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## The androgen receptor associates with the epidermal growth factor receptor in androgen-sensitive prostate cancer cells

L. Bonaccorsi<sup>a,\*</sup>, M. Muratori<sup>a</sup>, V. Carloni<sup>b</sup>, S. Marchiani<sup>a</sup>, L. Formigli<sup>c</sup>,  
G. Forti<sup>a</sup>, E. Baldi<sup>a</sup>

<sup>a</sup> *Dipartimenti di Fisiopatologia Clinica (Unità di Andrologia), Univerisity of Florence, Florence, Italy*

<sup>b</sup> *Dipartimenti di Medicina Interna, Univerisity of Florence, Florence, Italy*

<sup>c</sup> *Dipartimenti di Anatomia, Univerisity of Florence, Florence, Italy*

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### Abstract

Many recent evidences indicate that androgen-sensitive prostate cancer cells have a lower malignant phenotype that is in particular characterized by a reduced migration and invasion. We previously demonstrated that expression of androgen receptor (AR) by transfection of the androgen-independent prostate cancer cell line PC3 decreases invasion and adhesion of these cells (PC3-AR) through modulation of  $\alpha6\beta4$  integrin expression. The treatment with the synthetic androgen R1881 further reduced invasion of the cells without, however, modifying  $\alpha6\beta4$  expression on the cell surface, suggesting an interference with the invasion process in response to EGF. We investigated whether the presence of the AR could affect EGF receptor (EGFR)-mediated signaling in response to EGF by evaluating autotransphosphorylation of the receptor as well as activation of downstream signalling pathways. Immunoprecipitation studies demonstrated a reduction of EGF-induced tyrosine phosphorylation of EGFR in PC3-AR cells. In addition, EGF-stimulated PI3K activity, a key signalling pathway for invasion of these cells, was decreased in PC3-AR cells and further reduced by treatment with R1881, indicating decreased functionality of EGFR. An interaction between EGFR and AR has been demonstrated by immunofocal and co-immunoprecipitation analysis in PC3-AR cells, suggesting a possible interference of AR on EGFR signalling by interaction of the two proteins. In conclusion, our results suggest that the expression of AR by transfection in PC3 cells confers a less malignant phenotype by interfering with EGFR autophosphorylation and signalling in response to EGF leading to invasion through a mechanism involving an interaction between AR and EGFR.

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*Keywords:* Epidermal growth factor receptor; Androgen-sensitive prostate; Cancer cells

### 1. Introduction

Prostate cancer (PC) is the most common malignancy detected in men in western countries [1,2]. Although endocrine therapy is very useful when surgery is not feasible and leads to substantial periods of remission, progression from androgen dependence to independence always occurs in treated patients. Androgen-independent PC cells are insensitive to endocrine treatment and continue to grow and invade.

One of the striking characteristics of androgen-independent PC is represented by its higher invasive potential and malignant phenotype [3,4], that is in particular characterized by an increased migration and invasion. This pheno-

type, at least in part, can be explained by loss of androgen control of genes involved in limiting invasion [5].

We previously demonstrated that expression of androgen receptor (AR) by transfection of the androgen-independent prostate cancer cell line PC3, decreases adhesion, anchorage-independent growth and EGF-mediated Matrigel invasion of these cells (PC3-AR) respect to control cells (PC3-Neo, transfected only with the vector) through reduced expression of  $\alpha6\beta4$  integrin [3]. Similar results have been obtained by another group in a different cell line [4]. The treatment with the synthetic androgen R1881 further reduced Matrigel invasion of the cells [3,4], however, we have been unable to demonstrate an effect of the synthetic androgen on  $\alpha6\beta4$  expression on the cell surface [3], thus suggesting an alternative mechanism for decreasing invasive potential of the cells *in vitro*. We have thus evaluated the effect of the androgen on EGF-mediated signalling pathways leading to invasion in PC3-Neo and PC3-AR cells.

\* Corresponding author.

*E-mail address:* [l.bonaccorsi@dfc.unifi.it](mailto:l.bonaccorsi@dfc.unifi.it) (L. Bonaccorsi).

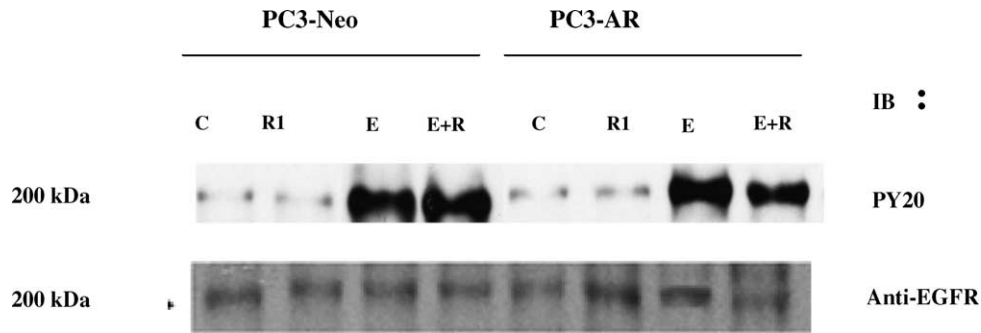


Fig. 1. Western blot analysis of PC3-Neo and PC3-AR cell total lysates using an anti-phosphotyrosine antibody (PY20) and an anti-EGF receptor antibody (anti-EGFR). Cells were stimulated or not with EGF (50 ng/ml, 10 min) in the presence or absence of the synthetic androgen R1881 (1 nM for 24 h). C: no stimulation.

### 1.1. EGFR autotransphosphorylation is reduced in PC3-AR cells

By Western blotting analysis, we examined tyrosine phosphorylation of EGFR in response to EGF. As shown in Fig. 1 tyrosine phosphorylation of EGFR in response to EGF was reduced in PC3-AR cells when R1881 was present. This result was also confirmed after EGFR immunoprecipitation and probing with anti-phosphotyrosine antibody (not shown). To ensure that EGFR expression was similar in PC3-AR and PC3-Neo cells, surface expression of the receptor was evaluated by FACScan analysis, which demonstrated no difference in EGFR expression in the two cell lines (not shown).

Overall, this result suggests an interference of the androgen with signalling pathways in response to EGF in PC3-AR cells.

### 1.2. The androgen receptor (AR) co-localize with EGFR at the plasma membrane

Emerging evidences indicate that besides its classical localization at the nuclear level, the AR may be targeted at the membrane level where interactions with proteins involved in growth factor signaling, such as src kinase family members [6], caveolin-1 [7] and PI3K [8] have been demonstrated. These studies prompted us to investigate whether AR can interact with EGFR in our cell line. The localization of AR and EGFR was investigated by confocal laser microscopy in PC3-AR cells stained for both proteins. As shown in Fig. 2, the AR (in red, middle panels) localizes both to the nucleus and the cytoplasm of PC3-AR cells, although after stimulation with R1881, increased location to the nuclei was evident. Interestingly, a striking co-localization (in yellow, right panels) of the AR with the EGFR (in green, left panels) at the plasma membrane level was present. No staining for AR was present in PC3-Neo cells (not shown). Co-immunoprecipitation studies, conducted in PC3-AR cells, confirmed interaction between the two proteins. Indeed, after immunoprecipitation with an

antibody against EGFR, a band at 110 kDa was detected by immunoblot analysis with an anti-AR antibody in PC3-AR cells (not shown). Co-immunoprecipitation between the EGFR and AR was also detected in the androgen-sensitive cell line LNCaP (not shown). Of interest, in this cell line, re-probing the membrane with an antibody against phosphotyrosine residues demonstrated that the treatment with R1881 determined a decrease in EGFR autophosphorylation in response to EGF. Overall, these results suggest that an interaction between EGFR and AR occurring in androgen-sensitive prostate carcinoma cells may be involved in determining the lower invasive potential of these cells [3,4].

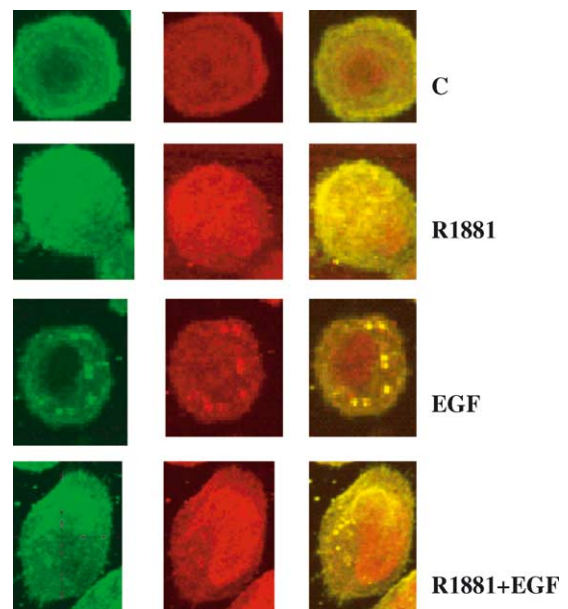


Fig. 2. Immunofluorescence analysis of the androgen receptor and the EGF receptor in PC3-AR cells. Cells pre-treated or not with the synthetic androgen R1881 (1 nM, 24 h), were plated on laminin-1 for 2 h and then stimulated with EGF (50 ng/ml) or vehicle (C) for 10 min. Cells were permeabilized and processed for double staining with rabbit polyclonal anti-AR (N-20) (red) and anti-EGFR (Ab-1) (green) antibodies. Yellow depicts co-localization of the two antibodies.

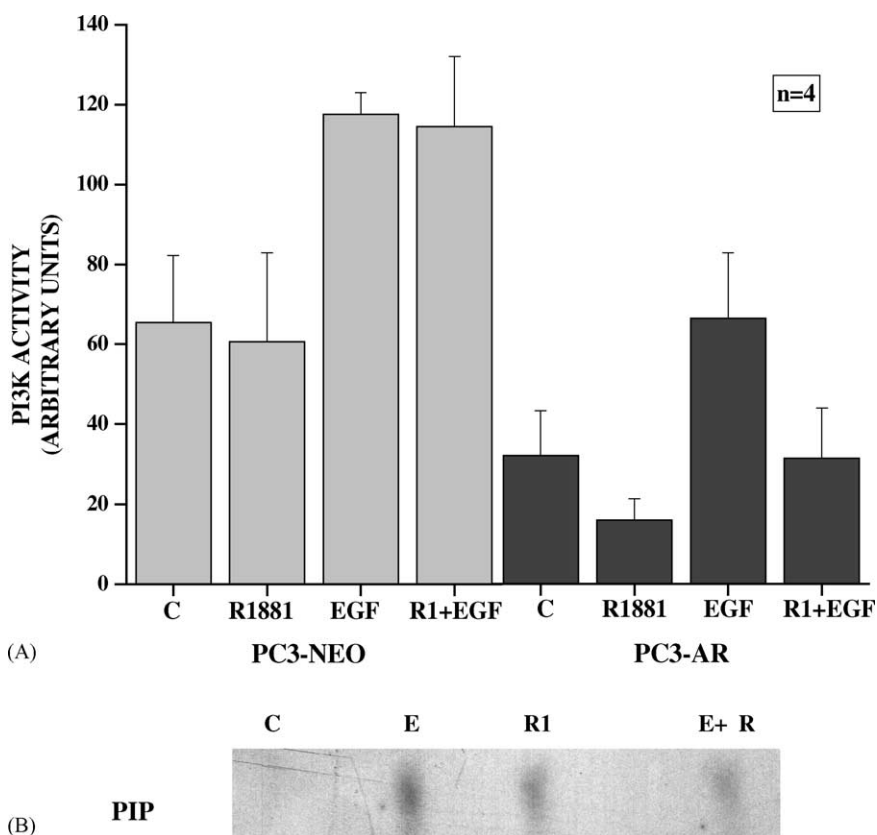


Fig. 3. PI3K activity in PC3-Neo and PC3-AR cells. Cells were stimulated or not with EGF (50 ng/ml, 10 min) in the presence or absence of the synthetic androgen R1881 (1 nM for 24h) and PI3K activity evaluated by immunokinase assay (13). Panel A: Mean  $\pm$  S.E.M. values of PI3K activity (arbitrary units) in four different experiments performed in the two cell lines. Panel B: Typical experiment performed in PC3-AR cells. PIP: phosphatidylinositol phosphate.

### 1.3. EGF-mediated PI3-kinase activation is reduced in PC3-AR cells

As mentioned above, EGF promotes the activation of distinct signaling pathways in cancer cells, including PI3K which finally leads to migration and metastasis [9]. To evaluate whether PI3K activation is involved in Matrigel invasion of PC3-Neo cells, invasion assays were performed in the absence or presence of the PI3K inhibitor LY294002 (80  $\mu$ M). We found that EGF-induced invasion through Matrigel was totally inhibited by the pretreatment (30 min) with LY294002 (not shown) indicating that the activation of PI3K pathway is essential for EGF-dependent invasion. To investigate whether PI3K activity was altered in PC3-AR cells, cell lysates from PC3-Neo and PC3-AR cells plated on laminin and stimulated or not with EGF (50 ng/ml) were immunoprecipitated with an anti-phosphotyrosine antibody to recruit the activated fraction of PI3K and assayed for their ability to phosphorylate L- $\alpha$ -phosphatidylinositol. As shown in Fig. 3A, which reports mean  $\pm$  S.E.M. values of PI3K activity (measured by quantification of the bands) of four different experiments, both basal and EGF-stimulated PI3K activity are reduced in PC3-AR cells respect to PC3-Neo. Pre-treatment with R1881 determined a further decrease of EGF-stimulated PI3K activity in PC3-AR cells, whereas it

was ineffective in PC3-Neo cells. Fig. 3B shows a typical experiment performed in PC3-AR cells. This result suggests that in the presence of the androgen receptor PI3K activity was reduced in line with the recent results demonstrating that the lost of androgens sensitivity such as in high passages LNCaP cells [10] and androgen-refractory LNCaP cells following androgen deprivation [11], leads to higher activity of PI3K respect to cells expressing a more differentiated phenotype, such as LNCaP cells at the early passages or androgen-sensitive LNCaP cells which maintain a much lower PI3K activity.

## 2. Conclusions

Androgen-insensitive prostate cancer cells are characterized by increased growth, adhesion, invasion and migration [1–4]. We show here that an interaction between the AR and the EGFR at the membrane level occurs in androgen-sensitive prostate cancer cell lines and show evidence that the reduced invasive properties of these cells are due to an interaction between the EGFR and AR, decreased signalling leading to EGFR autotransphosphorylation and lower PI3K activation in response to EGF. Whether this interaction is responsible for the reduced PI3K activity

detected in PC3-AR cells needs to be investigated, in any case our results suggest that in the presence of the androgen receptor PI3K activity was reduced according to the recent results demonstrating that the loss of androgens sensitivity leads to higher activity of PI3K respect to cells expressing a more differentiated phenotype, such as LNCaP cells at the early passages or androgen-sensitive LNCaP cells which maintain a much lower PI3K activity [10,11]. The interaction between the AR and EGFR as well as the reduced functionality of EGFR in PC3-AR cells leads to the hypothesis that membrane localization of the complex between AR-EGFR may determine a sequestration of the EGFR.

Whether the interaction between AR and EGFR is due to direct binding of the two proteins or is mediated by another protein needs to be investigated further. It is known from previous studies that AR is able to interact directly with c-src [6] and with PI3K [8]. In particular, the interaction with c-src is mediated by a proline-rich region present in the DNA binding domain of the receptor [6]. It is thus possible that another protein, able to interact with both the AR and the EGFR could mediate the interaction found in our experiments.

In conclusion, in androgen-sensitive prostate carcinoma cells the AR, localized at the plasma membrane, contribute to confer a less malignant phenotype of these cells both by reducing the expression and function of alpha6beta4 [3] and by interfering with EGFR signalling leading to invasion in response to EGF through a co-localization and interaction between AR and EGFR at the membrane [12].

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