Androgen receptor cytosine-adenine-guanine repeat polymorphisms modulate EGFR signaling in epithelial ovarian carcinomas

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Abstract

Objective. Length of a polymorphic cytosine-adenine-guanine (CAG) repeat in the androgen receptor (AR) may inversely correlate with AR activity. We have identified an association between short AR allelotypes and decreased survival in women with epithelial ovarian cancer. We hypothesize short AR allelotypes promote aggressive ovarian cancer phenotype through modulation of epidermal growth factor receptor (EGFR) signaling.

Methods. SKOV-3 cells were transfected with AR plasmids containing variable CAG repeat lengths, and AR activity was assessed through co-transfection with a luciferase plasmid. EGFR signaling was studied with Western blot analysis of EGFR, EGFR-p (phosphorylated), MAPK, and MAPK-p, and cellular proliferation examined by MTT assays. Data were analyzed using analysis of variance, Tukey–Kramer multiple comparison test, and Student's *t* test.

Results. We confirmed AR allelotype length inversely correlates with AR activity in epithelial ovarian cells; a 2.5% decrease in luciferase-fold activation was seen with each CAG unit increase ($p = 0.0002$). We observed inhibition of EGFR-p abundance with increasing abundance of transfected AR cDNA (89.2% and 39.9% for 3.0 and 6.0 μg, compared to 1.5 μg, $p = 0.03$). After transfection with short (CAG=14), median (CAG=21), and long (CAG=24) AR allelotypes, an inverse correlation was identified between abundance of MAPK-p and CAG repeat length ($p = 0.002$). Decrease in cellular abundance was also seen in cultures transfected with ARs of increasing CAG repeat length ($p < 0.0001$).

Conclusions. These data identify an inhibitory action of AR on EGFR signaling, and support research investigating AR/EGFR antagonism in the treatment of ovarian cancers.

Introduction

Significant evidence implicates aberrant androgen homeostasis in epithelial ovarian cancer biology. Androgens interact directly with a specific receptor (AR), and mediate control of cellular homeostasis, growth, and apoptosis in benign ovarian epithelial cells [1]. In malignant ovarian epithelium, immunocytochemical studies indicate that up to 95% of ovarian cancers express AR, suggesting that androgens play an important role in tumor biology [2,3].

Studies in prostate and kidney cell cultures indicate that length of a polymorphic cytosine-adenine-guanine (CAG) repeat sequence in *AR* inversely correlates with receptor transactivation function [4,5]. This CAG repeat sequence codes for a polyglutamine tract, which extends for a median length of 21 ± 2 repeats and normally ranges between 8 and 31 [6]. In an institutional cohort of 77 women with advanced stage papillary serous ovarian cancers, we observed a decreased incidence of optimal surgical cytoreduction (defined as <1 cm residual disease following resection) in patients with short *AR* alleles, as well as shortened progression-free and overall survival. Furthermore, multivariate analyses indicated that a

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short *AR* allele retained significance as an independent poor prognostic factor [7].

As the molecular mechanisms responsible for these clinical observations are not well defined, we have hypothesized that short *AR* alleles promote aggressive cancer biology through modulation of EGFR signaling. EGF is a potent mitogen for ovarian epithelial cells, and has been implicated in cancer development and progression [8]. Its receptor, EGFR, is a member of the ErbB receptor tyrosine kinase family, which plays key roles in malignant transformation and cancer biology. Expression of EGFR significantly correlates with greater surgical stage in epithelial ovarian carcinomas; furthermore, EGF and EGFR expressions are more intensive and diffuse in resistant carcinoma specimens obtained post-chemotherapy, when compared with specimens from the same patients prior to treatment [9]. Recent data have also identified aberrant EGFR signaling as a causative factor in modulating aggressive invasive phenotypes of ovarian carcinoma cells, including increased expression of laminin-1 and matrix metalloproteinase activity [10]. EGFR signaling may thus represent a candidate mechanism by which AR promotes aggressive epithelial ovarian cancer biology. In prostate carcinoma cells, androgens have been shown to up-regulate expression of EGFR and subsequently promote cellular proliferation [11]. Furthermore, immunoconfocal and co-immunoprecipitation analyses have also demonstrated an interaction between AR and EGFR, indicating interference by AR on EGFR signaling [12].

Our objectives of this study are to confirm, as proof of principle, the inverse correlation of *AR* CAG repeat length and AR activity, and to define the influence of AR and its CAG polymorphisms on EGFR signaling and malignant cell proliferation.

Methods

Plasmid constructs

We modified pcDNA3.1-hAR45, an *AR* expression plasmid with 45 CAG repeats, by replacing the CAG repeat with others amplified from patient DNAs whose lengths were identified in our previous study [7]. Segments containing entire CAG polymorphic regions were amplified using primers 5′-TAG GGC TGG GAA GGG-TCT AC-3′ and 5′-TCG AAG TGC CCC CTA AGT AA-3′ which anneal on either side of flanking native Eag I and Bfr I restriction sites. High-fidelity amplification was accomplished using Pfu DNA polymerase (Stratagene). Eag I and Bfr I digested amplicons were gel purified and ligated to the AR plasmid in place of the existing CAG 45 repeat prepared by the same two restriction enzymes (Invitrogen). The resulting plasmids were designated pcDNA3.1-hAR9, pcDNA3.1-hAR14, pcDNA3.1-hAR21, and pcDNA3.1-hAR24. Inserts were validated by sequencing.

Tissue culture

AR negative SKOV3 cells were cultured in McCoy's 5A medium with 10% charcoal-stripped FBS and 1× penicillin/streptomycin, and were subcultivated using standard trypsin/EDTA digestion as previously described [13]. Media reagents were purchased from Gibco and MediaTech. Transfections were accomplished using Superfect (Qiagen). Cultures were also treated with the indicated concentrations of EGF and DHT (Sigma).

Western blotting

Proteins were prepared from tissue cultures by lysis in Cell Lysis Buffer (Cell Signaling Technology) supplemented with additional protease inhibitors (Complete Mini, Roche). Proteins were separated in precast polyacrylamide

gels (Bio-Rad), transferred to Hybond (Amersham), and detected by ECL (Amersham). AR was detected using polyclonal AR antibody (N-20, Santa Cruz). Phosphorylated EGFR and total EGFR were detected using two distinct antibodies: polyclonal rabbit phospho-EGFR (tyr1068) antibody and EGFR antibody respectively (Cell Signaling Technology). Phosphorylated MAP kinase and total MAP kinase were also detected using two distinct antibodies: polyclonal phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibody and p44/p42 MAP kinase antibody (Cell Signaling Technology). β-actin was detected with actin mAb AC-40 (Sigma). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Densitometric studies were accomplished using ImageJ Analysis [14].

Assay of AR function

AR constructs were co-transfected with pAMAneo-Luc and pβGAL into SKOV3 cells. pAMAneo-Luc is a reporter plasmid harboring AR binding elements upstream of the firefly luciferase gene. pβGAL expresses β-galactosidase by a generic CMV promoter and was used to normalize transfection efficiency. After 24 h post-transfection, the culture media was replaced with media containing the indicated concentration of DHT or diluent. After an additional 48 h, cells were rinsed with PBS and lysed in Reporter Lysis Buffer (Promega). Experiments were performed in triplicate, and part of the lysate was used to detect luciferase using the Luciferase Assay System (Promega) with a luminometer, and part was used to detect β-galactosidase activity using a liquid assay, reported as Miller units [15]. Corrected luciferase values were reported as luciferase units per Miller units.

Proliferation assays

Cells were transfected as indicated and recovered 4 h, then plated in quadruplicate onto 96-well plates (1000 cells/well) in serum-free media. Cells were serum-starved 12 h and then treated with EGF, DHT or diluent for 72 h as indicated. The total diluent amount remained constant. Proliferation assays were accomplished using the CellTiter 96 AQ$_{ueous}$ Non-Radioactive Cell Proliferation Assay kit (Promega). Absorbance was read at 490 nm with a reference wavelength of 655 nm. AR was assessed by Western blotting in a small fraction of the transfected cultures to verify transfection.

Results

Length of the CAG repeat sequence in AR inversely correlated with receptor transactivation function in malignant epithelial ovarian cells

While several investigators have reported an inverse relationship between *AR* CAG repeat length and AR activity, none has confirmed this finding in epithelial ovarian cells. We thus utilized transient transfection to study 5 hAR plasmids containing CAG repeat sequences of 9, 14, 21, 24, and 45 in AR-null SKOV3. No luciferase activation was seen for untransfected cultures, consistent with our observations that SKOV-3 is AR-null. Furthermore, no luciferase activation was seen for transfected cultures in the absence of DHT. However, we identified a significant inverse relationship between CAG repeat length and AR transactivation function after treatment with 2.5 nM DHT ($p=0.0002$; Fig. 1). On average, we observed a 2.5% decrease in luciferase-fold activation for each additional unit increase in CAG repeat length.

Increasing AR abundance reduced EGFR activation

Before studying the influence of AR CAG polymorphisms on EGFR signaling, we initially sought to determine the effect
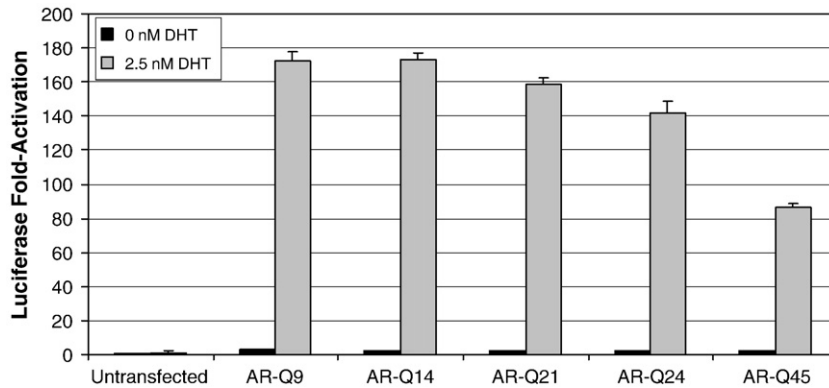
Fig. 1. Extension of the *AR* CAG repeat sequence reduces AR transactivation function. AR activity was assayed by fold-increase in luciferase activity after transient transfection of pcDNA3.1-hAR-9, -14, -21, -24, and -45 into AR-null SKOV3 cells and treatment with 25 nM DHT. A median 2.5% decrease in activity for each additional unit increase in CAG repeat length was observed (ANOVA, $p < 0.0002$).

of AR abundance on EGFR phosphorylation. Thus, we transiently transfected SKOV3 cells with 1.5, 3.0, and 6.0 μg of AR cDNA (using pcDNA3.1-hAR-21 to represent a median CAG repeat length). Cells were allowed to recover, serum-starved and then treated with 25 nM DHT and 100 ng/ml EGF for 10 min. Cells were harvested and subjected to Western blot analysis. As expected, greater AR abundance was observed with increasing concentrations of transfected AR cDNA (Fig. 2). However, cells transfected with 3.0 μg and 6.0 μg of AR cDNA demonstrated 89.2% and 39.9% of the abundance of EGFR-p

(normalized to actin) observed in cells transfected with 1.5 μg of AR cDNA (ANOVA, $p = 0.03$). Tukey–Kramer multiple comparison testing identified no statistical differences between abundance of EGFR-p in 1.5 versus 3.0 mg AR cDNA, nor in 3.0 versus 6.0 mg AR cDNA; however a statistical difference was identified between 1.5 and 6.0 mg AR cDNA ($q = 4.732$, $p < 0.05$).

AR CAG repeat length inversely correlated with MAPK phosphorylation

We have previously identified a correlation between short *AR* allelotypes and aggressive ovarian cancer biology. In order to characterize a molecular mechanism underlying these clinical observations, we studied the relative effect of 3 *AR* allelotypes (representing short, median, and long CAG repeat lengths) on EGFR signaling proteins. SKOV3 cells were transient transfected with pcDNA3.1-hAR-14, -21, and -24. Cells were then plated, allowed to adhere overnight, and then serum-starved for 16–24 h. Cells were then treated ±EGF and ±DHT for 10 min before cell lysates were prepared. Densitometric analysis of digitized Western blots revealed a non-significant linear decrease in the abundance of EGFR-p (corrected by abundance of EGFR) with *AR*s of increasing CAG repeat lengths. Specifically, SKOV-3 cells transfected with pcDNA3.1-hAR-21 and -24 and treated with both 10 nM DHT and 25 ng/mL EGF demonstrated 95% and 86% of the abundance of pEGFR/EGFR when compared to cultures expressing pcDNA3.1-hAR-14 ($p = 0.08$; Fig. 3A and B).

In order to examine downstream events in EGFR signaling, Western blots were probed with MAPK and MAPK-p antibodies. When treating with EGF alone, an increase in the abundance of MAPK-p (when corrected by abundance of MAPK) was identified in the AR-Q21 construct when compared to MAPK-p/MAPK in the AR-Q14 and -Q24 constructs. With treatment of both EGF and DHT, a significant linear decrease was observed in MAPK-p abundance with increasing CAG repeat length in *AR*. Specifically, abundance of MAPK-p/MAPK was 78% and 50% for pcDNA3.1-hAR-21 and -24, when compared to -14 ($p = 0.002$; Fig. 3C). Tukey–Kramer
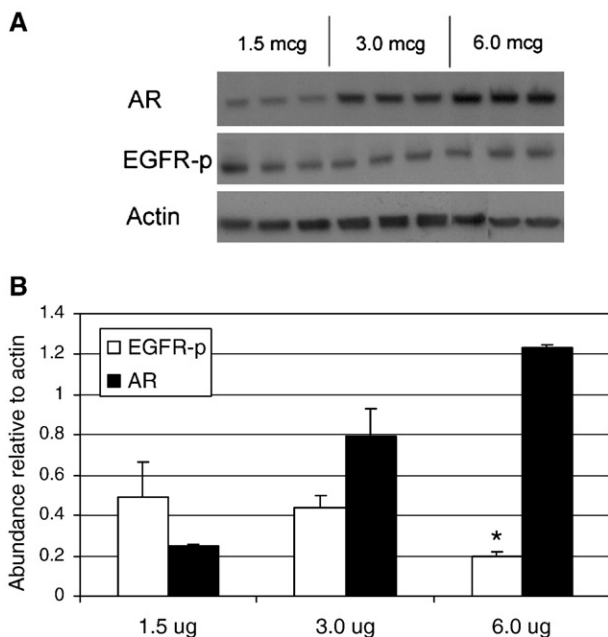

Fig. 2. Correlation of AR and EGFR in SKOV3 cells. (A) Immunoblotting of cells transfected as indicated with pcDNA3.1-hAR-Q21. (B) Densitometric representation of immunoblots. Bars represent means±standard deviations. Increasing abundance of AR corresponds to decreased phosphorylation of EGFR (ANOVA, $p = 0.03$). Black columns represent AR abundance, and white columns represent abundance of phosphorylated EGFR. Tukey–Kramer multiple comparison testing identified only a statistical difference between 1.5 and 6.0 mg AR cDNA ($q = 4.732$, $p < 0.05$).
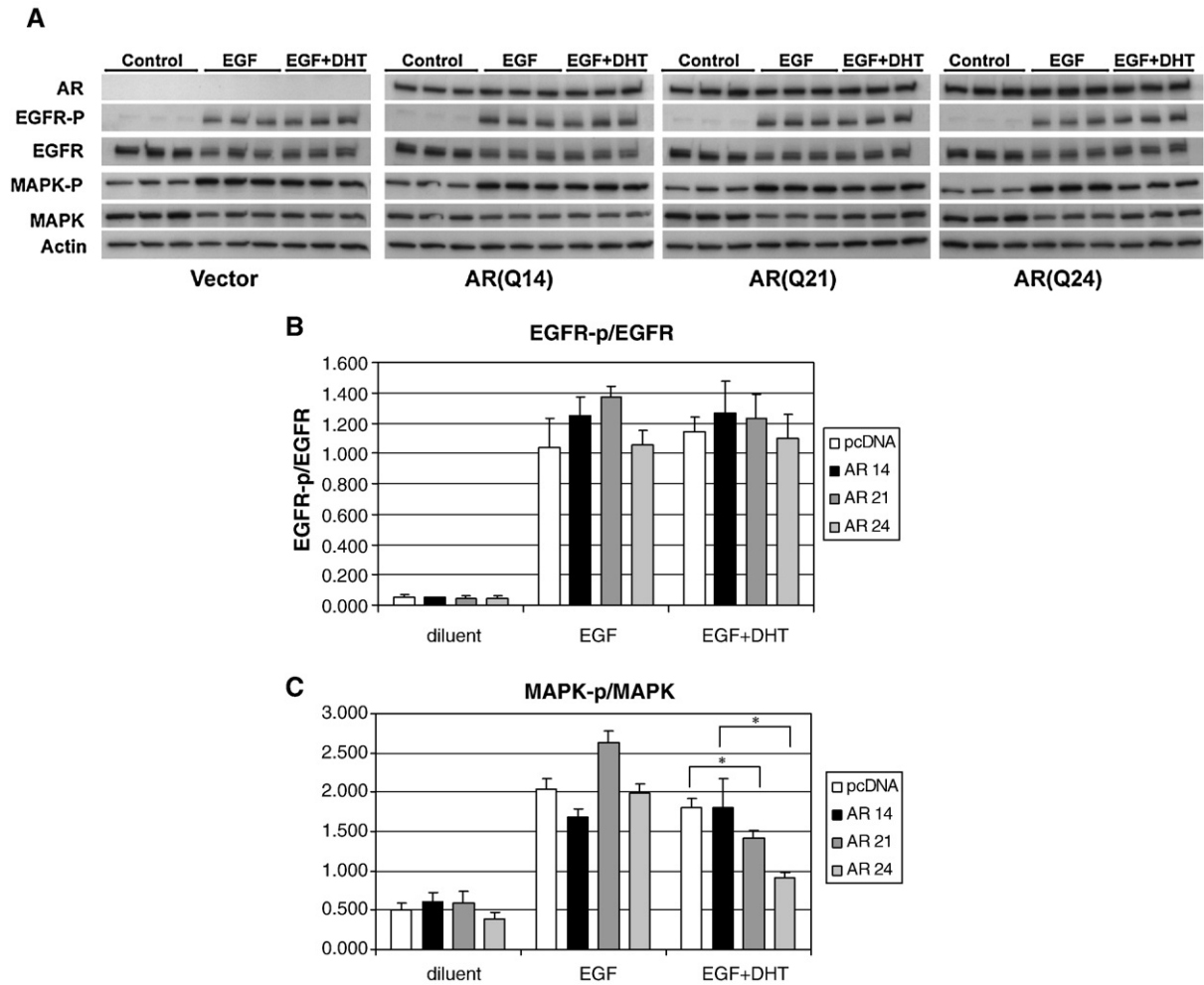
Fig. 3. Western blot analysis of transfected SKOV3 cultures with pcDNA3.1 (control), pcDNA3.1-hAR-14, -21, and -24 demonstrates a non-statistical linear decrease in phosphorylated EGFR and a statistical decrease in phosphorylated MAPK with increasing *AR* CAG repeat length. (A), digitized images of Western blot proteins stained for androgen receptor (AR), EGFR-p (phosphorylated), EGFR, MAPK-p (phosphorylated), MAPK, and actin. (B), comparison of relative EGFR-p abundance relative to EGFR. A non-significant trend is observed after treatment with both EGF and DHT, where decreasing EGFR-p abundance is seen with increasing *AR* CAG repeat length ($p=0.08$). (C), comparison of relative MAPK-p abundance relative to MAPK. A significant trend is observed after treatment with both EGF and DHT, where decreasing MAPK-p abundance is seen with increasing *AR* CAG repeat length ($p=0.002$). Tukey–Kramer multiple comparison testing revealed statistically significant differences between pcDNA versus AR-Q24 ($q=7.705$, $p<0.01$) and AR-Q14 versus AR-Q24 ($q=7.720$, $p<0.05$).

multiple comparison testing revealed statistically significant differences between pcDNA versus AR-Q24 ($q=7.705$, $p<0.01$) and AR-Q14 versus AR-Q24 ($q=7.720$, $p<0.05$).

AR CAG repeat length inversely correlated with cellular proliferation

To determine the influence of *AR* CAG repeat length on AR- and EGFR-mediated cellular proliferation, we expressed ARs of different CAG repeat lengths in SKOV-3 cells, treated cultures with and without DHT and EGF, and conducted MTT assays. The results indicate that there is a significant inverse correlation between cell proliferation and CAG repeat length. When comparing cultures transfected with pcDNA3.1 (control), pcDNA3.1-hAR-14, -21, and -24, and treated with DHT and EGF, significant reduction in cell abundance was observed with increasing CAG repeat length (Fig. 4, $p<0.0001$, ANOVA). AR-Q14, -Q21, and -Q24 demonstrated 86.1, 78.6, and 71.2%

of the cellular abundance observed for the pcDNA3.1 control. Tukey–Kramer multiple comparison testing also confirmed statistical differences between control versus AR-Q14; AR-Q14 versus AR-Q21; and AR-Q21 versus AR-Q24 ($q=6.621$, 10.299, 8.141 and $p<0.01$, 0.001, and 0.01, respectively). Several additional observations were also consistent within each transfected cell line. When compared to a no-treatment control ($-$DHT/$-$EGF), treatment with EGF alone ($-$DHT/$+$EGF) showed that EGF stimulated proliferation significantly in the control and all 3 AR constructs; this is consistent with its known mitogenic effect on epithelial ovarian cells ($p<0.01$ for pcDNA3.1, pcDNA3.1-hAR-14, -21, and -24). Treatment with DHT alone ($+$DHT/$-$EGF) did not stimulate proliferation significantly across all AR constructs, when compared to the no-treatment control. Treatment with both DHT and EGF ($+$DHT/$+$EGF) also stimulated proliferation significantly in the control and 3 AR constructs when compared to no treatment ($-$DHT/$-$EGF; $p<0.01$ for pcDNA3.1, pcDNA3.1-hAR-14,
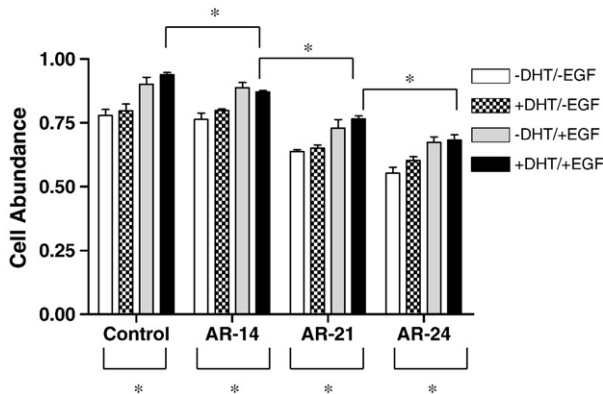
Fig. 4. MTT assays of transfected SKOV3 cultures with pcDNA3.1 (control), pcDNA3.1-hAR-14, -21, and -24 confirm an inverse correlation between *AR* CAG repeat length and cellular proliferation. The black columns indicate the significant reduction in cell abundance that was observed in cell cultures transfected with increasing CAG repeat length, after treatment with dihydrotestosterone (DHT) and EGF ($p < 0.0001$, ANOVA). Multiple comparison testing also confirmed statistical differences between control versus AR-Q14; AR-Q14 versus AR-Q21; and AR-Q21 versus AR-Q24 ($q = 6.621$, 10.299, 8.141 and $p < 0.01$, 0.001, and 0.01, respectively). In addition, treatment with EGF alone (−DHT/+EGF) showed that EGF stimulated proliferation significantly in the control and all 3 AR constructs ($p < 0.01$ for pcDNA3.1, pcDNA3.1-hAR-14, -21, and -24). Treatment with DHT alone (+DHT/−EGF) did not stimulate proliferation significantly across all AR constructs. Treatment with both DHT and EGF (+DHT/+EGF) also stimulated proliferation significantly in the control and 3 AR constructs when compared to no treatment (−DHT/−EGF; $p < 0.01$ for pcDNA3.1, pcDNA3.1-hAR-14, -21, and -24). Comparison of EGF treatment alone (−DHT/+EGF) compared to DHT and EGF treatment (+DHT/+EGF) did not reveal any statistical differences in cellular proliferation for any of the transfected cell lines.

-21, and -24). Finally, comparison of EGF treatment alone (−DHT/+EGF) compared to DHT and EGF treatment (+DHT/+EGF) did not reveal any statistical differences in cellular proliferation for any of the transfected cell lines. These data indicate that DHT is less mitogenic than EGF, and does not detectably contribute to EGF mitogenicity.

Discussion

We have hypothesized that enhanced androgen signaling, mediated by short *AR* CAG allelotypes, promotes aggressive epithelial ovarian cancer biology. To test this hypothesis, we initially confirmed, as proof of principle, the inverse correlation between *AR* CAG repeat length and AR transactivation function utilizing 5 *AR* expression plasmids in an epithelial ovarian cell model. Furthermore, we identified a novel inhibitory effect of AR on the abundance of phosphorylated EGFR. Finally, we distinguished inverse correlations between *AR* CAG repeat length and abundance of phosphorylated MAPK and cellular proliferation after treatment with DHT and EGF. While no statistically significant patterns between AR constructs could be identified for phosphorylated EGFR, we speculate that this could potentially result from small differences that could not be distinguished in these sets of experiments.

These data validate the growing body of literature supporting the functional consequences of CAG repeat expansion in *AR*. Previous studies have only examined CAG polymorphisms in

prostate, liver, and kidney cell cultures, and our study supports our prior clinical data implicating aberrant AR activity on clinical outcome in ovarian cancers. These data are further strengthened by the linear decrease observed in activity over 5 different polymorphic CAG repeat lengths; to date, only one other group have examined more than 3 CAG repeat lengths, and they report a similar 1.7% decrease in activity for each additional CAG repeat length in prostate cancer cell cultures [16].

This study is also the first to examine the molecular interactions between AR and EGFR in epithelial ovarian cancers. Previously, only correlative studies, using immunocytochemistry, have linked AR and EGFR expression in malignant ovarian epithelium, initially suggesting cross-talk between the two pathways in this disease [17]. An inhibitory role of AR on EGFR signaling has only recently been described in prostate cancer models. It is well established that in prostate cancer, androgens and AR are initially involved in tumorigenesis; however, once this disease progresses to a state of androgen independence, therapeutic options are limited and prognosis is invariably poor [18]. Recent studies indicate that re-expression of AR in androgen-independent prostate cancer cells decreases invasion and adhesion in response to EGF, and that transfection of AR reduces EGF-induced EGFR phosphorylation [12,19]. Furthermore, more recent data suggests that AR reduces the interaction of EGFR with key mediators of clathrin-mediated endocytosis. Reduction of EGFR internalization, with localization in a caveolar compartment, may promote an inactive state [20,21]. Taken together, these data indicate an inhibitory effect of AR on EGFR signaling in prostate cancer models, an effect we similarly demonstrate in malignant ovarian cancer cells transfected with median and long *AR* allelotypes.

Epidemiologic data suggest that the influence of heightened androgen activity is considered a risk factor for epithelial ovarian carcinogenesis, but its effect on tumor biology of ovarian epithelial cells remains poorly defined [reviewed in [1]]. Karlan et al. studied the effects of steroid hormones on two ovarian carcinoma cell lines, SKOV3 and CaOV-3, and did not demonstrate any differences in cellular proliferation with or without treatment with DHT [22]. Our findings are congruent with these observations, as we show only minimal differences in cell growth after treatment with DHT with or without EGF. From a clinical standpoint, treatment of recurrent, chemoresistant ovarian cancer with anti-androgen therapy has shown disappointing response rates [23,24]. We hypothesize that the AR–EGFR interaction in ovarian cancer biology is influenced by the length of the CAG polymorphism in AR; the effect of AR antagonism may be most pronounced in patients with short CAG polymorphisms, and those with median or long AR allelotypes may be less responsive to such therapies.

These data add to the growing body of evidence linking heightened androgenicity to the pathogenesis and tumor biology of epithelial ovarian cancers. Genotype analysis of AR may identify specific cohorts of patients who could potentially benefit from targeted anti-AR and/or anti-EGFR therapeutics. We have proposed studies in murine ovarian cancer models to further examine the potential benefits of AR/EGFR antagonism in cancers characterized by short or long *AR* CAG repeat lengths.

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