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Original Article

Androgen represses opioid growth factor receptor (OGFR) in human prostate cancer LNCaP cells and OGFR expression in human prostate cancer tissue

Hironobu Yamashita[1], Lauren Shuman[1], Joshua I Warrick[1], Jay D Raman[2], David J Degraff[1,2]

[1]*Department of Pathology, College of Medicine, The Pennsylvania State University, Hershey PA, USA;* [2]*Department of Surgery, Division of Urology, College of Medicine, The Pennsylvania State University, Hershey PA, USA*

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Abstract: Opioid receptors are G protein-coupled receptors that bind opioid ligands including endorphins and enkephalins. The existence of a number of opioid receptors, including the mu-opioid receptor (OPRM1), delta-opioid receptor (OPRD1), kappa-opioid receptor (OPRK1) and zeta-opioid receptor (OGFR) have been reported. However, the potential expression and role of these receptors on human prostate carcinogenesis is unknown. In the present study, we examined opioid receptor expression in human prostate cancer cell lines and in prostate cancer tissue. We observed using quantitative real-time PCR analysis that OGFR and OGFRL1 mRNA is expressed in all examined prostate cancer cell lines as well as in an immortalized, non-tumorigenic prostate epithelial cell line (RWPE-1). Conversely, OPRK1 mRNA expression was detected in a more limited number of cell lines (LNCaP and VCaP), while OPRD1 and OPRM1 mRNA expression was undetectable in all examined prostate cell lines. Interestingly, androgen sensitive LNCaP cells expressed high amounts of OPRK1, OGFR and OGFRL1 compared to other cell lines. Therefore, we investigated the effect of androgen on the mRNA expression of OPRK1, OGFR, OGFRL1 in the LNCaP cell line. Our results demonstrated that the synthetic androgen (R1881) represses mRNA of OPRK1, OGFR and OGFRL1 in a time-dependent manner. Furthermore, immunohistochemistry demonstrated OGFR is expressed at high levels in prostate cancer tissue compared to benign tissue, and that OGFR expression is high in undifferentiated and aggressive prostate cancer tissue. This is the first study showing OGFR and OGFRL1 are androgen repressed genes, and these results suggest a role for the opioid signaling axis in prostate cancer.

Keywords: Opioid receptor, OGFR, prostate cancer, androgen

Introduction

Prostate cancer (PCa) is the most common urologic malignancy in the United States. According to American Cancer Society (ACS), it is estimated that over 160,000 new cases of PCa will be diagnosed in 2018 in the United States with almost 30,000 resultant deaths. PCa expresses androgen receptor (AR) and initially grows in an androgen-dependent manner. Therefore, androgen deprivation therapy (ADT) is utilized initially for the treatment of advanced PCa [1, 2]. While initially effective, ADT almost invariably fails at various time points due to prostate cancer cells establishing a castrate-resistant phenotype with some elements of neuroendocrine differentiation [3]. Despite novel therapies, the cascade of CRPCa is often lethal, and it is therefore important to continue to identify molecular mechanisms which may underlie and impact the transition from androgen-dependent to CRPCa.

At present, several opioid receptors including the mu-opioid receptor (OPRM1, formerly MOR), delta-opioid receptor (OPRD1, formerly DOR), kappa-opioid receptor (OPRK1, formerly KOR) have been identified as classical opioid receptors. Classical opioid receptors (OPRD1, OPRK1, OPRM1) are G protein coupled receptors (GPCRs) for an array of opioid ligands, such as a dynorphins, enkepharins, endorphins, endomorphins etc [4]. OGFR was first identified as the zeta-opioid receptor [5-7]. However, OGFR has a distinct structure compared to other classical opioid receptors. OGFR structurally har-

bors bipartite nuclear localization signal [8]. Cumulative evidence shows that opioid growth factor (OGF) bound OGFR moves into the nucleus and binds close to heterochromatin, resulting in reduced cell proliferation via cell cycle control [9, 10]. Additionally, it have been reported that OGFR may have a tumor suppressor function [10].

Accumulating evidence indicates the potential relationships between opioid receptors and various cancers. It has been reported that OPRM1 plays a role as an oncogenic in lung cancer progression [11-13]. On the other hand, OPRK1 agonist reduces the growth of lung cancer, suggesting the role of OPRK1 as a tumor suppressor. Xenograft experiments using mice with deletion of OPRK1 gene demonstrated that loss of OPRK1 enhanced tumor growth of melanoma cells as well as lung cancer cells by suppressing angiogenesis [14, 15]. Furthermore, there are controversial observations that high nuclear expression of OPRK1 correlates with lymph node metastasis in esophageal squamous cell carcinoma and these patients show a poor prognosis [16], while decreased expression of OPRK1 correlates with shorter survival and indicates poor prognosis in hepatocellular carcinoma patients [17].

However, current knowledge regarding any potential relationship between components of the opioid signaling axis and PCa is relatively limited. Here, we investigated mRNA expression of the opioid receptors in human PCa cell lines, as well as an immortalized prostate epithelial cell line created from normal tissue. Because of the central role androgen signaling plays in prostate disease, we performed androgen treatment studies to examine androgen regulation of OGFR in our *in vitro* experiments. Furthermore, immunohistochemistry was used to analyze the expression of OGFR in human PCa tissue. Collectively, these studies suggest a role for the opioid signaling pathway in PCa.

Material and methods

Cell culture

All cells were purchased from the American Type Culture Collection (ATCC). The following cell lines were used in this study. RWPE-1 (ATCC, CRL-11609™), LNCaP (ATCC, CRL-1740™), VCaP (ATCC, CRL-2876™), PC3 (ATCC, CRL-1435™), DU145 (ATCC, HTB-81™), 22Rv1 (ATCC, CRL-2505™). Culture medium was used as follows: Keratinocyte Serum Free Medium (K-SFM) kit (RWPE-1), RPMI1640 medium with 10% FBS, (LNCaP and 22Rv1), DMEM medium with 10% FBS (VCaP), F-12K medium (PC3), Minimal Essential Medium with 10% FBS (DU145). All medium was supplemented with penicillin/streptomycin. All cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Androgen (R1881) treatment

A total of 4×10^5 LNCaP cells were seeded per well of 6 well plate (Corning). On the following day, medium was removed and starvation medium (RPMI1640 with phenol red free medium containing 10% charcoal stripped FBS) was added and incubated for 24 hrs. After starvation, fresh starvation medium containing R1881 (1 nM) was replaced and incubated for 0, 12, 24, 48 and 72 hrs.

RNA extraction and RT-qPCR

RNA was extracted using RNeasy kit (Qiagen) according to manufacturer protocol. To synthesize cDNA, reverse transcription was performed using M-MLV reverse transcriptase (Thermo Fisher Scientific). qRT-PCR reactions were performed using QuantaStudio7 Real-Time PCR System (Applied Biosystems) using a 96 well plate. Reactions of 20 μl per well were prepared by addition of 5 μl of cDNA, 10 μl of 2 × Taqman Gene Expression Master Mix (Applied Biosystems), 1 μl of 20 × Taqman probe and 4 μl of nuclease-free water. Taqman probes used in this study were as follows. KLK3 (Hs02576345_m1), OPRK1 (Hs00175127_m1), OPRD1 (Hs00538331_m1), OPRM1 (Hs01053957_m1), OGFR (Hs01071266_m1), OGFRL1 (Hs00226193_m1). Relative gene expression change was calculated by the deltadeltaCt method [18]. Eukaryotic18S rRNA (Thermo Fisher Scientific) was used as an endogenous control.

Immunohistochemistry

All human tissue studies were performed with Internal Review Board and/or intuitional approval. Immunohistochemistry (IHC) was performed using a human, de-identified PCa Tissue Microarray (PR1921, US Biomax, Inc.).

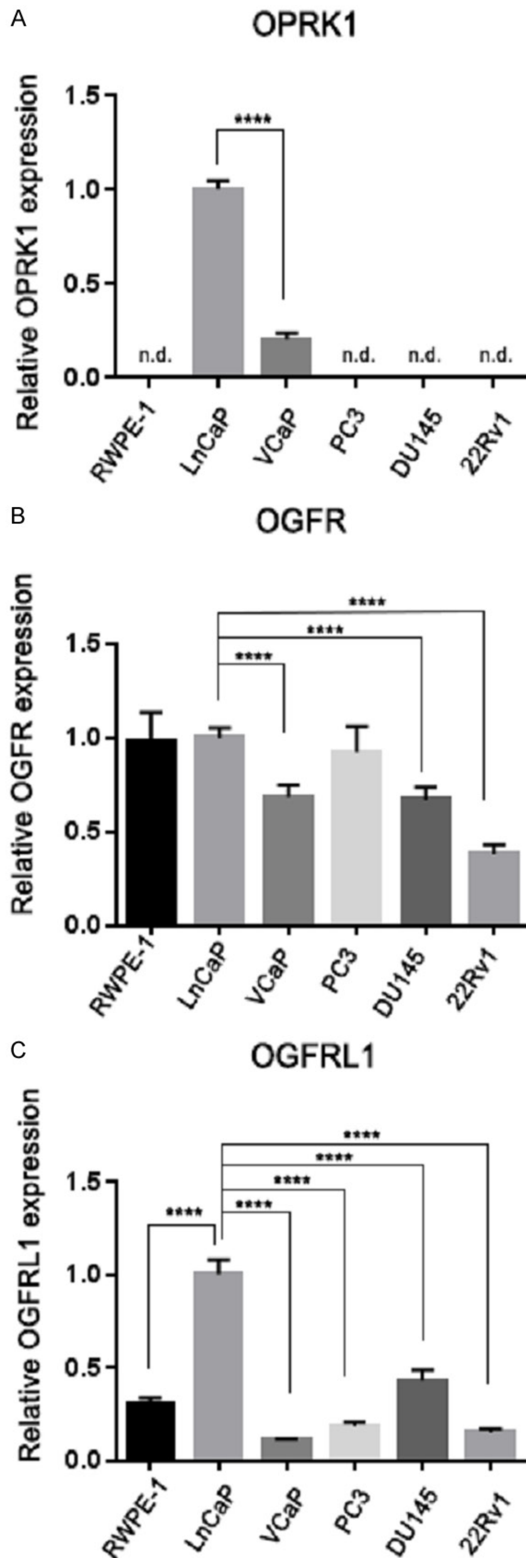

Figure 1. mRNA expression of Opioid receptors in human prostate cancer cell lines. qPCR analysis of opioid receptors (OPRK1, OPRD1, OPRM1, OGFR, OGFRL1) mRNA expression in 1 human prostate normal (RWPE-1) and 5 human prostate cancer cell lines (LNCaP, VCaP, PC3, DU145, 22Rv1). OPRK1 (A), OGFR (B), OGFRL1 (C) mRNA was detected, but OPRD1, OPRM1 mRNA was undetectable in all prostate cell lines. ****$P < 0.0001$, Student's t-test, n.d.: not detected in (A), ****$P < 0.0001$, one-way ANOVA with post hoc multiple comparison (Durnet) in (B and C).

Briefly, slides were deparaffinized and rehydrated through graded alcohols and washed in distilled water for 3 minutes. Antigen retrieval was performed by placing slides in 1% antigen unmasking solution (Vector Labs, Burlingame, CA) and heating slides at high pressure for 20 minutes in a pressure cooker (Cuisinart CPC-600). Slides were cooled to room temperature and washed 3 times for 10 minutes in phosphate-buffered saline (PBS) (pH 7.4). All incubations were performed at room temperature unless otherwise noted. Endogenous peroxidases were blocked by incubation in 1% hydrogen peroxide in methanol for 20 minutes, and slides were again washed 3 times for 10 minutes in PBS. Sections were incubated in PBS containing horse serum (Vector Labs) for 1 hour to reduce nonspecific antibody binding and then incubated overnight with OGFR primary antibody (1:200, Proteintech 11177-1-AP) in PBS containing horse serum at 4°C in a humidified chamber. Following overnight incubation, slides were washed 3 times for 10 minutes in PBS and sections were incubated in biotinylated secondary antibody diluted in PBS containing horse serum (1:200, Vector Labs) for 1 hour. Specific antibody binding was visualized using Vectastain Elite ABC Peroxidase kit (Vector Labs) according to the manufacturer protocol with diaminobenzidine substrate buffer as the chromogen (Thermo Scientific).

Statistical analysis

Statistics were performed using GraphPad Prism6 (GraphPad Software) or R version 3.3.2 (Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2017.). Results are expressed as the mean ± S.D. Data were analyzed by Student's t-test or one-way ANOVA with post hoc multiple comparison (Durnet) for comparing three or more groups of continuous variables. Differences with *p*-value ($P < 0.05$) were considered statistically significant. Wilcoxon rank sum test was used for comparing cytoplasmic and nuclear OGFR expression between benign and cancer tissue. Spearman's rank correlation coefficient was

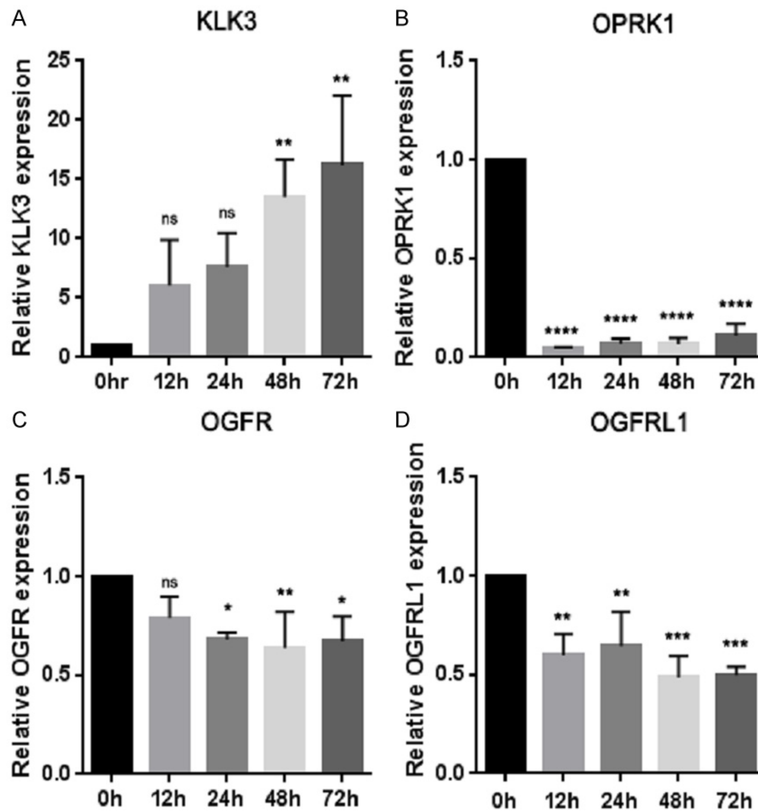

Figure 2. Androgen represses OGFR and OGFRL1 mRNA expression in LNCaP cells. qPCR analysis of time course OGFR and OGFRL1 mRNA levels in LNCaP cells after vehicle or R1881 treatment. Briefly, LNCaP cells were starved for 24 hr with medium containing charcoal-stripped FBS. After starvation, cells were treated with R1881 (1 nM). mRNA levels of OPRK1 (B), OGFR (C) and OGFRL1 (D) were measured at 0, 12, 24, 48, 72 hr. KLK3 (A) was used as a positive control for R1881. KLK3, OPRK1, OGFR, OGFRL1 expression were normalized by 18S, internal control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns: not significant, one-way ANOVA with post hoc multiple comparison (Durnet).

performed for Cytoplasmic/Nuclear OGFR expression between three Gleason grade groups (Gleason 3, 4, 5). Fisher's exact test of independence was also used for the above comparison, assigning tumors as positive based on cutoffs of Allred score > 3 for nuclear expression and cytoplasmic intensity > 1.

Results

LNCaP cells express high levels of OPRK1, OGFR and OGFRL1

In order to examine the expression of opioid receptors in PCa, we performed qPCR analysis on a panel of commonly utilized cell lines. While OPRK1 was detectable only in LNCaP and VCaP cells (**Figure 1A**), OGFR and OGFRL1 were detected in all cell lines analyzed (**Figure 1B**, **1C**). The expression of OPRM1 and OPRD1 was not observed in any studied cell lines (data not shown). Interestingly, LNCaP cells express significantly higher levels of OPRK1 and OGFRL1 compared to other cell lines, while they express significantly higher levels of OGFR when compared to VCaP, DU145 and 22RV1.

Androgen represses OPRK1, OGFR, OGFRL1 mRNA expression in LNCaP cells

LNCaP cells express a functional (albeit mutated) androgen receptor, and respond to androgen treatment by upregulating prostate specific antigen (PSA/KLK3). For this reason, LNCaP cells are perhaps the most widely used *in vitro* model for the study of androgen regulation in PCa research. Therefore, we utilized LNCaP cells to examine the potential regulation of opioid axis components by androgen.

As a positive control for our androgen treatment experiments, Q-RT-PCR results show R1881 increases KLK3 expression in a time-dependent manner (**Figure 2A**). Interestingly, our data shows OPRK1 is repressed as early as 12 hours after R1881 treatment (**Figure 2B**). In addition, both OGFR (**Figure 2C**) and OGFRL1 (**Figure 2D**) were also repressed following R1881 treatment. These results confirm previous reports identifying OPRK1 as an androgen-repressed gene, and further identify OGFR and OGFRL1 as androgen-repressed components of the opioid signaling pathway.

OGFR is overexpressed in PCa and associated with high Gleason score

Because our results suggested a possible role for OGFR in PCa, we utilized immunohistochem-

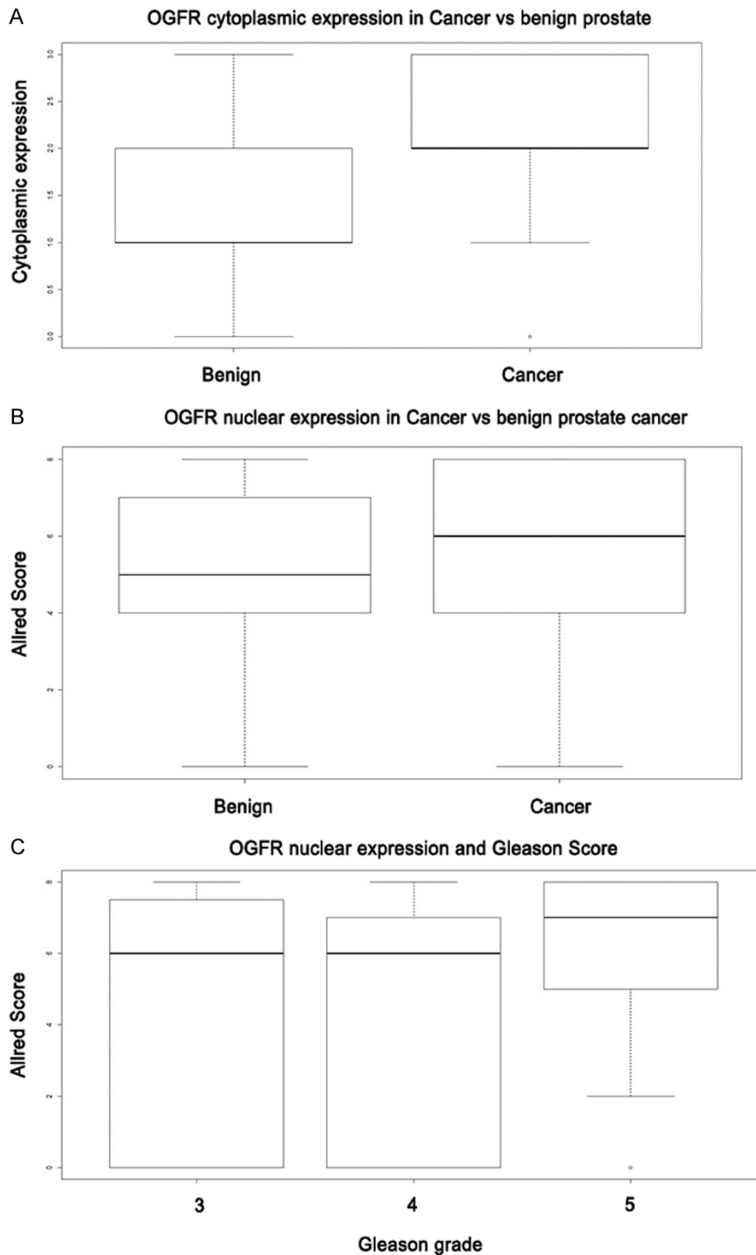

Figure 3. OGFR is expressed in human prostate cancer higher than prostate benign tissue. (A) Cytoplasmic OGFR expression in both human prostate benign and cancer tissue. P < 0.01, Wilcoxon rank sum (B) Nuclear OGFR expression in both human prostate benign and cancer tissue. (C) Nuclear OGFR expression and Gleason grade. Spearman's test; P < 0.01; r = 0.53.

Table 1. Cytoplasmic/Nuclear OGFR expression of human prostate benign and cancer tissues

	Benign (N = 32)		Cancer (N = 160)		*P* value
	OGFR+ (%)	OGFR- (%)	OGFR+ (%)	OGFR- (%)	
Cytoplasmic	15 (47%)	17 (53%)	117 (73%)*	43 (27%)	*P = 0.0059
Nuclear	27 (81%)	6 (19%)	119 (74%)	41 (26%)	P = 0.088

Cut off points for positive were Allred score greater than 3 and cytoplasmic intensity greater than 1, respectively. *P = 0.0059, Fisher's exact test of independence.

istry of a human PCa tissue microarray to further examine the expression of this pathway component in human tissue. We detected both nuclear and cytoplasmic staining of OGFR. Intensity of cytoplasmic expression was graded semi-quantitatively, ranging from 0 (no expression) to 3 (highest intensity). Nuclear expression was graded by the Allred score, a score based on adding expression intensity (range 0 to 3) to expression area (range 0 to 5) to give a score ranging from 0 to 8 [19]. Cytoplasmic and nuclear positively correlated with one another (Spearman's test; P < 0.01; r = 0.53), suggesting that fluctuations in OGFR expression impact both nuclear and cytoplasmic pools. Interestingly, benign tissue had decreased cytoplasmic OGFR expression relative to PCa tissue (**Figure 3A**: P < 0.01, Wilcoxon rank sum), but not significantly reduced nuclear staining (**Figure 3B**) (**Table 1**). Interestingly, nuclear expression of OGFR was correlated with Gleason grade (**Figure 3C**: Spearman's test; P < 0.01; r = 0.53), while cytoplasmic staining was not correlated (**Table 2**). These results suggest that OGFR expression is increased during malignant progression and is correlated with poorly differentiated PCa.

Discussion

The present study identifies OGFR and OGFRL1 as androgen-repressed genes in LNCaP human PCa cells. Moreover, immunohistochemical analysis using human PCa tissue shows OGFR at relatively high levels in poorly differenti-

Table 2. Correlation between Cytoplasmic/Nuclear OGFR expression and Gleason grade

Gleason Grade	Total	Cytoplasmic OGFR+ (%)	Cytoplasmic OGFR- (%)	*P* value	Nuclear OGFR+ (%)	Nuclear OGFR- (%)	*P* value
Gleason 3	16	11 (69%)	5 (31%)		10 (63%)	6 (37%)	
Gleason 4	74	60 (81%)	14 (19%)		53 (72%)	21 (28%)	
Gleason 5	60	40 (67%)	20 (33%)	P = 0.15	51 (85%)	9 (15%)	*P = 0.0015

Cut off points for positive were Allred score greater than 3 and cytoplasmic intensity greater than 1, respectively. *P = 0.0015, Fisher's exact test of independence.

ated disease, which often exhibits an aggressive clinical course.

In order to examine mRNA of opioid receptors in PCa cells, we used five human PCa cell lines as well as one immortalized, non-tumorigenic cell line derived from normal human prostate tissue. While mRNA expression of OPRD1 and OPRM1 were undetectable in all examined cell lines, mRNA expression of OPRK1 was detected only in LNCaP and VCaP PCa cell lines. Interestingly, mRNA expression of OGFR and OGFRL1 was ubiquitously detected. As LNCaP PCa cells are androgen sensitive and express high levels of OPRK1, OGFR and OGFRL1 compared to other cells, we used this line to investigate the effect of androgen treatment on OPRK1, OGFR and OGFRL1 mRNA expression. R1881 dramatically reduced OPRK1 mRNA expression in LNCaP cells as well as induction of the androgen responsive gene, KLK3 (PSA) mRNA expression. These results correspond to previous reported studies that R1881-stimulated full length androgen receptor suppressed OPRK1 [20] and that anti-androgen drug (bicalutamide) increased OPRK1 expression in LNCaP cells [21]. mRNA expression of OGFR and OGFRL1 were also significantly repressed by R1881 treatment in LNCaP cells although this repression was less than that of OPRK1. A recent report of transcriptional changes in liver tissue collected from male and female songbirds treated with androgen shows that OGFR expression was reduced in the livers of male birds treated with testosterone than those of male treated with the control [22, 23]. This recent report supports our finding that androgens repress OGFR expression in PCa cells.

OPRK1 also plays the role as a tumor suppressor in lung cancer and hepatocellular carcinoma [14, 17]. Considering the role of OPRK1 and OGFR as tumor suppressor, LNCaP cells may potentially grow by blocking OPRK1 or OGFR (OGFRL1) expression in an androgen dependent manner. While this needs to be tested, such a mechanism could have important implications for human disease.

Given the fact that OGFR reportedly functions as a tumor suppressor in cancers [10, 24-29], it seems puzzling that OGFR is expressed highly in PCa, especially in undifferentiated and aggressive disease. However, one explanation could be that OGFR expression increases during malignant progression, serving as a futile attempt to decelerate tumor progression. Interestingly, computational analysis using publically available data [30] demonstrated that OPRK1 and OGFR are mutated in 39% and 30% of neuroendocrine PCa cases, respectively. Therefore, pathologic alterations in OGFR may explain increased expression and/or altered function in PCa.

While this is the first study demonstrating that OGFR and OGFRL1 are androgen-repressed genes, and that OGFR is relatively overexpressed in poorly differentiated PCa, there are several limitations of this study. First, the mechanism by which opioid receptor signaling contributes to PCa pathology remains unknown. In addition, tissue studies reported here are based on retrospectively collected clinical samples, and we were unable to obtain clinical follow up data for these studies. Additional research is required to elucidate the mechanism of opioid receptor action in PCa, as well as any relationship to opioid signaling axis components and clinical outcome.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. David J Degraff, Department of Pathology, College of Medicine, The Pennsylvania State University, 500 University Drive, Hershey PA 17033, USA. Tel: 717-531-0003 Ext. 281295; Fax: 717-531-5021; E-mail: ddegraff@pennstatehealth.psu.edu

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