Abstract. We previously demonstrated that the inhibition of the epidermal growth factor receptor (EGFR) signalling affects the endocrine therapy responses of prostate cancer (PCa) cells and that bicalutamide (BCLT) is able to reinforce PI3K activity through mechanisms involving PTEN decrement and EGFR and Her2 activities. The aim of this study was to evaluate if the hormonal therapy with BCLT can affect the EGFR-targeted therapy using primary cultures obtained from 22 human PCa tissues harvested after radical prostatectomy (RP) in patients who received (n=10) BCLT and those that did not (n=12) as neoadjuvant hormone therapy (NHT). We demonstrated that cultures derived from PCa tissues harvested after NHT presented significantly higher EGFR and Her2 levels compared to cultures derived from control patients. However, cultures derived from patients with NHT were less sensitive to gefitinib when compared to cultures derived from control patients. Conversely, BCLT effectiveness did not seem to be different in the two groups and was partially additive with gefitinib in the NHT group and additive/synergistic in the control group. Cultures (8/22) were negative for the expression of the PTEN gene and we observed no differences in the two groups. Thus the different IC50 values observed for gefitinib and the partial additivity in the combination treatment with gefitinib and BCLT is influenced by EGFR/Her2 ratio because it was shown that EGFR inhibition was lower when Her2 is overexpressed. Taken together, our results indicate that anti-EGFR targeted therapies and a possible combination therapy involving gefitinib and BCLT should be performed early in naive patients when Her2 is not overexpressed and before the anti-androgenic hormone therapy induces such an undesirable effect.

Introduction

Prostate cancer (PCa) is a complex disease in which different clinical and biological phases of both the natural and treated history of the process may be characterized. To date, no effective therapeutic treatment prevents PCa progression to more advanced and invasive disease forms. At present, endocrine therapy represents the only suitable treatment, besides surgery, for patients with early-stage PCa. Unfortunately, the beneficial actions of existing endocrine measures are, in part, counteracted by the capacity of the tumor cells to eventually circumvent the use of steroid hormones, allowing them to continue to grow and progress despite such therapy. After a positive initial response, the tumors recur in an androgen-independent (AI) form that is unresponsive to additional androgen withdrawal. For these reasons, additional targets and therapies are needed in conjunction with androgen ablation. The pathways that underlie tumor progression, invasion and resistance to treatment in each clinical state are being increasingly understood and novel biological agents that target these pathways are in development or are now available for clinical testing. On the other hand, the establishment of new biomarkers is needed to identify high-risk patients as candidates for new (adjuvant) therapies and to find new surrogate endpoints to assess the efficacy of these treatments (1). In the past 10 years, conflicting results on HER-2/neu expression in prostate cancer were reported (2-8). Furthermore, it was proposed that Her2 and androgen receptors function synergistically in the absence of androgens (9-11). This fact suggests the existence of a cross-talk between the Her2/neu and androgen receptor pathways. However, treatment with the anti-Her2/neu antibody trastuzumab (12,13), which is efficient in breast cancers with
amplification-driven Her2/neu overexpression, as well as with pertuzumab, an anti-Her2 antibody which inhibits its heterodimerization (14,15) has failed in advanced prostate cancers despite experimental evidence of a favourable response in prostate cancer xenograft models (16,17). However, the reasons for treatment failure of anti-Her2 therapy are not entirely clear. One explanation may be the lack of Her2/neu gene amplification in prostate cancer and the low prevalence of Her2/neu overexpression (8).

Recent studies suggest that gefitinib induces cell cycle retardation and apoptosis and inhibits the growth of several types of human cancer cells expressing EGFR both in vitro and in vivo (18-30). In preclinical studies, gefitinib, a selective inhibitor of epidermal growth factor receptor-tyrosine kinase, produced growth inhibition in a wide variety of common solid tumor types including human PCa xenografts.

The study of prostate carcinogenesis and tumor progression is made difficult by the lack of appropriate in vitro and in vivo models. The most widely used cancer cell lines, which have been established from human metastatic lesions, do not accurately recapitulate the biological behaviour of primary tumors as compared to primary cultures generated from clinical PCa specimens. A procedure to propagate human prostatic epithelial cells in vitro for a limited number of cell generations has been developed by and optimized for prostate cancer cells from primary tumors. During the past fifteen years, in our laboratory we have processed more than 400 PCa generations has been developed by and optimized for prostate cancer cells from primary tumors. During the past fifteen years, in our laboratory we have processed more than 400 PCa and BPH tissue samples (31-33). To test the hypothesis that years, in our laboratory we have processed more than 400 PCa generations has been developed by and optimized for prostate cancer cells from primary tumors. During the past fifteen years, in our laboratory we have processed more than 400 PCa and BPH tissue samples (31-33). To test the hypothesis that the inhibition of the EGFR/HER1 signaling pathway may modify the antitumor effects of endocrine therapy, gefitinib and BCLT were administered to human PCa cells in primary cultures derived from tissue samples harvested by radical prostatectomy in patients who received or not BCLT treatment as neoadjuvant hormone therapy (NHT).

In this study, the effects of a combination treatment with gefitinib with BCLT, were determined on a series of 22 human PCa primary tissue cultures.

Materials and methods

Reagents and cell lines. All the materials for tissue culture were purchased from Hyclone (Cramlington, NE, USA). Plastic ware was obtained from Nunc (Roskilde, Denmark). EGF was purchased from Sigma Chemical (St. Louis, MO, USA). Charcoal/stripped foetal calf serum (CT/FCS) was obtained from Gibco (Gibco Brl, Gaithersburg, MD). Gefitinib and bicalutamide were obtained from AstraZeneca (Macclesfield, UK). Antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA) unless otherwise indicated. Akt/protein kinase B (PKB) activity was tested using a non-radioactive assay kit (StressXpress AKT/PKB Elisa kit) which was purchased from Stressgenne Bioreagents (Victoria, BC, Canada) on cell lysates after partial purification on a MonoQ exchange column (2 mg protein in 1 ml column) with 10 mM MOPS, pH 7.2, 25 mM b-glycerophosphate, 5 mM EGTA, 2 mM EDTA, 2 mM sodium orthovanadate and 2 mM dithiothreitol and eluted using a 12 ml linear NaCl gradient (0-0.8 M NaCl) with a flow rate of 0.5 ml/min. We collected fractions between 0.25 and 0.5 ml for analysis.

Patient selection, study design and treatment. Patients were recruited in our institution between January 2004 and March 2006. In this trial, the basic eligibility criterion was to have a histologically proven and operable (cT2/T3, N0, M0) PCa. Additional eligibility criteria were the absence of prior hormonal therapy including any form of therapy with 5α-reductase inhibitor and the absence of radiation and chemotherapy for PCa, no prior investigational agents and life expectancy >10 years. Patients were excluded if they had any prior malignancy within the last five years or had any other serious medical or psychiatric condition or illness that would not permit the patients to be managed according to the protocol. Our institutional review board approved the protocol and written informed consent was obtained before any medical treatment or BCLT before RP. Treated group subjects were assigned to receive medical therapy with 150 mg/d.i.e. BCLT before RP. Untreated group subjects received RP alone and were used as a control. During the treatment period, patients were evaluated for adverse effects (AEs) at baseline and every four weeks, until four weeks after treatment withdrawal. Baseline visit was performed at the time of randomization. We analysed a 22 patient cohort with clinically localized tumors that were surgically treated with RP. Among these, 12 patients received RP as initial treatment (group 1), whereas the other 10 patients received NHT for 4 months based on casodex (150 mg/d.i.e.) treatment (group 2). ‘BCLT (Casodex)’ and ‘Gefitinib (Iressa)’ are trademarks of the AstraZeneca group of companies.

Primary cultures from prostate specimens. Tissue samples were collected by needle biopsy or radical prostatectomy from patients attending the urology clinic at the University of L’Aquila Medical School, following standardised clinical procedures. Tissue samples were cultured in DMEM as previously described (31-33). Cultured cells were analysed for the presence of AR, EGFR, prostate-specific antigen (PSA) and telomerase activity, TRAP (34).

Immunoperoxidase staining. Cells were cultured in Lab-Tek Chamber slides (Nalgene Nunc International) and treated as described in the ‘Growth assays’ section. After 24-48 h of cell culture, cells were washed in PBS and fixed either in 4% PBS-paraformaldehyde for 5 min for immunostaining of membrane-associated antigens (EGFR/Her2) or in cold 1:1 acetone: methanol mixture in ice for 2 min for immunostaining of cellular or nuclear antigens (PSA and AR). Primary and secondary antibodies were used according to the manufacturer’s instructions.

RT-PCR for PTEN expression. Total RNA was extracted from cells with Trizol reagent according to the manufacturer’s instructions. An RT-PCR was carried out on Dnase Amp Grade (Gibco-Brl)-treated RNA using MuLV RT (50 units) in 100 mM Tris-HCl pH 8.3, 500 mM KCl, 5 mM MgCl₂, 0.5 μM each dNTP, 1 unit of RNasin, 500 pmol of random exon primers. Two microliters of cDNA were amplified in a 50 μl reaction volume containing 0.5 μM of sense and antisense primers, 2.5 U of Taq DNA polymerase (Applied Biosystems, Milan, Italy), 200 μM each dNTP and 1.5 mM MgCl₂. A co-amplification of GAPDH was performed.
Primer sequences used for PCR were: PTEN 3rd exon Fw 5’-ATATTCTCTGAAAAGCTCTGG-3’; PTEN Rev 5’-TTAATCGTGGTTAGAATCAA-3’; GAPDH Fw 5’-CAGACGTCCTGTGGAGTTCAG-3’; GAPDH Rev 5’-GACGGAACATTGGGGGTAG-3’. After amplification, 20 µl of the PCR reaction mixture was analysed by 1.2% agarose gel electrophoresis and stained with ethidium bromide.

Growth assays. Cells were seeded at a density of 2x10^4 cells per dish on 50-mm Petri dishes. Cells were allowed to attach and grow in 5% FCS DMEM for 24 h. After this time, cells were maintained in a culture medium containing androgens or subjected to androgen depletion. All other cells were treated with either 50 ng/ml EGF or different doses of gefitinib and/or the PI3K inhibitor, perifosine, at the recommended inhibition doses. Cells trypsinised and re-suspended in 20 ml saline were counted by a haemocytometer every 24 h (LabRecyclers, Gaithersburg, MD, USA) and 5 independent counts were performed for each dish. All experiments were conducted in triplicate. In order to evaluate the effective cell proliferation, we measured the uptake of [3H]-tymidine as follows. To calculate the inhibitory concentrations at 50% (IC50) of gefitinib, 2,500 cells were cultured in 96-well plates for 24-96 h. After adhesion (16 h), cells were grown in different culture conditions (see above). After 48-96 h, the cells were exposed for 4 h to thiazol blue (MTS, Promega Madison, WI, USA). The 96-well culture plates were then placed on a microplate shaker for 5 min and the absorbance of the converted dye was measured at the wavelength of 490 nm using a Biorad multiscan plate reader (Biorad, Richmond, CA, USA). Five replicate wells were used for each group. Inhibition curves were drawn by means of values obtained by OD percentages versus control for each concentration. IC50 values were calculated by the GraFit method (Erithacus Software Limited, Staines, UK) considering the slopes of inhibition curves obtained for each group of tests.

Apoptosis was evaluated by DNA fragmentation using a Titer TACS colorimetric assay kit (Trevigen Inc., Gaithersburg, MD, USA) and expressed as the percentage of cells that undergo apoptotic death.

Preparation of cell lysates and Western blot analysis. Following treatments, cells were washed with cold PBS and immediately lysed with 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM p-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin). The nuclear extracts were collected as described: briefly, cells were scraped in 1 ml PBS-EDTA and centrifuged at 3000 rpm. Pellets were re-suspended in 1 ml harvest buffer containing 10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA and 0.5% Triton X-100 and incubated on ice for 5 min. Cells were pelleted at 1000 rpm with a table top swinging microfuge, washed and re-suspended in 1 ml of 10 mM HEPES pH 7.9 containing 10 mM KCl, 0.1 mM EDTA and 0.1 mM EGTA. Lysates were electrophoresed in 7% SDS-PAGE and separated proteins transferred to nitrocellulose and probed with the appropriate antibodies using the conditions recommended by the suppliers. The EGFR or ERK activation status was analysed using phosphospecific antibodies, blots were stripped and reprobed with the appropriate antibody for assessment of total ERK or EGFR expression.

Statistics. Statistical analysis was performed using SPSS 11.0 software (SPSS, Inc., Chicago, IL). P values <0.05 were considered to indicate statistical significance. All statistical tests were two-tailed. Continuous variables were analyzed using a Wilcoxon-Mann Whitney Rank Sum Test or Student's t-test when appropriate. Differences in the categorical variables between groups were compared with the Chi-square test or Fisher's exact test when the tables were too sparse. The Kruskal Wallis test was used to compare apoptosis in primary cultures.

Results

Effect of gefitinib and its correlation with EGFR, Her2 and PTEN expression. Epithelial prostate primary cultures showed a homogeneous expression for AR and EGFR as indicated in Fig. 1 (panels A and B). Her2/neu positivity was present in 20-60% of cells (Fig. 1C). In addition, we showed that EGFR expression, by Western blotting analysis (Fig. 2), was higher, but not in a statistically significant manner, in cultures derived from patients from the NHT group when compared to the control (UNT) patients (2.5 normalized Arbitrary Densitometric Units (ADU)±0.3 vs 1.7 ADU±0.3, P=0.075). EGFR
was basally phosphorylated in 8/12 (66.7%) and in 9/10 (90%) of primary cultures derived from the UNT and NHT groups, respectively (P=0.430, NS) and the levels of phosphorylation were higher in the NHT group when compared to the UNT group (0.88 ADU±0.37 vs 1.94 ADU±0.36, P=0.059), but this difference was not statistically significant. Her2 expression (Fig. 2) was similar in primary cultures derived from NHT patients when compared to UNT patients (1.1 ADU±0.4 for both groups). Her2 was basally phosphorylated in 6/12 (50%) and in 9/10 (90%) of cultures derived from the control and NHT groups, respectively (P=0.122, NS) and the levels of p-Her2 were significantly higher in the NHT patients when compared to the UNT patients (0.2 ADU±0.8 vs 1.8 ADU±0.8, P<0.001). The blockade of EGFR/Her2 signalling pathways by gefitinib was able to reduce cell proliferation with IC50 ranging between 0.1-1.0 μM as indicated in Fig. 2. IC50 mean value of gefitinib, calculated for all 22 cell cultures independent of the treatment of patients, was 0.62 μM±0.74 (mean ± SD). When we considered the neoadjuvant pre-operative treatment, we did not observe any statistically significant difference for this parameter (0.59 μM±0.97 vs 0.67 μM±1.16, P=0.608, NS). We then performed a correlation analysis between EGFR levels and IC50 values for gefitinib, but we did not observe any statistical relationship (R=0.241; P=0.280). Thus, the effectiveness of gefitinib was not dependent upon EGFR levels, but we did not observe any significant correlation (R=0.241; P=0.252).

Considering the PTEN expression, we observed that gefitinib was more effective in PTEN positive cultures derived from the UNT and NHT groups, respectively (P=0.430, NS) and the levels of phosphorylation were higher in the NHT group when compared to the UNT group (0.88 ADU±0.37 vs 1.94 ADU±0.36, P=0.059), but this difference was not statistically significant. Her2 expression (Fig. 2) was similar in primary cultures derived from NHT patients when compared to UNT patients (1.1 ADU±0.4 for both groups). Her2 was basally phosphorylated in 6/12 (50%) and in 9/10 (90%) of cultures derived from the control and NHT groups, respectively (P=0.122, NS) and the levels of p-Her2 were significantly higher in the NHT patients when compared to the UNT patients (0.2 ADU±0.8 vs 1.8 ADU±0.8, P=0.001). The blockade of EGFR/Her2 signalling pathways by gefitinib was able to reduce cell proliferation with IC50 ranging between 0.1-1.0 μM as indicated in Fig. 2. IC50 mean value of gefitinib, calculated for all 22 cell cultures independent of the treatment of patients, was 0.62 μM±0.74 (mean ± SD). When we considered the neoadjuvant pre-operative treatment, we did not observe any statistically significant difference for this parameter (0.59 μM±0.97 vs 0.67 μM±1.16, P=0.608, NS). We then performed a correlation analysis between EGFR levels and IC50 values for gefitinib, but we did not observe any statistical relationship (R=0.241; P=0.280). Thus, the effectiveness of gefitinib was not dependent upon EGFR levels, but we did not observe any significant correlation (R=0.241; P=0.252).

Considering the PTEN expression, we observed that gefitinib was more effective in PTEN positive cultures derived from 22 PCa tissues of patients who underwent NHT (10 patients) or not (12 patients) as described in Material and methods. Each lane was loaded with 50 μg of proteins from total cell extracts and normalized in each experiment with a common cell extract with known EGFR and Her2 content (we used conventionally DU145 cell extract). EGFR and Her2 were expressed also as a ratio between their levels in each culture. PTEN expression was evaluated both RT-PCR and Western blotting and indicated in the figure as presence (+) or absence (−) of PTEN protein. IC50 values from gefitinib were calculated as described (lower panel).

Figure 2. Western Blot analysis of EGFR/p-EGFR and Her2/p-Her2 expression levels in primary cultures derived from 22 PCa tissues of patients who underwent NHT (10 patients) or not (12 patients) as described in Material and methods. Each lane was loaded with 50 μg of proteins from total cell extracts and normalized in each experiment with a common cell extract with known EGFR and Her2 content (we used conventionally DU145 cell extract). EGFR and Her2 were expressed also as a ratio between their levels in each culture. PTEN expression was evaluated both RT-PCR and Western blotting and indicated in the figure as presence (+) or absence (−) of PTEN protein. IC50 values from gefitinib were calculated as described (lower panel).
BCLT showed no additive (4/10, 40%) or partially additive (6/10, 60%) effects in cultures derived from the NHT group whereas the effects were additive (5/12, 41.7%) or synergistic (7/12, 58.3%) in cultures derived from the control group, suggesting that the molecular modifications induced by hormonal therapy (EGFR, Her2 increased expression and PTEN loss acceleration) can negatively influence the effectiveness of dual inhibition. This suggestion was particularly reinforced observing data on apoptosis evaluated as DNA fragmentation using a Titer TACS colorimetric kit (Fig. 3). We observed that apoptosis was increased in a dose-dependent manner by BCLT without any significant differences between UNT and NHT groups of cultures. Similarly, gefitinib (GFTN) sensitively stimulated the apoptotic death both in the UNT and NHT group. The NHT group showed a lower apoptotic death although the difference was not statistically significant. Moreover combination treatments showed synergistic effects in the UNT group whereas these were only additive in the NHT group.

**Discussion**

We previously demonstrated that AR-transfected DU145 cells treated with an antiandrogen, such as hydroxyflutamide, were more sensitive to the action of EGFR-tyrosine kinase inhibitor, PKI166 (39) due to the increased expression of EGFR. Thus, we hypothesized that the treatment with antiandrogens could effectively increase the sensitivity versus gefitinib. For these purposes, we verified the action of gefitinib in primary cultures derived from prostate cancer tissues that patients were treated with the sole radical prostatectomy or with neoadjuvant therapy based on bicalutamide treatment. The aim of this work was to evaluate if the hormonal therapy with BCLT can affect the EGFR targeted therapy. Previously, we demonstrated that expression of EGFR and Her2 increases significantly after hormone therapy performed in a neoadjuvant regimen (40). Here, we demonstrated that epithelial prostate cells in primary cultures derived from samples harvested from PCa after NHT showed higher levels of Her1 and Her2 compared to control PCa cultures as well as the levels of activated form of receptors are increased also if the differences were statistically not significant probably due to the small number of cultures examined. The block of the EGFR/Her2 signalling pathways by gefitinib were able to reduce cell proliferation and we did not observe statistically significant differences between cultures derived from NHT and UNT patients suggesting that globally the effects of gefitinib were maintained. Although the difference in PTEN expression was not statistically significant, we observed that 3/12 (25%) cultures were negative for PTEN expression in UNT group and 5/10 (50%) in the NHT group and gefitinib was more effective in PTEN-positive respect to PTEN-negative cultures as indicated previously (35). We observed that the effects of gefitinib were not related to EGFR levels, as previously observed by us (35) and others (36,37) as to Her2 levels. However, considering both Her2 expression and EGFR/Her2 ratio in the more responder PTEN-positive population, we observed that the effectiveness of gefitinib was inversely related to Her2 levels as well as to EGFR/Her2 ratio suggesting that when PTEN is present, the blockade of the EGFR pathway is more effective if the levels of Her2 were low and if the EGFR/Her2 ratio was high.

The role of c-erbB-2 in prostate cancer is also controversial, contrary to this receptor's clinical importance only for advanced breast cancer (41). Both c-erbB-1 and c-erbB-2 immunoreactivity were shown to be associated with an unfavorable prognosis in a homogeneous series of hormonally untreated cancer patients, in particular if these two receptors are combined (42).

The effectiveness of BCLT did not seem to be different in the two groups and was partially additive with gefitinib in NHT group and additive/synergistic in the control group. Thus, the different IC50 values observed for gefitinib and the partial additivity in the combination treatment with gefitinib and bicalutamide can be influenced by EGFR/Her2 ratio because it was shown that EGFR inhibition was lower when Her2 is overexpressed. However, it needs to be considered that the AR expression could slow down the EGFR phosphorylation due its membrane co-localization as observed from Bonaccorsi and co-workers (43). In addition we observed...
that the combination treatment regimens show higher effects in cultures derived from UNT in respect to NHT patient groups suggesting that the molecular modifications induced by hormonal therapy (EGFR, Her2 increased expression and PTEN loss acceleration) can negatively influence the effectiveness of dual inhibition. The data suggest that anti-EGFR targeted therapies and combination therapeutic approach involving gefitinib and BCLT should be performed earlier in naive patients when Her2 is not overexpressed and before any possible hormone therapy induces this event.

References


