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Original Article

Latrophilin-1 and latrophilin-2 as androgen receptor-responsive G protein-coupled receptors promote bladder cancer progression

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Abstract: Objectives: To investigate the functional role of latrophilin-1 (LPHN1; encoded by the *ADGRL1* gene) and latrophilin-2 (LPHN2; encoded by the *ADGRL2* gene), members of the G protein-coupled receptor family, in relation to androgen receptor (AR) signaling, in the outgrowth of bladder cancer. Methods: Human bladder urothelial carcinoma cell lines were subjected to real-time PCR, western blotting, chromatin immunoprecipitation, MTT assay, and wound-healing assay. Immunostaining was also performed on a set of bladder cancer tissue microarrays consisting of transurethral resection specimens. Results: In bladder cancer cells with endogenous or exogenous AR expression, dihydrotestosterone markedly up-regulated *ADGRL1*/LPHN1 and *ADGRL2*/LPHN2 expression. Chromatin immunoprecipitation confirmed AR binding to the promoter regions of *ADGRL1* and *ADGRL2*. Additionally, LPHN ligands (*e.g.* α-latrotoxin, FLRT3) induced their expression. Knockdown of LPHN1 or LPHN2 via shRNA virus infection significantly reduced cell viability and migration, while the stimulatory effects of LPHN ligands on cell viability were more significant in AR-negative or AR-knockdown lines than in corresponding AR-positive lines. Immunohistochemical analysis in surgical specimens further showed that LPHN1 overexpression (*i.e.* moderate/strong) in muscle-invasive tumors (n = 62) independently predicted poorer disease-specific survival following radical cystectomy (hazard ratio 2.662, $P = 0.031$). Analysis of The Cancer Genome Atlas (TCGA) dataset (n = 305, stage II-IV bladder cancer) also revealed that high *ADGRL2* expression was associated with significantly worse overall survival. Conclusion: These findings suggest that LPHN1 and LPHN2 function as downstream effectors of AR and contribute to the progression of bladder cancer.

Keywords: ADGRL1, ADGRL2, androgen receptor, bladder cancer, LPHN1, LPHN2

Introduction

Urinary bladder cancer, of which the most common histology is urothelial carcinoma, remains one of the most prevalent malignancies, particularly among men [1, 2]. The global burden of bladder cancer-related mortality appears to be rising significantly, with an estimated 165,100 deaths in 2012 [1] and 220,349 reported in 2022 [2]. Although bladder tumors are often detected as non-invasive lesions which may not be fatal, these patients initially present with non-invasive disease carry a high risk of recurrence, and in some cases, progression to invasive disease following transurethral surgery. More concerningly, muscle-invasive bladder cancer is often associated with metastatic disease, as reflected by a 5-year survival rate of only 9.1% [3], despite the emergence of new targeted therapy options [4, 5]. Thus, there is a critical need to identify key molecular targets or signaling pathways that drive the progression of urothelial cancer.

Recent evidence highlights the role of androgen receptor (AR), a member of the nuclear receptor superfamily, in the pathogenesis and progression of urothelial cancer. Specifically, activation

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of AR signaling has been shown to promote tumor growth and confer resistance to standard non-surgical treatments for bladder cancer including chemotherapy, intravesical BCG immunotherapy, and radiotherapy [6-9]. It has been well established that androgens enhance, while anti-androgens or AR inhibitors suppress, the proliferation, migration, and invasion of AR-positive bladder cancer cell lines [6, 7, 9, 10]. However, the precise molecular mechanisms through which AR modulates the growth of urothelial cancer remain incompletely understood.

Latrophilins (LPHNs) are a subgroup of the highly conservative G protein-coupled receptors, originally identified as binding proteins for latrotoxin (LTX), a neurotoxin found in the venom from black widow spiders (*e.g. Latrodectus genus*) [11-13]. Of the three known human isoforms, LPHN2 is ubiquitously expressed, whereas LPHN1 and LPHN3 are abundant particularly in the brain [13-15]. Nonetheless, the biological functions of LPHNs and their encoded genes (*i.e. ADGRL*s) are largely unknown, particularly in neoplastic conditions. Notably, in our DNA microarray analysis [16], *ADGRL3* was identified as one of the genes that were considerably down-regulated in an AR-knockdown subline, compared with control AR-positive bladder cancer UMUC3 cells. Furthermore, we recently demonstrated that androgen treatment or AR overexpression led to up-regulation of LPHN3 expression in bladder cancer cells and thereby promoted their growth [17]. The present study aimed to determine if LPHN1 and LPHN2 could also induce the progression of urothelial cancer as downstream effectors of AR signaling.

Materials and methods

Cell lines

Human bladder urothelial carcinoma cell lines, 647V, 5637, and UMUC3, were originally obtained from the American Type Culture Collection. Stable sublines expressing human full-length wild-type AR (*i.e.* 647V-AR [18], 5637-AR [19]) or an AR-targeted short hairpin RNA (shRNA) (*i.e.* UMUC3-AR-shRNA [19]) were established in our previous studies. Similarly, LPHN1-shRNA lentiviral particles (sc-45408-V, Santa Cruz Biotechnology) or LPHN2-shRNA lentiviral particles (sc-60919-V, Santa Cruz Biotechnology) were infected and stably expressed in 647V, 647V-AR, 5637-AR, or UMUC3 cells. All parental cell lines and derivative sublines were maintained in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 units/mL each), and eventually cultured in phenol red-free medium supplemented with 5% charcoal-stripped fetal bovine serum for androgen treatment experiments or standard 5% fetal bovine serum for other experiments.

Chemicals and antibodies

Dihydrotestosterone was purchased from Sigma-Aldrich, α-LTX from Alomone Labs, and recombinant human FLRT3 protein from R&D Systems. Primary antibodies obtained from Santa Cruz Biotechnology included LPHN1 (clone A-4), LPHN2 (clone E-3), and GAPDH (clone 6C5).

Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA was extracted from cultured cells using TRIzol (Invitrogen) and reverse transcribed with oligo-dT primers and Omniscript reverse transcriptase (Qiagen). Real-time PCR was performed using the primer sets specific for *ADGRL1* (forward, 5'-GGTGAACTCACAGGTCATCGCA-3'; reverse, 5'-CCGAGTAGTTCCAGAAGGAGCA-3'), *ADGRL2* (forward, 5'-GAGCAGTTGGTGGACATCCTTG-3'; reverse, 5'-GGTCTCAGAAGGTTGTCCACTG-3'), *AR* (forward, 5'-TGAAAGCCATGCTACTCTTCAG-3'; reverse, 5'-GCAGCTCTCTCGCAATAGGC-3'), and *GAPDH* (forward, 5'-AAGGTGAAGGTCGGAGTCAAC-3'; reverse, 5'-GGGGTCATTGATGGCAACAATA-3'), as we previously described [6, 20].

Western blotting

Western blotting analysis was performed, as we recently described [21, 22]. Briefly, total proteins (30 μg) obtained from the cell extracts were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane (Thermo Fisher Scientific) electronically, blocked, and incubated with a specific antibody [*i.e.* LPHN1 (dilution 1:100), LPHN2 (dilution 1:100), GAPDH (dilution 1:5000)] overnight at 4°C and a secondary antibody (*i.e.* anti-mouse or anti-rabbit IgG HRP-linked antibody; Cell Signaling Technology) for 1 hour at room temperature. Chemiluminescent signals generated by a Clarity

Western ECL Substrate (Bio-Rad) were detected by an imaging system (ChemiDoc™ MP, Bio-Rad).

Chromatin immunoprecipitation (ChIP) assay

Potential AR-binding sites in the promoters of *ADGRL1* and *ADGRL2* were predicted using LASAGNA-Search 2.0 (https://biogrid-lasagna.engr.uconn.edu/lasagna_search/; see **Figure 1C**). A ChIP assay was then performed, using the Magna ChIP kit (Sigma-Aldrich), as we recently described [21-23]. Soluble chromatin from the cell lysates was immunoprecipitated with an anti-AR antibody (clone 441; Santa Cruz Biotechnology) or normal mouse IgG control (sc-2025; Santa Cruz Biotechnology). DNA was then extracted and subjected to PCR using the following primer set for *ADGRL1* (forward, 5'-TGCACAACCCTTCCAGATCT-3'; reverse, 5'-TTTCCTTCCTTTCGCCTCCT-3') or *ADGRL2* (forward, 5'-TTGTGTACCTGGCCACTAATA-3'; reverse, 5'-AATGAGGGACAGCGCAA-3').

MTT assay

Cell viability was assessed using the MTT assay, as we recently described [21, 22]. Briefly, cells (3-8 × 10^3/well) seeded in 96-well tissue-culture plates, cultured for 96 hours, and incubated with 0.5 mg/mL MTT solution (Sigma-Aldrich) for 4 hours were subjected to measure the absorbance at a wavelength of 570 nm with background subtraction at 630 nm.

Wound-healing assay

Cell migration was assessed using a scratch wound-healing assay, as we recently described [21, 22]. Briefly, cells grown to ≥ 90% confluence in 6-well tissue-culture plates were scratched with a 200-μL pipette tip and cultured in serum-free medium for 24 hours. Wound closure was analyzed by photographing cells at 0 and 24 hours, and the normalized cell-free area (24-h/0-h) was quantitated using ImageJ (National Institute of Health).

Immunohistochemistry

A set of bladder tissue microarray (TMA) had previously been constructed upon appropriate approval by the Institutional Review Boards at the University of Rochester Medical Center and The Johns Hopkins Hospital [24], and all the procedures were performed in accordance with the guidelines of the Declaration of Helsinki. Immunostaining was performed on 5-μm sections from the bladder TMA consisting of transurethral resection specimens, using a primary antibody to LPHN1 (dilution 1:100) or LPHN2 (1:100), as we previously described [17, 21, 25]. All stains were then scored by multiplying the percentage of immunoreactive cells (scale 0-4) and staining intensity (scale 0-3), as we previously described [23]. The final immunoreactive scores (range 0-12) were categorized as negative (0; score 0-1), weakly positive (1+; score 2-4), moderately positive (2+; score 6-8), and strongly positive (3+; score 9-12).

Public database analysis

The R2 Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) was used to assess the prognostic value of *ADGRL1* and *ADGRL2* expression in patients with bladder cancer.

Statistical analysis

Numerical and categorized data were analyzed using Student's *t*-test and Fisher's exact test (two-tailed), respectively. Progression-free survival and cancer-specific survival in patients with muscle-invasive bladder cancer were calculated by the Kaplan-Meier method and compared by the log-rank test. A Cox regression model was applied for multivariable analysis. All statistical analyses were performed using EZR [26] (R version 4.0.2; and the R Foundation for Statistical Computing) or Prism version 10.3.1 (GraphPad Software). A *P* value of less than 0.05 was considered to be statistically significant.

Results

Impact of androgen/AR on LPHNs

We first examined the association between AR activity and *ADGRL*/LPHN expression in human bladder cancer cells. Quantitative RT-PCR analysis revealed that dihydrotestosterone treatment significantly increased the levels of *ADGRL1* and *ADGRL2* expression in AR-positive lines, UMUC3 and 5637-AR, but not in an AR-knockdown subline (**Figure 1A**). Consistently, western blotting demonstrated that dihydrotestosterone markedly elevated LPHN1 and LPHN2 expression in the AR-positive 647V-AR line (**Figure 1B**).

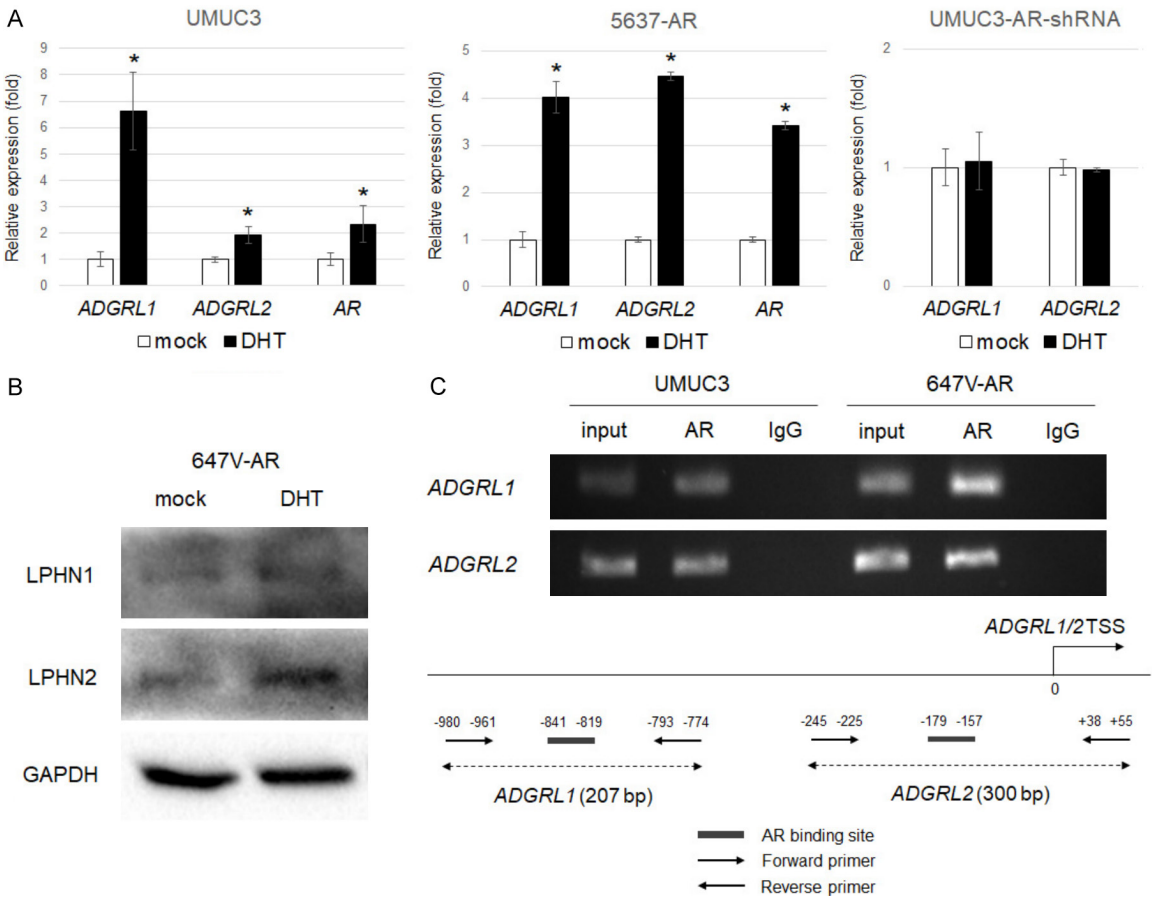

Figure 1. Associations between AR signaling and LPHN1/LPHN2 expression in bladder cancer cells. A. Real-time RT-PCR of *ADGRL1* and *ADGRL2* in UMUC3, 5637-AR, and UMUC3-AR-shRNA cultured for 24 hours with ethanol (mock) or 10 nM dihydrotestosterone (DHT). The expression of *ADGRL1*, *ADGRL2*, or *AR* normalized to that of *GAPDH* and representing the mean (± SD) of triplicate determinants is presented relative to that of mock treatment. **P* < 0.05 (vs. mock treatment). B. Western blotting of LPHN1 and LPHN2 in 647V-AR cultured for 24 hours with ethanol (mock) or 10 nM DHT. GAPDH served as a loading control. C. The ChIP assay, using UMUC3 and 647V-AR cell lysates immunoprecipitated with an anti-AR (or IgG as a negative control). The DNA fragments PCR-amplified with sets of the primers specific for the promoters of *ADGRL1* and *ADGRL2* were electrophoresed on 1% agarose gel. Fractions of the mixture of protein-DNA complex (*i.e.* 1% of total cross-linked, reserved chromatin prior to immunoprecipitation) were used as "input" DNAs.

Given the identification of putative AR binding sites in the promoter regions of *ADGRL1* and *ADGRL2*, we performed a ChIP assay (**Figure 1C**). DNA fragments from UMUC3 and 647V-AR cells immunoprecipitated with an anti-AR antibody (or normal IgG) were amplified using primers specific to the *ADGRL1* and *ADGRL2* promoters. PCR products corresponding to the predicted binding sites were detected only in precipitants precipitated with the AR antibody.

Impact of LPHNs on cell growth

Next, we assessed the effects of two known LPHN ligands, α-LTX (**Figure 2A**) and FLRT3 (**Figure 2B**), on *ADGRL1/ADGRL2* expression in bladder cancer cells. Quantitative RT-PCR in UMUC3 cells revealed that both ligands, particularly at higher concentrations, significantly up-regulated *ADGRL1* and *ADGRL2* expression. We then assessed the effects of these ligands on cell proliferation using MTT assay. Consistent with our previous findings [17], α-LTX and FLRT3 induced the cell viability of AR-positive UMUC3 (**Figure 2C**) and 647V-AR (**Figure 2D**). Notably, their effects were even more pronounced in AR-knockdown UMUC3-AR-shRNA cells (α-LTX: 83% vs. 22% increase; FLRT3: 77% vs. 33% increase) and AR-negative 647V cells (α-LTX: 42% vs. 20% increase; FLRT3: 65% vs. 11% increase).

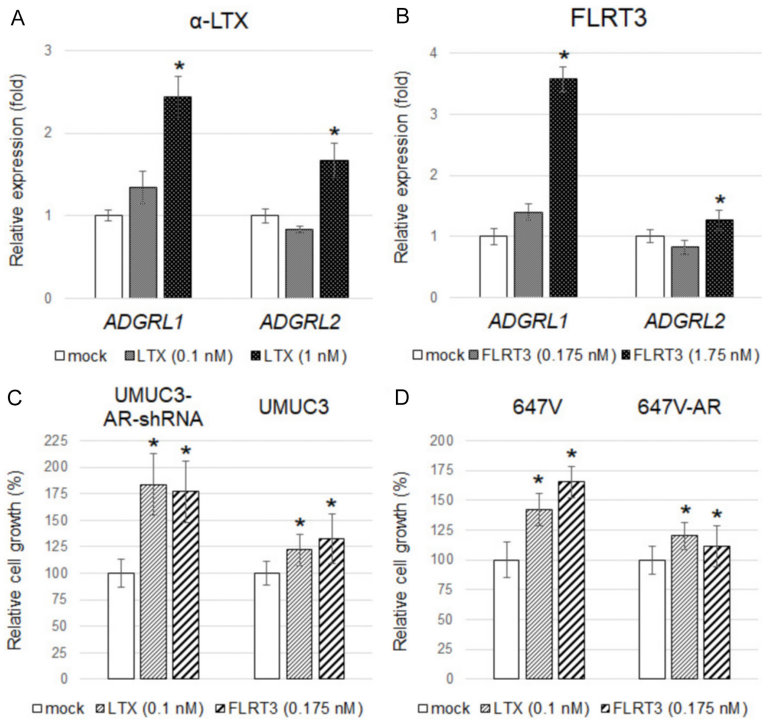

Figure 2. Effects of ligand treatment on the expression of *ADGRL1* and *ADGRL2* in bladder cancer cells (A, B) and their viability (C, D). Real-time RT-PCR of *ADGRL1* and *ADGRL2* in UMUC3 cultured for 24 hours with ethanol (mock) vs. 0.1-1 nM α-LTX (A) or 0.175-1.75 nM FLRT3 (B). The expression of *ADGRL1* and *ADGRL2* normalized to that of *GAPDH* and representing the mean (± SD) of triplicate determinants is presented relative to that of mock treatment. MTT assay in UMUC3-AR-shRNA vs. UMUC3 (C) or 647V vs. 647V-AR (D) treated with ethanol (mock), 0.1 nM α-LTX, or 0.175 nM FLRT3 for 72 hours. Cell viability presented relative to that of mock treatment in each subline represents the mean (± SD) from three independent experiments. **P* < 0.05 (vs. mock treatment).

To further clarify the functional role of LPHNs, we generated UMUC3 and 647V sublines stably expressing LPHN1-shRNA or LPHN2-shRNA (**Figure 3A**). MTT assay demonstrated that knockdown of LPHN1 (in 647V only) or LPHN2 (in both lines) led to a significant reduction in cell viability (**Figure 3B**). Similarly, knockdown sublines of 647V-AR and 5637-AR were established (**Figure 3C**). A scratch wound-healing assay demonstrated that knockdown of LPHN1 (in 647V-AR only) or LPHN2 (in both lines) significantly impaired the cell migration, compared with control sublines (**Figure 3D**).

Expression of LPHNs in surgical specimens

We immunohistochemically stained for LPHN1 and LPHN2 in a total of 137 bladder tumor samples. Positive signals for both proteins were detected predominantly in the cytoplasm of tumor cells (**Figure 4A**).

Associations between the expression levels (*e.g.* 0 vs. 1+/2+/3+, 0/1+ vs. 2+/3+) of LPHN1 (**Table 1**) or LPHN2 (**Table 2**) and clinicopathologic parameters were then analyzed. High-grade tumors were significantly more often immunoreactive for LPHN1 (95.7% vs. 80.0%; *P* = 0.005) and LPHN2 (78.3% vs. 46.7%; *P* < 0.001), compared with low-grade tumors. Additionally, LPHN2 was more frequently (*P* = 0.031) expressed in the tumors from male patients (72.6%) than in female tumors (51.6%). No significant differences in LPHN1 or LPHN2 expression were observed between non-muscle-invasive vs. muscle-invasive tumors or between muscle-invasive tumors without vs. with lymph node metastasis.

Univariate survival analysis was then conducted to determine possible associations of LPHN1 or LPHN2 expression in 62 muscle-invasive tumors with oncologic outcomes following radical cystectomy. Patients with LPHN1-positive tumor had a significantly greater risk of disease progression (*P* = 0.047; **Figure 4B**). Similarly, moderate or strong LPHN1 expression was associated with significantly worse cancer-specific survival (*P* = 0.019; **Figure 4C**). No strong correlations were found between LPHN2 levels and patient outcomes (**Figure 4D** and **4E**).

To evaluate whether the expression of LPHNs was an independent predictor of poorer prognosis, multivariable analysis of clinicopathologic factors was performed (**Table 3**). In the Cox proportional hazards model, moderate to strong LPHN1 expression (hazard ratio = 2.662, 95% confidence interval = 1.096-6.465, *P* = 0.031), as well as advanced pathological stage

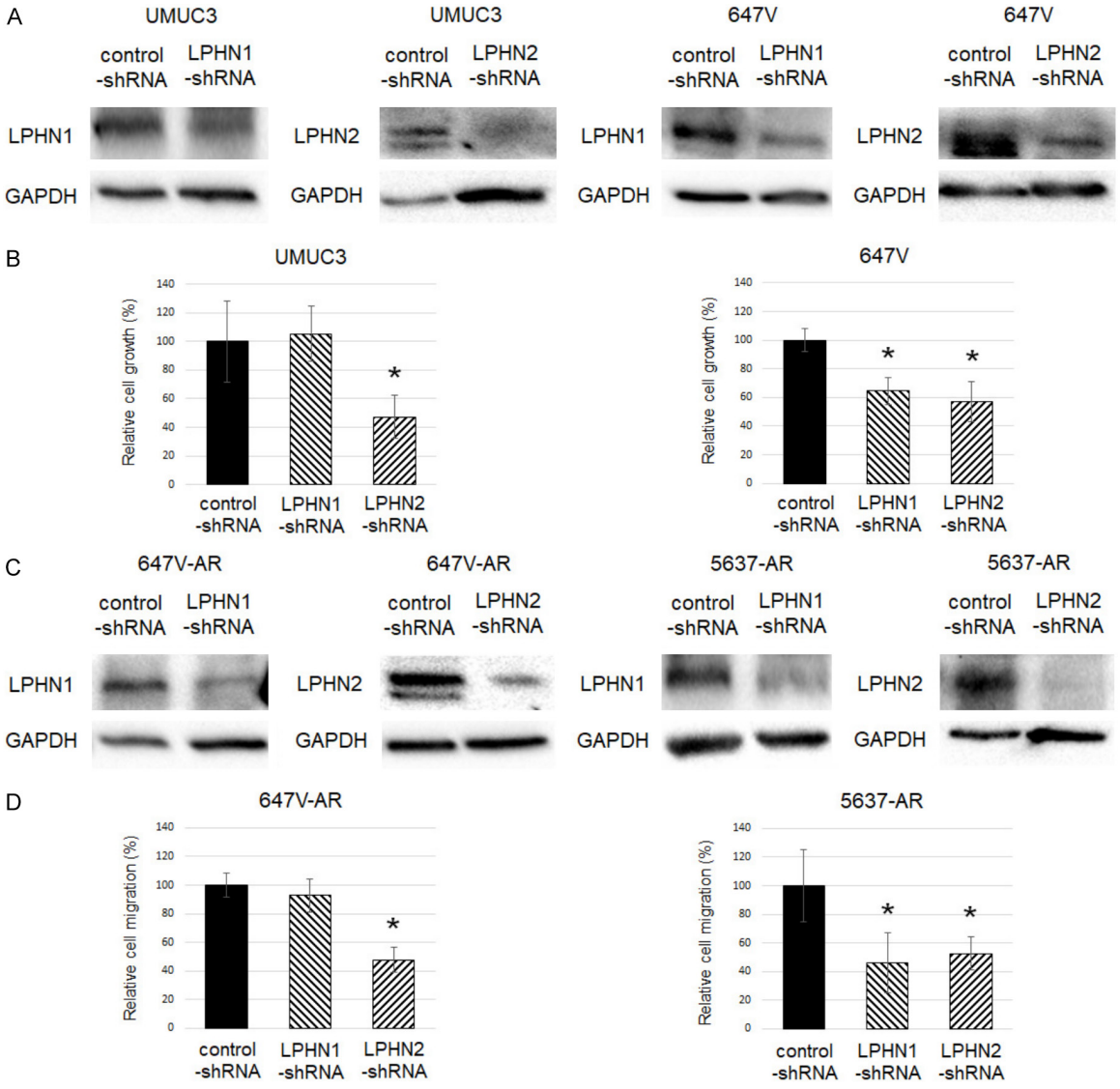

Figure 3. Effects of LPHN1/LPHN2 knockdown on the growth of bladder cancer cells. A. Western blotting of LPHN1 and LPHN2 in stable sublines expressing control-shRNA vs. LPHN1-shRNA or LPHN2-shRNA. GAPDH served as a loading control. B. MTT assay in control vs. LPHN1 or LPHN2 knockdown sublines cultured for 120 hours. Cell viability presented relative to that of control-shRNA cells represents the mean (± SD) from three independent experiments. *$P < 0.05$ (vs. control-shRNA subline). C. Western blotting of LPHN1 and LPHN2 in stable sublines expressing control-shRNA vs. LPHN1-shRNA or LPHN2-shRNA. GAPDH served as a loading control. D. Wound-healing assay in control vs. LPHN1 or LPHN2 knockdown sublines cultured for 24 hours after scratching. Cell migration as the width of the wound area presented relative to that of control cells represents the mean (± SD) from three independent experiments. *$P < 0.05$ (vs. control-shRNA subline).

(pT3-4 vs. pT2), was independently associated with worse cancer-specific survival.

Finally, a publicly available database was searched to further determine the prognostic relevance of *ADGRL1* and *ADGRL2* expression in bladder cancer. The Cancer Genome Atlas (TCGA) data in 305 patients with stage II-IV bladder cancer showed that high expression of *ADGRL1* (**Figure 4F**) or *ADGRL2* (**Figure 4G**) was associated with a marginally ($P = 0.063$) or significantly ($P < 0.001$), reduced overall survival, respectively.

Discussion

G protein-coupled receptors are known to mediate a wide range of physiological and pathological processes [13, 27, 28]. However, limited data exists regarding the involvement of LPHNs,

Table 1. Correlations of LPHN1 expression with clinicopathologic features of bladder cancer

	N	Negative (0)	Positive (1+)	Positive (2+/3+)	*P* (0 vs 1+/2+/3+)	*P* (0/1+ vs 2+/3+)
Sex					0.297	0.840
Male	106	12 (11.3%)	42 (39.6%)	52 (49.1%)		
Female	31	1 (3.2%)	14 (45.2%)	16 (51.6%)		
Tumor grade					0.005	0.146
Low*	45	9 (20.0%)	18 (40.0%)	18 (40.0%)		
High	92	4 (4.3%)	38 (41.3%)	50 (54.3%)		
pT stage					0.142	0.494
Non-muscle-invasive (pTa* or pT1)	75	10 (13.3%)	30 (40.0%)	35 (46.7%)		
Muscle-invasive (pT2-4)	62	3 (4.8%)	26 (41.9%)	33 (53.2%)		
Lymph node metastasis in pT2-4 cases					0.545	0.102
Negative (pN0 or pNX)	42	3 (7.1%)	20 (47.6%)	19 (45.2%)		
Positive	20	0 (0%)	6 (30.0%)	14 (70.0%)		

*Includes cases with papillary urothelial neoplasm of low malignant potential.

Table 2. Correlations of LPHN2 expression with clinicopathologic features of bladder cancer

	N	Negative (0)	Positive (1+)	Positive (2+/3+)	*P* (0 vs 1+/2+/3+)	*P* (0/1+ vs 2+/3+)
Sex					0.031	0.405
Male	106	29 (27.4%)	58 (54.7%)	19 (17.9%)		
Female	31	15 (48.4%)	13 (41.9%)	3 (9.7%)		
Tumor grade					< 0.001	0.140
Low*	45	24 (53.3%)	17 (37.8%)	4 (8.9%)		
High	92	20 (21.7%)	54 (58.7%)	18 (19.6%)		
pT stage					0.142	0.494
Non-muscle-invasive (pTa* or pT1)	75	32 (42.7%)	34 (45.3%)	9 (12.0%)		
Muscle-invasive (pT2-4)	62	12 (19.4%)	37 (59.7%)	13 (21.0%)		
Lymph node metastasis in pT2-4 cases					0.306	0.740
Negative (pN0 or pNX)	42	10 (23.8%)	24 (57.1%)	8 (19.0%)		
Positive	20	2 (10.0%)	13 (65.0%)	5 (25.0%)		

*Includes cases with papillary urothelial neoplasm of low malignant potential.

a subgroup of G protein-coupled receptors, in malignant tumors [13, 29-32]. We recently demonstrated that LPHN3 promoted the development [21] and growth [17] of urothelial cancer. We herein investigated the functional role of LPHN1 and LPHN2 in the progression of bladder cancer, in relation to AR signaling.

Similar to LPHN3, we found that androgen treatment in AR-positive bladder cancer cells significantly induced the expression of *ADGRL1*/LPHN1 and *ADGRL2*/LPHN2. A ChIP assay in bladder cancer cells further confirmed that wild-type AR could directly bind to *ADGRL1* and *ADGRL2* at their promoter regions. These results indicate that LPHN1 and LPHN2 represent direct transcriptional targets of AR signaling in bladder cancer cells.

LTX, a neurotoxin naturally found in widow spider venom, is known to bind and activate all three LPHNs [11-13], while FLRT3, a member of the fibronectin leucine rich transmembrane protein family, is an endogenous ligand at least for LPHN3 [13, 14, 33, 34]. In bladder cancer cells, we demonstrated that not only α-LTX but also FLRT3 induced the expression of both *ADGRL1* and *ADGRL2*. Nonetheless, it should be further determined whether these ligands can induce the expression of *ADGRL1*/LPHN1 and *ADGRL2*/LPHN2 through a feedback loop.

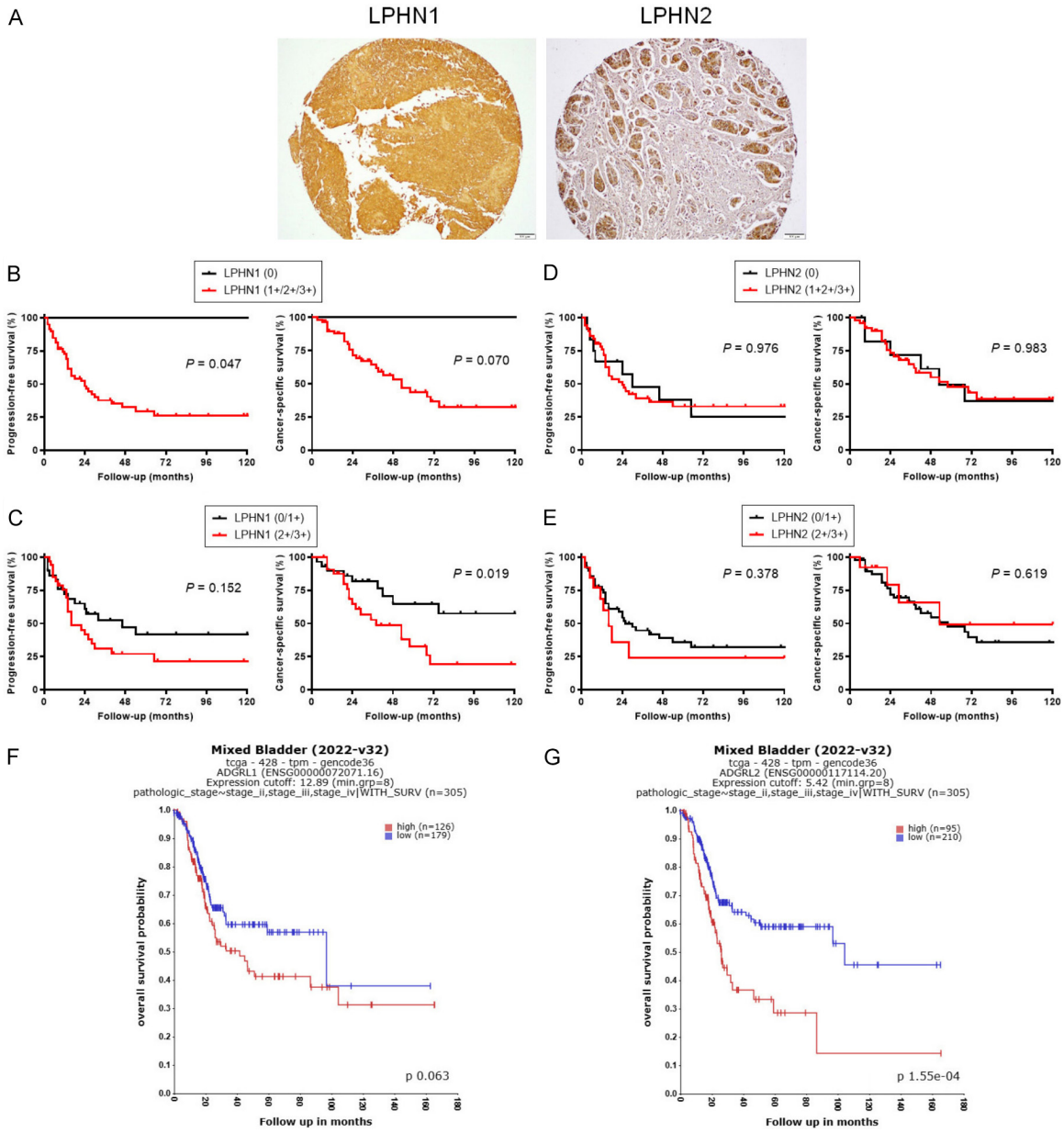

Figure 4. Expression of LPHN1/*ADGRL1* and LHPN2/*ADGRL2* in bladder cancer specimens. (A) Representative images of LPHN1 and LPHN2 immunoreactivity. Kaplan-Meier curves for progression-free survival and cancer-specific survival in patients with LPHN1 (0) vs. LPHN1 (1+/2+/3+) (B), LPHN1 (0/1+) vs. LPHN1 (2+/3+) (C), LPHN2 (0) vs. LPHN2 (1+/2+/3+) (D), or LPHN2 (0/1+) vs. LPHN2 (2+/3+) (E) muscle-invasive tumor. Kaplan-Meier curves for overall survival in patients with stage II-IV *ADGRL1*-high vs. *ADGRL1*-low tumor (F) or stage II-IV *ADGRL2*-high vs. *ADGRL2*-low tumor (G).

ADGRL3/LPHN3 has been suggested to induce the growth of several types of neoplastic diseases, such as ependymoma [32], bladder cancer [17], and prostate cancer [25], as well as resistance to chemotherapy in acute myeloid leukemia [30]. In addition, the increased exon inclusion in the *ADGRL2* gene has been detected in muscle-invasive bladder cancers [35]. However, there is no other evidence to support that *ADGRL1*/LPHN1 or *ADGRL2*/LPHN2 promotes urothelial cancer progression. Meanwhile, we previously demonstrated that α-LTX and FLRT3 could induce the proliferation of bladder [17] or prostate [25] cancer cells. In line with LPHN3 data [17], knockdown of LPHN1 or LPHN2 in bladder cancer cells resulted in the reduction of their viability and migration. These findings suggest that LPHN1 and LPHN2 func-

Table 3. Multivariable analysis for cancer-specific survival in patients undergoing radical cystectomy

	HR	95% CI	*P*
Age	1.021	0.980-1.065	0.322
Sex (Male vs. female)	1.097	0.399-3.020	0.858
pT (2 vs. 3-4)	3.779	1.314-10.87	0.014
Lymph node metastasis	1.121	0.404-3.110	0.826
LPHN1 (0/1+ vs. 2+/3+)	2.662	1.096-6.465	0.031
LPHN2 (0 vs. 1+/2+/3+)	1.180	0.439-3.171	0.743

CI, confidence interval; HR, hazard ratio.

tion as promoters of bladder cancer progression. However, limitations in the present study include the lack of rescue experiments that determine whether LPHN1/LPHN2 overexpression restores the cell growth and counteracts the effect of AR inhibition. Instead, when we compared the effects of LPHN ligands on the cell viability of AR-negative (647V) or AR-knockdown (UMUC3-AR-shRNA) lines vs. corresponding AR-positive lines (647V-AR/UMUC3), much stronger induction was observed in the latter cells where the levels of endogenous *ADGRL1*/LPHN1 and *ADGRL2*/LPHN2 expression should be lower. Nonetheless, further investigation is warranted to elucidate the downstream signal pathways of LPHNs.

The status of *ADGRL1*/LPHN1 and *ADGRL2*/LPHN2 expression in urothelial tumors remained uncertain. We immunohistochemically assessed the expression of LPHN1 and LPHN2 in a set of TMA consisting of transurethral resection specimens showing various tumor grades and stages. The positive rates of LPHN1 and LPHN2 expression were significantly higher in high-grade tumors than in low-grade tumors. However, there were no significant differences in the levels of LPHN1 or LPHN2 expression between non-muscle-invasive vs. muscle-invasive tumors or muscle-invasive tumors without vs. with lymph node metastasis. Notably, LPHN1 positivity (*i.e.* 1+/2+/3+) and overexpression (*i.e.* 2+/3+) were associated with significantly higher risks of postoperative progression and cancer-specific mortality, respectively, in patients with muscle-invasive disease undergoing radical cystectomy. Multivariate analysis of clinicopathologic factors further identified LPHN1 overexpression in muscle-invasive tumors as an independent predictor of cancer-specific mortality. In our immunohistochemistry data, however, no prognostic value of LPHN2 expression in patients with muscle-invasive tumor was observed. Instead, analysis of TCGA data indicated that high *ADGRL2* expression was strongly associated with worse overall survival in patients with stage II-IV bladder cancer. These findings in surgical specimens may strongly support our *in vitro* data indicating that LPHN1/*ADGRL1* and LPHN2/*ADGRL2*, potentially as a consequence of AR activation, induce the progression of urothelial cancer.

In conclusion, our findings demonstrate that LPHN1 and LPHN2 are downstream effectors of AR in bladder cancer cells and promote their growth. Alongside our previous results implicating LPHN3 [17], overexpression and/or activation of LPHNs may represent a key underlying mechanism by which AR signaling drives bladder cancer progression. Accordingly, therapeutic strategies targeting LPHNs, either alone or in combination with AR inactivation, may hold promise for advanced urothelial cancer. Furthermore, LPHN1/LPHN2 overexpression may serve as a useful prognostic biomarker, particularly in patients with muscle-invasive bladder cancer. Future studies are needed to not only validate our present results but also delineate the molecular mechanisms through which LPHNs promote urothelial cancer progression.

Disclosure of conflict of interest

None.

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