EGF promotes neuroendocrine-like differentiation of prostate cancer cells in the presence of LY294002 through increased ErbB2 expression independent of the phosphatidylinositol 3-kinase-AKT pathway

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An increased neuroendocrine (NE) cell population in prostate cancer is associated with higher aggressiveness and recurrence after androgen-deprivation therapy, although the mechanism responsible is unknown. In this study, we report that the treatment of LNCaP cells with epidermal growth factor (EGF) in the presence of LY294002, an inhibitor of the phosphoinositol 3-kinase (PI3K)-AKT pathway, induced an increase of levels and activity of ErbB2. Under these conditions, we also observed cell survival and NE differentiation. When we treated with Wortmannin, another PI3K inhibitor, or we knocked down PI3K or AKT isoforms in the presence of EGF, ErbB2 up-regulation was not observed, suggesting that the increase of ErbB2 induced by EGF plus LY294002 is not mediated by the PI3K-Akt pathway. Other targets of LY294002 were also discounted. We also show that ErbB2 up-regulation is directly involved in neuroendocrine differentiation but not in cell survival as ErbB2 levels increased in parallel with NE differentiation marker levels, whereas ErbB2 knockdown reduced them; other NE differentiation inducers also increased the ErbB2 levels and the immunohistochemical analysis of prostate cancer samples showed colocalization of ErbB2 and chromogranin A. We found that, in LNCaP cells, EGF in combination with LY294002 increased ErbB2 levels by a PI3K/AKT-independent mechanism and that this increase was associated with the acquisition of a NE phenotype. These results suggest that is worth reconsidering ErbB2 as a drug target in prostate cancer and this should be kept in mind when designing new clinical schedules for the treatment of this disease.

Introduction

Prostate cancer is one of the most common malignancies among men in the western world and a major health problem in many industrialized countries. The tumor is initially androgen dependent in the majority of cases, so endocrine manipulation is the first-line therapy for metastatic and locally advanced cancers and often leads to regression of the disease (1). However, this period of remission is invariably followed by tumor relapse, and the cancer is commonly referred to as androgen-independent prostate cancer. At this stage, available treatment options are only palliative (2,3). Therefore, an understanding of what drives progression to androgen independence is critical. In fact, the prostate is known to be dependent not exclusively on androgens but also on growth factors and neuropeptides secreted by neuroendocrine (NE) cells that maintain normal prostate function and play a role in the development of pathological conditions (4). In prostate tumour lesions, cells appear showing features similar to NE cells and are called NE-like cells. These cells lack nuclear androgen receptors (5) and thus represent an androgen-insensitive cell phenotype in the prostate. In fact, long-term androgen-ablation therapy tends to select for prostate tumour populations that are enriched in NE cells (6,7). NE cells can lead to the development and growth of androgen-refractory prostate tumours through the secretion of neuropeptides that induce the proliferation of adjacent carcinoma cells in an androgen-depleted condition (8). Those tumours with an increased NE cell population are often more aggressive and have a poorer prognosis (9,10). Therefore, it has been hypothesized that NE differentiation is an intermediate step between the androgen-dependent and the androgen-independent stages (8). However, the molecular mechanisms of NE cell origin and enrichment remain an enigma.

One of the most important mitogenic factors known to regulate normal prostate function is epidermal growth factor (EGF) (11,12). EGF binds specifically to ErbB1 or epidermal growth factor receptor (EGFR), the prototypical member of the ErbB family of receptors that also includes ErbB2 (HER2, neu), ErbB3 (HER3) and ErbB4 (HER4) (13,14). Ligand binding to a cognate ErbB receptor induces receptor homodimerization and heterodimer formation, leading to stimulation of the intrinsic tyrosine kinase activity of the receptor. These activated receptor tyrosine kinase complexes activate, in turn, a number of cytoplasmic signalling pathways, including the mitogen-activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K)/Akt pathways (14). Although ErbB2 is a ligandless receptor, it appears to be the preferred heterodimerization partner of all ErbB proteins and is one of the best-studied genes involved in human malignancy (15,16). There have been conflicting results relating to ErbB2 protein expression in prostate cancer, but recently a study of a long cohort of patients has demonstrated that low-level ERBB2 overexpression occurs at a significant frequency in prostate cancer and is associated with rapid tumour cell proliferation and poor prognosis (17). Several studies have raised the possibility that ErbB2 overexpression contributes to androgen independence of prostate cancer, promoting the transcriptional activity of androgen receptor (18–20).

In a previous report, our group showed that PI3K/Akt is a modulator of the role of EGF in prostate cancer cells (21). The PI3K/Akt pathway is constitutively active in LNCaP cells due to a mutation in the PTEN tumour-suppressor gene, a negative regulator of this pathway (22). This pathway is responsible for cell survival in the absence of androgens because treatment with the PI3K inhibitor LY294002 leads to apoptosis (23). This apoptotic effect can be antagonized by different ligands of EGFR, including EGF (24). Consistent with these data, we found that EGF rescued LNCaP cells from LY294002-induced apoptosis and promoted NE differentiation, suggesting that abrogation of PI3K/Akt signalling changed the proliferative role of EGF to one of the differentiation (21). The aim of the present study was to clarify the molecular mechanisms by which EGF in combination with LY294002 promotes NE differentiation.

Materials and methods

Cell culture

The prostate cancer cell line LNCaP was obtained from the American Type Culture Collection and cultured in RPMI medium containing antibiotics and 7% fetal bovine serum (Invitrogen, Prat de Llobregat, Barcelona, Spain).

Abbreviations: CK2, casein kinase 2; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; NE, neuroendocrine; NSE, neuron-specific enolase; PBS, phosphate-buffered saline; PI3K, phosphoinositol 3-kinase; siRNA, small interfering RNA; TMRM, tetramethylrhodamine methyl ester.
EGF, wortmannin (W), interleukin-6 (IL-6) were purchased from Sigma Aldrich Ibérica S.A., (Madrid, Spain). Forskolin (Fsk) was purchased from Merck (Darmstadt, Germany). Trastuzumab was obtained from Roche Farma (Madrid, Spain). The corresponding antibodies used for these analyses included the following: anti-chromogranin A, anti-ephrin-erbB2 (Y1248 clone PN2A), anti-erbB2 (clone 3B5) and anti-neu-specific enolase (NSE) from Lab Vision Corporation Neomarkers (Thermo Scientific, Fremont, CA); anti-phospho-erbB2 (Y877) from SAB, signalway antibody (Pearland, TX); anti-phospho-AKT (S473), anti-AKT1 (1H10), anti-AKT2 (SB5), anti-phospho-EGFR (Y1068) and anti-phospho-p44/42 MAPK (T202/Y204) from Cell Signalling Technology (Izasa S.A., Barcelona, Spain); anti-phospho-AKT (T308) and anti-Erk from Santa Cruz Biotechnology (Quimonig S.L., Madrid, Spain); anti-EGFR from BD Biosciences (San Agustín de Guadalix, Madrid, Spain); anti-jun-tubulin (clone TUB2.I) from Sigma (Sigma–Aldrich Ibérica S.A.); Alexa dye-conjugated secondary antibodies from Invitrogen (Prat de Llobregat, Barcelona, Spain).

**Western blot analysis**

Cells were treated as indicated in the figure legends. They were then collected, washed twice in cold phosphate-buffered saline (PBS) and solubilised with 50 mM Tris–HCl buffer (pH 7.5) containing 140 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.3 mg/ml soybean trypsin inhibitor and 0.1 mM phenylmethylsulphonyl fluoride (buffer A) in the presence of 1% Triton X-100 and 0.5 mM sodium orthovanadate. The mixture was gently agitated for 30 min at 4°C and then centrifuged at 18 500g for 20 min. Soluble proteins (50–90 μg) were resolved on 7.5% sodium dodecyl sulphate polyacrylamide gels, transferred to a nitrocellulose membrane and immunoblotted with primary antibodies. Immunoreactive proteins were visualized by the ECL immunodetection system (Pierce, Rockford, IL) with horseradish peroxidase-conjugated secondary antibodies and quantified using the Image Scion programme (Scion Corporation, Frederick, MD).

For immunoprecipitation, soluble proteins (400–600 μg) were incubated for 2 h at 4°C with an anti-EGFR protein antibody. Protein G-sepharose (Sigma–Aldrich Ibérica S.A.) was then added, and samples were rotated for 1 h more. Immunoprecipitates were then washed thrice with buffer A and resuspended in Laemmli sample buffer. The samples were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrotheropically transferred to a nitrocellulose membrane. Western blot analyses were performed as described above.

**Immunocytochemistry**

Cells were fixed with 4% paraformaldehyde, permeabilised in 0.1% Triton X-100 in PBS for 10 min and stained with primary antibodies indicated followed by Alexa Fluor 633-conjugated anti-rabbit antibody or Alexa Fluor 488-conjugated anti-human antibody. In order to perform nuclear staining, cells were incubated with 4’,6-diamidino-2-phenylindole. Fluorescent images were acquired using a Leica TCS-SP5 confocal microscope.

**Transfection of siRNAs**

The small interfering RNAs (siRNAs) targeting ErbB2, p110alpha (PI3K), Akt1 and Akt2 genes were synthesized by Ambion Transfections were performed with the SuperScript II reverse transcription kit (Invitrogen). Real-time PCR was performed using the Fast Start Taqman Probe Master and Universal ProbeLibrary (Roche) and the following primers: ErbB2 variant 1 primers: sense, 5′-CCCTGACACCTCTGATA-3′ and antisense, 5′-GGGAACCTGGAATCCTAC-3′; chromogranin B primers: sense, 5′-ATCTCT- GGCGAGTTACAG-3′ and antisense 5′-CACGCAAACCTGCCTTC-3′; and 18S primers: sense, 5′-TCGGGAGTTGGAATTTGC-3′ and antisense, 5′-GAGAGGAGGCGCAGAAC-3′. All assays were performed using three separate samples of reverse transcriptase product. Messenger RNA (mRNA) expression was normalized using the standard curve method (25).

**Reverse transcription–PCR analysis**

Total RNA was isolated from LNCaP cells after treatment by using TRIzol Reagent (Invitrogen) according to the manufacturer’s recommendations, and complementary DNA was produced with the SuperScript II reverse transcription kit (Invitrogen). PCR was performed using the Taq polymerase (Biotools, Madrid, Spain) and the following primers: ErbB2 variant 1 primers: sense, 5′-GGGGGCAGAAGAAAGTTGAT-3′ and antisense, 5′-ATGGTCGTTGCGGAAAGTTGAT-3′; and antisense, 5′-ATAGAGGTGTCCGAAGGCTGCCC-3′; 18S primers: sense, 5′-ACCTTTTCTTCAATGATGTTGGC-3′ and antisense, 5′-CTAGGGTTGTTGTCAAGTGTA-3′. The mRNA expression was analysed in DNA polyacrylamide/TAE gels with ethidium bromide staining.

**Experimental subjects and immunohistochemistry**

Since our purpose was to perform an exploratory immunohistochemical screening, the paraffin-embedded samples were selected at random from a larger series of 120 cases, most of which had Gleason scores ≥7. Seven samples from benign prostatic hyperplasia and 22 from prostate cancers, with a larger series of 120 cases, most of which had Gleason scores ≥8 (n = 1), 6 (n = 1), 7 (n = 11), 8 (n = 5) and 9 (n = 4), were used. None of the patients received neoadjuvant or adjuvant therapy. Approval for the study was obtained from the local ethical committee. The high grade intraepithelial neoplasia present in the selected samples were also analysed. Five micrometre sections were obtained, deparaffinised with xylene and rehydrated in a graded ethanol series. Antigen retrieval was performed with 10 mM citrate buffer for 3 min in a pressure cooker. Endogenous fluorescence was quenched by incubating the samples with 4% sodium borohydride in Tris buffered saline pH 7.4 for 60 min. Non-specific immunoreactions were prevented by incubation with blocking-A solution (3% normal donkey serum and 0.05% triton X-100) in Tris buffered saline, pH 7.5 for 30 min. For ErbB2 detection, we used either a monoclonal rabbit anti-ErbB2 antibody or a humanized anti-ErbB2 antibody (Trastuzumab) diluted in the same blocking solution overnight. After washing, samples were incubated with a mouse monoclonal anti-chromogranin A and incubated with Alexa dye-conjugated secondary antibodies for 1 h. An additional 30 min incubation with blocking-A solution was performed for trastuzumab immunostaining before the second anti-human antibody to minimize the background due to endogenous human immunoglobulins. After washing with Tris buffered saline, samples were coverslipped with non-fade mounting medium and analysed by confocal microscopy. Additionally, a double immunohistochemical staining method was used to confirm the results of the immunofluorescence analysis. Briefly, the anti-ErbB2 antibody was detected by the classical streptavidin–biotin complex method (streptABC, Dako diagnostic S.A., Barcelona, Spain) and 3′-diaminobenzidine as chromogen, and the chromogranin A antibody was detected using a second
antibody conjugated with alkaline phosphatase (Chemicon, Millipore Iberica S.A.U., Madrid, Spain) and AP-red as chromogen (Zymed, Invitrogen).

The immunohistochemical staining pattern for ErbB2 was scored in four categories (0, 1−, fewer than 1% positive cells; 2+, 1–10% positive cells; 3+, more than 10% positive cells). Samples scored as 2+ or 3+ were classified as highly immunopositive, and those scoring 0 or 1+ were grouped as negatively weakly immunopositive. For chromogranin A, positive or negative scores were determined by means of detecting immunopositive groups of cells within the tumour. Terminally differentiated chromogranin A cells within normal glands were not included for scoring purposes, although the possibility of its immunopositive reaction with ErbB2 was also considered.

Data analysis

Results are presented as the mean ± standard error of mean of three independent experiments, which were compared using a paired sample t-test. A Wilcoxon test for paired samples was used to compare real-time PCR data. In both tests, differences were considered statistically significant for values of P < 0.05.

Results

EGF in combination with LY294002 increases ErbB2 levels in LNCaP cells

Previous results from our group have demonstrated that EGF in the presence of LY294002 was unable to inhibit NE differentiation or stimulate cell proliferation (21) but probably rescues LNCaP cells from LY294002-induced apoptosis. To confirm this, apoptosis was determined by flow cytometry using the TMRM probe. As shown in Supplementary Figure S1 (available at Carcinogenesis Online), the presence of EGF prevented LY294002 from reducing the percentage of TMRM-negative cells. Under these conditions, the expression of NSE and chromogranin A and B, markers previously used to assess the extent of NE differentiation in LNCaP cells (26), was increased.

Together, these data show that combined treatment with EGF and LY294002 induces cell survival and NE differentiation in LNCaP cells. Therefore, our objective was to investigate the mechanism underlying this effect. EGF binds to EGFR and promotes the formation of EGFR/EGFR homodimers and EGFR/ErbB2 heterodimers, so we examined the effect of EGF in the presence of LY294002 on the levels and degree of phosphorylation of EGFR and ErbB2. As shown in Figure 1A, EGFR levels decreased by ~60% after EGF treatment but recovered somewhat in the presence of EGF and LY294002. EGFR downregulation was expected and could be due to ligand-induced degradation, a mechanism to which ErbB2 is resistant. However, ErbB2 levels increased sharply when LNCaP cells were incubated with EGF and LY294002, these being three times that detected in untreated cells. EGF or LY294002 did not modify the ErbB2 levels. The ErbB3 levels were not modified by any of the treatment. In all treatments, significant inhibition of PI3K activity by LY294002 was confirmed by dephosphorylation of Akt Ser 473 and Thr 308, surrogate indicators of this activity.

This increase of ErbB2 was detected by long-term incubation and was maintained over time (Supplementary Figure S2 is available at Carcinogenesis Online). The ErbB2 levels peaked at 18 h after treatment with EGF and LY294002 and were maintained after 24 h. The increase of ErbB2 was also dose dependent with respect to EGF and LY294002 (Supplementary Figure S3 is available at Carcinogenesis Online).

In order to confirm that ErbB2 up-regulation also increased the ErbB2 levels in the plasma membrane, we measured the presence
of ErbB2 expression at the cell surface by flow cytometry. As shown in Figure 1B, EGF in combination with LY294002 increased the surface expression of ErbB2 by ~100%.

We have demonstrated that in LNCaP cells, EGF activates EGFR and ErbB2 through the formation of EGFR-ErbB2 heterodimers (21). To provide direct additional evidence of ErbB2 functionality, we incubated the LNCaP cells with EGF alone or in combination with LY294002 and analysed its ability to heterodimerize with EGFR and the phosphorylation status of both receptors. As shown in Figure 1C, the treatment with EGF and LY294002 doubled the amount of ErbB2 coimmunoprecipitated with EGFR in comparison with basal conditions and EGF treatment. This was accompanied by higher tyrosine phosphorylation of EGFR and ErbB2 (Figure 1D). Finally, we analysed p42/44 MAPK activity. This kinase is activated by ErbB receptors in several biological processes. MAPK activity also increased upon treatment with EGF and LY294002. Strikingly, the activation of MAPK increased in parallel with that of ErbB2, suggesting a direct relationship between the two effects (Supplementary Figure S2 is available at Carcinogenesis Online). The increase of ErbB2 levels was not observed in DU145 and PC3 androgen-insensitive cells treated with EGF plus LY294002 (Supplementary Figure S4 is available at Carcinogenesis Online).

These findings indicate that EGF in combination with LY294002 sharply raises the levels of ErbB2 and its activity in LNCaP cells, as is suggested by its localization at the cell surface, the formation of more EGFR/ErbB2 heterodimers and the activation of MAPK.

**EGF in combination with LY294002 increases ErbB2 through a PI3K/AKT-independent mechanism**

The involvement of NE differentiation in the progression of prostate cancer prompted us to study further the mechanisms involved in the increase in ErbB2 induced by EGF in the presence of LY294002. To confirm the effect of LY294002, wortmannin, another structurally unrelated inhibitor of PI3K, was used. In contrast to what was observed with LY294002, wortmannin in the presence of EGF reduced ErbB2 levels at concentrations that effectively inhibited Akt phosphorylation (Figure 2A). However, if under these conditions we add LY294002, ErbB2 levels increase again, reaching similar levels to those obtained in the presence of EGF plus LY294002. Based on these results, we tested the effects of LY303511, an inactive LY294002 analogue. LY303511 contains a one-atom substitution that abolishes its ability to inhibit PI3K activity (27). As shown in Figure 2B, LY303511 did not inhibit PI3K and did not modify the ErbB2 levels. Due to the contradictory results obtained with these inhibitors, we directly depleted PI3K using a small interfering RNA (siRNA p110α) and Akt1 and Akt2. The p110α isoform is required for PI3K activation in response to receptor tyrosine kinase stimulation in LNCaP cells and Akt1 and Akt2 are Akt isoforms expressed in these cells, as is suggested by its localization at the cell surface, the formation of more EGFR/ErbB2 heterodimers and the activation of MAPK.

**Fig. 2.** EGF in combination with LY294002 increases ErbB2 by a PI3K/AKT-independent mechanism. LNCaP cells were cultured in RPMI with 7% fetal bovine serum for 2 days and then placed in serum-free medium for 24 h. (A) The cells were then treated with EGF (10^−8 M), alone or in combination with LY294002 (20 μM), wortmannin (100 nM) or LY294002 plus wortmannin. Cellular lysates were monitored by immunoblotting using the indicated specific antibodies. (B) Serum-starved cells were treated with EGF (10^−8 M), alone or in combination with LY294002 (20 μM) or LY30 (20 μM). Cellular lysates were monitored by immunoblotting using the indicated specific antibodies. (C) LNCaP cells were transfected with siRNA against p110α (PI3K) or control RNA (SC); 48 h post-transfection, the cells were treated with EGF (10^−8 M) for 24 h. The p110α mRNA levels were analysed by reverse transcription–PCR (left). ErbB2 protein levels and Akt phosphorylation (S473 and T308) were analysed by western blot (right). (D) LNCaP cells were transfected with siRNA against Akt1 (siRNA 1), Akt2 (siRNA 2) or both together (siRNA Akt 1+2) or control RNA (SC); 48 h post-transfection, the cells were treated with EGF (10^−8 M) for 24 h. ErbB2, Akt1 and Akt2 protein levels were analysed by western blot. (E) Serum-starved cells were treated with EGF (10^−8 M), alone or in combination with LY294002 (20 μM), rapamycin (10 nM) or LY294002 plus rapamycin. Cellular lysates were monitored by immunoblotting using the indicated specific antibodies. (F) Serum-starved cells were treated with EGF (10^−8 M), alone or in combination with TBB (2.5 μM). ErbB2 levels were analysed by western blot. β-tubulin was used as an equal loading marker. Each figure is representative of three different experiments, all of which exhibited similar trends.
cells. We observed that the depletion of p110α (Figure 2C), Akt1 or the codepletion of Akt1 and Akt2 (Figure 2D) reduced ErbB2 levels, whereas the depletion of Akt2 did not modify them (Figure 2D). Together, these data clearly show that the increase of ErbB2 levels induced by EGF in the presence of LY294004 is independent of the PI3K-Akt pathway.

LY294002 is also an inhibitor of mTORC1. mTORC1 is a critical mediator of the canonical pathway of PI3K/Akt and is constitutively active in LNCaP cells as we show in Supplementary Figure S5 (available at Carcinogenesis Online). In fact, we observed that 56k, a direct substrate of mTORC1, is phosphorylated at Thr 389 in basal conditions and in presence of EGF, an effect that is completely inhibited by LY294002 alone or in combination with EGF. To test whether mTORC1 was implicated in the increase of ErbB2 induced by EGF plus LY294002, we used rapamycin, a specific inhibitor of this complex. We observed that EGF in presence of rapamycin did not modify significantly ErbB2 levels (Figure 2E). However, ErbB2 levels increased again by the addition of LY294002. The phosphorylation of Akt at Ser 473 was only inhibited when LY294002 is present.

Other target of LY294002 such as casein kinase 2 (CK2) was discounted. We observed that EGF in presence of TBB, a specific inhibitor of CK2, did not increase ErbB2 levels (Figure 2F).

**ErbB2 levels increase due to greater gene transcription**

To clarify the mechanism used by EGF to increase ErbB2 levels in the presence of LY294002, the next step was to determine whether this increase was due to reduced protein degradation, increased transcription or both.

To evaluate whether EGF plus LY294002 regulates ErbB2 protein stability, we blocked translation of ErbB2 mRNA with cycloheximide in EGF plus LY294002-treated cells (Figure 3A). The half-life of ErbB2 protein was not modified, being similar in EGF-treated or EGF- and LY294002-treated cells, clearly indicating that the increase of ErbB2 induced by EGF in the presence of LY294002 was not due to a lower level of degradation of ErbB2.

We further analysed the effect of EGF plus LY294002 on ErbB2 expression at the transcriptional level. We observed that EGF in the presence of LY294002 induced a 5-fold increase of ErbB2 mRNA (Figure 3B). These data clearly show that accumulation of ErbB2 protein induced by EGF plus LY294002 is due to the increase of ErbB2 gene transcription without modification in protein degradation.

**Increase in ErbB2 is responsible for the NE differentiation of LNCaP cell induced by EGF plus LY294002**

To assess the involvement of ErbB2 in survival and NE differentiation of LNCaP cells detected in the presence of EGF plus LY294002, we knocked down the expression of ErbB2 by RNA interference and analysed the changes in apoptosis and differentiation markers. Decreased ErbB2 levels were confirmed by western blot (Figure 4A). The depletion of ErbB2 in the presence of EGF did not modify the percentage of cells that were TMRM-negative or in sub-Go/G1 (Figure 4B), indicating that the increase of ErbB2 levels was not involved in the survival of LNCaP cells induced by EGF in the presence of LY294002. We also observed that ErbB2 knockdown had no effect on apoptosis induced by LY294002. We further analysed expression of NSE and chromogranin A and B. The depletion of ErbB2 in the presence of EGF and LY294002 reduced the levels of NSE protein, chromogranin B mRNA (Wilcoxon test $P < 0.01$) and chromogranin A immunofluorescence (Figure 4C). Together, these data show that the increase in ErbB2 is directly involved in NE differentiation but not in cell survival induced by EGF in the presence of LY294004 in LNCaP cells.

We also examined whether other classically employed NE differentiation inducers (28) also modify ErbB2 levels. As shown in Figure 4D, the treatment of LNCaP cells with forskolin or interleukin 6 also increased ErbB2 levels.

**Colocalization of ErbB2 and chromogranin A in human prostate cancer**

In an attempt to confirm the association of ErbB2 expression and the NE phenotype in prostate cancer in vivo, we analysed a small cohort of samples from patients with prostate cancer for ErbB2 and chromogranin A protein expression using immunohistochemical procedures. ErbB2 was expressed at moderate–high levels (score 2+/3+) in 31% $(n = 7)$ of the prostate cancer samples. Only one of the samples scored 3+, with >80% positive cells. The same samples

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![Image](https://academic.oup.com/carcin/article-abstract/33/6/1169/2463703/suppl/DC1)

**Fig. 3.** ERBB2 levels increase due to a greater gene transcription. (A) LNCaP cells were cultured in RPMI with 7% fetal bovine serum for 2 days and then placed in serum-free medium for 24 h. The cells were then treated with EGF (10^{-8} M), alone or in combination with LY294002 (20 μM); after 18 h, cells were treated with 100 μg/ml cycloheximide (CHX) for 2, 4 or 8 h and the cell lysates were immunoblotted with the indicated antibodies. A representative image of six independent experiments is shown (top). Densitometric analysis of six independent experiments: error bars represent the standard error of the mean (SEM) (bottom). (B) Serum-starved cells were treated with EGF (10^{-8} M), alone or in combination with LY294002 (20μM), and the relative amount of ErbB2 mRNA was analysed by reverse transcription–PCR (RT–PCR) (top) and quantified by real-time RT–PCR (bottom). Error bars represent the standard error.
were found to contain groups of tumour cells that were immunopositive for chromogranin A, with the highest number of chromogranin A cells observed in the case that scored 3++. The analysis of the dual labelling for ErbB2/chromogranin A showed a variable degree of colocalization among samples, but all of them in which ErbB2 was detected had ErbB2-immunopositive chromogranin A cells. The greatest number of ErbB2/chromogranin A cells was observed in two of the samples that scored 2+ and in the sample that scored 3+. Colocalization was not observed in the samples scored as negative/low for ErbB2 or in those from patients diagnosed with benign prostatic hyperplasia, where just weak ErbB2 immunoreactivity was observed mainly in the basal compartment of some normal glands but not in normal terminally differentiated chromogranin A- NE cells. Chromogranin A/ErbB2 immunoreaction was usually observed in tumour cells grouped into small clusters (Figure 5G and I), although in some tumours most of the cells within the abnormal glands were immunopositive (Figure 5H). Not only apparently undifferentiated clustered cells but also some morphologically differentiated chromogranin A+ cells dispersed within the tumours showed concomitant expression of ErbB2 (Figure 5I). High-grade prostatic intraepithelial

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neoplasia were frequently observed in the samples, and the colocalization of chromogranin A and ErbB2 was again observed (Figure 5J), suggesting that the expression of ErbB2 might be an early event in the process of acquiring the NE phenotype in some prostate cancers.

Discussion

An increased NE cell population in cancer prostate is often associated with a more aggressive disease and recurrence after androgen-deprivation therapy, suggesting that NE cells play a crucial role in cancer prostate progression (8,10). However, the mechanism responsible for the increase in NE cells in advanced prostate cancer is unknown. Therefore, it is essential to determine the mechanism responsible for NE differentiation in prostate cancer if we are then to identify possible therapeutic targets for treatment of advanced prostate cancer.

We have reported that EGF prevents NE differentiation of LNCaP cells induced by androgen depletion by a mechanism that requires constitutive Akt activation (21). The PI3K/Akt pathway is also required for cell survival in the absence of androgens since treatment with the PI3K inhibitor LY294002 leads to apoptosis (23). EGF antagonizes this apoptotic effect (24). Thus, EGF in the presence of the PI3K inhibitor LY294002 induces cell survival and NE differentiation, processes directly involved in the progression of prostate cancer.

In this study, we report for the first time that NE differentiation, but not survival of LNCaP cells, is mediated by the increase in the ErbB2 levels induced by treatment with EGF and LY294002. This increase of

Fig. 5. Colocalization of ErbB2 and chromogranin A in human prostate cancer. Confocal microscopy images from tumours with advanced Gleason scores, stained with anti-ErbB2 (A–C, green fluorescence) and with anti-chromogranin A (D–F, red fluorescence); G–I: corresponding superimposed images showing the yellow/orange fluorescence in cells expressing both proteins. J: Transmitted light microscopy image from a sample stained following the double immunohistochemical chromogen-based method; ErbB2 in brown, chromogranin A in red. CgA/ErbB2 colocalization in tumour cells grouped into small clusters (arrows); tumour gland showing CgA/ErbB2 colocalization in most of the cells (asterisk). Some morphologically differentiated CgA+ cells dispersed within the tumours showed concomitant expression of ErbB2 (arrowhead). High-grade prostatic intraepithelial neoplasia (J) showing the colocalization of CgA (diffuse red cytoplasmic signal) and ErbB2 (brown signal at the plasma membranes).
ErbB2 is due to greater gene transcription by a PI3K-independent mechanism. ErbB2 is functional under these experimental conditions, as shown by its localization in plasma membrane, the ability to heterodimerize with EGFR, greater phosphorylation and MAPK activation. Although EGFR is the specific receptor for EGF, we did not observe similar changes in its levels. The treatment with EGF and LY294002 only reverted the EGFR downregulation induced by EGF; this reversion could be due to the increase in ErbB2. In fact, it has been demonstrated that EGFR is downregulated upon ligand binding. Conversely, ErbB2 is resistant to these mechanisms and its overexpression has been reported to inhibit endocytosis of the EGFR (29). Consistent with these findings, the EGFR- and LY294002-induced ErbB2 increase and the more abundant formation of the heterodimer may be responsible for the recovery of EGFR levels. This ErbB2 up-regulation is independent of the PI3K/Akt pathway. While LY294002 and wortmannin indeed inhibited this pathway, LY294002 but not wortmannin increased ErbB2 levels. Furthermore, the depletion of PI3K, AKT1 or AKT2 genes did not increase but rather decreased ErbB2 levels in the presence of EGF. Surprisingly, the addition of LY294002 to EGF plus wortmannin-treated cells increased ErbB2 levels again, suggesting that LY294002 and not PI3K inhibition is required to upregulate ErbB2. LY294002 and wortmannin also inhibit mTOR. mTORC1 is one of the complex formed by mTOR and is a critical mediator of PI3K/Akt; the involvement of this complex was also ruled out because the rapamycin in the presence of EGF did not upregulate ErbB2. LY294002 inhibits other targets such as DNA-dependent protein kinase and CK2 (30). Wortmannin also inhibits DNA-dependent protein kinase and CK2 is inhibited by LY30 and TBB (30–32). The negative results from these inhibitors confirmed that none of the known targets for LY294002 was involved in the increase of ErbB2 induced by LY294002 in the presence of EGF.

To clarify the molecular mechanism by which EGF plus LY294002 induces ErbB2 expression, and given that the increase of ErbB2 was observed to occur after a long incubation time, we analysed the stability of ErbB2 protein and the levels of its mRNA. We found that ErbB2 stability was not modified although the levels of ErbB2 mRNA were increased. It has been reported that ErbB2 expression is increased by NF-kB (33,34) and is inhibited by oestrogen receptor (35). Interestingly, recent reports have shown that LY294002 regulates the activity of both transcription factors through a PI3K-independent mechanism. In monocytic cells, LY294002 enhances cytokine-mediated NF-kB activation (36). On the other hand, LY294002 inhibits anti-oestrogenic effects by binding directly to the oestrogen receptor (37). The isoform β of the oestrogen receptor is expressed in most metastases of prostate cancer (38) and LNCaP cells (39). These findings lead us to speculate that the ErbB2 increase induced by EGF in the presence of LY294002 is mediated by NF-kB or oestrogen receptor. Further experimentation is necessary to investigate this hypothesis.

The results clearly demonstrate the essential role of ErbB2 up-regulation in the NE phenotype acquisition induced by the combined treatment with EGF and LY294002. This finding is supported by several pieces of evidence. First, ErbB2 levels increased in parallel with NE differentiation marker levels, whereas ErbB2 depletion reduced them. Secondly, different NE differentiation inducers also increased the ErbB2 levels. Thirdly, ErbB2 and chromogranin A colocalize in prostate cancer tissue. Despite many studies of model systems and clinical specimens, the role of ErbB2 and the mechanisms by which they become activated in prostate cancer are under intense scrutiny. Several clinical studies showed that, unlike breast cancer, excess ErbB2 protein synthesis or decreased protein degradation might take place in the absence of gene amplification (17,40,41). Moreover, ErbB2 protein overexpression is involved in the emergence of androgen-independent prostate cancer. Experimental evidence for this possibility has been demonstrated in prostate cancer patients and in LNCaP cells, where ErbB2 can activate androgen receptor in a ligand-independent fashion, enhancing the androgen-independent growth of prostate cancer (42). The direct relationship between increased ErbB2 levels and the induction of NE differentiation described in this report might represent an alternative mechanism by which ErbB2 overexpression promotes the emergence of androgen-independent prostate cancer. Providing evidence for this possibility, Quin et al. (43) showed that ErbB2 is a critical component of interleukin-6 signalling; interleukin-6 is one of the best-known inducers of NE differentiation. Moreover, the increase of ErbB2 induced by EGF in combination with LY294002 was not detected in DU145 and PC3 cells, which are androgen-insensitive. However, we analysed a small cohort of patients and clearly showed that higher ErbB2 levels in some prostate cancer sections were accompanied by chromogranin A expression. Even so, a large study is needed to confirm the association between ErbB2 and NE differentiation and the clinical context in which it occurs.

ErbB2 up-regulation is not involved in the survival of LNCaP cells induced by EGF in the presence of LY294002 because ErbB2 depletion does not modify the percentage of dead cells. In a previous report, we showed that EGF binds to EGFR and promotes the formation of EGFR/EGFR homodimers and EGFR/ErbB2 heterodimers and that the downregulation of ErbB2 levels inhibits the formation of heterodimers and promotes the homodimers. Furthermore, Terrig et al. (24) showed that ErbB1 activity is essential for protection from the apoptotic effects of LY294002 in LNCaP cells. Thus, it is possible that knockdown of ErbB2 could promote the liberation of EGFR and EGFR/EGFR homodimerization. These homodimers could mediate the effect of EGF on cell survival in the presence of LY294002 and suggest that the absence of ErbB2 could be compensated by EGFR.

In summary, we have demonstrated that in LNCaP cells, EGF in the presence of LY294002 increases the levels and activity of ErbB2 by a PI3K/AKT-independent mechanism. ErbB2 up-regulation is not responsible for cell survival but induces the acquisition of the NE phenotype.

The results of this study raise a number of questions about the use of inhibitors of therapeutic targets in prostate cancer. LY294002, as a PI3K/AKT inhibitor, is a potent inducer of cell death in prostate cancer although it has not progressed to clinical trials due to its unfavourable pharmacokinetic properties and toxicity (44), it has been used to develop new drugs that are now being tested in clinical trials as cancer therapy. However, the use of these inhibitors to induce apoptosis could upregulate ErbB2 and increase the population of mitogenic factor-secretor NE cells than maintain the growth potential of tumour cells. The unequivocal association of ErbB2 overexpression with NE differentiation suggests that we should reconsider ErbB2 as a drug target in prostate cancer, possibly with modified approaches.

Supplementary material
Supplementary Figures S1–S5 can be found at http://carcin.oxfordjournals.org/.

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References
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