcription [27,28,30,32]. In hormonally driven malignancies such as prostate cancer, levels of JMJD2A and LSD1 are altered in patient tumors, and a functional role for each in mediating AR-dependent prostate cancer cell proliferation has been described [28,39,40]. Recent studies implicate human bladder carcinogenesis as a hormonally driven disease. [13,18,19] Here, we performed immunohistochemical analysis in 129 clinical bladder specimens to test whether protein levels of either JMJD2A or LSD1 are also altered during

bladder carcinogenesis. We observed significant differences in JMJD2A and LSD1 coregulator levels between malignant and benign urothelium. Lower levels of both proteins were detected with primary tumor progression, including detrusor muscle invasion and extravesical extension. JMJD2A reduction also correlated with lymph node metastasis, lymphovascular invasion, and presence of carcinoma *in situ*, all independent predictors of worse patient outcomes after radical cystectomy [5–7]. Together, our data support reduced expression of these lysine demethylating AR coregulators during bladder cancer progression. Interestingly, the expression patterns observed in this study are different from those reported for prostate tissue, where JMJD2A and LSD1 are upregulated during tumorigenesis and cancer progression, respectively [28,40]. Thus, LSD1 and JMJD2A appear to play different roles in bladder *versus* prostate carcinogenesis.

We also investigated AR protein levels in bladder tissue. Similar to JMJD2A and LSD1, lower AR levels were observed during primary tumor progression to invasion. Consistent with prior reports from our group and Tuygun *et al*, we found AR reduction to be associated with the acquisition of the muscle-invasive phenotype [19,20]. In contrast to these reports, Mir and colleagues recently failed to detect any association between stage and loss of AR expression [45]. However AR was not detected in the majority of bladder cancer specimens examined regardless of stage in this study though the authors acknowledge a limitation to their study was that the majority of samples examined were muscle invasive tumors [45]. Of note, we observed reduced AR levels in malignant *versus* benign urothelium, suggesting downregulation during tumorigenesis (Table 2). While other studies have noted little or no AR expression in benign urothelium [18,20], we [19] and Mir et al [45] detected AR expression in benign urothelium. Finally, we detected a novel association between lower AR levels and lymph node metastasis. As with the coregulators, these findings are in contrast to the persistent AR expression observed during prostate cancer progression/metastasis and further suggest a novel function for the AR signaling complex in bladder carcinogenesis [46].

In prostate cancer cells, expression of several AR coregulators is described as being under AR regulation [47–50]. In this study, we observed only very weak correlations between levels of AR and levels of JMD2A or LSD1 in bladder tissue. Similarly, we detected JMJD2A and LSD1 transcripts in bladder cancer cell lines regardless of whether or not the AR transcript was present. We thus conclude that JMJD2A and LSD1 expression in bladder is likely not regulated by the AR in bladder cells.

The expression patterns observed in this study support a complex function for AR and its associated coregulator protein complexes in bladder tissue. In prostate cancer, conventional understanding is that AR activity promotes proliferation, however recent studies suggest a greater complexity of AR signaling, including the ability to suppress cancer growth cell proliferation under specific circumstances [24,51]. The frequent loss of AR and JMJD2A in early and advanced bladder cancers suggests these proteins may not be required for initiation and/or progression of disease and may indicate a potential role in impairing or delaying carcinogenesis. However, the functional significance of AR and JMJD2A loss with regards to effects on androgen signaling remains to be determined. With LSD1, the dynamic expression pattern consistent with early stage upregulation and advanced stage downregulation suggests a more complex role for LSD1 in bladder carcinogenesis. This complexity may relate to the fact that LSD1 also regulates additional signaling pathways implicated in human cancers, most notably those of p53 [31] and estrogen receptor, both of which are implicated in bladder carcinogenesis [29,39,52]. Thus, LSD1 represents a point of convergence of multiple, distinct signaling pathways implicated in human cancer. Regardless, it is possible that higher LSD1 levels during early bladder carcinogenesis may

be important in altering the function of AR transcriptional complexes and compensating in part for lower AR levels.

To further elucidate the contribution of the AR-lysine demethylase complex to bladder carcinogenesis we have described the association of AR and coregulator protein levels with multiple clinical variables, including known risk factors for diagnosis and/or cancer-specific death. We report a novel association between decreased JMJD2A and active patient smoking, the most common risk factor for bladder tumorigenesis and also a poor prognostic trait [2]. A link between AR signaling and smoking has been suggested by various clinical observations, such as lower testosterone levels or worse oncologic responses to androgen-deprivation therapy among active tobacco users [53,54]. A specific mechanism is unknown but may relate to the ability of AR complexes to regulate N-acetyltransferases, enzymes necessary for bioactivation of tobacco carcinogens [55]. Lower levels of JMJD2A in this study also trended towards association with female gender [2,8–10], an additional poor prognostic features among bladder cancer patients, although this finding did not reach statistical significance (p=0.06). Nevertheless, this observation is provocative and raises the intriguing possibility that alterations in levels of sex-steroid hormone receptor coregulators may contribute to the worse female prognosis, given levels of the sex-steroid hormone receptors themselves appear similar between the genders [20]. It remains to be determined whether JMJD2A also interacts with the estrogen receptor, as already demonstrated for other AR-coactivators including LSD1 [27]. Finally, this study also uncovers a novel association between AR-complex signaling and preoperative chemotherapy. Specifically, higher AR protein levels were associated with the receipt of preoperative intravesical chemotherapy with mitomycin-C. The efficacy of chemotherapeutic drugs is well known to be negatively affected by cell cycle arrest, and the function of AR as key regulator of G1-S cell-cycle transition could therefore be important in modifying drug response.

The prognostic significance of reduced AR protein during clinical bladder cancer progression has not been well characterized. Tuygun *et al* found no association between AR reduction and disease recurrence. However nearly half of the patients in that study were excluded from analysis due to poor follow-up, and an association with patient survival was not investigated [20]. While we observed no prognostic value for lower AR or LSD1 levels with regards to disease recurrence or patient survival, we found JMJD2A staining intensity to be predictive of overall patient survival, as 100% of patients with at least moderate JMJD2A staining intensity remained alive at the time of the study, while over 80% of patients with JMJD2A-negative tumors had died within less than 2 years of surgery. Thus, consistent with its association with numerous adverse clinicopathologic parameters, JMJD2A loss appears to be an accurate marker of particularly aggressive favorable bladder disease. JMJD2A immunostaining in clinical bladder specimens may thus have utility in facilitating the identification of high-risk patients who may benefit from more aggressive therapeutic approaches, including immediate radical cystectomy, extended lymphadectomy, and neoadjuvant or adjuvant systemic therapies.

An additional promising clinical application for JMJD2A and LSD1 in bladder cancer is their ability to serve as novel targets for epigenetic pharmacotherapies [27,33–38]. The recent groundbreaking discovery that histone lysine demethylation could be biochemically achieved under physiologic conditions has ignited efforts to develop drugs that target this activity for reversal of aberrant epigenetic modifications associated with carcinogenesis [33,37]. Crystal structures of LSD1 reveal the protein to belong to the amine oxidase enzyme superfamily, and the ability of the MAOI class of clinical drugs to inhibit LSD1 has been confirmed in prostate cancer models [27]. Here we show that an MAOI, tranylcypromine, effectively suppresses AR-dependent transcription and growth of bladder cancer cell lines. Furthermore, tranylcypromine demonstrated higher efficacy in the AR-

positive cell lines, consistent with growth inhibition being attributable to disruption of AR-KDM complex signaling. Thus the potential future application of KDM-targeting therapeutics will require a more detailed understanding of the role of the AR-lysine demethylase complex in bladder carcinogenesis, as well as knowledge of the AR-status of individual bladder tumours.

We have found increased tumor LSD1 levels relative to normal urothelium. These findings support AR lysine-demethylase coregulators as potential therapeutic targets to delay or suppress bladder carcinogenesis. Efforts to develop drugs targeting LSD1 as well as JMJD2 members with greater specificity are currently underway [33–38]. Huang and associates recently exploited the structural similarity of LSD1 and polyamine oxidases to identify several inhibitors of LSD1, which effectively reversed aberrant genetic silencing implicated in tumorigenesis, consistent with an oncogenic role for LSD1 [33,34]. Similarly, identification of small molecule inhibitors with antiproliferative activity and selectivity for LSD1 was recently described by Ueda and colleagues [38]. Our findings suggest that agents such as these may hold promise in the future treatment of bladder cancer patients.

CONCLUSION

Limited knowledge is available regarding endocrine signaling pathways in bladder carcinogenesis compared to other hormonally regulated solid tumors such as prostate and breast cancers. This study provides the first investigation to our knowledge on the combined roles of the LSD1-JMJD2 histone lysine-demethylase coregulators and AR in bladder cancer. We show that alterations in JMJD2A and LSD1 occur during clinical bladder carcinogenesis, with reduction in both occurring during cancer stage progression in an AR-independent manner. JMJD2A reduction correlates with numerous pathologic and clinical variables conferring a poor patient prognosis, and JMJD2A itself lends important prognostic value with regards to patient survival, providing a novel biomarker for bladder cancer patient risk stratification. Increased LSD1 levels in malignant *versus* benign urothelium suggest a complex role for this protein in bladder carcinogenesis that may include an oncogenic function. Accordingly, we show that pharmacologic inhibition of LSD1 effectively suppresses androgen induced transcription and bladder cancer cell growth, supporting the biologic efficacy of targeting the AR-complex in this tissue type. Altogether, this study supports a role for AR signaling in human bladder carcinogenesis and has identified a novel contribution of lysine-demethylase AR coregulators in this process. The ability to target lysine-demethylase coregulators with specificity and thus modulate aberrant epigenetic gene regulation may hold promise for future intravesical and systemic pharmacotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

|	
AR	Androgen receptor
FAD	flavin adenine dinucleotide
JMJD2A	jumanji-domain-2A
KDM	lysine demethylases
LSD1	lysine specific demethylases

MAOI monoamine oxidase inhibitor

Acknowledgments

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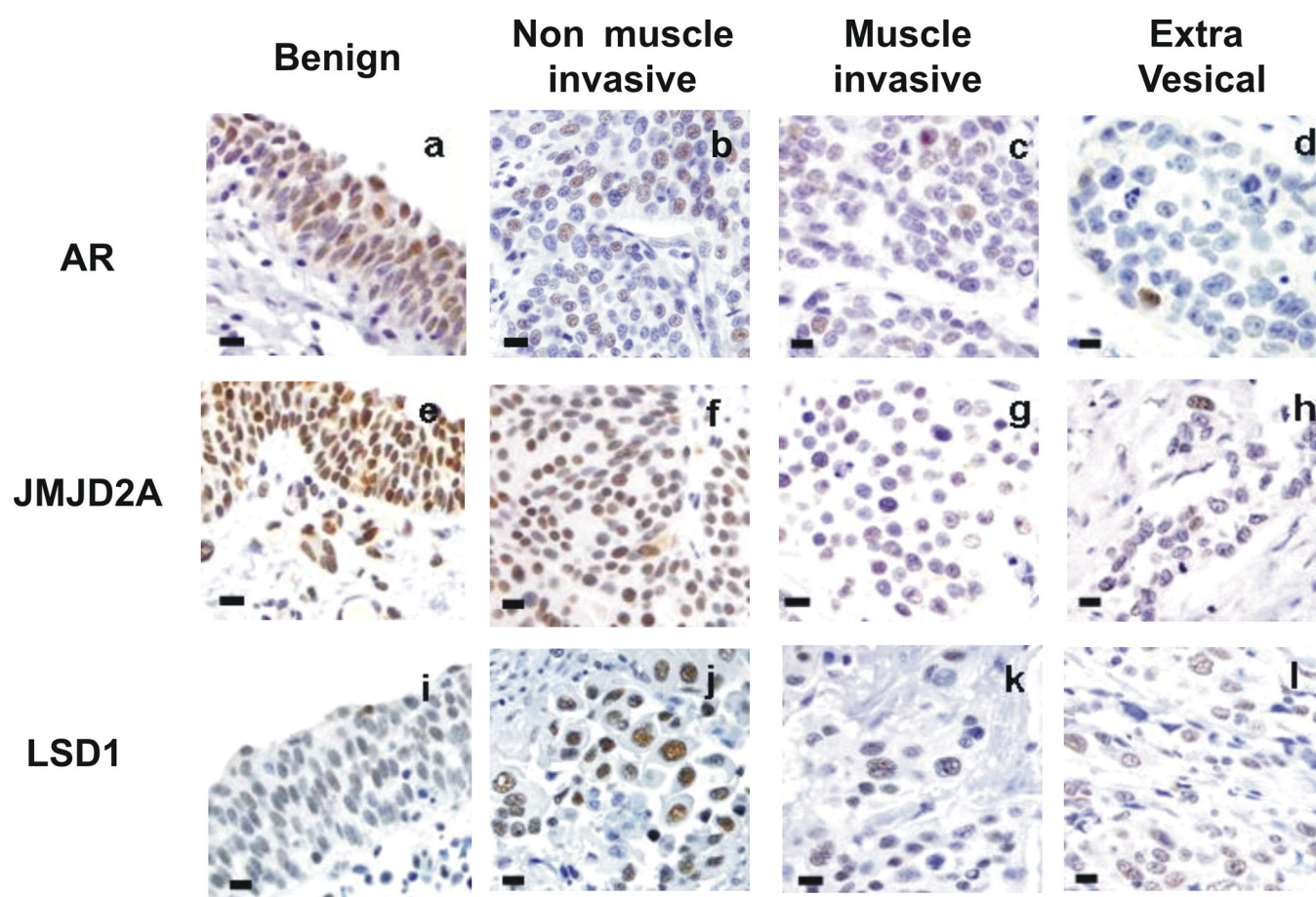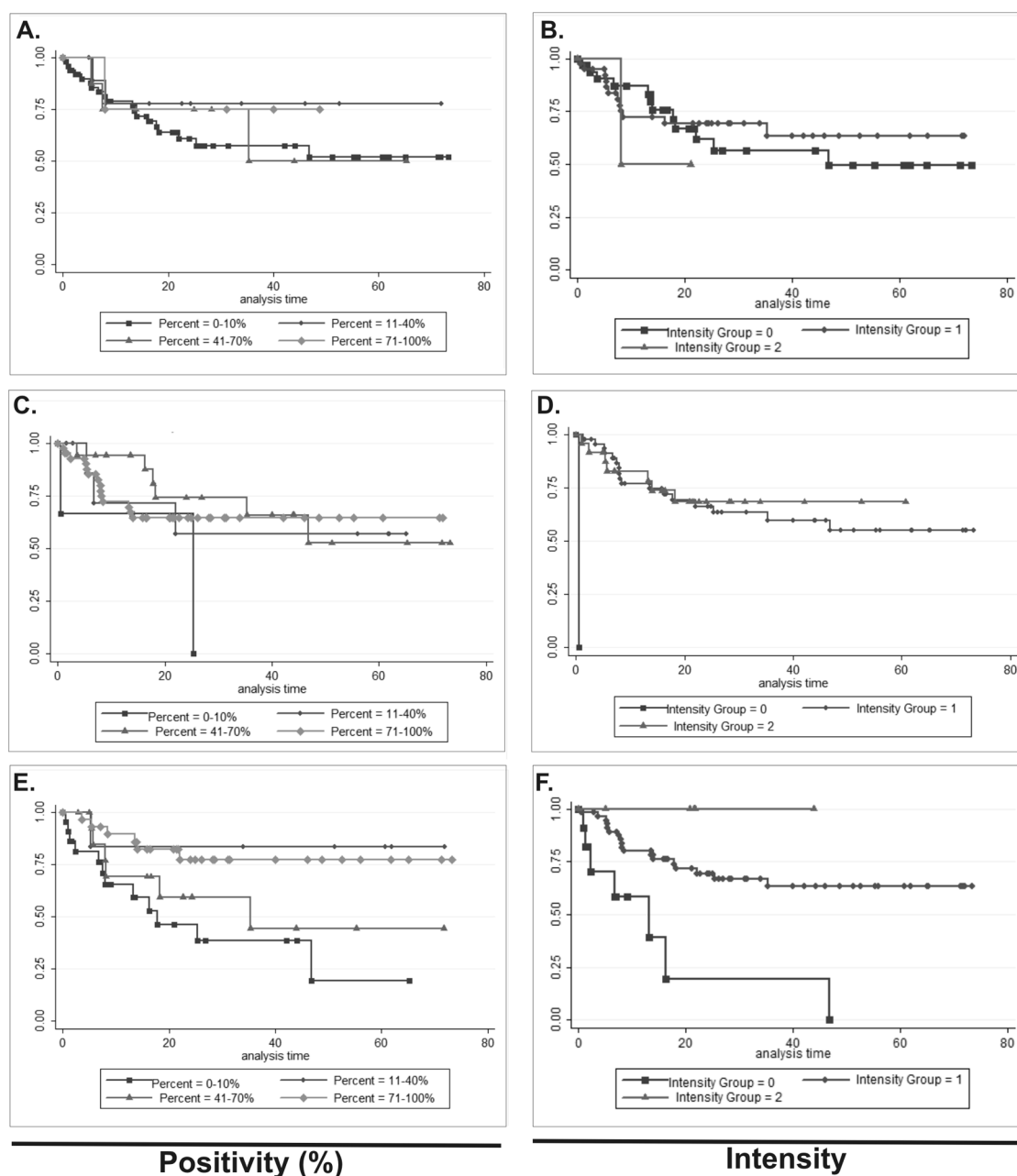
Figure 1. Immunohistochemical staining
Representative immunohistochemical staining for AR (a-d), LSD1 (d-f) and JMJD2A (g-i)in benign urothelium, non-muscle invasive urothelial carcinoma (pTis, pTa, pT1), muscle invasive carcinoma (pT2) and extravesical (pT3) carcinoma. (All images taken at 400X total magnification; bar – 20 microns)
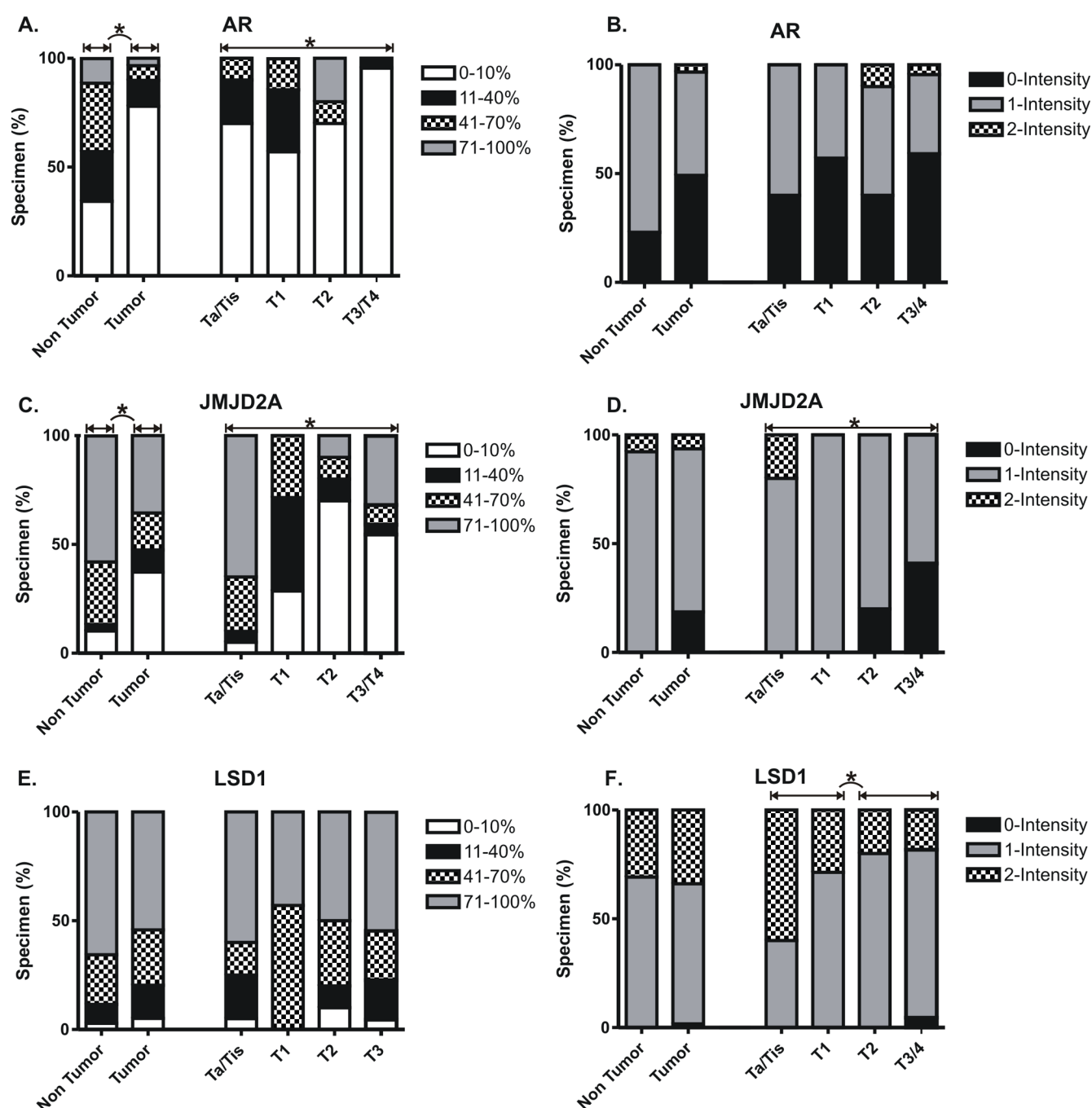
Figure 2. Distribution of staining positivity and intensity by pathological tumor stage
Separate scores were given to the stained tissue sections based on: a) tissue positivity, *i.e.*, the percentage of tissue staining positive (0–100%), and b) the intensity of positively staining cells (0– none, 1– weak, 2– moderate, and 3– strong). Only nuclear staining was considered positive, and a minimum of 100 tumor nuclei were counted for each case. A summary of statistically significant differences in staining positivity and intensity are indicated (*). Please refer to Table 2 for detailed statistical analysis.

Overall Survival

Figure 3. Kaplan Meier survival estimates
Kaplan-Meier overall survival estimates for 72 cystectomy patients, stratified by immunohistochemical staining positivity and intensity for AR (A-B), LSD1(C-D) and JMJD2A (E-F) in bladder tumors.
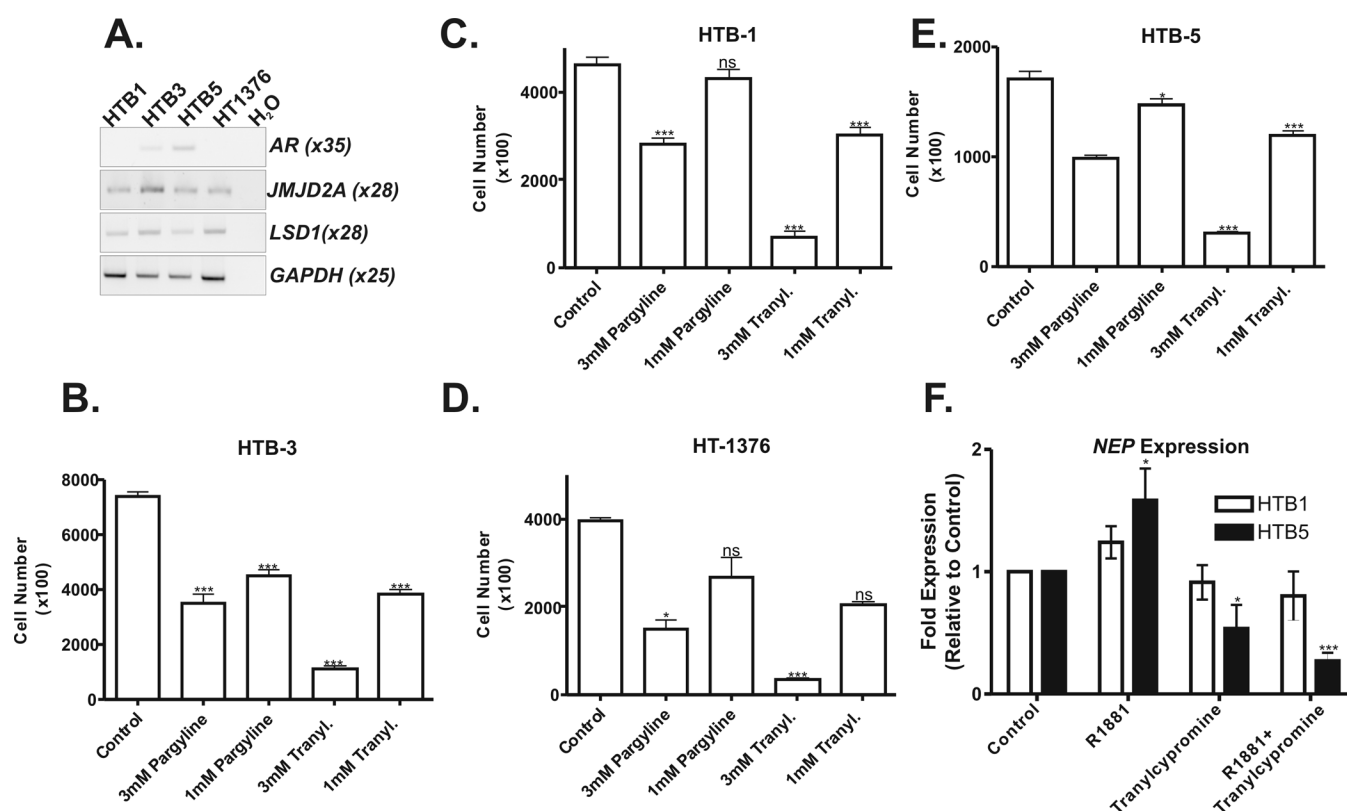
Figure 4. RT-PCR expression detection of *AR* and *AR* coregulators in human bladder cancer cell lines and effects of monoamine oxidase inhibition on bladder cancer cell proliferation and transcription

Confirmation of mRNA expression of AR, JMJD2A, LSD1 in bladder cancer cell lines (A). Full length gels are presented in supplemental figure 1. The number of PCR cycles employed for each gene is indicated. *GAPDH* served as an internal control for cDNA integrity. Comparison of effects of 3mM and 1mM pargline and tranylcypromine (Tranyl) on proliferation of HTB-1 (B), HTB-5 (C), HTB-3 (D) and HT-1376 (E) bladder cancer cells. Quantitative RT-PCR was used to assess the effects of tranylcypromine on androgen regulated transcription (F). Neutral endopeptidase (*NEP*) is a representative AR-regulated gene expressed in both HTB1 and HTB5 bladder cancer cells. Bladder cells were treated with tranylcypromine (50µM) for 48 hours prior to transcriptional activation with the R1881 (1nM) synthetic androgen for 30 minutes. R1881 induces *NEP* expression in AR-positive HTB5 cells. This effect of R1881 on *NEP* expression in HTB5 cells is impaired by tranylcypromine. Neither R1881 nor tranylcypromine alter *NEP* expression in AR-negative HTB1 cells, suggesting the effects of tranylcypromine are dependent upon the presence of a functional AR-KDM complex.(*:$p<0.05$, ***$p<0.01$, ns: not significant).

Table 1

Cystectomy patient characteristics

TOTAL PATIENTS, n= (%)	72 (100)

AGE, years
    mean +/− s. d.
    median
    range
	66.4 +/− 10.0
	66.6
	43.3 – 88.8

GENDER, n= (%)
    Female
    Male
	21 (29)
	51 (71)

RACE, n= (%)
    Caucasian
    Other
	66 (92%)
	6 (8%)

BMI, kg/m2
    mean +/− s. d.
    median
	26. 8 +/− 4. 8
	26. 5

SMOKING HISTORY, n= (%)	56 (74)
ACTIVE SMOKER, n= (%)	15 (21)
PRIOR PELVIC IRRADIATION, n= (%)	8 (11)
PRIOR INTRAVESICAL CHEMOTHERAPY, n= (%)	21 (29)
PRIOR NEOADJUVANT SYSTEMIC CHEMOTHERAPY, n= (%)	11 (15)
PRE-OPERATIVE HYDRONEPHROSIS, n= (%)	20 (28)

PATHOLOGIC STAGE, n= (%)
    pT0
    pTa
    pTis
    pT1
    pT2
    pT3
    pT4
	13 (18)
	9 (13)
	11 (15)
	7 (10)
	10 (15)
	11 (15)
	11 (15)

LYMPH NODE METASTASES, n= (%)	18 (25)

N-STAGE
    N1
    N2
    N3
	10 (14)
	5 (7)
	3 (4)

LYMPHOVASCULAR INVASION, n= (%)	15 (21)
PERINEURAL INVASION, n= (%)	6 (8)
CONCOMITANT CARCINOMA IN SITU (CIS), n= (%)	34 (47)

POST-OPERATIVE FOLLOW UP INTERVAL, months
    mean +/− s. d.
    median
	26. 3 +/− 21. 2
	21. 2

POST-OPERTATIVE RECURRENCE, n= (%)	19 (26)
POST-OPERATIVE DEATH FROM BLADDER CANCER, n= (%)	15 (21)

Table 2

Association of JMJD2A, LSD1 and AR levels with patient clinicopathologic traits, disease recurrence and cancer-specific mortality in tumor specimens

Fishers exact p-values are shown for each statistical association. Dark shading = significant (p<0.05). Superficial (Ta, T1, CIS) and muscle invasive (T2, T3, T4) bladder cancers. NMI:non muscle invasive; MI= muscle invasive ; BC: bladder confined, EVE; extravsical.

Marker	JMJD2A		LSD1		AR	
	% Positivity	Intensity	% Positivity	Intensity	% Positivity	Intensity
	p value		p value		p value	
CLINICAL						
Age	0.101	0.817	0.560	0.294	0.638	0.821
Gender	0.557	0.06	0.200	0.582	0.961	0.900
Race	0.121	0.911	0.565	0.821	0.686	0.926
Smoking: Any History	0.344	1	0.533	0.669	0.705	1
Active	**0.027**	**0.001**	0.562	0.755	0.606	0.317
Body Mass Index	0.535	0.114	0.273	0.595	0.998	0.37
Prior Pelvic Radiation	0.241	0.787	0.831	1	1	0.428
Hydronephrosis	0.736	0.065	0.856	0.573	0.619	0.537
Intravesical Chemotherapy	0.813	0.298	0.969	0.289	**0.009**	0.189
Neoadjuvant Chemotherapy	0.664	0.465	0.09	0.141	0.637	0.666
HISTOPATHOLOGIC						
Malignant vs. Benign	**<0.001**	**0.012**	0.493	0.096	**<0.001**	**<0.001**
Stage:						
Overall	**<0.001**	**<0.001**	0.639	0.121	**0.014**	0.443
Superficial vs Invasive	**<0.001**	**<0.001**	0.574	**0.006**	0.358	0.35
NMI vs MU	**0.001**	**<0.001**	1	**0.012**	**0.028**	0.382
BC vs EVE	0.177	**0.001**	0.939	**0.046**	0.092	0.376
Lymphovascular Invasion	0.062	**0.016**	1	0.129	0.762	0.532
Perineural Invasion	0.417	0.097	0.777	0.165	0.496	0.496
Presence of CIS	**0.033**	1	0.649	0.485	0.894	0.191
Positive Bladder Margin	0.687	0.125	0.426	0.565	1	0.619
Lymph Node: Positivity	**0.034**	0.141	0.225	0.084	**0.02**	0.166

Marker	JMJD2A			LSD1			AR		
	% Positivity	Intensity		% Positivity	Intensity		% Positivity	Intensity	
	p value			p value			p value		
Density	0.088	0.309		0.204	0.112		0.256	0.082	
Stage	0.022	0.028		0.306	0.155		0.675	0.276	
SURVIVAL									
Disease-free survival	0.799	0.409		0.372	0.494		0.284	0.409	
Cancer Specific Survival	0.896	0.761		0.885	0.810		0.665	0.456	
Overall Survival	0.064	**0.033**		0.740	0.402		0.766	0.745	

Table 3

Correlation of levels of AR coregulators to levels of AR and to each other

Expression of each marker was assessed for correlation using the Spearman rank correlation test. Spearman correlation coefficient and p values are shown for each correlation.

		JMJD2A Positivity (%)	JMJD2A Intensity	AR Positivity (%)	AR Intensity	LSD1 Positivity (%)	LSD1 Intensity
JMJD2A Positivity (%)	Correlation coefficient	1	0.511	0.171	0.161	0.201	0.117
	p-value		<0.001	0.053	0.070	0.023	0.187
JMJD2A Intensity	Correlation coefficient		1	0.168	0.254	0.177	0.066
	p-value			0.057	0.004	0.046	0.461
AR Positivity (%)	Correlation coefficient			1	0.569	0.193	0.077
	p-value				<0.001	0.029	0.387
AR Intensity	Correlation coefficient				1	0.209	0.080
	p-value					0.018	0.369
LSD1 Positivity (%)	Correlation coefficient					1	0.440
	p-value						<0.001