The significance of Her2 on androgen receptor protein stability in the transition of androgen requirement in prostate cancer cells

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PROSTATE CANCER IS AN AGE-RELATED CARCINOMA and the most commonly diagnosed malignancy among men (28). Although prostate-specific antigen (PSA), a biomarker of hypertrophy in the prostate gland, helps to identify prostate cancer in the early stages, the disease still has a high mortality rate. Traditionally, gonadectomy is the main therapeutic procedure for androgen-dependent prostate cancer. Once the cancer escapes from androgen dependence and becomes androgen independent, radio- or chemotherapies are subsequently applied. Unfortunately, the treatment of hormone-refractory prostate cancer at this stage is often ineffective, and the mechanisms of prostate cancer progression in this stage remain to be elucidated.

Therefore, it is imperative to understand the androgen requirement transition and to develop strategies for prolonging the survival of patients with recurrent and hormone-refractory prostate cancer.

The androgen receptor (AR), a member of the steroid receptor family, plays a decisive role in the development of the prostate gland and in the pathogenesis and progression of prostate cancer. AR binds to androgen response elements (AREs), thereby mediating androgen-regulated gene expression (12). A growing number of clinical investigations show amplifications of AR and AR-regulated genes in hormone-refractory prostate cancer, which suggests that the AR signaling pathway is still activated and important in limiting androgen concentrations (14). Previous research indicates that elevated AR expression levels correlate to anti-androgen therapy resistance (3). The cross-talk between receptor tyrosine kinases with their cognate ligands and AR signaling in the transition of hormone requirement of prostate cancer has also been addressed (6, 11, 29). Additionally, Her2/ErbB3 signals have been suggested to stabilize AR proteins and to increase the interaction of AR to promoter/enhancer regions of the AR-regulated gene in androgen-dependent prostate cancer cells (25).

Here, we established an androgen-independent prostate cancer cell line, LNCaPdccc, by incubating LNCaP cells in androgen-deprived conditions. We tried to take advantage of this cell modeling strategy to determine how prostate cancer cells maintain AR protein levels and activation in an androgen-free environment. Indeed, we observed several characteristics that markedly changed after androgen deprivation. Importantly, our data showed that LNCaPdccc cells were more sensitive to Her2 inhibition with the increase of AR phosphorylation in parental LNCaP cells. Taken together, our data demonstrate that Her2 plays an important role in the support of AR protein stability in the transition of androgen requirement in prostate cancer cells. We hope these findings will provide novel insight into the treatment of hormone-refractory prostate cancer.

Materials and Methods

Hsu FN, Yang MS, Lin E, Tseng CF, Lin H. The significance of Her2 on androgen receptor protein stability in the transition of androgen requirement in prostate cancer cells. Am J Physiol Endocrinol Metab 300: E902–E908, 2011. First published March 1, 2011; doi:10.1152/ajpendo.00610.2010.— Androgen ablation therapy is the most common strategy for suppressing prostate cancer progression; however, tumor cells eventually escape androgen dependence and progress to an androgen-independent phase. The androgen receptor (AR) plays a pivotal role in this transition. To address this transition mystery in prostate cancer, we established an androgen-independent prostate cancer cell line (LNCaPdccc), by long-term screening of LNCaP cells in androgen-deprived conditions, to investigate changes of molecular mechanisms before and after androgen withdrawal. We found that LNCaPdccc cells displayed a neuroendocrine morphology, less aggressive growth, and lower expression levels of cell cycle-related factors, although the cell cycle distribution was similar to parental LNCaP cells. Notably, higher protein expression of AR, phospho-Ser81-AR, and PSA in LNCaPdccc cells were observed. The nuclear distribution and protein stability of AR increased in LNCaPdccc cells. In addition, cell proliferation results exhibited the biphasic nature of the androgen (R1881) effect in two cell lines. On the other hand, LNCaPdccc cells expressed higher levels of Her2, phospho-Tyr1221/1222-Her2, ErbB3, and ErbB4 proteins than parental LNCaP cells. These two cell lines exhibited distinct responses to Her2 activation (by heregulin treatment) on Her2 phosphorylation and Her2 inhibition (by AG825 or Herceptin treatments) on proliferation. In addition, the Her2 inhibitor more effectively caused AR degradation and diminished AR Ser81 phosphorylation in LNCaPdccc cells. Taken together, our data demonstrate that Her2 plays an important role in the support of AR protein stability in the transition of androgen requirement in prostate cancer cells. We hope these findings will provide novel insight into the treatment of hormone-refractory prostate cancer.

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Lake Placid, NY), AR (sc-13062 and sc-7305; Santa Cruz Biotechnology), PSA (sc-7316; Santa Cruz Biotechnology), α-tubulin (05-829; Upstate Biotechnology), poly(adenosine 5′-diphosphate ribose)polymerase (06-557; Upstate Biotechnology), phospho-

Trypan blue assay. LNCaP and LNCaPdccc cells were seeded in a 24-well plate in complete culture medium. Cells were trypsinized, stained with 0.2% trypan blue (Sigma), and counted by hemocytometer to distinguish the live and dead cells.

Analysis of cell cycle distribution. Propidium iodide staining was used for DNA content measurement. Cancer cells, which were trypsinized and fixed in 70% ethanol, were washed once with PBS and treated with RNase A for 30 min, followed by staining with propidium iodide (0.1% sodium citrate, 0.1% Triton X-100, and 20 μg/ml propidium iodide). DNA content was measured using flow cytometry (FACS Calibur). The percentage of cells in each phase of the cell cycle was analyzed by the software Cell Quest (2a).

Statistics. All values are given as means ± SE. The Student t-test was used to compare the quantified cell proliferation and blotting results. A difference between two means was considered statistically significant when P < 0.05 (16a).

RESULTS

Comparisons of characteristics of LNCaPdccc vs. parental LNCaP cells. The LNCaPdccc cells displayed dendritic-like morphology and neuroendocrine differentiation compared with parental LNCaP cells (Fig. 1A). In addition, the growth curves of the two cell lines were determined by cell counting (Fig. 1B). The comparison of doubling time (Fig. 1B, inset) showed that LNCaPdccc cells grew much slower than parental LNCaP cells. Using flow cytometry, the differences of cell cycle distribution between parental LNCaP and LNCaPdccc cells were identified. The data showed that the S phase distribution of LNCaPdccc cells was markedly higher than that of parental LNCaP cells, although the G1 and G2/M phase distributions of two cell lines were similar (Fig. 2A). Therefore, it is interesting to understand the levels of cell cycle-related proteins expressed in the two cell lines. The results revealed that the protein levels of Cdk1, cyclin A, cyclin B1, and cyclin D1 were all lower in LNCaPdccc cells (Fig. 2B), which might explain why LNCaPdccc cells grew slowly.

AR-related features in two cell lines. Compared with parental LNCaP cells, LNCaPdccc cells expressed higher protein levels of phospho-Ser81-AR, AR, and PSA (AR-regulated gene; Fig. 3A). In addition, protein fractionation was utilized to investigate the subcellular distribution of AR proteins. Notably, compared with parental LNCaP cells, LNCaPdccc cells contained higher levels of nuclear AR protein (Fig. 3B), indicating that ARs in LNCaPdccc cells are still activated even in the absence of androgen. Then, cycloheximide (CHX) was used to block protein synthesis, and the degradation of existing proteins was monitored. The result exhibited that AR proteins in LNCaPdccc cells were more stable than those in parental LNCaP cells (Fig. 3C). The individual control value of each cell line was 100%. The quantitative results from triplicate independent experiments were used to assess the mean percentages of AR protein degradation compared with controls. After a 6-h treatment with CHX, the AR degradation percentage of parental LNCaP (quantitative ratio) was 27%, which was significantly higher than 11% of LNCaPdccc cells (Fig. 3D). Subsequently, the cell proliferation in response to androgen treatment was investigated using the MTT assay. Parental LNCaP cell proliferation was sensitive to synthetic androgen R1881 under steroid-deprived conditions, especially at limiting concentrations (0.1 and 1 nM). However, the proliferation of LNCaPdccc cells was inhibited at low concentrations (Fig. 4).
The result illustrates that the proliferation of LNCaPdcc cells is androgen independent.

Her2-related features in two cell lines. According to previous research, there is a correlation between AR and Her2 signals in androgen-dependent prostate cancer cells (25). Therefore, the protein expression of Her2 and its activation partners ErbB3 and ErbB4 in the two cell lines was investigated. The data showed that LNCaPdcc cells expressed higher levels of phospho-Tyr1221/1222-Her2, Her2, ErbB3, and ErbB4 (Fig. 5A). Because Her2 activation was correlated to its phosphorylation status, the quantitative ratio of p-Tyr1221/1222-Her2/Her2 shown in Supplemental Fig. S1 (Supplemental Material for this article can be found online at the AJP-Endocrinology and Metabolism website) implies that Her2 is more active in LNCaPdcc cells than in parental LNCaP cells. In addition, both parental LNCaP and LNCaPdcc cells were treated with 10 ng/ml heregulin β1 (HRGβ1; ligand of Her2/ErbB3 and Her2/ErbB4) in a time course manner. HRGβ1-induced Her2 activation in parental LNCaP cells was detected 1 h after treatment and then quickly dropped; however, activation in LNCaPdcc cells was delayed (Fig. 5B). These data suggest that the kinetics of Her2 activation is different in the two cell lines. To understand the physiological functions of Her2 in different cell lines, the effects of Her2 inhibitors on the proliferation of the two cell lines were evaluated using the MTT assay. AG825 and Herceptin (monoclonal antibody of Her2 for clinical use) were used to treat both cell lines. Parental LNCaP cell proliferation was low in response to both Her2 inhibitors, whereas LNCaPdcc cell proliferation declined significantly in response to Her2 inhibition (Fig. 5C). These findings may be due to the high levels of Her2 in LNCaPdcc cells. Accordingly, Her2 levels in LNCaPdcc cells could more strongly affect LNCaPdcc proliferation compared with parental LNCaP cells.

AR stability in LNCaPdcc cells depends on high Her2 activation. AR is a protein with a short half-life that tends to be degraded through the ubiquitin-proteasome pathway (30). It has been reported that AR proteins can be stabilized by Her2/ErbB3 activation (25). In addition, our data indicated that AR protein levels correlated to Her2 activation in both cell lines (data not shown). To determine whether Her2 is involved in the increased AR stability associated with the transition to androgen independence of prostate cancer (Fig. 3C), Her2 inhibition (by AG825) was performed and the AR stability of the two cell lines monitored. The results showed that Her2
inhibition accelerated AR degradation in LNCaPdcc cells (Fig. 6A and B), although the initial level of AR protein in LNCaPdcc cells was still higher than in parental LNCaP cells (time = 0; Fig. 6A). After a 9-h treatment with CHX, the AR degradation percentage of LNCaPdcc (quantitative ratio) was 70%, which is significantly higher than 50%, as observed in parental LNCaP cells (Fig. 6B). Furthermore, Ser81 phosphorylation of AR has been reported to be responsible for receptor stability (23). Corresponding to previous research (25), Her2 inhibitor effectively reduced AR Ser81 phosphorylation in both cell lines (Fig. 6C). Notably, LNCaPdcc cells were more sensitive to AG825 treatment on the inhibition of AR Ser81 phosphorylation (40% inhibition in LNCaPdcc cells vs. 20% inhibition in parental LNCaP cells). Taken together, higher Her2 activation may play an important role in AR protein stability through phosphorylation of Ser81 in LNCaPdcc cells.

**DISCUSSION**

Prostate carcinoma is a leading cause of death in males. Because the prostate is an androgen-dependent gland, androgen ablation therapy is the most frequent strategy used to suppress prostate tumor pathogenesis. Nevertheless, cancer cells eventually escape androgen dependence and progress to an androgen-independent phenotype. The treatment for hormone-refractory prostate cancer remains clinically challenging. As prostate cancer progresses, the AR emerges as an important prognostic determinant. AR controls cell cycle, cell proliferation, inhibition of apoptosis, regulation of angiogenic growth factors, and stimulation of cellular migration (5). To investigate the mechanisms of AR modulation in therapy-resistant prostate cancer progression following androgen withdrawal, we established a novel androgen-independent prostate cancer subline, LNCaPdcc, which approximates the situation in patients with hormone-refractory prostate carcinoma.

Initially, LNCaPdcc cells revealed a dendritic-like morphology (Fig. 1A) and a lower growth rate (Fig. 1B), indicating the adaptation of LNCaPdcc cells to androgen-free conditions. Notably, AR proteins of LNCaPdcc cells were more active in the absence of androgen, as evidenced by higher levels of AR Ser81 phosphorylation, PSA proteins (Fig. 3A), and nuclear AR proteins (Fig. 3B) in LNCaPdcc cells. These findings may be due to the excessive recruitment of coactivators (10) or cross-talk between several polypeptide growth factors and cognate receptors (24, 31) in the androgen requirement transition of prostate cancer. Additionally, cyclin D1 was reported to interact predominantly with the NH2-terminal domain of AR, and this interaction depends on the presence of the AR 23FxxL27 motif, which

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**Fig. 2. Analyses of cell cycle distribution and cell cycle-related protein expression in both cell lines. A: cells were stained with propidium iodide for 30 min, followed by the analysis of flow cytometry, as described in MATERIALS AND METHODS (n = 3). The average distribution of the cell cycle is indicated. The values of error bars were given as means ± SE; **P < 0.01 vs. parental LNCaP cells; **B: immunoblotting was performed, and specific antibodies were utilized to investigate the expression levels of the indicated proteins. β-Actin served as an internal control.**

**Fig. 3. Comparisons of androgen receptor (AR)-related proteins, AR subcellular distribution, and AR stability between the 2 cell lines. A: immunoblotting was performed, and specific antibodies were utilized to investigate the levels of protein expression and phosphorylation. B: protein fractionation was performed on LNCaP and LNCaPdcc cell lysates. AR proteins were immunoblotted in both nuclear and cytosolic fractions. Polyclonal antisera against AR, poly(adenosine 5’-diphosphate ribose)polymerase (PARP), and α-tubulin served as markers for the nuclear and cytosolic fractions, respectively. C: LNCaP and LNCaPdcc were treated with cycloheximide (CHX; 10 μg/ml) for 0, 3, 6, or 9 h. Endogenous AR protein degradation was monitored by immunoblotting. The numbers below the gel images represent the fold change normalized to the control group of parental LNCaP cells. D: the quantitative results revealed the mean percentages of AR protein degradation compared with the individual control. The independent immunoblot experiment was repeated 3 times. Data are represented as means ± SE; *P < 0.05 vs. parental LNCaP cells.**
is also important for interaction between the NH2 and COOH termini of AR. Through this motif, cyclin D1 protein prevents the interaction between the two termini of AR, consequently inhibiting AR activity (2). Our data revealed that cyclin D1 proteins dramatically declined in LNCaPdcc cells (Fig. 2B), illustrating that the decrease in cyclin D1 levels might help to increase AR activation. In addition, we found that parental LNCaP and LNCaPdcc cells displayed altered proliferation indices in response to androgen stimulation (Fig. 4). LNCaPdcc (characterized by us), LNCaP-abl [generated by Culig et al. (7)], and LNCaP 104-R [established by Kokontis et al. (15)] share the same proliferative response features to androgen. Cellular proliferation of all three androgen-independent prostate cancer cell lines generated from similar strategy was inhibited by androgen (R1881) treatment. Previous research indicated that the androgenic repression of androgen-independent prostate cancer cells (LNCaP 104R) is due to the accumulation of the Cdk inhibitor p27kip1, which results in Cdk2 inhibition and cell cycle arrest (16). Thus, parental LNCaP and LNCaPdcc cells showed a biphasic proliferative response to androgen (Fig. 4). In addition, it has been reported that the AR in LNCaP cell line is a T877A mutant that can be activated not only by androgens but also by nonandrogenic steroid hormones and antiandrogens (35). Our unpublished data showed that parental LNCaP cell proliferation was stimulated significantly by estradiol benzoate (EB; synthetic estrogen) in a dose-dependent manner (Fig. 2A), suggesting that the AR Ser81 site modification of AR has also been discussed extensively (8). The existence of AR Ser81 phosphorylation is correlated to AR protein stability (23). On the other hand, the Her2-ErbB3 axis has been reported to provide signals to AR, which protects AR protein stability (25). This research also demonstrates that the androgen-induced Ser81 phosphorylation of AR is declined by a small molecule Her2 inhibitor, PKI-166 (25). Additionally, our findings indicated that AR protein levels seem to be positively regulated by Her2 activity but not by epidermal growth factor receptor activation (data not shown). These results suggest the existence of a specific and enhanced regulation between Her2 activation and AR stability in LNCaPdcc cells. Our findings also indicated that AR Ser81 phosphorylation was inhibited by Her2 inhibitors (Fig. 6C), suggesting that the AR Ser81 site

Fig. 5. Comparison of Her2-related issues between the 2 cell lines. A: immunoblotting was performed, and specific antibodies were utilized to investigate the levels of protein expression and phosphorylation in LNCaP and LNCaPdcc cells. B: heregulin β1 (HRGβ1) was used to treat both cell lines at the concentration of 10 ng/ml in a time course manner (0, 1, 12, and 24 h) under serum-free conditions. Immunoblotting was performed, and specific antibodies were utilized to investigate the levels of protein expression and phosphorylation in LNCaP and LNCaPdcc cells. C: the cells were seeded separately in 96-well plates, as described in Fig. 4. After cells attached, AG825 (25 μM) and Herceptin (20 ng/ml) were added in respective complete medium. Cell proliferation was analyzed using the MTT assay (n = 8). Control values of cell proliferation were set at 100%. The error bars represent means ± SE. **P < 0.01 vs. control group of LNCaP cells; #P < 0.01 and #P < 0.05 vs. control group of LNCaPdcc cells.

Fig. 4. Difference of androgen sensitivity on proliferation of the 2 cell lines. The cells were seeded separately in 96-well plates at densities of 1.5 × 10⁶ (LNCaP) and 2 × 10⁶ cells/well (LNCaPdcc) in steroid-deprived medium. After 48 h, R1881 (synthetic androgen) was added to the medium at concentrations of 0, 0.1, 1, or 10 nM for 4 days. Cell proliferation was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (n = 8). The control value of cell proliferation was set at 100%. The error bars represent means ± SE. **P < 0.01 vs. control group of LNCaP cells; #P < 0.01 and #P < 0.05 vs. control group of LNCaPdcc cells.

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is a downstream substrate of the Her2 pathway. In regard to Her2 downstream serine-threonine kinases, it has been reported that Akt/protein kinase B is not the kinase that phosphorylates AR Ser81 site due to the analysis of phosphorylation consensus sequence sites (25). Although the Ser81 site occurs in the consensus sequence of protein kinase C (PKC), PKC inhibitors fail to reduce AR Ser81 phosphorylation (9). Several kinases are predicted candidates for phosphorylating the AR Ser81 site, such as Cdk1, Cdk5 (4), and Erk (33). However, Cdk1 activation is inhibited by Her2 via phosphorylation of the Tyr15 site (34). Moreover, Cdk1 proteins diminished in our LNCaPdccc cells (Fig. 2B), illustrating that the increasing levels of Her2-dependent AR Ser81 phosphorylation might be irrelevant to Cdk1 activity. In contrast, we have reported that Cdk5 activity is elevated by Her2 activation through Tyr15 phosphorylation in thyroid cancer cells (18). In addition, we found that Cdk5 is also reported to modulate androgen production (19) and cell fate of prostate cancer (17, 20). With regard to Erk, we found that both phospho-Erk1/2 and Erk1 levels increased in LNCaPdccc cells compared with parental LNCaP cells (Supplemental Fig. S2). The specific kinases that are regulated by Her2 and are responsible for AR Ser81 phosphorylation need to be investigated further.

According to the results in Fig. 3, LNCaPdccc cells displayed higher levels of AR Ser81 phosphorylation and longer half-lives of AR proteins in an androgen-stripped environment. Coincidentally, LNCaPdccc cells expressed higher levels of phospho-Her2 and Her2 proteins (Fig. 5A). Using Her2 inhibitors, Her2 in LNCaPdccc cells was more sensitive to its inhibitors and resulted in drops of either AR Ser81 phosphorylation or AR protein stability (Fig. 6). Notably, comparing the quantification ratio after a 9-h treatment of CHX, as seen in Figs. 3C and 6A, the relative AR levels of LNCaPdccc cells were obviously lower after Her2 inhibition (1.28 vs. 0.31), whereas the relative AR levels of parental LNCaP cells were similar (0.69 vs. 0.68). These results suggest that Her2 not only plays a role as a growth factor receptor but also protects AR protein stability through Ser81 phosphorylation in LNCaPdccc cells after cells escape androgen requirement.

In conclusion, we used a newly established prostate cancer cell subline, LNCaPdccc, to elucidate different characteristics and protein expression patterns compared with parental LNCaP cells with androgen-independent features. We first found that, in LNCaPdccc cells, Her2 activation becomes more important to protect AR protein stability through Ser81 phosphorylation and thus modulates cell proliferation. Based on previous studies indicating Her2 protein overexpression and/or gene amplification in prostate cancer patients, especially in the androgen-independent phase of the disease (27, 32), we used Her2 modulation in our androgen-independent tumor model to demonstrate the significance of Her2 on AR protein stability in prostate cancer cells after androgen withdrawal. We hope our findings will be helpful in understanding the transition to androgen independence. Furthermore, we also propose that the Her2-AR axis could become a diagnostic and therapeutic target in hormone therapy-resistant prostate cancer in the near future.

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DISCLOSURES

The authors have no conflicts of interest to declare.
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