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ErbB-2 signaling plays a critical role in regulating androgen-sensitive and castration-resistant androgen receptor-positive prostate cancer cells

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Abstract

While androgen deprivation therapy (ADT) reduces tumor burden, autocrine growth factor loops such as human epidermal growth factor receptor 2 (HER2/ErbB-2/neu) have been proposed to contribute to prostate cancer (PCa) survival and relapse. However, the role of ErbB-2 in regulating androgen-sensitive (AS) and castration-resistant (CR) cell proliferation remains unclear. Here, we determined the role of ErbB-2 in PCa progression and survival under steroid-reduced conditions using two independent PCa cell progression models. In AR-positive androgen-independent (AI) PCa cells that exhibit the CR phenotype, *ErbB-2* was constitutively activated, compared to corresponding AS PCa cells. In AS LNCaP C-33 cells, androgen-induced *ErbB-2* activation through ERK1/2 mediates PCa cell proliferation. Further, the ErbB-2-specific but not EGFR-specific inhibitor suppresses basal and androgen-stimulated cell proliferation and also blocks ERK1/2 activation. ErbB-2 ectopic expression and cPacP siRNA transfection of LNCaP C-33 cells each increases ErbB-2 tyrosine phosphorylation, correlating with increased AI PSA secretion and cell proliferation. Conversely, trapping ErbB-2 by transfected endoplasmic reticulum-targeting ScFv5R expression vector abolished DHT-induced LNCaP C-33 cell growth. Moreover, inhibition of ErbB-2 but not EGFR in AI LNCaP C-81 and MDA PCa2b-AI PCa cells significantly abolished AI cell growth. In contrast to androgens via ErbB-2/ERK1/2 signaling in

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Conflicts of Interest

No potential conflicts of interest were disclosed.

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AS PCa cells, the inhibition of ErbB-2 abrogated AI cell proliferation by inhibiting the cell survival protein Akt in those AI cells. These results suggest that *ErbB-2* is a prominent player in mediating the ligand-dependent and -independent activation of AR in AS and AI/CR PCa cells respectively for PCa progression and survival.

Keywords

ErbB-2; androgen sensitivity; androgen-independent; cPacP; ERK1/2

1. Introduction

Androgens play a vital role in diverse biological activities such as masculinization and virilization, as well as in the development and progression of prostate cancer (PCa). PCa is the most commonly diagnosed solid malignancy and the second leading cancer-related death in US males [1]. Currently, androgen deprivation therapy (ADT) is the gold standard treatment for metastatic PCa patients. Eventually, resistance to ADT develops, and cancer progresses to castration-resistant (CR) PCa, which is associated with a limited life span of approximately about 18 months. For CR PCa patients, the most available treatments are only palliative. Hence, understanding the mechanism leading to castration-resistance is critical and has been the subject of intensive research in the past decade.

Experimental analyses reveal that over 90% of CR PCa patients express androgen receptor (AR) and androgen-response genes, indicating continued androgens/AR signaling in the castrated levels of androgens [2–4]. Several studies have established that PCa cells acquire the CR phenotype by adapting the AR pathway in part to the lower levels of androgens by AR mutation, amplification and variation from splicing, deregulation of cofactors and intratumoral androgen production [5–7]. Furthermore, results of clinical studies indicate that autocrine growth factor loop such as epidermal growth factor (EGF) receptor (EGFR/ERBB1) and its family member ERBB2 could also activate AR and enhance CR PCa progression [8–10].

The EGFR family members consist of EGFR (HER1), ErbB-2 (HER2/Neu), ErbB-3 (HER3) and ErbB-4 [11]. Classically, EGFR's are activated by the ligand binding which induces receptor dimerization and stimulation of the intrinsic receptor tyrosine kinase (RTK) activity. These RTK complexes can activate mitogen-activated protein kinase (MAPK) and phosphoinositol 3'-kinase (PI3K)/Akt pathways [12]. These sustained ligand-dependent or -independent activation of EGFR family member result in uncontrolled proliferation of cancer cells and hence these RTK has long been considered a promising therapeutic target [13]. In PCa, most PCa cells do not express ErbB4, and ErbB3 lacks intrinsic kinase activity; hence both EGFR and ErbB2 can play an important functional role in the pathogenesis [12].

Several studies showed EGFR expression level may be elevated in a subset of PCa [14–16]. Concurrently, other studies showed higher levels of ErbB-2 protein in some prostatic tumors than normal controls [17–19]. Analyses of clinical specimens showed that ErbB-2 protein may be elevated in a subset of CR PCa [20–23]. Conversely, inhibition of ErbB-2 results in

the suppression of xenograft tumor growth and decreases AR transcriptional activity [24, 25]. In addition, results of a recent phase II study showed combined inhibition of EGFR and ErbB-2 exhibit significant activity in a small subset of patients [26]. All these studies suggest the EGFR family members such as EGFR or ErbB-2 contribute to CR PCa cell growth and correlate with poor patients' prognosis. In spite of significant progress in PCa research, it is still unclear whether EGFR or ErbB-2 activation/overexpression plays a critical role in androgen-stimulated cell proliferation and CR PCa progression. Further, cross talk between androgens and EGFR/ErbB-2, and the biochemical mechanism that governs ErbB-2 activation in androgen-sensitive (AS) and CR PCa cells remain unclear.

One of the critical needs is the availability of a cell model that faithfully recapitulates human CR PCa progression because these cell models can serve as useful tools for rapid discovery of underlying mechanisms and for the evaluation of alternate therapies. In the present study, we used LNCaP and MDA PCa2b cell progression model as an experimental cell model to investigate the role of EGFR/ErbB-2 in driving CR phenotype. Both cell models include AS and respective AI/CR PCa cells. The AI cells exhibit the biochemical property of CR PCa including proliferation and PSA secretion under androgen-reduced conditions [27, 28]. With the use of ErbB-2 and EGFR inhibitors, we have shown the role of ErbB-2 in androgen-induced PCa cell proliferation. We have further shown that inhibition of ErbB-2 with AG879 significantly blocks androgen stimulation and CR PCa cell growth. The data from this cell model supports the notion that despite improved ADT, PCa cells in part depend on non-canonical RTK-mediated AR signaling. Hence, combination therapy of ADT together with ErbB-2 specific inhibitor may provide an attractive therapeutic strategy for combating CR mechanisms.

2. Materials and Methods

2.1. Reagents

RPMI 1640 medium, glutamine, gentamicin, and trypsin/EDTA reagents were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Regular and charcoal/dextran-treated certified fetal bovine sera (FBS) were obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Protein molecular weight standard markers, acrylamide, and the protein assay kit were obtained from Bio-Rad (Hercules, CA). scFv5R cDNA and vector alone transfected cells were as described [29, 30]. Polyclonal Abs recognizing all three isoforms of Shc protein and anti-phospho-EGFR (Y1173) Ab were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antiphospho-ErbB-2 (pY1221/2), antiphospho-ErbB-2 (pY1248) and antiphospho-Akt (S473) Abs were from Anaspec (Fremont, CA). Anti-Akt Abs was purchased from Biolabs (Ipswich, MA). Monoclonal anti-p-Tyr Ab (4G10), antiphospho-ERK1/2, antiphospho-p38MAPK and anti-p38MAPK Abs were from Cell Signaling Technology (Danvers, MA). AG879 and AG1478 were from Millipore Corporation (Temecula, CA). Polyclonal anti-ErbB-2 (C-18), anti-EGFR, anti-AR, anti-PSA, anti-SHP1 (SH-PTP1), anti-SHP-2 (SH-PTP2), anti-cyclin D1, anti-cyclin B1, anti-ERK1/2, horseradish peroxidase-conjugated anti-rabbit, anti-goat and anti-mouse IgG Abs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti- β -actin Ab and 5 α -

dihydrotestosterone (DHT) were obtained from Sigma (St Louis, MO). An enhanced ECL detection system was purchased from Pierce (Rockford, IL).

2.2. Cell culture and cell proliferation assay

Human prostate carcinoma cell line LNCaP and MDA PCa2b were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were split once per week, which was defined as one passage. LNCaP cells with passage numbers less than 33 were designated as C-33 cells, those with numbers greater than 80 as C-81 cells [27, 31]. While C-33 cells are AS and C-81 cells are AI and exhibit the CR PCa phenotype; both cells express functional AR. LNCaP PCa cells were routinely maintained in the regular medium, i.e., phenol red-positive RPMI 1640 medium supplemented with 5% (v/v) FBS, 2 mM glutamine, and 50 µg/mL gentamicin. For steroid-reduced (SR) medium, cells were maintained in phenol red-free RPMI 1640 medium containing 5% (v/v) charcoal/dextran-treated FBS, 2 mM glutamine and 50 µg/mL gentamicin [32].

MDA PCa2b cells were cultured in BRFF-HPC1 medium containing 20% FBS, 2 mM glutamine and 50 mg/mL gentamicin. MDA PCa2b cells express AR and are sensitive to androgens. Upon continuous passage in BRFF-HPC1 medium containing 20% FBS, androgen independent MDA PCa2b-AI cells were established and these cells proliferate under androgen-reduced conditions [33, 34]. For SR condition, MDA PCa 2b cells were cultured in phenol red-free BRFF-HPC1 medium containing 5% cFBS, 2 mM glutamine and 50 mg/mL gentamicin [35]. AG879 and AG1479 were dissolved in DMSO as 1,000× concentrated stock solutions, stored at −20°C, and diluted in the respective culture media at the time of use.

For cell proliferation assay, cells were plated in the respective regular culture medium for 3 days followed by cultured in SR condition for additional 48 hours. For proliferation assay, cells were trypsinized and counted after steroid starvation for 48 hours or treating with ErbB-2 and EGFR inhibitors for additional 24 hours in SR condition. Control cells were treated with equal amount of DMSO. The cell proliferation was determined by cell counting after trypan blue exclusion assay by Nexcelom Cellometer Auto T4. The ratio of cell growth was calculated by normalizing the cell number to that of the control cells. Results shown are an average of two-three sets of independent experiments performed in duplicates or triplicates. Results were expressed as mean±SE and were considered statistically significant if $p < 0.05$.

2.3. Transient transfection of ErbB-2 cDNA, siPacP and ErbB-2-specific Fab fragment in LNCaP C-33 PCa cells

The Myc-His-tagged ErbB-2 expression vector was obtained as described in our previous report [36]. For transient transfection, LNCaP C-33 cells were plated at the densities of 2×10^5 cells/well in a 6-well plate and cultured in RPMI 1640 medium containing 5% FBS for 48 hours. After forty-eight hours, 1 µg/well of plasmid DNA was mixed with Lipofectamine reagent and introduced into LNCaP cells by following manufacturer recommendation. Six hours after the incubation, the culture medium was replaced by RPMI 1640 containing 10% FBS, and incubated for an additional 16 h.

The PACP specific siRNA was synthesized as described previously. Briefly, two different oligonucleotides: siPACP-78, 5'-GCCTTAGCCTTGGCTTCTT-3'; siPACP-126, 5'-GTGTACTAGCCAAGGAGTT-3' were used for the present experiments based on efficient knockdown of PACP [37]. A negative control siRNA containing scramble oligos was also included to eliminate non-specific changes in gene expression profiles due to siRNA delivery. For transient transfection, LNCaP C-33 cells were plated at the densities of 2×10^5 cells/well in a 6-well plate and cultured in RPMI 1640 medium containing 5% FBS for 48 hours. The siRNAs were then diluted in OPTI-MEM medium to a final concentration of 30 nM and was then overlaid onto the cells. 48 hours after incubation, the cells were collected and further processed for western blot analysis to determine the relative expression.

ErbB-2-impaired scFv5R LNCaP cells that stably express the scFv5R antibody to trap ErbB-2 in endoplasmic reticulum (ER), and pcDNA vector-transfected LNCaP control cells were kind gifts from Dr Hsing-Jien Kung at the University of California Davis Cancer Center, Sacramento, CA, USA [38, 39]. For DHT treatment, cells were cultured for a time period as indicated in the figure legends with SR containing 5% charcoal/dextran-treated FBS [31, 36, 40]. Cell cultured in the presence or absence of DHT was trypsinized and counted at day 4 and was normalized to the control.

2.4. Western blot analysis

Immunoblot analysis was performed as described previously [31, 40]. Briefly, cultured cells were harvested by scrapped with ice-cold HEPES-buffered saline, centrifuged, pellets were lysed using ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40) containing protease and phosphatase inhibitors. Cell lysates were resolved by SDS-PAGE (7.5% to 12% acrylamide) and transferred to nitrocellulose membranes. The proteins of interest were visualized by an ECL detection system. For total protein detection, the membranes of respective phosphoproteins were stripped and re-probed with respective antibody for total proteins. For detecting β -actin as a loading control, the membranes were re-probed with monoclonal β -actin Ab.

2.5. Anchorage-independent cell growth assay

The anchorage-independent growth of cells was determined by soft agar analysis as described previously [40, 41]. Briefly, 5×10^3 cells were seeded in 0.25% agarose on the top of a base layer containing 0.3% agarose. One day after seeding, cell clumps containing more than one cell were excluded from the study and the cells were regularly fed with fresh medium containing respective treatment compounds once in every three days. Colony number was counted after 4 weeks of incubation at 37°C. Alternatively, the colonies were stained with 0.1% crystal violet solution containing 20% methanol and counted.

2.6. Statistical analysis

Each set of experiments was performed in duplicates or triplicates, as specified in the figure legend or experimental design, repeated at least two or three times as independent experiments, and the mean and standard error values of experimental results were calculated. Student's t test was used for comparison between each group. $p < 0.05$ was considered statistically significant [40].

3. Results

3.1. Profiling of ErbB-2 tyrosyl phosphorylation and its downstream signaling in androgen-sensitive vs. castration-resistant/androgen-independent LNCaP and MDA PCa2b cells

We investigated the role of ErbB-2 involving in the CR phenotype of PCa utilizing the AR-positive PCa cell progression model, including AS cells and its corresponding CR cells in LNCaP and MDA PCa2b cells. Importantly, LNCaP C-81 and MDA PCa2b-AI cells exhibit many biochemical properties of clinical CR PCa cells and are thus suitable for serving as a model system in analyzing the molecular profiling in CR PCa cells [6, 27, 31, 33, 34, 37, 40, 42]. We first analyzed the activation profiling of ErbB-2 and EGFR and their downstream signaling by immunoblotting in those cells grown in both regular and SR culture conditions.

In regular culture conditions, as shown in Fig. 1A, both AS and CR cells express respectively similar levels of AR. In those cells, only ErbB-2, not EGFR, was hyper-activated as shown by Y1221/2 phosphorylation in both CR LNCaP C-81 and MDA PCa2b-AI cells, higher than their respective AS cells. Importantly, similar ErbB-2 and EGFR protein levels were seen between AS and respective CR cells (Fig. 1A). We determined the downstream signaling by analyzing the activation status of Akt, ERK1/2, and p38 MAPK in these cells. As shown in Fig. 1A, in both CR cells, Akt, ERK1/2 and p38 MAPK were activated by phosphorylation, higher than their respective AS cells. In addition, the cell cycle regulators, cyclin B1 and cyclin D1 protein levels were highly elevated, positively correlating with ErbB-2 tyrosine phosphorylation; while cPacP protein, a prostate-specific tumor-suppressor gene (TSG), decreased (Fig. 1A; [43]). The increased cell growth correlates with elevated levels of secreted PSA in culture media (Fig. 1A) [27, 39].

To mimic clinical conditions under androgen ablation therapy, we performed analyses in cells cultured in SR conditions. As shown in Fig. 1B, in LNCaP C-81 cells, the overall tyrosyl phosphorylation level and the phosphorylation of Y1221/2 but not Y1112 or Y1248 of ErbB-2 were greatly elevated, whereas ErbB-2 protein levels remained the same. In parallel, when compared with AS cells, in MDA PCa2b-AI cells, ErbB-2 overall tyrosine phosphorylation and Y1221/2 and Y1248 phosphorylation, but not Y1112, were greatly increased, correlating with cell growth (Fig. 1C). The increased cell growth by cell number counting (Fig. 1C) and cell cycle analysis (data not shown) and also ErbB-2 tyrosine phosphorylation level (Figs. 1A and 1B) negatively correlate with decreased cPacP level in both LNCaP C-81 and MDA PCa2b-AI cells (Figs. 1A and 1B). In summary, in CR PCa cells, cPacP decreased; while ErbB-2 Y1221/2 phosphorylation and PCa cell proliferation are increased in both regular and SR conditions.

3.2. Involvement of ErbB-2 signaling in androgen-induced LNCaP C-33 cell proliferation

We analyzed the involvement of the EGFR family members in DHT-induced PCa cell growth stimulation by culturing AS LNCaP C-33 cells in the presence or absence of 10 nM DHT with inhibitors to EGFR (AG1478) or ErbB-2 (AG879). As shown in Fig. 2A, in AS LNCaP C-33 cells, 10 nM DHT increased cell growth by about 80 % upon 48 hour treatment (column 1 vs 2, $P < 0.001$). 1 μ M ErbB-2 inhibitor AG879 ($IC_{50} = 1 \mu$ M)

significantly suppressed the basal cell growth by about 40% (column 1 vs. 3) and completely blocked the DHT-induced cell proliferation (column 2 vs. 4). In comparison, EGFR inhibitor AG1478 (IC₅₀ = 3 nM) at the same 1 μ M concentration had no effect on the basal C-33 cell growth in SR conditions (column 1 vs. 5) despite significantly blocking DHT-induced C-33 cell growth (column 2 vs. 6). The results clearly indicate that androgen effect on PCa cell growth is at least in part mediated through the EGFR/ErbB-2 signaling pathway.

To investigate ErbB-2 tyrosine phosphorylation signaling in androgen-stimulated PCa cell proliferation, AS LNCaP C-33 cells were treated with 10 nM DHT in the presence or absence of ErbB-2 inhibitor AG879. Androgen treatment greatly increased the phosphorylation of ErbB-2 at Y1221/2, but not Y1248, and also ERK1/2 and p38 MAPK phosphorylation (Fig. 2B, lane #2 vs. #1). In addition, androgen-induced cell growth (Fig. 2A) is clearly reflected by increased cell cycle protein levels of cyclin B1 and cyclin D1 (Fig. 2B, lane #2). ErbB-2 inhibitor AG879 decreased ErbB-2 tyrosine phosphorylation and ERK1/2 and p38 MAPK phosphorylation and also cyclin B1 and cyclin D1 protein levels in the presence and absence of DHT (Fig. 2B, lanes #3&4 vs. #1&2), correlating with decreased cell growth (Fig. 2A, columns #3&4).

To clarify the role of EGFR in androgen-stimulated cell growth, LNCaP C-33 cells were treated with EGFR specific inhibitor AG1478 in the presence or absence of androgens. EGFR inhibitor AG1478 had only marginal effect on basal ErbB-2 phosphorylation and also cyclin B1 and cyclin D1 levels in the absence of androgens (Fig. 2B, lane #5). Nevertheless, AG1478 could block DHT-induced pY1221/2 of ErbB-2 and cyclin B1 and D1 protein levels (Fig. 2B, lane #6). Interestingly, AG1478 inhibited ERK1/2 phosphorylation but not p38 MPK phosphorylation. The data (Fig. 2A & 2B) together indicate that in AS PCa cells, ErbB-2 plays the predominant role in mediating the androgen-induced proliferation of PCa cells.

To rule out the possibility of EGFR inhibitor AG1478 being non-functional, e.g., below the optimal concentration, we treated LNCaP C-33 cells with human recombinant EGF (rhEGF) in presence or absence of AG1478 and determined the activation of EGFR and its downstream ERK1/2 phosphorylation. Our results clearly showed that EGFR inhibitor AG1478 completely abrogated EGF-induced EGFR and ERK1/2 phosphorylation (Fig. 2C, lane #2 vs. #4). For comparison, ErbB-2 inhibitor AG879 did not have an effect on rhEGF-induced EGFR and ERK1/2 phosphorylation (Fig. 2C; lane #2 vs. #6) despite that AG879 inhibited the basal ERK activation (lane #1 vs. #5, long exposure). Taken together, the data support the notion that ErbB-2 via ERK1/2 signaling plays a critical role in androgen-stimulated PCa cell proliferation.

3.3. Effect of endoplasmic reticulum-trapped ErbB-2 on androgen-induced growth of AS LNCaP C-33 cells

To test further the hypothesis that ErbB-2 plays a predominant role among ErbB family members in regulating androgen-stimulated signaling of PCa cells, we obtained LNCaP C-33 cells that were transfected with scFv5R expression vector in which the de novo biosynthesized ErbB-2 protein retained in the endoplasmic reticulum, and those cells would not have the fully processed functional ErbB-2 molecule at plasma membrane [29, 38, 39]. Fig.

2D clearly showed that while loss of functional ErbB-2 protein at plasma membrane by trapping ErbB-2 in endoplasmic reticulum did not affect the basal cell growth; the loss of functional ErbB-2 at plasma membrane significantly abolished androgen-induced cell proliferation. Thus, the expression of functional ErbB-2 plays a critical role in regulating androgen-stimulated cell proliferation.

3.4. Effect of ErbB-2 overexpression on androgen-independent proliferation of AS LNCaP C-33 cells

We examined the role of ErbB-2 signaling in AI growth by transfecting LNCaP C-33 cells with Myc-His tagged ErbB-2 cDNA [44]. Cells were then maintained in SR conditions, followed by analyzing its activation by tyrosyl phosphorylation and also ERK1/2 phosphorylation. Control cells were transfected with the vector alone. The expression of ectopic ErbB-2 proteins was detected by immunoblot using anti-Myc Ab (Fig. 3A). As shown in Fig. 3A, in SR conditions, the exogenous ErbB-2 was activated as evidenced by increased overall tyrosine phosphorylation level with a pY(4G10) Ab (Fig. 3A). In those ErbB-2 overexpressed cells, ERK1/2 was also activated by increased phosphorylation level (Fig. 3A). Importantly, elevated ErbB-2 expression led to AI PSA secretion in conditioned media (Fig. 3A) and correlated with increased cell growth rate (Fig. 3B). Thus, ErbB-2 activation by elevated expression can lead to AI PSA secretion and cell proliferation under SR conditions.

3.5. Effect of PAcP knockdown on ErbB-2 tyrosine phosphorylation and the androgen-independent growth of AS LNCaP C-33 cells

Given the fact that cPacP can function as a prostate-specific tumor suppressor with decreased expression in prostate carcinomas [40, 43], we determined the effect of decreased PAcP expression on tyrosine phosphorylation of ErbB-2 and AI cell growth. LNCaP C-33 cells were transiently transfected with two independent PAcP siRNA's and then maintained in SR media. The PAcP protein level and ErbB-2 activation profile were analyzed by immunoblotting and the cell growth was determined by cell number counting. To determine the specificity of siPacP to PAcP, we also analyzed the expression level of two other protein tyrosine phosphatases, Src homology 2 domain-containing PTPs SHP1 and SHP2, in PAcP-knockdown cells. Transfection of two independent PAcP siRNA resulted in decreased cPacP protein level, respectively; while they did not cause a decrease of SHP1 and SHP2 protein levels instead both SHP-1 and SHP-2 were slightly elevated (Fig. 3C). In PAcP-knockdown cells, the overall tyrosine phosphorylation and pY1221/2 levels but not pY1248 of ErbB-2 were elevated (Fig. 3C). The decrease of endogenous PAcP expression in C-33 cells resulted in up to about 30% increase of cell growth in SR conditions by cell number counting, correlating with elevated cell proliferation marker PCNA in both siPacP-78 and siPacP-126 transiently transfected cells (Fig. 3D). Thus, decreased cPacP protein as seen in clinical PCa is associated with increased ErbB-2 tyrosyl phosphorylation and PCa cell proliferation in SR conditions.

3.6. Effect of ErbB-2 inhibitor on ErbB-2 and its downstream signaling in CR PCa cell proliferation

To determine the role of ErbB-2 and its signaling in CR cell growth, we first assessed ErbB-2 inhibitor on C-81 cell growth and ErbB-2 activation by phosphorylation in SR conditions because LNCaP C-81 cells exhibit the CR PCa phenotype [6, 42]. ErbB-2 inhibitor AG879 significantly blocked CR cell proliferation by cell number counting (Fig. 4A), which is supported by the decrease of positive cell cycle proteins cyclin B1 and cyclin D1 and also growth regulator p66Shc protein (Fig. 4B, lane #2). Unexpectedly, while ErbB-2 phosphorylation level and also that of EGFR were decreased in AG879-treated C-81 cells; AG879 treatment did not significantly alter ErbB-2 downstream signaling, including the activation status of ERK1/2 and p38 MAPK (Fig. 4B, lane #2). Nevertheless, in AG879-treated C-81 cells, Akt was inactivated shown by decreased phosphorylation (Fig. 4B, lane #2). In those cells, the AR activity was decreased as indicated by the decrease of cellular PSA level. In comparison, AG1478 alone did not have a significant effect on CR growth of LNCaP C-81 cells (Fig. 4A) despite the fact that EGFR phosphorylation was completely blocked with partial inhibition on ERK1/2, p38 MAPK and Akt phosphorylation (Fig. 4B, lane #3). Furthermore, in AG1478-treated LNCaP C-81 cells, cellular PSA level was not changed, indicating AG1478 has no effect on AR activity. The combination of AG879 plus AG1478 did not have a significant, added effect on cell proliferation or cell signaling. In summary, ErbB-2, but not EGFR, plays a critical role in regulating CR proliferation of AR-positive C-81 cells.

We further examined the role of ErbB-2 in CR PCa cell proliferation in MDA PCa2b-AI cells [33, 34]. ErbB-2 inhibition by AG879 treatment significantly suppressed CR growth of MDA PCa2b-AI cells, which was also indicated by decreased cyclin B1, cyclin D1 and p66Shc protein levels in SR culture conditions (Figs. 4C & 4D, lane #2). The levels of ErbB-2 phosphorylation at Y1221/2 and Y1248 were decreased in AG879-treated cells, compared with that of control cells. ErbB-2 total protein was also decreased. In AG879-treated cells, EGFR phosphorylation but not protein level was decreased (Fig. 4D, lane #2). Further, the decreased cellular PSA level indicated the decrease of AR activity.

On the other hand, EGFR inhibitor AG1478 did not have a significant effect on CR growth of MDA PCa2b-AI cells (Fig. 4C), cyclin B1, cyclin D1 or p66Shc protein levels (Fig 4D, lane #3) despite the fact that AG1478 significantly abrogated EGFR phosphorylation (Fig. 4D, lane #3). AG1478 did not affect ErbB-2 protein or its phosphorylation (Fig. 4D, lane #3). The combined treatment with AG879 and AG1478 exhibited a slightly more potent effect on ErbB-2 signaling but not cell proliferation. Therefore, the data taken together clearly support the notion that in AR-positive, CR PCa cells, ErbB-2-dependent tyrosine phosphorylation signaling impacts cell proliferation through a mechanism independent of EGFR protein under SR conditions.

3.7. Effect of ErbB-2 inhibitor AG879 on LNCaP C-81 cell tumorigenicity

Since ErbB-2 plays a critical role in the proliferation of AR-positive, CR PCa cells under SR conditions, we investigated whether ErbB-2 inhibitor can decrease the tumorigenicity of LNCaP C-81 cells by performing colony formation assay in soft agar. As shown in Fig. 5,

the LNCaP C-81 control cells formed colonies in all plated cells, and AG879 treatment markedly decreased colony number and size ($p<0.05$), whereas, AG1478 had much less effect on colony formation including colony size and number. We further examined whether the combination of two inhibitors has added effects on inhibiting tumorigenicity. As shown in Fig. 5, combination of AG879 and AG1478 only had a marginal added inhibitory effect than AG879 alone. Thus, inhibition of ErbB-2 leads to reduced tumorigenicity of AR-positive, CR PCa cells.

4. Discussion

ADT is still the gold standard treatment for metastatic PCa. Introduction of anti-androgens and the recent developments of the second-generation antiandrogens such as MDV3100 and abiraterone acetate which respectively target androgen synthesis and androgen/androgen receptor signaling, significantly improve the median overall survival in PCa [45, 46]. Although these agents can improve the overall survival for a few months, frequently, tumor relapses and PCa cells survive. Deregulation of RTK signaling cascades including EGFR and ErbB-2 can activate the downstream signaling cascades such as PI3K/Akt and is shown to be associated with the development of CR progression of PCa cells. Nevertheless, despite recent advances in PCa research and in part due to the lack of suitable cell models, the role of ErbB-2 vs. EGFR in androgen-stimulated proliferation is still not fully understood. Furthermore, the investigation on the role of EGFR and ErbB-2 activation and/or overexpression in the development of CR PCa cells is inconsistent [14–20]. Therefore, in the present study, we used two independent *in vitro* cell models which respectively include AS and CR cells and recapitulates clinical PCa progression from AS to CR stage to determine the role of EGFR vs. ErbB-2 activation in androgen-stimulated proliferation and CR PCa cell growth.

In this study, we first showed the correlation of these two cell models to clinical PCa progression and then determined the role of ErbB-2 signaling in androgen-stimulated AS LNCaP C-33 cells (Figs. 1 and 2). Western blot analyses show that androgens preferentially activate ErbB-2 by Tyr1221/2 phosphorylation but not Tyr1248 (Fig. 2B), indicating that Tyr1221/2 phosphorylation is a potential site of mediating androgen-regulated PCa cell growth.

To elucidate the mechanistic role of ErbB-2 vs. EGFR and downstream signaling in mediating androgen-regulated processes, we used ErbB-2 and EGFR specific small molecule inhibitors. Androgen-induced ErbB-2 activation and cell growth are completely blocked by ErbB-2 inhibitor AG879 (Fig. 2A and 2B). These results are consistent with previous observations that ErbB-2 activation plays an important function in regulating androgen-stimulated proliferation of PCa cells [36]. In androgen-activated ErbB-2 cells, ERK1/2 and p38 MAPK are also activated by phosphorylation, and androgen-stimulated cell proliferation is also supported by elevated cyclin B1 and cyclin D1 protein levels (Fig. 2B, and Refs. [47]. The pharmacological inhibition revealed that both basal and androgen-induced ERK1/2 and p38 MPK activation are significantly inhibited by ErbB-2 inhibitor, correlating with abolished cell growth and decreasing cyclin B1 and cyclin D1 proteins to undetectable levels (Fig. 2B). In parallel, we also investigated the effect of EGFR-dependent

signaling by treating LNCaP C-33 cells with EGFR specific inhibitor AG1478. Interestingly, under steroid-reduced conditions, EGFR inhibitor AG1478 is able to block androgen-stimulated proliferation but not the basal cell growth (Fig. 2A), and it also reduces the basal and androgen-induced ERK1/2 phosphorylation but to a lesser degree on p38 MAPK phosphorylation and cyclin B1 protein (Fig. 2B). It has been shown that EGFR can regulate AR nuclear translocation [48] and transactivation [49]. It is thus possible that in the presence of androgens, AG1478 (EGFR inhibitor) inhibits the AR nuclear translocalization [48] and transactivation [49]; therefore AG1478 blocks androgen-induced cell proliferation and signaling. Nevertheless, further experiments are required to elucidate the mechanism that AG1478 inhibits DHT-induced AS PCa cell growth and signaling.

To further investigate androgens via ErbB-2 regulating cell proliferation, we obtained LNCaP cells that were transfected with scFv5R expression vector, which expresses Fab fragment of Ab to ErbB-2 with an ER localization domain and thus can trap de novo synthesized ErbB-2 protein in ER. Those cells do not have functional ErbB-2 protein at the plasma membrane [38]. In those ErbB-2-trapped cells, androgen-induced PCa cell proliferation was blocked, but not the basal cell growth (Fig. 2D). Conversely, we analyzed the effect of increased ErbB-2 expression on androgen sensitivity by transfecting LNCaP C-33 cells with ErbB-2 cDNA. The increased expression of functional ErbB-2 protein is significantly associated with ERK1/2 activation and androgen-independent PSA secretion and cell proliferation (Figs. 3A and 3B). Since prostate-specific tumor suppressor cPacP dephosphorylates ErbB-2 [31, 37, 40], knockdown cPacP expression in LNCaP C-33 cells by transfected with cPacP siRNA resulted in increased ErbB-2 tyrosine phosphorylation and CR cell proliferation (Figs. 3C & 3D). It should be noted that only about 25 % growth stimulation is at least in part due to the poor transfection efficiency of LNCaP C-33 cells with only about 10% efficacy. Importantly, cPacP-knockdown cells proliferated in SR conditions. Together, the data support the notion that ErbB-2 plays a critical role in regulating androgen sensitivity. The data further support the notion that androgen stimulation signaling is primarily mediated by ErbB-2 via both ERK1/2 and p38 MAPK; while EGFR is involved in transducing androgen stimulation through ERK1/2 pathway.

To determine the role of ErbB-2 vs. EGFR in mediating the proliferation signal of CR PCa cells, we used LNCaP C-81 and MDA PCa2b-AI PCa cells. Although those cells express a similar level of functional AR, both LNCaP C-81 and MDA PCa2b-AI cells show the constitutive activation of ErbB-2 and its downstream MAPK and Akt when compared to the corresponding AS PCa cells (Fig. 1). Furthermore, in androgen-deprived conditions, those CR PCa cells show higher PSA secretion and cell proliferation, a hallmark of CR PCa phenotype [30, 36, 50, 51]. These results support the notion that AR-positive LNCaP C-81 and MDA PCa2b-AI cells are suitable to serve as a clinic-relevant model to investigate the role of ErbB-2 vs. EGFR signaling in CR PCa progression under androgen deprivation therapy.

Under androgen-deprived conditions and in the presence of ErbB-2 inhibitor AG879, the growth of LNCaP C-81 and MDA PCa 2b-AI cells was significantly attenuated (Figs. 4A & 4C). In those AG879-treated cells, ErbB-2 and its downstream signaling were also effectively blocked. While AG879 treatment blocked Akt activation by phosphorylation in

LNCaP C-81 cells with a lesser degree in MDA PCa 2b-AI cells, AG879 essentially had no inhibition on ERK1/2 activation (Fig. 4), opposite to the observation in AS PCa cells (Fig. 4 vs. Fig. 2). Furthermore, AG879 treatment also decreases AR protein and activity levels in LNCaP C-81 and MDA PCa2b-AI cells. It is possible that inhibition of ErbB-2 by AG879 can decrease AR protein stability through inhibition of Akt. Supportively, overexpression of a constitutively active, myristoylated Akt (myr-Akt) in LAPC4 PCa cells markedly raised AR protein levels and PSA production in vitro and in vivo [52]. Further, it has been shown that ErbB-2-mediated signal can stabilize AR protein and optimize the binding of AR to androgen response element's (ARE's) in AR-regulated genes [52]. These results indicate that in androgen-deprived conditions, the activation of ErbB-2 via its downstream Akt, but not ERK1/2 pathway, for the survival of these CR PCa. The results corroborate observations of increased ErbB-2 and Akt activation in clinical PCa specimens [17, 19, 47, 53].

However, EGFR was neither activated nor elevated in both CR cells than corresponding AS cells even under regular culture media containing androgenic activity (Fig. 1). While EGFR inhibitor AG1478 can inhibit EGFR activation by phosphorylation in CR cells; EGFR inhibitor has no effect on CR cell proliferation, supported by no significant change on cyclin B1 or cyclin D1 protein levels or tumorigenicity (Figs. 4A & 4C). Furthermore, EGFR inhibitor has no effect on Akt or ERK1/2 activation in CR cells. The data indicate that EGFR activation in CR cells does not play a critical role; instead it is apparently a bystander effect by ErbB-2 activation. Together, our results support the notion that ErbB-2 activation plays a critical role in the progression of CR PCa cells under androgen-deprived conditions. Our data further indicate that ErbB-2 activation has differential roles in regulating AS and CR PCa cell proliferation, i.e., ErbB-2 activation via ERK1/2 cascades regulates androgen-stimulated AS cells, whereas in CR cells, ErbB-2 activates Akt pathway for the survival of those PCa cells.

In summary, utilizing two clinic-relevant PCa cell models, for the first time, we clearly demonstrate the critical role of ErbB-2 activation but not EGFR signaling in regulating the proliferation of both AS and CR PCa cells. As shown in Fig. 6, in AS/androgen-dependent cells, androgens activate ErbB-2 signaling through ERK1/2 pathway; while in AI/CR cells, ErbB-2 signaling can take over androgen dependency and promote PCa cell proliferation, which is dependent on PI3K/Akt pathway to a lesser extent on ERK1/2 cascades. The present results together with previous observations suggest that controlling ErbB-2 pathway is an attractive combinational therapeutic approach together with second generation antiandrogen agents such as enzalutamide and abiraterone acetate to overcome CR PCa.

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References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin.* 2014; 64:9–29. [PubMed: 24399786]

2. Sharifi N. New agents and strategies for the hormonal treatment of castration-resistant prostate cancer. *Expert Opin Investig Drugs*. 2010; 19:837–846.
3. Lonergan PE, Tindall DJ. Androgen receptor signaling in prostate cancer development and progression. *J Carcinog*. 2011; 10:20. [PubMed: 21886458]
4. Shiota M, Yokomizo A, Fujimoto N, Naito S. Androgen receptor cofactors in prostate cancer: potential therapeutic targets of castration-resistant prostate cancer. *Curr Cancer Drug Targets*. 2011; 11:870–881. [PubMed: 21762076]
5. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*. 2001; 1:34–45. [PubMed: 11900250]
6. Dillard PR, Lin MF, Khan SA. Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol. *Mol Cell Endocrinol*. 2008; 295:115–120. [PubMed: 18782595]
7. Devlin HL, Mudryj M. Progression of prostate cancer: multiple pathways to androgen independence. *Cancer Lett*. 2009; 274:177–186. [PubMed: 18657355]
8. Craft N, Shostak Y, Carey M, Sawyers CL. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nat Med*. 1999; 5:280–285. [PubMed: 10086382]
9. Reese DM, Small EJ, Magrane G, Waldman FM, Chew K, Sudilovsky D. HER2 protein expression and gene amplification in androgen-independent prostate cancer. *Am J Clin Pathol*. 2001; 116:234–239. [PubMed: 11488070]
10. Guyader C, Ceraline J, Gravier E, Morin A, Michel S, Erdmann E, de PG, Cabon F, Bergerat JP, Poupon MF, Oudard S. Risk of hormone escape in a human prostate cancer model depends on therapy modalities and can be reduced by tyrosine kinase inhibitors. *PLoS One*. 2012; 7:e42252. [PubMed: 22879924]
11. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J*. 2000; 19:3159–3167. [PubMed: 10880430]
12. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001; 2:127–137. [PubMed: 11252954]
13. Ratan HL, Gescher A, Steward WP, Mellon JK. ErbB receptors: possible therapeutic targets in prostate cancer? *BJU Int*. 2003; 92:890–895. [PubMed: 14632841]
14. Di LG, Tortora G, D'Armiento FP, De RG, Staibano S, Autorino R, D'Armiento M, De LM, De PS, Catalano G, Bianco AR, Ciardiello F. Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clin Cancer Res*. 2002; 8:3438–3444. [PubMed: 12429632]
15. Harper ME, Goddard L, Glynn-Jones E, Peeling WB, Griffiths K. Epidermal growth factor receptor expression by northern analysis and immunohistochemistry in benign and malignant prostatic tumours. *Eur J Cancer*. 1995; 31A:1492–1497. [PubMed: 7577078]
16. Hernes E, Fossa SD, Berner A, Otnes B, Nesland JM. Expression of the epidermal growth factor receptor family in prostate carcinoma before and during androgen-independence. *Br J Cancer*. 2004; 90:449–454. [PubMed: 14735192]
17. Fox SB, Persad RA, Coleman N, Day CA, Silcocks PB, Collins CC. Prognostic value of c-erbB-2 and epidermal growth factor receptor in stage A1 (T1a) prostatic adenocarcinoma. *Br J Urol*. 1994; 74:214–220. [PubMed: 7921940]
18. Kuhn EJ, Kurnot RA, Sesterhenn IA, Chang EH, Moul JW. Expression of the c-erbB-2 (HER-2/neu) oncoprotein in human prostatic carcinoma. *J Urol*. 1993; 150:1427–1433. [PubMed: 8105108]
19. Myers RB, Srivastava S, Oelschlager DK, Grizzle WE. Expression of p160erbB-3 and p185erbB-2 in prostatic intraepithelial neoplasia and prostatic adenocarcinoma. *J Natl Cancer Inst*. 1994; 86:1140–1145. [PubMed: 7913137]
20. Sanchez KM, Sweeney CJ, Mass R, Koch MO, Eckert GJ, Geary WA, Baldrige LA, Zhang S, Eble JN, Cheng L. Evaluation of HER-2/neu expression in prostatic adenocarcinoma: a requested for a standardized, organ specific methodology. *Cancer*. 2002; 95:1650–1655. [PubMed: 12365012]

21. Gu K, Mes-Masson AM, Gauthier J, Saad F. Overexpression of her-2/neu in human prostate cancer and benign hyperplasia. *Cancer Lett.* 1996; 99:185–189. [PubMed: 8616823]
22. Montironi R, Mazzucchelli R, Barbisan F, Stramazzotti D, Santinelli A, Scarpelli M, Lopez BA. HER2 expression and gene amplification in pT2a Gleason score 6 prostate cancer incidentally detected in cystoprostatectomies: comparison with clinically detected androgen-dependent and androgen-independent cancer. *Hum Pathol.* 2006; 37:1137–1144. [PubMed: 16938518]
23. Minner S, Jessen B, Stiedenroth L, Burandt E, Kollermann J, Mirlacher M, Erbersdobler A, Eichelberg C, Fisch M, Brummendorf TH, Bokemeyer C, Simon R, Steuber T, Graefen M, Huland H, Sauter G, Schlomm T. Low level HER2 overexpression is associated with rapid tumor cell proliferation and poor prognosis in prostate cancer. *Clin Cancer Res.* 2010; 16:1553–1560. [PubMed: 20179235]
24. Mellingerhoff IK, Tran C, Sawyers CL. Growth inhibitory effects of the dual ErbB1/ErbB2 tyrosine kinase inhibitor PKI-166 on human prostate cancer xenografts. *Cancer Res.* 2002; 62:5254–5259. [PubMed: 12234993]
25. Liu Y, Majumder S, McCall W, Sartor CI, Mohler JL, Gregory CW, Earp HS, Whang YE. Inhibition of HER-2/neu kinase impairs androgen receptor recruitment to the androgen responsive enhancer. *Cancer Res.* 2005; 65:3404–3409. [PubMed: 15833875]
26. Whang YE, Armstrong AJ, Rathmell WK, Godley PA, Kim WY, Pruthi RS, Wallen EM, Crane JM, Moore DT, Grigson G, Morris K, Watkins CP, George DJ. A phase II study of lapatinib, a dual EGFR and HER-2 tyrosine kinase inhibitor, in patients with castration-resistant prostate cancer. *Urol Oncol.* 2013; 31:82–86. [PubMed: 21396844]
27. Igawa T, Lin FF, Lee MS, Karan D, Batra SK, Lin MF. Establishment and characterization of androgen-independent human prostate cancer LNCaP cell model. *Prostate.* 2002; 50:222–235. [PubMed: 11870800]
28. Yuan TC, Veeramani S, Lin FF, Kondrikou D, Zelivianski S, Igawa T, Karan D, Batra SK, Lin MF. Androgen deprivation induces human prostate epithelial neuroendocrine differentiation of androgen-sensitive LNCaP cells. *Endocr Relat Cancer.* 2006; 13:151–167. [PubMed: 16601285]
29. Beerli RR, Wels W, Hynes NE. Intracellular expression of single chain antibodies reverts ErbB-2 transformation. *J Biol Chem.* 1994; 269:23931–23936. [PubMed: 7929040]
30. Veeramani S, Yuan TC, Chen SJ, Lin FF, Petersen JE, Shaheduzzaman S, Srivastava S, MacDonald RG, Lin MF. Cellular prostatic acid phosphatase: a protein tyrosine phosphatase involved in androgen-independent proliferation of prostate cancer. *Endocr Relat Cancer.* 2005; 12:805–822. [PubMed: 16322323]
31. Lin MF, Meng TC, Rao PS, Chang C, Schonthal AH, Lin FF. Expression of human prostatic acid phosphatase correlates with androgen-stimulated cell proliferation in prostate cancer cell lines. *J Biol Chem.* 1998; 273:5939–5947. [PubMed: 9488733]
32. Lin MF, Lee MS, Garcia-Arenas R, Lin FF. Differential responsiveness of prostatic acid phosphatase and prostate-specific antigen mRNA to androgen in prostate cancer cells. *Cell Biol Int.* 2000; 24:681–689. [PubMed: 11023645]
33. Yuan TC, Lin FF, Veeramani S, Chen SJ, Earp HS III, Lin MF. ErbB-2 via PYK2 upregulates the adhesive ability of androgen receptor-positive human prostate cancer cells. *Oncogene.* 2007; 26:7552–7559. [PubMed: 17563746]
34. Chen SJ, Karan D, Johansson SL, Lin FF, Zeckser J, Singh AP, Batra SK, Lin MF. Prostate-derived factor as a paracrine and autocrine factor for the proliferation of androgen receptor-positive human prostate cancer cells. *Prostate.* 2007; 67:557–571. [PubMed: 17221842]
35. Chou YW, Zhang L, Muniyan S, Ahmad H, Kumar S, Alam SM, Lin MF. Androgens upregulate Cdc25C protein by inhibiting its proteasomal and lysosomal degradation pathways. *PLoS One.* 2013; 8:e61934. [PubMed: 23637932]
36. Meng TC, Lee MS, Lin MF. Interaction between protein tyrosine phosphatase and protein tyrosine kinase is involved in androgen-promoted growth of human prostate cancer cells. *Oncogene.* 2000; 19:2664–2677. [PubMed: 10851066]
37. Chuang TD, Chen SJ, Lin FF, Veeramani S, Kumar S, Batra SK, Tu Y, Lin MF. Human prostatic acid phosphatase, an authentic tyrosine phosphatase, dephosphorylates ErbB-2 and regulates prostate cancer cell growth. *J Biol Chem.* 2010; 285:23598–23606. [PubMed: 20498373]

38. Qiu Y, Ravi L, Kung HJ. Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells. *Nature*. 1998; 393:83–85. [PubMed: 9590694]
39. Lee MS, Igawa T, Yuan TC, Zhang XQ, Lin FF, Lin MF. ErbB-2 signaling is involved in regulating PSA secretion in androgen-independent human prostate cancer LNCaP C-81 cells. *Oncogene*. 2003; 22:781–796. [PubMed: 12569372]
40. Lin MF, Lee MS, Zhou XW, Andressen JC, Meng TC, Johansson SL, West WW, Taylor RJ, Anderson JR, Lin FF. Decreased expression of cellular prostatic acid phosphatase increases tumorigenicity of human prostate cancer cells. *J Urol*. 2001; 166:1943–1950. [PubMed: 11586265]
41. Muniyan S, Chou YW, Ingersoll MA, Devine A, Morris M, Otero-Marrah VA, Khan SA, Chaney WG, Bu XR, Lin MF. Antiproliferative activity of novel imidazopyridine derivatives on castration-resistant human prostate cancer cells. *Cancer Lett*. 2014; 353:59–67. [PubMed: 25050738]
42. Fredericks WJ, Sepulveda J, Lai P, Tomaszewski JE, Lin MF, McGarvey T, Rauscher FJ III, Malkowicz SB. The tumor suppressor TERE1 (UBIAD1) prenyltransferase regulates the elevated cholesterol phenotype in castration resistant prostate cancer by controlling a program of ligand dependent SXR target genes. *Oncotarget*. 2013; 4:1075–1092. [PubMed: 23919967]
43. Muniyan S, Ingersoll MA, Batra SK, Lin MF. Cellular prostatic acid phosphatase, a PTEN-functional homologue in prostate epithelia, functions as a prostate-specific tumor suppressor. *Biochim Biophys Acta*. 2014; 1846:88–98. [PubMed: 24747769]
44. Lee MS, Igawa T, Lin MF. Tyrosine-317 of p52(Shc) mediates androgen-stimulated proliferation signals in human prostate cancer cells. *Oncogene*. 2004; 23:3048–3058. [PubMed: 14990987]
45. Tran C, Ouk S, Clegg NJ, Chen Y, Watson PA, Arora V, Wongvipat J, Smith-Jones PM, Yoo D, Kwon A, Wasielewska T, Welsbie D, Chen CD, Higano CS, Beer TM, Hung DT, Scher HI, Jung ME, Sawyers CL. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science*. 2009; 324:787–790. [PubMed: 19359544]
46. Attard G, Reid AH, Yap TA, Raynaud F, Dowsett M, Settatree S, Barrett M, Parker C, Martins V, Folkard E, Clark J, Cooper CS, Kaye SB, Dearnaley D, Lee G, de Bono JS. Phase I clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms that castration-resistant prostate cancer commonly remains hormone driven. *J Clin Oncol*. 2008; 26:4563–4571. [PubMed: 18645193]
47. Mukherjee R, McGuinness DH, McCall P, Underwood MA, Seywright M, Orange C, Edwards J. Upregulation of MAPK pathway is associated with survival in castrate-resistant prostate cancer. *Br J Cancer*. 2011; 104:1920–1928. [PubMed: 21559022]
48. Orio F Jr, Terouanne B, Georget V, Lumbroso S, Avances C, Siatka C, Sultan C. Potential action of IGF-1 and EGF on androgen receptor nuclear transfer and transactivation in normal and cancer human prostate cell lines. *Mol Cell Endocrinol*. 2002; 198:105–114. [PubMed: 12573820]
49. Gregory CW, Fei X, Ponguta LA, He B, Bill HM, French FS, Wilson EM. Epidermal growth factor increases coactivation of the androgen receptor in recurrent prostate cancer. *J Biol Chem*. 2004; 279:7119–7130. [PubMed: 14662770]
50. Veeramani S, Chou YW, Lin FC, Muniyan S, Lin FF, Kumar S, Xie Y, Lele SM, Tu Y, Lin MF. Reactive oxygen species induced by p66Shc longevity protein mediate nongenomic androgen action via tyrosine phosphorylation signaling to enhance tumorigenicity of prostate cancer cells. *Free Radic Biol Med*. 2012; 53:95–108. [PubMed: 22561705]
51. Meng TC, Lin MF. Tyrosine phosphorylation of c-ErbB-2 is regulated by the cellular form of prostatic acid phosphatase in human prostate cancer cells. *J Biol Chem*. 1998; 273:22096–22104. [PubMed: 9705354]
52. Mellinghoff IK, Vivanco I, Kwon A, Tran C, Wongvipat J, Sawyers CL. HER2/neu kinase-dependent modulation of androgen receptor function through effects on DNA binding and stability. *Cancer Cell*. 2004; 6:517–527. [PubMed: 15542435]
53. Grasso AW, Wen D, Miller CM, Rhim JS, Pretlow TG, Kung HJ. ErbB kinases and NDF signaling in human prostate cancer cells. *Oncogene*. 1997; 15:2705–2716. [PubMed: 9400997]

Highlights

ErbB-2 was constitutively activated in androgen-independent PCa cells.

Androgen-induced ErbB-2 activation mediates PCa cell proliferation through ERK/MAPK.

cPacP siRNA transfection increases ErbB-2 tyrosine phosphorylation and cell proliferation.

ErbB-2 is a prominent player in mediating the ligand-dependent and -independent activation of PCa cells.

Fig. 1. ErbB tyrosine phosphorylation signaling in androgen-sensitive (AS) vs. androgen-independent (AI) LNCaP and MDA PCa2b cells. **A.** Regular culture conditions: LNCaP (2×10^4 cells/cm²) and MDA PCa2b (4×10^4 cells/cm²) cells were plated in their respective culture medium for three days, and then fed with fresh medium for another 48 hours. Cells were collected in ice-cold saline, lysed and analyzed for AR, p-ErbB-2 (Tyr1221/2), p-ErbB-2 (Tyr1248), ErbB-2, p-EGFR (Tyr1173), EGFR, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p38MAPK (Thr180/Tyr182), p38 MAPK, cPacP, sPSA, cyclin B1 and cyclin D1 protein levels. β -actin protein level was used as a loading control. The conditioned medium was collected after 24 h culturing and analyzed for secreted PSA by Western blotting. A Coomassie-blue-stained protein band on the nitrocellulose membrane was used as a loading control. **B.** Steroid-reduced culture conditions: LNCaP and MDA PCa2b cells were cultured for 3 days under the same conditions described above, and then fed with fresh steroid-reduced medium for 48 hours. Cells were collected in ice-cold saline, lysed and analyzed for p-ErbB-2(Y4G10), p-ErbB-2 (Tyr1221/2), p-ErbB-2 (Tyr1248), p-ErbB-2 (Tyr1112), ErbB-2 and cPacP. **C.** Steroid-reduced culture conditions: AS and AI LNCaP and MDA PCa2b cells were seeded at a density of 3×10^4 cells/cm² in six-well plates in regular culture medium for 3 days. Forty eight hours after steroid starvation in steroid-

reduced (SR) medium, cells were then trypsinized and counted for cell growth analyses. The results presented were representative from three independent sets of experiments. * $p < 0.05$.

Fig. 2.

Effect of ErbB-2 inhibitor on androgen-induced LNCaP C-33 cell proliferation and signaling. **A.** LNCaP C-33 cells were seeded at a density of 4×10^4 cells/cm² in six-well plates in regular culture medium for 3 days. Forty eight hours after steroid starvation in steroid-reduced (SR) medium, cells were then treated with 10 nM DHT in the presence or absence of ErbB-2 inhibitor (1 μ M AG879) or EGFR inhibitor (1 μ M AG1478). After 48 hours of treatment, cells were then trypsinized and counted for cell growth analyses. The relative ratio of cell number in the experimental group to that of the control group was calculated for cell proliferation. The results presented were mean $\pm$ SE; $n=2 \times 3$. Groups were statistically significant with control, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **B.** LNCaP C-33 cells were seeded, cultured in SR conditions and treated with DHT plus AG879 or AG1478 as described above. After 24 hours of treatment, cells were collected by scraping in ice-cold saline, lysed and western blotting was performed to determine p-ErbB-2 (Tyr1221/2), p-

ErbB-2 (Tyr1248), ErbB-2, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p38MAPK (Thr180/Tyr182), p38 MAPK, Shc, cyclin B1, cyclin D1, protein levels. β -actin protein level was used as a loading control. **C.** LNCaP C-33 cells were seeded in regular culture medium for 3 days and then steroid starved for 48 hours in steroid-reduced (SR) containing 2% charcoal-treated FBS. Cells were treated with 10 ng/ml EGF in the presence or absence of ErbB-2 (1 μ M AG879) or EGFR (1 μ M AG1478) inhibitors for 30 minutes. Cells were collected in ice-cold saline, lysed and analyzed for p-EGFR (Tyr1173), EGFR, p-ERK1/2 (Thr202/Tyr204) protein levels. β -actin protein level was used as a loading control. The results presented were representative from two sets of independent experiments. **D.** LNCaP C-33 cells stably transfected with ScFv5R vector and control cells transfected with vector alone were plated in regular culture medium for three days and then steroid starved for two days. Cells were then fed with fresh steroid-reduced medium with or without DHT for 4 days with a change of fresh SR media at 48 hours. After 4 days, cell growth was determined by cell number counting. The relative ratio of cell number in the DHT-treated group to that of the control group was calculated for cell proliferation. The results presented were mean $\pm$ SE; n=3. *p<0.05.

Fig. 3.

Effect of ErbB-2 elevation and cPACP knockdown on ErbB-2 phosphorylation and androgen-independent growth of AS LNCaP C-33 cells. **A.** LNCaP C-33 cells were transiently transfected with Myc-tagged ErbB-2 cDNA. Control cells were transfected with the vector alone (Vec). The transfected cells were cultured in regular culture medium for 72 hours, and then fed with SR medium for another 48 hours. Cells were analyzed for ErbB-2 expression and activation and ERK1/2 activation. The conditioned medium was collected after 24 h culturing and analyzed for secreted PSA by Western blotting. A Coomassie-blue-stained protein band on the nitrocellulose membrane was used as a loading control. **B.** ErbB-2 cDNA-transfected LNCaP C-33 cells were cultured in regular culture medium for 72 hours, and then fed with SR medium for another 48 hours. Cells were the collected by

trypsinization and counted for cell growth analysis. **C.** LNCaP C-33 cells were seeded in regular culture medium for 2 days and then transfected with siPacP oligos. Control cells were transfected with scrambled oligos (Oligo). The transfectants were cultured in SR medium containing 10 % charcoal-treated FBS for additional 48 hours. Cells were harvested, lysed, and analyzed for cPacP and tyrosine phosphorylation levels of ErbB-2 and SHP-1 and SHP-2 protein levels. Membranes were stripped and probed for ErbB-2. β -actin protein level was used as a loading control. **D.** LNCaP C-33 cells transfected with siPacP oligos were cultured for 48 hours in SR medium as described above. Cell were the collected by trypsinization and counted for cell growth analysis. $n = 2 \times 3$. * $p < 0.05$ vs. Control. Inset: Western bot analyses of cPacP and PCNA protein levels in the lysate from cells for growth determination. β -actin protein level was used as a loading control.

Fig. 4.

ErbB-2 and EGFR signaling in castration-resistant (CR) PCa cell growth. **A.** LNCaP C-81 cells at a density of 2×10^4 cells/cm² were seeded in 6-well plates in regular culture medium for 3 days. Cells were then steroid starved for 48 hours in steroid-reduced (SR) containing 2% charcoal-treated FBS. After fed with fresh SR medium, cells were treated with either 1 μ M AG879 or AG1478 or both. After 24 hours, cells were trypsinized and cell number was determined by counting. The relative ratio of cell number in the ErbB-2 and EGFR inhibitor treated group to that of the control group was calculated for cell proliferation. The results presented were mean $\pm$ SE; $n=2 \times 3$. * $p < 0.05$; ** $p < 0.01$. **B.** LNCaP C-81 cells were seeded in T25 flasks and maintained under the same conditions as described above. After 24 hours of treatment with inhibitors, cells were collected in ice cold saline, lysed and analyzed for p-ErbB-2 (Tyr1221/2), p-ErbB-2 (Tyr1248), ErbB-2, Shc, cyclin B1, cyclin D1, p-Akt (Ser473), Akt, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p38MAPK (Thr180/Tyr182) and p38 MAPK protein levels. β -actin protein level was used as a loading control. The results presented were representative from two individual set of experiments. **C.** MDA PCa2b-AI cells at a density of 4×10^4 cells/cm² were seeded in 6-well plates in regular culture medium for 3 days. Cells were then steroid starved for 48 hours in steroid-reduced (SR) containing 5% charcoal-treated FBS. After fed with fresh SR medium, cells were treated with either 1 μ M AG879 or AG1478 or both for 24 hours. After 24 hours, cells were trypsinized and cell growth was determined. The relative ratio of cell number in the ErbB-2 and EGFR inhibitor treated group to that of the control group was calculated for cell proliferation. The results presented were mean $\pm$ SE; $n=2 \times 3$. * $p < 0.05$. **D.** MDAPCa2b AI cells were seeded in T25 flasks and maintained under the same conditions as described above. After 24 hours of

treatment with inhibitors, cells were collected in ice cold saline, lysed and analyzed for p-ErbB-2 (Tyr1221/2), p-ErbB-2 (Tyr1248), ErbB-2, Shc, cyclin B1, cyclin D1, p-Akt(Ser473), Akt, p-ERK1/2 (Thr202/Tyr204), ERK1/2, p-p38MAPK (Thr180/Tyr182), p38 MAPK protein levels. β -actin protein level was used as a loading control. The results presented were representative from two independent sets of experiments.

Fig. 5.

Effects of ErbB-2 and EGFR inhibitors on the *in vitro* tumorigenicity of LNCaP C-81 cells.

A. LNCaP C-81 cells at the densities of 5×10^3 cells were plated on the top layer containing 0.25% agarose with a bottom layer of 0.3% agarose in 35 mm dishes. After 24 hours, dishes were checked for any double cells. Dishes with single cells were then randomly assigned to different groups, treated with either 1 μ M AG879 or AG1478 or both for every three days. At the end of four weeks, the colonies grown were stained and counted. **A.** Representative images of colony formation. Bottom: microscope enlarged images. **B.** Number of colonies in respective groups (expressed as total colony number). The results presented were mean $\pm$ SE; n=3. *p<0.05, **p<0.01.

Fig. 6.

Schematic representation of ErbB-2 signaling in AS/androgen-dependent and castration-resistant/AI PCa cell proliferation. **Left panel:** In AS/androgen-dependent PCa cells, in the absence of androgen, the activation by phosphorylation of ErbB-2 is kept in control by the high level of prostate-specific cPacP. The addition of androgens decreases cPacP and thus ErbB-2 is activated by tyrosine phosphorylation which in turn activates ERK1/2 and p38 MAPK and increases PCa cell proliferation and tumorigenicity. **Right Panel:** The progression of AS PCa cells towards advanced CR/androgen-independent PCa is accompanied by early decrease/loss of cPacP expression in PCa cells, resulting in hyper-tyrosyl-phosphorylation of ErbB-2/HER-2/neu. ErbB-2 activation can in turn activate downstream PI3K/Akt signaling for the survival and proliferation of CR PCa cells under androgen-ablated conditions. Abbreviation: Akt, Protein kinase B; AR, Androgen receptor; cPacP, cellular prostatic acid phosphatase; CR, Castration-resistance; DHT, 5 α -dihydrotestosterone; ERK, Extracellular signal-regulated kinase; ErbB-2, Epidermal growth factor receptor-2; P13K, Phosphatidylinositol 3 kinase; Hsp, Heat shock protein.