Dihydrotestosterone upregulates the expression of epidermal growth factor receptor and ERBB2 in androgen receptor-positive bladder cancer cells

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Abstract

Androgen receptor (AR) signals play important roles in bladder carcinogenesis and tumor progression. Activation of the epidermal growth factor receptor (EGFR) family, including EGFR and ERBB2, leads to bladder cancer cell growth and correlates with poor patients’ prognosis. However, cross talk between AR and EGFR/ERBB2 pathways in bladder cancer remains poorly understood. In AR-positive bladder cancer UMUC3 and TCC-SUP cells, dihydrotestosterone (DHT) increased the expression of EGFR and ERBB2 both in mRNA and in protein levels, and an anti-androgen hydroxyflutamide antagonized the effect of DHT. The necessity of AR was confirmed by silencing the receptor, using short hairpin RNA (shRNA), in UMUC3 cells, as well as by expressing the receptor in AR-negative 5637 cells. Of note were much higher basal levels of EGFR and ERBB2 in UMUC3-control-shRNA than in UMUC3-AR-shRNA and those of EGFR in 5637-AR than in 5637-V. DHT additionally upregulated the levels of phosphorylation of EGFR (pEGFR) and its downstream proteins AKT (pAKT) and ERK1/2 (pERK), induced by EGF treatment, in AR-positive cells. Immunohistochemistry on cystectomy specimens showed strong associations between expressions of AR and EGFR (P=0.0136), pEGFR (P=0.0041), ERBB2 (P=0.0331), or pERK (P=0.0274), but not of pAKT (P=0.5555). The Kaplan–Meier and log-rank tests further revealed that positivity of AR (P=0.0005), EGFR (P=0.2425), pEGFR (P=0.1579), ERBB2 (P=0.2997), or pERK (P=0.1270) and negativity of pAKT (P=0.0483) were associated with tumor progression. Our results indicate that AR activation upregulates the expression of EGFR and ERBB2 in bladder cancer cells. AR signals may thus contribute to the progression of bladder cancer via regulation of the EGFR/ERBB2 pathways.

Introduction

Urinary bladder cancer is the fourth most commonly diagnosed malignancy in males in the United States, accounting for 6.7% of all cancer cases (Jemal et al. 2010). However, in females, the morbidity of bladder cancer is much lower, accounting for 2.4% of all cancer cases. The classical concept has been tempting to attribute the sex-related differences in the risk of bladder cancer to environmental or lifestyle factors, such as industrial chemicals and cigarette smoke. Nonetheless, excess risk of bladder cancer in men persisted after controlling for these carcinogenic factors (Hartge et al. 1990, Jemal et al. 2010). We have recently shown molecular evidence for the discrepancy indicating that androgen receptor (AR) signaling pathway is involved in bladder cancer (Miyamoto et al. 2007, Johnson et al. 2008). Castrated male and wild-type female mice had a lower incidence of bladder cancer induced by a chemical carcinogen than wild-type male mice. In addition, AR knockout completely prevented mice from bladder cancer development. We and others also showed that

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androgens increased cell proliferation of AR-positive bladder cancer lines \textit{in vitro} and \textit{in vivo}, and that anti-androgen treatment or downregulation of AR abolished the effect of androgens (Miyamoto et al. 2007, Johnson et al. 2008, Boorjian et al. 2009, Wu et al. 2010). Thus, AR signals likely promote bladder carcinogenesis as well as cancer progression.

It is well known that in a variety of malignancies, activation of the epidermal growth factor receptor (EGFR) family, including EGFR and ERBB2, contributes to tumorigenesis and tumor progression. In bladder cancer, frequent overexpression and/or gene amplification of \textit{EGFR/ERBB2} have been reported, which correlates with higher tumor grade/stage and poorer clinical outcome (Neal et al. 1990, Lipponen & Eskelinen 1994, Orlando et al. 1996, Miyamoto et al. 2000, Jimenez et al. 2001, Latif et al. 2004).

Indeed, the EGFR pathway has been shown to play a critical role in cell proliferation, apoptosis, differentiation, migration, and angiogenesis in bladder cancer (Bellmunt et al. 2003, MacLaine et al. 2008). Ultimately, the efficacy of targeted therapy with novel agents directed at EGFR signaling pathway has been assessed in bladder cancer (Bellmunt et al. 2003, Latif et al. 2004, Black et al. 2007, Bhuvaneswari et al. 2009).


In contrast, little is known about the relationship between AR and EGFR family in bladder cancer. The purpose of this study was to investigate the involvement of androgens/AR signaling in the EGFR/ERBB2 pathways in bladder cancer cells.

### Materials and methods

#### Cell culture and chemicals

Human urothelial carcinoma cell lines UMUC3, TCC-SUP, and 5637, and human embryonic kidney cell line 293T (all obtained from the American Type Culture Collection, Manassas, VA, USA) were maintained in appropriate medium (Mediatech, Manassas, VA, USA; DMEM for UMUC3, TCC-SUP, and 293T; RPMI-1640 for 5637) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. Cells were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS at least 18 h before experimental treatment. We obtained dihydrotestosterone (DHT) and EGF from Sigma; hydroxyflutamide (HF) from Schering (Kenilworth, NJ, USA); cycloheximide from MP Biomedical (Solon, OH, USA); and PD168393 from Calbiochem (San Diego, CA, USA).

#### Stable cell lines with AR and AR-short hairpin RNA

To establish a stable AR knockdown cell line, UMUC3 directly transfected with a retrovirus vector pMSCV/U6-AR-short hairpin RNA (shRNA) or pMSCV/U6-control-shRNA, using GeneJuice transfection reagent (Novagen, Gibbstown, NJ, USA), was selected using 3 μg/ml puromycin (Sigma), as described previously (Miyamoto et al. 2007). In addition, a full-length wild-type human AR cDNA was subcloned into PWPI plasmid (Addgene, Cambridge, MA, USA), and the lentivirus-based vector (PWPI-AR/PWPI-control) with pMD2.G packaging and psPAX2 envelope plasmids (lentivirus:packaging:envelope = 2:1:1) was co-transfected into 293T cells, using GeneJuice. After 48 h of transfection, the target cells (5637) were cultured in the presence of viral supernatant containing 8 μg/ml polybrene (Millipore, Billerica, MA, USA) for 6 h. Flow cytometry was used to obtain pure cell line overexpressing AR (5637-AR) or vector only (5637-V).

#### Reporter gene assay

Bladder cancer cells at a density of 50–60% confluence in 24-well plates were co-transfected with 250 ng of MMTV-luc reporter plasmid DNA and 2.5 ng of PRL-TK-luc plasmid DNA, using GeneJuice, as described previously (Miyamoto et al. 2003). After 6 h of transfection, the medium was replaced with another medium supplemented with charcoal-stripped FBS in the presence or absence of ligands (DHT, HF, or both) for 24 h. Cells were harvested, lysed, and
assayed for luciferase activity, which was determined using a Dual-Luciferase Reporter Assay kit (Promega, Madison, WI, USA) and luminometer (TD-20/20, Turner BioSystems, Sunnyvale, CA, USA).

**Cell proliferation assay**

We used the MTT (methyl thiazolyl diphenyl tetrazolium bromide) assay to assess cell viability, as described previously (Canacci et al. 2011). Cells (3 × 10^5) seeded in 96-well tissue culture plates were incubated with medium supplemented with charcoal-stripped FBS containing ethanol or ligands (DHT and/or HF). The media were refreshed every other day. After 4 days of treatment, we added 10 μl MTT (Sigma) stock solution (5 mg/ml) to each well with 0.1 ml of medium for 4 h at 37 °C. Then, we replaced the medium with 100 μl DMSO, incubated for 5 min at room temperature, and measured the absorbance at a wavelength of 570 nm with background subtraction at 655 nm.

**Reverse transcription and real-time PCR**

Total RNA (0.5 μg) isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) was reverse transcribed using 1 μmol/l oligo (dT) primers and 4 units of Omniscript reverse transcriptase (Qiagen, Valencia, CA, USA) in a total volume of 20 μl. Real-time PCR was then performed in 15 μl system by using SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), as described previously (Miyamoto et al. 2007). The primer sequences are given as below: EGFR (forward, 5'-CCAAGGCCACG-GTAACAA-3'; reverse, 5'-ACATAACCAGCCACCT-CC-3'); and ERBB2 (forward, 5'-TGACACCTAGCGGAGCGAT-3'; reverse, 5'-GGGGGATGTGTTTTCCCTCAA-3'). GAPDH (forward, 5'-CTCCTCCA-CCTTTTGACGCTG-3'; reverse, 5'-ATAGAGCTTGGACAA-3') was used as an internal control.

**Western blot**

Protein extraction and western blot were performed as described previously (Miyamoto et al. 2003) with minor modifications. Briefly, equal amounts of protein (50 μg) obtained from cell extracts were separated in 10% SDS–PAGE and transferred to polyvinylidene difluoride membrane (Millipore) by electroblotting using a standard protocol. Specific antibody binding was detected, using HRP detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, Rockford, IL, USA). Anti-AR (N20) (diluted 1:1000), anti-EGFR (diluted 1:100), anti-ERBB2 (diluted 1:100), anti-ERK1/2 (diluted 1:1000), and anti- β-actin (diluted 1:1000) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphorylation of EGFR (pEGFR) (Tyr1068) (diluted 1:1000), anti-AKT (diluted 1:1000), anti-pAKT (Ser473) (diluted 1:1000), and anti-pERK1/2 (Thr202/Tyr204) (diluted 1:1000) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

**Bladder tissue microarray and immunohistochemistry**

Appropriate approval from the Institutional Review Board of the University of Rochester Medical Center was obtained prior to construction and use of the tissue microarray (TMA). Bladder cancer TMA was constructed from 24 formalin-fixed paraffin-embedded cystectomy tissue specimens retrieved from the Surgical Pathology archives, using 1.0 mm cores of representative tumor lesions. These 24 specimens were obtained from 19 men and 5 women, with a mean age at cystectomy of 66.2 years (range 49–86 years) and a mean follow-up after the surgery of 8.3 months (range 3–20 months). All the cases were histologically diagnosed as high-grade urothelial carcinoma. These included 2 pTis, 3 pT1, 4 pT2, and 15 ≥ pT3 tumors, and 12 node-negative and 12 node-positive tumors.

Immunohistochemical staining was performed as described previously (Miyamoto et al. 2007, Canacci et al. 2011) with minor modifications. Briefly, TMA sections (4 μm thick) were deparaffinized in xylene, rehydrated in a graded ethanol series, and incubated in 3% hydrogen peroxide to block endogenous peroxidase. Slides were incubated overnight at 4 °C with the same antibodies as utilized for western blot: anti-AKT (Thr202/Tyr204) (diluted 1:1000), anti-EGFR (diluted 1:100), anti-ERBB2 (diluted 1:100), anti-AKT (diluted 1:1000), and anti-pERK1/2 (diluted 1:1000). We then incubated the samples with a broad spectrum secondary antibody (Invitrogen). After being rinsed in PBS, the slides were incubated with diaminobenzidine (Invitrogen), and finally counterstained with hematoxylin. These stains were manually scored by one pathologist (H M) blinded to patient identity. German Immunoreactive score (0–12) was calculated, only in tumor cells, by multiplying the percentage of immunoreactive cells (0%, 0–1%, 1–10%, 1–11–50%, 2; 51–80%, 3; 81–100%, 4) by staining intensity (negative, 0; weak, 1; moderate, 2; strong, 3). Scores were considered as follows: negative (0) versus positive (2–12) for AR; negative (0–8) versus positive (for overexpression) (9–12) for EGFR/ERBB2; and negative (0–4) versus positive (6–12) for pEGFR/pAKT/pERK.

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Statistical analyses

Differences in variables with a continuous distribution across dichotomous categories (i.e. luciferase activity, estimated cell proliferation) were analyzed by Student’s t-test. Differences in protein expression between the two groups from human tissue samples were analyzed by Fisher’s exact test or χ² test. Progression-free survival rates in patients were calculated by the Kaplan–Meier method, and comparison was made by log-rank test. P values <0.05 were considered to be statistically significant.

Results

Androgen mediates AR transactivation and cell proliferation in bladder cancer

It was found that two bladder cancer cell lines UMUC3 and TCC-SUP express AR (Miyamoto et al. 2007, Boorjian et al. 2009). We additionally showed that androgens increased AR transcriptional activity and cell proliferation in these two cell lines, and an anti-androgen HF antagonized the effects of androgens (Miyamoto et al. 2007). Using a reporter gene assay, we first confirmed the functional activity of AR in another bladder cancer cell line 5637 with a full-length wild-type AR stably expressed by lentivirus. Luciferase activity was determined in the cell extracts with transfection of a plasmid (MMTV-ARE-luc) containing an androgen response element (ARE) as a reporter of AR-mediated transcriptional activity and treatment of DHT and/or HF. As shown in Fig. 1A, DHT treatment increased luciferase activity to more than 70-fold in 5637-AR over mock treatment, and HF showing partial agonist activity (approximately sixfold) could block DHT-induced AR transcriptional activity. In AR-negative 5637-V, the basal activity with mock treatment was ~55% of that in 5637-AR (P=0.016), and DHT and HF showed only marginal effects on AR transcription. We next performed the MTT assay to evaluate the effects of androgen on cell proliferation of AR-positive and AR-negative bladder cancer cell lines. As shown in Fig. 1B, in 5637-AR cells, DHT increased cell growth by ~60% in 4 days (lane 1 vs 2, P=0.003) and HF at least partially blocked the DHT effect (lane 2 vs 4). In contrast, DHT and/or HF only marginally affected the growth of 5637-V cells (lane 7 vs 8–10). Additionally, using a specific inhibitor of EGFR, PD168393, we assessed the contribution of the EGFR pathway to DHT-induced growth stimulation. As expected, PD168393 reduced the cell growth by ~73% (Fig. 1B, lane 1 vs 5, P<0.001). In the presence of PD168393, the growth induction rate by DHT was decreased to 15% (lane 5 vs 6, P=0.455) (vs 60% increase without the inhibitor described above), suggesting that androgen effect on cell growth is at least partially mediated through the EGFR pathway. In 5637-V cells, PD168393 showed a lower rate (up to 54%) of growth suppression (Fig. 1B, lane 7 vs 11, P=0.009), and DHT did not stimulate the cell growth (lane 11 vs 12, P=0.853).

Androgen upregulates EGFR and ERBB2 gene transcription

We assessed changes in EGFR and ERBB2 mRNA levels following androgen treatment in AR-positive bladder cancer cells by real-time reverse transcription...
(RT)-PCR. As shown in Fig. 2A, DHT treatment for 24 h increased EGFR levels to 1.7- and 1.9-fold in UMUC3 and TCC-SUP cell lines respectively, compared with mock treatment. Similarly, DHT treatment resulted in up to 1.7-fold increase in ERBB2 levels in both cell lines. As expected, HF blocked the effect of DHT on the expression of EGFR and ERBB2 in these cell lines.

Ligand-activated AR is known to regulate the expression of its target genes either by serving as a transcription factor via directly binding to their regulatory sequences or by modulating other transcription factors (Nelson et al. 2002, Pignon et al. 2009). To validate the mechanism responsible for androgenic regulation of ERBB2 and EGFR gene expression, we introduced cycloheximide prior to DHT treatment to block protein neosynthesis. As shown in Fig. 2B, both at 1 and 10 nM, DHT alone was found to increase EGFR and ERBB2 transcript abundance in both cell lines in a dose-dependent manner. In cycloheximide-treated cells, DHT lost its effect on influencing EGFR or ERBB2 transcription. These results suggest that androgen-mediated increase in ERBB2 and EGFR mRNA levels requires novel protein synthesis and, therefore, androgens could indirectly stimulate gene expression of EGFR and ERBB2.

**Androgen upregulates EGFR and ERBB2 protein expression**

We also examined the effect of androgen on EGFR and ERBB2 expression in protein levels in UMUC3 and TCC-SUP cell lines. Cell extracts upon androgen/anti-androgen treatment were analyzed by western blot, using an antibody to EGFR or ERBB2. In accordance with their mRNA levels shown above, DHT increased EGFR and ERBB2 protein expression and HF antagonized the DHT effect (Fig. 3A). AR protein expression was similarly regulated by DHT and HF in these cells.

The AR not only functions as a transcription factor but also is shown to modulate the stability of protein (Perry & Tindall 1996). We again blocked the neosynthesis of protein, using cycloheximide pretreatment, and the degradation of EGFR and ERBB2 proteins at different time points in the presence or absence of DHT was determined in UMUC3 and TCC-SUP cells. As shown in Fig. 3B, there was no significant difference in the ratios of their protein degradation in the presence and absence of DHT. These findings indicate that androgen treatment has little influence on the stability of both EGFR and ERBB2 proteins in bladder cancer cells.

**AR pathway is necessary for regulation of EGFR and ERBB2 expression by androgen**

To further investigate whether upregulation of EGFR and ERBB2 expression by androgen is dependent on AR, stable bladder cancer cell lines (i.e. UMUC3-AR-shRNA versus UMUC3-control-shRNA and 5637-AR versus 5637-V) were analyzed by western blotting.
We confirmed that the expression of AR-shRNA in AR-positive UMUC3 by retrovirus effectively silenced endogenous AR (Fig. 4A). Basal levels of EGFR and ERBB2 were much lower in UMUC3-AR-shRNA than in UMUC3-control-shRNA. AR-shRNA also abolished the effect of DHT on upregulation of EGFR and ERBB2. Interestingly, HF obviously increased EGFR and ERBB2 proteins via unknown pathway.

Protein levels of EGFR and ERBB2 were also compared in 5637-AR and 5637-V. Overexpression of AR lead to a dramatic increase in basal levels of EGFR, whereas it showed marginal effects on basal levels of ERBB2 (Fig. 4B). As expected, DHT increased EGFR and ERBB2 protein expression in 5637-AR, but not in 5637-V. EGFR and ERBB2 levels were marginally augmented by HF in AR-negative 5637-V cells.

Thus, it is likely that the AR is necessary for androgenic upregulation of EGFR and ERBB2 expression in bladder cancer cells.

**Androgen promotes pEGFR and its downstream proteins**

To examine whether AR activation influences the function of the EGFR pathway, we next investigated the phosphorylation status of EGFR and its downstream proteins AKT and ERK1/2 in AR-positive (UMUC3, 5637-AR) and AR-negative (5637-V) bladder cancer cells treated with DHT, EGF, and/or PD168393. In the presence or absence of EGF and PD168393, DHT increased the levels of EGFR in UMUC3 (Fig. 5A) and 5637-AR (Fig. 5B), but not in 5637-V (Fig. 5C). Furthermore, we detected eruption of pEGFR, pAKT, and pERK, but not EGFR, after introduction of EGF in all three bladder cancer cell lines, which was blocked by PD168393. However, additional increases in pEGFR, pAKT, and pERK were observed only in AR-positive UMUC-3 and 5637-AR cells after DHT treatment. In the presence of PD168393, DHT also slightly induced the levels of pAKT in UMUC3 cells, but not in 5637-AR and 5637-V cells. DHT and/or EGF did not affect the levels of AKT and ERK in these cells.

**Immunoreactivity in bladder cancer tissue samples**

We next performed Immunohistochemical stains for AR, EGFR, pEGFR, ERBB2, pAKT, and pERK in 24

![Figure 3](image3.png) Regulation of ERBB2 and EGFR protein expression by androgen in bladder cancer cell lines. (A) UMUC3 or TCC-SUP cells were treated with ethanol (mock), 1 nM DHT, and/or 1 μM HF for 24 h. Equal amounts of protein extracted from each cell line were immunoblotted for EGFR (170 kDa), ERBB2 (185 kDa), or AR (110 kDa), as indicated. β-actin (43 kDa) served as the internal control. (B) UMUC3 or TCC-SUP cells were pretreated with 25 μg/ml cycloheximide for 30 min, and proteins were extracted at different time points after ethanol/1 nM DHT treatment for 0–24 h, as indicated. Western blots for EGFR, ERBB2, and β-actin were then performed.

We confirmed that the expression of AR-shRNA in AR-positive UMUC3 by retrovirus effectively silenced endogenous AR (Fig. 4A). Basal levels of EGFR and ERBB2 were much lower in UMUC3-AR-shRNA than in UMUC3-control-shRNA. AR-shRNA also abolished the effect of DHT on upregulation of EGFR and ERBB2. Interestingly, HF obviously increased EGFR and ERBB2 proteins via unknown pathway.
radical cystectomy specimens with high-grade urothelial carcinoma (Fig. 6). The results of the staining and correlations with pathological stage are summarized in Table 1. Overall, positive signals were found in 8 (33% for AR), 12 (50% for EGFR), 14 (58% for pEGFR), 17 (71% for ERBB2), 13 (54% for pAKT), and 13 (54% for pERK) cases. There were no statistically significant correlations between each staining and presence of muscle invasion (≤T1 vs ≥T2) or lymph node metastasis, except an inverse correlation between pAKT positivity and tumor invasiveness (P = 0.0303) and a positive correlation between pERK and node metastasis (P = 0.0498). No significant association between gender of the patients and AR (P = 0.1028), EGFR (P = 0.1584), pEGFR (P = 0.0751), ERBB2 (P = 0.4625), pAKT (P = 0.1118), or pERK (P = 0.1118) was found. AR expression was then analyzed in comparison with respective stains: there were strong associations with EGFR (P = 0.0136), pEGFR (P = 0.0041), ERBB2 (P = 0.0331), or pERK (P = 0.5555) (Table 1). Similarly, EGFR overexpression significantly correlated with the expression of pEGFR (P = 0.0005), ERBB2 (P = 0.0343), or pERK (P = 0.0498), but not with that of pAKT (P = 0.5000).

To assess possible associations between each expression and disease progression, we then performed the Kaplan–Meier analysis coupled with log-rank test. Of the 24 patients with a mean follow-up of 8.3 months, 8 (33%) developed recurrent/metastatic tumors after radical surgery. As shown in Fig. 7A, AR positivity was significantly associated with tumor progression (P = 0.0005). Status of EGFR (P = 0.2425; Fig. 7B), pEGFR (P = 0.1579; Fig. 7C), ERBB2 (P = 0.2997; Fig. 7D), and pERK (P = 0.1270; Fig. 7F) tended to correlate with progression. Conversely, pAKT positivity was associated with significantly better prognosis (P = 0.0483; Fig. 7E).

Discussion

There is increasing evidence to indicate that the AR, as a ligand-regulated transcription factor, significantly contributes to the development and progression of bladder cancer (Miyamoto et al. 2007, Johnson et al. 2008, Wu et al. 2010). Dysregulation of the EGFR/ERBB2 signaling pathway is well known to play an important role in the progression of bladder cancer (Neal et al. 1990, Lipponen & Eskelinen 1994, Orlando et al. 1996, Miyamoto et al. 2000, Jimenez et al. 2001, Bellmunt et al. 2003, Latif et al. 2004, MacLaine et al. 2008). However, the cross talk between AR and EGFR/ERBB2 pathways is uncharacterized in

![Figure 5](image-url)
bladder cancer, although it has been widely studied in prostate cancer (Culig et al. 1994, Scher et al. 1995, Craft et al. 1999, Signoretti et al. 2000, Di Lorenzo et al. 2002, Liu et al. 2005, Mukherjee & Mayer 2008, Pignon et al. 2009). In this study, we provide evidence that androgens regulate EGFR and ERBB2 expression via the AR pathway in bladder cancer cells.

In prostate cells, the AR likely has opposing effects on the regulation of EGFR expression: downregulation in benign cells and upregulation in malignant cells (Brass et al. 1995, Itoh et al. 1998, Traish & Morgentaler 2009). In prostate cancer cells, androgens were also reported to reduce EGFR protein expression (Mukherjee & Mayer 2008). The underlying mechanism responsible for the distinct effects needs to be determined. As shown for the regulation of AR activity by AKT in LNCaP prostate cancer line (Lin et al. 2003), such opposing effects might be dependent on passage number of the cells in which AR status could be different (e.g. expression level and sensitivity to ligands). ERBB2 is generally repressed by androgens in prostate cancer cells (Berger et al. 2006, Pignon et al. 2009). In this study, we showed in bladder cancer cells that androgen could induce the expression of both EGFR and ERBB2. The upregulation was observed in two bladder cancer cell lines with endogenous AR and an additional cell line exogenously expressing the AR. We further confirmed the necessity of the AR pathway for the upregulation, using anti-androgen treatment and RNA interference strategy. In addition, using an EGFR inhibitor PD168393, we showed that androgen-induced growth stimulation of AR-positive bladder cancer cells could be mediated via the EGFR pathway. PD168393 is believed to be a selective inhibitor of EGFR tyrosine kinase activity (Fry et al. 1998). However, treatment with higher doses of PD168393 (e.g. 200 nM) resulted

Figure 6 Immunohistochemistry of AR (A), EGFR (B), pEGFR (C), ERBB2 (D), pAKT (E), or pERK (F) in bladder cancer tissue samples. Original magnification, 400×.
Table 1 Expression of androgen receptor (AR), epidermal growth factor receptor (EGFR), phosphorylation of EGFR (pEGFR), ERBB2, pAKT, and pERK in 24 bladder cancer tissue microarrays

<table>
<thead>
<tr>
<th></th>
<th>Positive cases (%)</th>
<th>≤ T1 (n=5)</th>
<th>≥ T2 (n=19)</th>
<th>N(−) (n=12)</th>
<th>N(+) (n=12)</th>
<th>AR(−) (n=16)</th>
<th>AR(+) (n=8)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>AR</td>
<td>8 (33)</td>
<td>2 (40)</td>
<td>6 (32)</td>
<td>2 (17)</td>
<td>6 (50)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>EGFR</td>
<td>12 (50)</td>
<td>2 (40)</td>
<td>10 (53)</td>
<td>0.5000</td>
<td>5 (42)</td>
<td>7 (55)</td>
<td>0.3021</td>
<td>5 (31)</td>
</tr>
<tr>
<td>pEGFR</td>
<td>14 (58)</td>
<td>4 (80)</td>
<td>10 (53)</td>
<td>0.2826</td>
<td>7 (67)</td>
<td>9 (75)</td>
<td>0.6549</td>
<td>6 (38)</td>
</tr>
<tr>
<td>ERBB2</td>
<td>17 (71)</td>
<td>4 (80)</td>
<td>13 (63)</td>
<td>0.5375</td>
<td>8 (67)</td>
<td>9 (75)</td>
<td>0.5000</td>
<td>9 (56)</td>
</tr>
<tr>
<td>pAKT</td>
<td>13 (54)</td>
<td>5 (100)</td>
<td>8 (42)</td>
<td>0.0303</td>
<td>8 (67)</td>
<td>5 (42)</td>
<td>0.2068</td>
<td>9 (56)</td>
</tr>
<tr>
<td>pERK</td>
<td>13 (54)</td>
<td>2 (40)</td>
<td>11 (58)</td>
<td>0.4146</td>
<td>4 (33)</td>
<td>9 (75)</td>
<td>0.0498</td>
<td>6 (38)</td>
</tr>
</tbody>
</table>

N, lymph node metastasis.

in similar rates of growth suppression between 5637-AR and 5637-V cells (data not shown) that exhibit distinct basal levels of EGFR (Fig. 4B), postulating the possibility of the inhibition of other pathways by this inhibitor.

It is worth noting that AR knockdown in UMUC3 and AR overexpression in 5637 resulted in significant decrease and increase respectively, in the basal levels of EGFR (both cell lines) and ERBB2 (UMUC3 only). These findings may raise the possibility of the constitutive activity of the AR. However, the presence of mutations in the endogenous AR gene, leading to its androgen-independent activation often found in prostate cancer, has not been described in bladder cancer cell lines. Furthermore, there were no significant differences in the basal levels of AR transcriptional activity and cell growth rate between UMUC3/TCC-SUP with and without AR knockdown (Miyamoto et al. 2007) and between 5637-AR and 5637-V (Fig. 1). The 5637 cell line exhibited a relatively high basal level of ERBB2 protein, and AR overexpression in this line may, therefore, increase ERBB2 expression only marginally in the absence of androgens (Fig. 4B). However, the precise reason for the difference in the effect of AR expression on ERBB2 levels between UMUC3 and 5637 cells remains uncertain.

Although several putative AREs in an EGFR promoter region were identified by a computer analysis, their functionality in reporter vectors has not been demonstrated (Quandt et al. 1995, Pignon et al. 2009). Chromatin immunoprecipitation assays showed that androgens induced the recruitment of RNA polymerase II to the EGFR proximal promoter in prostate cancer cells (Pignon et al. 2009), suggesting the stimulation of EGFR gene transcription by androgen-activated AR. However, it remains to be determined whether the AR directly binds to the promoter. In this study in bladder cancer cells, blocking of protein neosynthesis, using cycloheximide pretreatment, abolished androgen-mediated increase in EGFR and ERBB2 transcripts, suggesting that androgens act through an androgen-dependent intermediary. Ligand-activated AR is known to have dual functions in regulating target proteins either by increasing transcription or by decreasing degradation. The regulation of EGFR expression by androgens was shown to be at the transcriptional level in prostate cancer cell lines (Traish & Morgentaler 2009). Our protein stability assays showing marginal effects of androgen suggest that the AR functions only as a transcription factor in regulation of both EGFR and ERBB2 in bladder cancer cells.

EGF stimulates tyrosine phosphorylation of its receptor by homodimerization of EGFR and activation of receptor tyrosine kinases (Chen et al. 1987). The Ras– Raf– MAPK and PI3K–AKT– GSK pathways are considered to be the main traditional downstream of EGFR (Anderson et al. 1990). In addition to increased expression/activity of EGFR, these downstream signaling cascades are frequently deregulated in neoplasms, leading to tumorigenesis and tumor progression (Bacus et al. 1996, Craven et al. 2003). We observed that androgen treatment for 24 h increased pEGFR levels, particularly with EGF treatment, only in the presence of functional AR. Similar increases in the levels of pAKT and pERK, but not in those of total AKT and ERK1/2, were observed in androgen-treated AR-positive bladder cancer cells. Although direct interactions between the membranous AR and the growth factor receptors are possible mechanisms of this cross talk, shorter durations of androgen treatment (e.g. 1 h) resulted in little induction in the expression or activation of the receptors (data not shown), suggesting the classic genomic effects of androgens on EGFR/ERBB2 in bladder cancer cells. Moreover, androgen treatment in the presence of the EGFR inhibitor still slightly increased the levels of pAKT expression in UMUC3 cell line, but not in 5637-AR or 5637-V cell line, postulating the involvement of non-EGFR pathway(s) in UMUC3 cells.
In prostate and breast cancers where androgenic regulation of the EGFR/ERBB2 pathways was shown, AR expression was found to significantly correlate with ERBB2 overexpression (Brys et al. 2004, Micello et al. 2010, Park et al. 2010). To investigate whether AR and EGFR/ERBB2 or phosphorylated forms of their downstream proteins co-express in bladder cancers, we immunohistochemically stained for AR, EGFR, pEGFR, ERBB2, pAKT, and pERK. As expected, there was a statistically significant association between EGFR overexpression and pEGFR expression as well as the status of ERBB2 or pERK. AR expression was then found to strongly correlate with the expression of EGFR, pEGFR, ERBB2, or pERK. These correlations strongly support the potential cross talk between AR and EGFR/ERBB2 pathways in bladder cancer.

Additionally, we analyzed the relationship between the immunostaining and tumor stage (i.e. degree of invasion and lymph node metastasis). In our cohort, pAKT positivity showed an inverse association with deep invasion, confirming a recent report demonstrating significantly higher levels of pAKT in non-invasive bladder cancers than in invasive tumors (Schultz et al. 2011). However, earlier studies demonstrated similar overexpression of pAKT between non-invasive and invasive bladder tumors yet induction of invasive capacity of cancer cell lines via the PI3K/AKT pathway (Wu et al. 2004, Knowles et al. 2009). This study also analyzed and compared the prognostic value of respective expression, using Kaplan–Meier survival curves and log-rank test. Surprisingly, patients with AR-positive tumor had significantly higher risks of progression compared with those with AR-negative tumor. There are controversial data as to the correlation of AR expression in bladder cancer with tumor aggressiveness (Boorjian et al. 2004, 2009, Miyamoto et al. 2007, Mir et al. 2010, Tuygun et al. 2011). Few studies in which outcome differences among patients with muscle invasive disease were not analyzed uninversely correlated with the expression of AR, pEGFR, ERBB2, pAKT, and pERK.

Figure 7 Progression-free survival rates in patients with bladder cancer. The Kaplan–Meier analysis was performed according to the expression of AR (A), EGFR (B), pEGFR (C), ERBB2 (D), pAKT (E), or pERK (F), and comparisons were made by log-rank test.
demonstrated a decrease in AR expression in higher grade/stage tumors (Boorjian et al. 2004, 2009, Tuygun et al. 2011). In contrast, a recent study analyzing 473 patients has revealed that AR positivity was significantly higher in muscle invasive tumors than in non-muscle invasive tumors (Mir et al. 2010). Nonetheless, in this study, there was no statistically significant difference in cancer-specific survival among patients with T2 disease. Our pilot study analyzing 33 superficial bladder carcinomas, using a quantitative RT-PCR method, showed that recurrence-free survival in patients with high AR-expressing tumors tended to be lower than that in patients with low AR-expressing tumors (Miyamoto et al. 2007). This study is the first to show a strong relationship between AR status and tumor progression. Because EGFR and ERBB2 were frequently overexpressed in aggressive bladder tumors (Neal et al. 1990, Lipponen & Eskelinen 1994, Orlando et al. 1996, Miyamoto et al. 2000, Jimenez et al. 2001, Latif et al. 2004) as discussed, we analyzed only high-grade, mostly invasive, urothelial carcinomas. The expression of EGFR, pEGFR, ERBB2, and pERK also tended to predict tumor progression. In addition to numerous previous studies on EGFR and ERBB2, a recent study has shown a possibility of pERK as a prognosticator in muscle invasive bladder tumors (Karlou et al. 2009). Indeed, there was a statistically significant association between pERK positivity and the presence of lymph node metastasis in our cohort. We further found that higher pAKT expression in bladder cancer predicted a better prognosis. However, this finding was inconsistent with not only our current data showing an increase in pAKT expression by androgen in AR-positive bladder cancer cells but also published evidence suggesting tumor progression via activation of PI3K/AKT signals (Wu et al. 2004, Knowles et al. 2009). Underlying mechanisms responsible for the protective roles of AKT activation in bladder cancer progression remain unclear. Therefore, further studies including larger patient cohorts with longer follow-up are needed to validate these initial results.

In conclusion, our study demonstrates that androgen upregulates the levels of EGFR and ERBB2 expression, as well as the activity of EGFR signaling, through the AR pathway in bladder cancer cells. Thus, AR signals may play an important role in the regulation of the EGFR/ERBB2 pathways, leading to the progression of bladder cancer. These results may significantly enhance previous findings suggesting the involvement of the AR pathway in bladder cancer and the consequence of androgen deprivation as a potential therapeutic approach.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Y Zheng and K Izumi performed the experiments and data analysis. J L Yao provided bladder cancer TMA and evaluated patient information. H Miyamoto conceived of the study, coordinated and supervised the project, and also evaluated immunohistochemical staining. Y Zheng drafted the manuscript and H Miyamoto edited it.

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