Short Androgen Receptor Allele Length Is a Poor Prognostic Factor in Epithelial Ovarian Carcinoma

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ABSTRACT

Purpose: Epidemiological evidence implicates a heightened androgenic state in women with epithelial ovarian cancer. Androgen activity may be modulated by altered expression or activity of the androgen receptor (AR) or AR polymorphisms. Exon 1 of the AR gene contains a polymorphic (CAG) sequence whose length is inversely correlated with transcriptional activity.

Experimental Design: Differential expression of AR mRNA and protein was examined in 46 primary cultures of normal human ovarian surface epithelium (HOSE) and malignant Cedars-Sinai ovarian cancer (CSOC) ovarian epithelial cells. AR allele length was characterized by genotyping in 77 ovarian cancer specimens.

Results: AR mRNA expression was higher in CSOC primary cultures (1.58 ± 0.17) when compared with HOSE (1 ± 0.09, P = 0.005), but protein expression was not statistically different. CAG repeat lengths were shorter in CSOC (20.6 ± 1.2) than in HOSE (23.4 ± 0.9, P = 0.04). Patients with an AR allele containing ≤19 CAG repeats had a shorter time to recurrence (5.5 versus 19.4 months, P < 0.0001) and overall survival (9 versus 32.6 months, P = 0.0007). There was no correlation between AR allelotype and age of diagnosis, stage, or grade; however, a short CAG length ≤19 repeats was associated with decreased surgical cytoreducibility (44.4 versus 10.3%, P = 0.035). Multivariate analyses confirmed a short AR allele as an independent prognostic factor (P = 0.02).

Conclusions: These data support epidemiological evidence linking heightened androgenicity to the pathogenesis and tumor biology of epithelial ovarian cancer.

INTRODUCTION

Significant evidence implicates alterations in androgen hormone homeostasis in the pathogenesis and progression of epithelial ovarian carcinomas. Heightened androgenicity, in particular, has been associated with increased risk of ovarian cancer [reviewed by Risch (1)]. Significant elevation in serum androgen levels has been identified in women years before their diagnosis of epithelial ovarian cancer, and testosterone has been shown to stimulate the growth of ovarian epithelial tumors in animal models (2, 3). Furthermore, immunohistochemical studies indicate that ≥95% of epithelial ovarian cancers express AR (4, 5). Conservation of AR expression after malignant transformation suggests that androgens function in ovarian cancer biology. From a functional perspective, ovarian adenocarcinomas express high levels of AR-associated protein 70, a coactivator that enhances the transactivational potential of AR (6). Androgens may also promote ovarian cancer progression indirectly by modulating tumor growth factor-β receptor expression, which may in turn disrupt normal cellular tumor growth factor-β-mediated growth inhibition (7).

The actions of androgens are mediated via the AR, encoded by a gene located on the X chromosome. There is a single known AR species, in contrast to ERs and PRs, which are both expressed as two functionally distinct subtypes (ER-α and ER-β and PR-A and PR-B, respectively). However, polymorphisms of AR, because of variable lengths of a trinucleotide CAG repeat sequence in exon 1, have been shown to modulate receptor activity levels. An inverse relationship has been found between allele length and transcriptional transactivation function; longer AR polyglutamine lengths encoded by more CAG repeats (ranging between 8 and 31) are associated with lower transcriptional activity and vice versa (8, 9). Following the Lyon hypothesis of random X inactivation, each cell expresses only one AR allele; the ovarian epithelium, like all tissues, is a mosaic of cells expressing one of the two inherited alleles (10, 11). Increased androgen activity modulated by expression of shorter AR alleles has been suggested as a possible causative factor in hormone-responsive tumors. Shorter AR allelotype has been associated with increased risk of prostate cancer and has been correlated with a younger age at ovarian cancer diagnosis in a cohort of Ashkenazi Jewish women (12, 13).

AR allele length polymorphisms may also contribute to a more aggressive tumor biology. In prostate cancers, men harboring a short allele with ≤19 CAG repeats demonstrate significantly higher grade tumors, more aggressive early stage cancers, and an unfavorable prognosis (14). Analysis of AR
allelotype in women with breast cancer identified a 6% reduction in the risk of death with every CAG repeat increase (15). Although the significance of AR allelotype has not been investigated in ovarian cancer, known clinical and pathological factors that predict prognosis and survival include age at diagnosis, stage and grade of disease, and surgical cytoreducibility (16, 17). Feasibility of optimal tumor cytoreduction, defined as residual disease <1 cm in maximum diameter at the completion of initial surgery, may reflect inherent properties of the specific tumor biology (18).

Previous investigations of AR in ovarian neoplasia have been limited. One study of four benign and four malignant primary cultures reported AR transcript expression to be lost in malignant cultures (19). A singular report of CAG length polymorphisms in epithelial ovarian carcinoma compared germ-line, but not tumor, DNA to that of normal women; furthermore, they did not examine allele length with respect to clinical parameters, such as cytoreducibility or survival (20). We hypothesize that the effect of androgens, mediated by AR expression patterns and/or activity, may be heightened in transformed ovarian epithelial cells and contribute to a more aggressive tumor biology. The purpose of this study was to perform a detailed characterization of AR mRNA and protein expression patterns in a large cohort of primary normal and malignant epithelial ovarian cell cultures and investigate the relationship between AR activity and tumor biology by examining AR allelotype in the context of clinicopathological factors and survival.

MATERIALS AND METHODS

Cell Cultures. Twenty-three primary HOSE cultures and 23 primary CSOC cultures were evaluated. HOSE primary cultures were initiated from surface scrapings of normal ovaries removed from women undergoing surgical oophorectomy for benign indications. CSOC cultures were derived from macro-dissected, minced tumor tissue incubated with collagenases as described previously (21). All tissues were collected from the Gynecologic Oncology laboratory at Cedars-Sinai Medical Center after obtaining signed informed consent under an Institutional Review Board-approved protocol. Ovarian histopathology was confirmed by pathological review. HOSE cultures were early passages (4–7) in medium 199 and MCDB 105 (1:1; Sigma-Aldrich Corp., St. Louis, MO), and CSOC cultures were early passages (3–8) in McCoy’s 5A media (Sigma-Aldrich Corp.). Media were supplemented with 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin, and cultures were grown at 37°C in 5% CO₂. The histological nature and homogeneity of these epithelial cell cultures were confirmed by immunohistochemical characterization (21).

Tumor Samples. Malignant tissue from primary ovarian specimens from 77 women who signed informed consent was identified with gross and histological review and snap frozen at time of initial cytoreductive surgery. All samples were histologically confirmed as exclusively epithelial ovarian carcinoma by a pathologist specializing in gynecologic oncology.

RNA Isolation and Reverse Transcriptase-PCR. Total cellular RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH). RNA was quantitated by spectrophotometric analysis. After DNase treatment to remove contaminating genomic DNA, 2 μg of total RNA were reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Subsequently, 5 μl of the resulting 20-μl cDNA samples were used in each PCR reaction.

Novel primer sequences for AR were designed and used for PCR amplification (forward, 5’-CTC-TCT-CAA-GAG-TTT-CTT-TGG-CT-3’; reverse, 5’-CAC-ATG-CCA-AGA-GAT-GAT-CTC-TGC-3’). Hot start PCR using AmpliTaq Gold DNA polymerase (Perkin-Elmer Life Sciences, Inc., Boston, MA) was used for all amplifications. The reaction mixtures were preheated at 95°C for 5 min before thermal cycling. The routine PCR program was 35 cycles of 30 s at 94°C, 30 s at 63°C (annealing temperature for AR primers), and 30 s at 72°C. The amount of RNA in each sample was standardized by PCR amplification of the housekeeping gene GAPDH. GAPDH was amplified for 25 cycles at an annealing temperature of 55°C using primers published previously (22). PCR products were resolved on 2% NuSieve agarose gel containing 10 μg/ml ethidium bromide. Gel images were digitized under UV transillumination, and PCR products were quantitated by densitometric analysis performed using One-Dscan software (Scanalytics, Billerica, MA). Representative products were sequenced to confirm PCR product identity.

Protein Extraction and Western Blot Analysis. Protein lysates of primary cell cultures were prepared using 400 μl of lysis buffer (1% deoxycholate, 0.1% Triton X-100, 0.1% SDS, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 20 mM sodium P2O5, 2 mM Na3VO4, 0.15 mM aprotinin, 10.8 mM leupeptin, 0.44 mM pepstatin, and 1.65 mM antipain) per T75 culture flask. Protein concentration was quantitated using the BCA Protein Assay (Pierce Biotechnology, Rockford, IL). Fifty μg of each protein lysate were separated by SDS-PAGE and electro-transferred to Hybond-P membrane (Amersham-Pharmacia Biotech, Piscataway, NJ) for Western blot analysis. AR was stained with 1 μg/ml rabbit polyclonal antibody (N-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in diluted blocking buffer [PBS, 0.05% Tween 20 (Santa Cruz Biotechnology, Inc.), and 5% nonfat milk]. After incubating overnight at 4°C in blocking buffer, membranes were incubated for 1 h at room temperature in primary antibody, washed, and incubated in the goat antirabbit horseradish peroxidase-labeled secondary antibody according to the manufacturer’s instructions (Vectorstain Elite; Vector Laboratories, Burlingame, CA). The immunolabeled proteins were visualized by incubating in diaminobenzidine [10 mg in 10 ml 50 mM sodium phosphate buffer (pH 7.4)] and quantitated using digitized gel images as above. Androgen-responsive LNCaP prostate cancer cell lysates were used as positive control.

AR CAG Repeat Length Determination. In primary cultures, CAG repeat length sequences of the expressed AR allele were determined using cDNA. In tumor tissues, genomic DNA was isolated from snap frozen tumor tissues using standard procedures (23). Sequences encompassing the polyglutamine CAG repeat region were amplified by hot start PCR using primers flanking the CAG sequence (5’-TCC-AGA-ATC-TGT-TCC-AGA-GCG-TGC-3’ and 5’-GCT-GTG-AAG-GTT-GCT-GTT-CCT-CAT-3’) as described by Giovannucci et al. (14). Primers were labeled with fluorescein aminomethane to determine sequence length using laser activated fluorescent dye
technologies (ABI 377 PRISM and associated software; Applied Biosystems, San Mateo, CA). Representative PCR products were independently sequenced to confirm number of CAG repeat lengths and product identity.

**Statistical Analysis.** For all quantitative analyses, mean HOSE mRNA and protein levels were standardized to 1, and all experimental values were adjusted accordingly. Data are reported as mean ± SE. mRNA and protein data from primary cultures, AR allele length, and clinicopathological factors were analyzed with Student’s t test and Pearson’s correlation. Multivariate analyses examined AR allelotypes as a continuous and dichotomous variable using the Cox proportional hazards model. A short AR allele was defined as ≤19 CAG repeat sequences, because this cutoff has been shown to be associated with higher incidences of benign prostatic hypertrophy and more aggressive prostatic carcinomas (14, 24). A long AR allele was defined as ≥28 CAG repeat sequences, consistent with cutoffs identifying modifications in breast cancer risk (25). A short mean AR allele was defined as ≤20 CAG repeat sequences, the length reported as the mean of heterozygotic CAG repeat sequences in a large cohort of women with breast cancer (20). Progression-free and overall survival were analyzed using the methods of Kaplan and Meier. Statistical significance was determined according to the conventional P of <0.05.

**RESULTS**

To determine differences in AR expression between malignant and normal ovarian epithelium, we examined 23 HOSE and 23 CSOC primary cultures. All CSOC cultures were derived from advanced stage, high-grade tumors of papillary serous histology (Table 1). The majority were stage IIIIC (20 of 23 or 87%) and grade 3 (21 of 23 or 91.3%). The average age of women contributing tissue was 49.2 years (range 33–71) for the HOSE cultures and 62.4 years for the CSOC cultures (range 45–82). Within the benign cohort, 73.9% (17 of 23) of women were premenopausal. All women (23 of 23) in the malignant cohort were postmenopausal. These differences are consistent with the average age at ovarian cancer diagnosis, as well as the average age of women undergoing oophorectomy for benign gynecologic diseases at our institution.

AR mRNA and protein were detected in all 23 HOSE and 23 CSOC cultures examined (Fig. 1). The mean expression level of AR mRNA was statistically higher in CSOC (1.58 ± 0.17) when compared with HOSE (1 ± 0.09, P = 0.05). Protein expression levels were not statistically different between CSOC and HOSE (0.68 ± 0.09 in CSOC and 1 ± 0.18 in HOSE, P = n.s.). When stratified by menopause status, no statistical differences were identified in HOSE cultures derived from pre and postmenopausal women for AR mRNA (1.13 ± 0.64 versus 1.22 ± 0.12, respectively) or for AR protein expression (1.06 ± 0.09 versus 0.94 ± 0.01, respectively). Comparisons between mRNA and protein levels between CSOC cultures and pre and postmenopausal HOSE cultures did not yield any statistical differences (data not shown).

To ascertain potential differences in AR activity between normal and malignant ovarian epithelial cells, we performed allelotype analysis of the expressed AR allele from cDNA generated from mRNA isolated from the 23 HOSE and 23 CSOC cultures. Mean CAG repeat lengths were significantly shorter in CSOC (20.6 ± 1.2 trinucleotide repeats) than in HOSE (23.4 ± 0.9 trinucleotide repeats, P = 0.04).

To determine whether a correlation between AR activity and clinical behavior of ovarian cancer exists, we determined CAG allele lengths from tumor tissue of 77 women with epithelial ovarian carcinoma and analyzed these findings with respect to time to recurrence, overall survival, and clinicopathological prognostic indicators. The range of allele lengths detected in tumors and frequency of occurrence of each CAG repeat length are shown in Fig. 2. Fifty-two samples were informative for two heterozygotic alleles (67.5%), whereas 25 samples were informative for one homozygotic allele (32.5%). The mean number of CAG repeats was 23.6 ± 0.7 and ranged

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**Table 1** Characteristics of the 23 tumors used to establish CSOC primary cultures

<table>
<thead>
<tr>
<th>Factor</th>
<th>n</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillary serous histology</td>
<td>23</td>
<td>100%</td>
</tr>
<tr>
<td>Grade 2</td>
<td>2</td>
<td>9%</td>
</tr>
<tr>
<td>Grade 3</td>
<td>21</td>
<td>91%</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>20</td>
<td>87%</td>
</tr>
<tr>
<td>Stage IVA</td>
<td>3</td>
<td>13%</td>
</tr>
</tbody>
</table>

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![Fig. 1](https://example.com/fig1.png) A. AR mRNA expression in normal (Lanes 1–7) and malignant (Lanes 8–15) primary ovarian epithelial cell cultures. Mean HOSE values were standardized to 1 for purposes of statistical analysis. A 1.58-fold increase in expression was identified in malignant compared with benign primary cultures (C). GAPDH mRNA levels were characterized as an internal control of mRNA quantity in all HOSE and CSOC primary cultures. B. expression profile of AR protein from HOSE primary cultures (Lanes 1–4) and CSOC primary cultures (Lanes 5–8) as determined by Western analysis. No differences in protein expression levels were identified between benign and malignant primary cultures. Prostate carcinoma cell line LNCaP (Lane 9) was included as a positive control.
between 8 and 31 CAG repeats. To determine whether allele length influences tumor biology, we examined time to recurrence and overall survival in patients stratified by AR allele.

Patients with a short AR allele containing 19 CAG repeats demonstrated a significantly shorter time to recurrence (progression-free survival of 5.5 versus 19.4 months, \( P < 0.0001 \)) and overall survival (9 versus 32.6 months, \( P = 0.0007 \); Fig. 3) compared with patients without a short allele. To determine whether longer allele lengths conversely impart a less aggressive tumor biology, we examined time to recurrence and overall survival in patients with a long allele (\( \geq 28 \) CAG repeats) versus those without a long allele; there were no differences in either time to recurrence (17.6 versus 17.3 months, \( P = 0.96 \)) or overall survival (34.1 versus 43.3 months, \( P = 0.27 \); Fig. 4).

Epithelial ovarian tissues reflect a mosaic of cells expressing both AR alleles. To evaluate the global effect of allele length on overall AR activity, average length of the two alleles was calculated. A short average allele length (19 CAG repeats) was found to be associated with shorter time to recurrence (8.9 versus 19 months, \( P = 0.035 \)) but not with overall survival (37.3 versus 43.3 months, \( P = 0.15 \); Fig. 5).

Known clinicopathological prognostic factors were analyzed via univariate analysis to identify associations with AR genotype. There was no correlation between CAG repeat length and age at ovarian cancer diagnosis, stage of disease, or tumor grade (Table 2). However, patients with one AR allele with 19 CAG repeats were found to have disease less amenable to optimal surgical cytoreduction (10.3% had residual disease \( \leq 1 \) cm, compared with 44.4% of patients without a short AR allele, \( P = 0.035 \)). Multivariate analysis was performed using the Cox regression hazards model (Table 3). Age, stage, grade, and cytoreducibility were not significant prognostic factors in this cohort. However, the presence of a short AR allele (\( \leq 19 \) CAG repeats) retained prognostic significance as an independent predictor of overall survival (\( P = 0.02 \)).

Previous studies have indicated that a short CAG allele is associated with earlier age of ovarian cancer diagnosis in Ashkenazi Jewish women (13). This ethnic group was represented by 27 of 77 (35%) patients in our cohort. When comparing age at diagnosis in this subcohort, women with shorter alleles of \( \leq 19 \) CAG repeats were diagnosed at an earlier age (54 versus 61.7 years) than women with longer alleles. However, the few Ashkenazim with a short AR allele (\( n = 3 \)) could not power any
epithelial cultures and evaluated studied AR expression in normal and malignant primary ovarian cancer biology in advanced disease. To test this hypothesis, we transformed ovarian epithelial cells and effect a more aggressive AR expression patterns and/or activity, may be heightened in ian surface epithelial cell cultures to recapitulate the ovarian carcinomas. The ability of early passaged primary ovar-based cohort of tumor specimens from women with epithelial origin for epithelial ovarian carcinomas, with expected ex-}

DISCUSSION

We hypothesized that the effects of androgens, mediated by AR expression patterns and/or activity, may be heightened in transformed ovarian epithelial cells and effect a more aggressive cancer biology in advanced disease. To test this hypothesis, we studied AR expression in normal and malignant primary ovarian epithelial cultures and evaluated AR allele using a hospital-based cohort of tumor specimens from women with epithelial ovarian carcinomas. The ability of early passaged primary ovar-

statistical evaluation. In the remainder of the cohort, no differences in mean age at diagnosis were seen in non-Jewish women with a short allele (61.7 years) compared with those without a short allele (59.9 years).

multiples of 77 tumors revealed a range of AR allele types with 8–31 CAG repeats, consistent with the range reported in germ-line DNA of women both with and without ovarian cancer (20). We found that the presence of a short AR allele (≤19 CAG repeats) in ovarian tumors was associated with a significantly decreased time to recurrence and overall survival. Univariate analyses identified a statistically significant association between the presence of a short AR allele and decreased surgical cytoreducibility. The ability to optimally cytoreduce epithelial ovarian cancers may reflect intrinsic properties ascribed to tumor biology, as also stage and grade (18). Although optimal surgical cytoreduction, stage, and grade are all established prognostic indicators for ovarian carcinoma, the retention of AR allele as the sole independent indicator of survival on multivariate analyses suggests that heightened AR activity may be a significant underlying contributor to an aggressive and/or invasive tumor phenotype.

A preponderance of evidence implicates a monoclonal origin for epithelial ovarian carcinomas, with expected expression of one singular AR allele in all tumor tissues (29, 30). Although our genotype analysis in tumor tissues does not distinguish which AR allele is expressed, our data demonstrate a strong independent correlation of short allele type with overall survival. This correlation is strengthened by our in vitro data demonstrating that CSOC cultures express a shorter average AR CAG length polymorphism than HOSE.

Table 2 Univariate analyses of clinicopathologic characteristics and AR allele in 77 tumor tissues

<table>
<thead>
<tr>
<th>CAG repeat length</th>
<th>n</th>
<th>&lt;19</th>
<th>≥20</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>60.4</td>
<td>60.8</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>9 (100%)</td>
<td>61 (90%)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td>38 (89%)</td>
<td>58 (85%)</td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Suboptimal cytoreduction</td>
<td>4 (44%)</td>
<td>7 (10%)</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

* Not statistically significant.

Table 3 Cox regression analysis of the contribution of CAG repeat length and known prognostic factors to overall survival in 77 ovarian adenocarcinoma patients

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>SE</th>
<th>Risk ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short CAG allele</td>
<td>1.26</td>
<td>0.56</td>
<td>3.53</td>
</tr>
<tr>
<td>Age</td>
<td>0.00</td>
<td>0.02</td>
<td>1.00</td>
</tr>
<tr>
<td>Stage</td>
<td>0.42</td>
<td>1.02</td>
<td>1.51</td>
</tr>
<tr>
<td>Grade</td>
<td>-0.12</td>
<td>0.81</td>
<td>0.89</td>
</tr>
<tr>
<td>Suboptimal cytoreduction</td>
<td>-0.78</td>
<td>0.49</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Fig. 5 Effect of short average AR allele (≤20 CAG repeats) on time to recurrence and overall survival. In A, a short average allele length was found to be associated with deceased time to recurrence (progression-free survival 8.9 versus 19 months, P = 0.004). In B, overall survival was not statistically shorter in patients with a short mean AR allele (37.3 versus 43.3 months, P = 0.15).
cultures. We hypothesize that the effect of AR allelotype on tumor biology may result from a direct autocrine effect of enhanced AR transactivational activity in tumor cells. Alternatively, expression of a shorter, and presumably more functionally active, AR allele by the mosaic of adjacent benign cells in the tumor milieu may affect tumor biology through an indirect or paracrine mechanism.

In a cohort of Ashkenazi Jewish women with epithelial ovarian carcinoma, Levine and Boyd reported that women harboring a short AR allele were diagnosed an average of 7.2 years earlier than patients who did not carry a short allele (13). They did not find any association between AR allelotype and the presence of a BRCA mutation. When stratified by ethnicity, our data reflected a similar 7.2 year younger age at diagnosis for the three Ashkenazi Jewish women with a short AR allele, but statistical significance could not be assessed. In our ethnically diverse cohort, differences in age were not observed between patients with long and short AR allelotypes. Taken together, these data suggest that the age of ovarian cancer diagnosis in Ashkenazi Jewish women may be influenced by factors associated with excess androgenic stimulation because of AR allelotype.

These data add to the growing body of evidence linking heightened androgenicity to the pathogenesis and tumor biology of epithelial ovarian cancers. Our findings suggest that increased AR activity, rather than receptor overexpression, modulates the actions of androgens in ovarian neoplasia and tumor growth. Functional studies characterizing hormone activity and response pathways may reveal specific mechanisms in which androgens function in ovarian cancer biology.

ACKNOWLEDGMENTS

We thank Hang Tran, Dr. Elizabetta Nemeth, and Dr. Kent Taylor of the Cedars-Sinai Medical Center genotyping core for their assistance.

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