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Androgen receptor and androgen-responsive gene FKBP5 are independent prognostic indicators for esophageal adenocarcinoma

Short Title: AR signalling in esophageal adenocarcinoma

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ABSTRACT

Background: Esophageal adenocarcinoma is a male dominant disease, but the role of androgens is unclear.

Aims: To examine the expression and clinical correlates of the androgen receptor (AR) and the androgen-responsive gene FK506 binding protein 5 (FKBP5) in esophageal adenocarcinoma.

Methods: Expression of AR and FKBP5 was determined by immunohistochemistry. The effect of the AR ligand 5α-dihydrotestosterone (DHT) on the expression of a panel of androgen-responsive genes was measured in AR-positive and AR-negative esophageal adenocarcinoma cell lines. Correlations in expression between androgen-responsive genes were analysed in an independent cohort of esophageal adenocarcinoma tissues.

Results: There was AR staining in 75 of 77 cases (97%), and FKBP5 staining in 49 (64%), all of which had nuclear AR. Nuclear AR with FKBP5 expression was associated with decreased median survival (451 versus 2800 days), and was an independent prognostic indicator (HR 2.894, 95% CI 1.396 to 6.002, $p = 0.0043$) in multivariable Cox proportional hazards models. DHT induced a significant increase in expression of the androgen-responsive genes FKBP5, HMOX1, FBXO32, VEGFA, WNT5A and KLK3 only in AR-positive cells in vitro. Significant correlations in expression were observed between these androgen-responsive genes in an independent cohort of esophageal adenocarcinoma tissues.

Conclusion: Nuclear AR and expression of FKBP5 is associated with decreased survival in esophageal adenocarcinoma.

Keywords: Adenocarcinoma of the Esophagus, Androgen Receptors, FK506 binding protein 5, Steroids, Prognosis

BACKGROUND

Esophageal adenocarcinoma (EAC) is a dismal disease with a relative five year survival rate of 14% [1]. Its incidence has increased more rapidly than any other cancer over the last four decades in the West, but most markedly in males [2-4]. The major risk factors are gastro-esophageal reflux disease and obesity, leading to the only described precursor lesion for the cancer, Barrett's esophagus (BE). The reported ratio of males to females ranges from 7 - 10 to 1 [4]. This ratio is highest in younger patients and lower in older patients [4], which is in part accounted for by an approximately 20 year delay in onset in females for BE [5] and EAC [6].

The high ratio of males with this cancer, and the change in the ratio with age, suggests a role for the sex steroid hormones: their concentrations differ between males and females, and change over the lifespan. Serum estrogen and progesterone levels cycle about a relatively high mean in the adult female, and drop abruptly at menopause. Serum androgen levels are high in young adult males, and decline progressively throughout adulthood. However, evidence that these hormones play a role in EAC is limited. The male dominance could be, at least partly, explained by a protective effect of estrogens in females which is lost after menopause. Estrogen receptors have been reported in esophageal tissue [7, 8], and there are reports which suggest that estrogen is inhibitory to EAC cell lines [9].

Alternatively, androgens could be involved in the biology of this cancer. There have been relatively few studies of androgens or androgen receptor (AR) signalling in EAC. Serum androgens have been reported to be elevated in both BE [10] and EAC [11]. Three previous studies investigated AR protein expression in EAC, but they examined relatively small patient cohorts, produced conflicting results, did not examine if AR was functional, and

reported no associations with survival [8, 11, 12]. Two epidemiological reports support a role for androgens. Prostate cancer patients given anti-androgen therapy had a statistically significant 30% risk reduction for EAC [13], and gastro-esophageal cancer was positively associated with a family history of prostate cancer [14].

The androgen signalling cascade is activated by androgens, particularly testosterone and its metabolite 5α-dihydrotestosterone (DHT), which bind to the AR in the cytoplasm. The activated AR translocates to the nucleus and binds to androgen response elements in the genome. This binding may then result in the up- or down-regulation of transcription of androgen-responsive genes, such as FK506 binding protein 5 (FKBP5) [15-17], heme oxygenase 1 (HMOX1) [18], F-box protein 32 (FBXO32) [19], wingless-type MMTV integration site family, member 5A (WNT5A) [20], vascular endothelial growth factor A (VEGFA) [21] and kallikrein-related peptidase 3 (KLK3) [22]. The actual genes whose expression is altered is influenced by the interaction of AR and various co-regulators and is tissue and context dependent. FKBP5 expression is often used as an indicator of functional AR signaling, as in prostate cancer studies where it reflects better than any other AR target gene androgen levels after either short-term or long-term androgen deprivation therapy [23].

Given the conflicting data on AR expression in EAC, and the lack of information as to whether, when present, it is functional, the specific aim of this study was to investigate AR expression and signalling in EAC. Associations between expression of AR and FKBP5 and clinicopathological parameters, including overall survival, were examined using multivariable Cox proportional hazards models to adjust for confounding parameters. The effect of DHT on the expression of androgen-responsive genes was assessed in AR-negative and AR-positive esophageal cancer cell lines. Correlations between the expression levels of

putative androgen-responsive genes were assessed using tissues from an independent cohort of patients with BE and EAC.

MATERIALS AND METHODS

Tissue microarrays and immunohistochemistry

Specificity of all antibodies was confirmed by Western immunoblot, which included both positive and negative controls. Each antibody labeled a single band at the expected molecular weight. Antibodies then were optimized with control tissue blocks before application to the tissue microarrays. A tissue microarray composed of one or more representative cores from 77 cases of EAC was constructed as previously described [24]. None of the patients had been given preoperative chemotherapy or radiotherapy. Sequential 4 μm sections were mounted on polylysine-coated slides, dewaxed and rehydrated. Antigen retrieval was performed by heating the sections for 5 min in 10 mmol/L citrate buffer (pH 6) in a microwave pressure cooker. After cooling to room temperature, sections were immunostained using an Autostainer Plus (Dako, Glostrup, Denmark). Sections were incubated for 60 min with either 1:50 rabbit anti-human AR (clone N-20, raised against the first 20 amino acids of the N-terminus of AR) polyclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or 1:400 rabbit anti-human FKBP5 (FKBP51, clone H-100) polyclonal IgG (Santa Cruz Biotechnology Inc.). Slides were then incubated with MACH 4 Universal Horseradish Peroxidase-Polymer (Biocare Medical, Concord, CA, USA). Liquid 3,3-diaminobenzidine (Dako) was used as the chromogen, and sections were counterstained with Meyer's haematoxylin. The staining was scored by an experienced gastrointestinal pathologist (ARR) and ES. Expression of AR was scored separately in the cytoplasm and the nucleus as positive (present in ≥ 5% of the tumor epithelial cells) or negative. Expression of FKBP5 was scored

as positive (present in ≥ 5% tumor epithelial cells) or negative.

Cell lines

The EAC cell lines OE33, OE19 and JH-EsoAd1 were maintained in RPMI-1640, and FLO-1 in DMEM, supplemented with 10% foetal bovine serum, 4 mmol/L L-glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin. The esophageal squamous cell line TE7 was similarly maintained in RPMI-1640 plus supplements. All cells were incubated at 37^{o}C with 5% CO_2 in air.

Stable transduction of cell lines with androgen receptor

The AR gene was amplified from the expression vector pCMV-AR3.1 using Gateway cloning compatible primers (Supplementary Table S1) and transferred into pLV411 plasmid using the Gateway cloning system, as previously described [25]. Stably transduced cells were selected using two rounds of fluorescence activated cell sorting for green fluorescent protein. The mock transduced OE33 and AR expressing cell line (OE33-AR) were maintained in phenol red free media supplemented with 10% dextran-coated charcoal-stripped foetal bovine serum, 4 mmol/L L-glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin (stripped medium).

***In vitro* transactivation assay**

Cells were seeded at 15,000 cells per well in 96-well plates in stripped medium and incubated for 24 h. Cells were transiently transfected with either 50 ng of the synthetic minimal androgen-responsive luciferase probasin-driven promoter tk81-PB3 (PB3-luc) or 50 ng of PB3-luc and 2.5 ng of the androgen receptor expression vector pCMV-AR3.1 (AR) and incubated for 4 h, as previously described [26]. Cells were treated with either vehicle (V;

0.1% ethanol), 10 nmol/L DHT, 10 mmol/L of the anti-androgen bicalutamide (B), or 10 nmol/L DHT and 10 mmol/L B (DHT + B) in stripped medium and incubated for 16 to 20 h. Cells were lysed and luciferase activity was measured using a FLUOstar Optima (BMG Labtech, Ortenberg, Germany). Whole-cell lysates from six replicate wells were pooled and analysed for protein expression by Western immunoblot.

Western immunoblot analysis

Cells were seeded at 2 x 10^5 cells per well in 6-well plates in stripped medium and incubated for 72 h. Cells were treated with either V, or 10 nmol/L DHT for 16 h. Whole-cell lysates were prepared and 15 μg of protein was resolved by denaturing electrophoresis on 4-15% Mini-Protean TGX precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA), transferred to Hybond-C membrane (Amersham Biosciences, Castle Hill, NSW, Australia), and immnostained using 1:10,000 rabbit anti-human AR (N-20) polyclonal IgG, 1:4000 rabbit anti-human FKBP5 (H-100) polyclonal IgG, and 1:5000 mouse anti-human β-actin (clone AC-15) polyclonal IgG1 (Sigma-Aldrich, St Louis, MO). Immunoreactivity was detected using the appropriate horseradish peroxidase-conjugated IgG and visualized using enhanced chemiluminescence (Amersham).

Measurement of gene expression by quantitative real-time reverse-transcription PCR

Cells were seeded in stripped medium at 5 x 10^5 cells per well in 6-well plates, and incubated for 24 h. Cells were treated with either V or 10 nmol/L DHT in stripped medium and incubated for 4, 8 or 24 h. Total RNA was isolated using the RNeasy Mini Kit with on-column DNase I digestion (Qiagen, Hilden, Germany). Total RNA (1 μg) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) in a final volume of 20 μL. Gene expression was determined using iQ SYBR Green Supermix (Bio-Rad

Laboratories) in a final volume of 10 μL, containing 0.1 μL of cDNA and a final concentration of 0.2 μmol/L of each forward and reverse primer (Supplementary Table S1). Triplicate reactions were performed using a CFX (Bio-Rad Laboratories) at 95°C for 3 min, then 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 30 s, followed by a final extension of 72°C for 1 min. The products were melted to confirm specificity. Normalized fold expression (ΔΔCq) was calculated using β-actin (ATCB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference genes using the CFX software.

Statistical analysis

The statistical software used was SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and Prism 6.0d for Macintosh (GraphPad Software, San Diego CA, USA; www.graphpad.com). Hazard ratios (HR), 95% confidence intervals (CI) and *p* values were calculated from univariate and multivariable Cox proportional hazards models. The proportional hazards assumption was found to be upheld for each univariate and multivariable regression. Initially each confounder that had a significant HR in univariate analysis ($p < 0.1$) was included in the multivariable model with the predictor being AR nuclear localization or FKBP5 expression or AR nuclear localization and FKBP5. However, there were too few observations to account for the ten covariates. Therefore, backwards stepwise elimination was performed. The confounder with the highest *p* value was eliminated, one at a time, until the final most parsimonious model had all confounders with $p < 0.05$ or $p < 0.2$ depending on the model. Normalized fold expression data were compared using unpaired t-test. Correlations between androgen-responsive genes in esophageal tissues were determined using linear regression. All statistics were considered significant when the two-tailed $p < 0.05$.

RESULTS

Expression of AR and FKBP5 in esophageal adenocarcinoma tissues

The protein expression of AR and FKBP5 was investigated by immunohistochemistry in resection tissue from 77 cases of EAC (Fig. 1). Low to medium intensity staining of AR in tumor epithelial cells was observed in 75 of the 77 cases (97.4%). Nuclear localisation was observed in 70 cases (90.9%). There was nuclear only staining in seven cases (9.1%), cytoplasmic only in five (6.5%), and both nuclear and cytoplasmic in 63 (81.8%).

Low to high intensity staining of FKBP5 in tumor epithelial cells was observed in 49 cases (63.6%). All of the FKBP5 positive cases also had nuclear localisation of AR. Of the 28 cases that did not express FKBP5, 21 had nuclear localisation of AR and seven did not. There was a significant association between FKBP5 expression and AR nuclear localisation (p = 0.0005). These data suggest that in primary EAC epithelial cells, nuclear localisation of the AR is necessary but not sufficient for FKBP5 expression.

Clinical significance of AR and FKBP5 in esophageal adenocarcinoma

To determine the clinical significance of the expression of AR and FKBP5, we examined associations with clinicopathological data which was available for 76 of the cases. The median age of these patients at surgery was 64 years (range 36 to 81), the median follow-up time was 865 days (range 37 to 4,661), and the 5-year overall survival rate was 36.7%.

Nuclear localisation of AR was significantly associated with the presence of BE (Supplementary Table S2; p = 0.0009). It was detected in all tissues from patients who had co-existing BE, but only 76.7% of tissues from patients without BE. There was no significant

difference in AR staining for patient age or gender. Patients with nuclear AR had a median overall survival of 671 days compared to 1,321 days for those without (Fig. 2A).

Similarly, the expression of FKBP5 was more prevalent in patients with BE observable on endoscopy or in the resection specimen (Supplementary Table S2; $p = 0.0495$). Patients with FKBP5 expression had a median overall survival of 451 days compared to 1,338 days for those that were FKBP5-negative (Fig. 2B). For those patients who were FKBP5-negative but had nuclear AR (nuc AR+/FKBP5-), the median overall survival was 2,800 days (Fig. 2C).

To investigate the difference between hazards of dying, univariate and multivariable Cox proportional hazards models were used. In univariate models neither AR nuclear localization nor FKBP5 expression were associated with a significant difference in risk of death (Supplementary Table S3). In multivariable models when adjusting for confounders, AR nuclear localization (HR 3.290, 95% CI 1.125 to 9.620, $p = 0.0296$) and FKBP5 expression (HR 3.043, 95% CI 1.417 to 6.531, $p = 0.0043$) were associated with a significant increase in risk of death (Supplementary Table S3). For the subset of patients who had AR nuclear localization, FKBP5 expression was not associated with a significant difference in risk of death in the univariate model (Table 1; HR 1.829, 95% CI 0.904 to 3.701, $p = 0.0930$). However, in the multivariable model, after adjusting for confounders, patients who had AR nuclear localization and FKBP5 expression had 2.9 times the hazard of dying (Table 1; HR 2.894, 95% CI 1.396 to 6.002, $p = 0.0043$).

AR and FKBP5 in esophageal cancer cell lines

The expression of AR and FKBP5 protein was measured in esophageal cancer cell lines (Fig. 3A). AR was not detected, nor induced by DHT, in OE33, OE19, JH-EsoAd1, FLO-1 or TE7.

FKBP5 expression was low in OE33, OE19, JH-EsoAd1 and TE7, higher in FLO-1, and not upregulated by DHT in any of these cell lines.

Functional AR activity was not measured by transactivation assay in cell lines which were transiently transfected just with the synthetic minimal androgen responsive luciferase probasin-driven promoter tk81-PB3 (PB3-luc; Fig. 3B and 3C). No luciferase activity was induced over a broad concentration range of DHT (0.01 to 1000 nmol/L) in OE33 or at 10 nmol/L in OE19, JH-EsoAd1 and FLO-1. However, transient co-transfection of both the AR expression vector pCMV-AR3.1 (AR) and the PB3-luc resulted in DHT induced luciferase expression (Fig. 3B and 3C). Expression of AR in these transiently co-transfected cells was confirmed by Western immunoblots (data not shown). Luciferase activity was dependent on the concentration of DHT, and was blocked by the anti-androgen bicalutamide. These results show that although functional AR was not expressed in the cell lines, they were competent for AR signalling.

In order to examine the effect of AR signalling we stably transduced OE33 cells with AR, designating them OE33-AR. Expression of AR was confirmed by Western immunoblot (Fig. 3A) and AR function was confirmed by transactivation assay (Fig. 3D). Treatment with DHT did not alter FKBP5 mRNA expression in the untransduced, AR-negative, OE33 cells (Fig. 3E), but did induce a time-dependent increase in OE33-AR (Fig. 3F). Furthermore, the abundance of FKBP5 protein steady state levels in the OE33-AR cells was increased by DHT (Fig. 3A).

Androgen-responsive genes in AR-positive cell line and esophageal tissues

To further explore the effect of functional AR in cell lines, we measured the effect of DHT on the expression of a panel of putative, clinically relevant androgen-responsive genes. Androgen-responsive genes have not been defined in EAC, so we measured expression of genes known to be androgen-responsive in other tissues and cell lines. DHT significantly increased the expression of HMOX1 (23-fold), FBXO32 (19-fold), WNT5A (4-fold), and VEGFA (3-fold), and induced the expression of KLK3 in the AR-positive OE33-AR, but not in the AR-negative OE33 (Fig. 4).

To determine if this panel of androgen-responsive genes was also altered in an independent cohort of esophageal tissues, we looked for correlations between the genes in a publicly available transcriptional microarray dataset [27]. There were significant correlations between FKBP5 and each of the genes in the panel in EAC (Fig. 5). In contrast, there was no significant correlation in esophageal squamous mucosa (SQ) and the only correlations in BE were observed for FBXO32 and KLK3.

DISCUSSION

We observed AR protein expression in tumor epithelial cells in 75 of 77 patients with EAC. There was nuclear localisation in 91% of these. The androgen-responsive gene FKBP5 was expressed in 64% of these tissues, but only in those which also had nuclear localisation of AR. Expression of either AR or FKBP5 was associated with decreased overall survival by multivariable analysis. We created an AR-positive EAC cell line, OE33-AR, by stably transducing the gene for AR into the AR-negative OE33. We found that DHT induced a time-dependent increase in FKBP5 expression in the OE33-AR cells, but not the AR-negative OE33. Also, DHT increased expression of the androgen-responsive genes HMOX1,

FBXO32, WNT5A, VEGFA and KLK3. Correlations between the expression of these androgen-responsive genes were observed in an independent cohort of EAC tissues, consistent with functional AR being expressed in EAC.

Ours is the largest cohort to date used to investigate AR protein expression in EAC. Three previous studies of AR expression in EAC have produced conflicting results. Focal staining was reported in one of 20 patients [8], in the tumor epithelial cells in five of 11 patients with no stromal expression [12], and in the stroma in 13 of 18 patients with no expression in the tumor epithelial cells [11]. In contrast, we observed a significantly higher incidence of AR expression and nuclear localisation in EAC tumor epithelial cells than the previous reports. There are several possible explanations for the discrepancy. There may be differences in the sensitivity of the staining methods or reporting thresholds, particularly as the abundance of AR in EAC is relatively low compared to, for example, prostate or breast cancer. Two of the studies used a different antibody to ours [11, 12], and although these two studies used the same antibody, one reported no staining of AR in the tumor epithelial cells, the other staining in 45% of cases. Variability of positivity and staining intensity between studies is not unusual. AR is expressed across a wide range of cancers, but for most cancers, just as with EAC, the published rates of expression vary widely, for reasons that are not clear [28].

To determine if the AR signalling pathway was functional in EAC, we stained for the androgen-responsive gene FKBP5. Expression was only found in a subset of tumors which had nuclear localization of AR, suggesting that AR activation was required, but not sufficient, for FKBP5 expression. This was consistent with our cell line data, where DHT did not alter FKBP5 expression in the AR-negative EAC cell lines, but did in the AR-positive cell line, OE33-AR.

One explanation for our survival data is that the expression of FKBP5 is a marker of a functional AR signalling pathway which alters the expression of one or more genes which then reduce overall survival. In the nuclear AR-positive, FKBP5-negative cells, the AR pathway might not be functional, or is regulating different androgen-responsive genes from those in the FKBP5-positive tissues. This is consistent with recent studies which show that AR signalling is not a simple ligand-receptor-bind to specific DNA receptor elements model. Rather AR, like other steroid receptors, derives cell-specific transcription activity from interactions with various co-regulators and DNA-binding proteins that regulate receptor binding, and lineage-specific chromatin organization [29]. Alternatively, FKBP5 itself may influence survival, but in our tissues it is only expressed in cells with a functional AR signalling pathway, while in other contexts it may be expressed as a result of progestin or glucocorticoid signalling.

Overexpression of FKBP5 has been reported in a range of solid tumors [30], including melanoma [31], glioma [32], colon [33], and prostate [34-37]. FKBP5 can inhibit apoptosis and promote cell proliferation in normal, premalignant and malignant tissues. In melanoma, expression correlated with tumor aggressiveness and was maximal in metastatic lesions [31] and in glioma expression correlated with stage and overall patient survival [32]. In contrast, down-regulation of FKBP5 has been reported in pancreatic cancer, and decreased expression resulted in hyperphosphorlyation of Akt and decreased cell death following genotoxic stress in cell lines [38]. These reports do not detail the AR status of the cancer tissues. Thus FKBP5 may either be acting as a surrogate marker of a particular AR activated set of genes, or it may be the responsible gene itself.

None of the four common EAC cell lines we examined expressed AR. Lack of AR expression in cultured cell lines does not mean that the receptor was not present in the primary tissue from which the cell line was derived. Protein expression of steroid receptors, such as AR, present in cells of the primary tissue are frequently lost from the cells following culture, by mechanisms that are not clearly understood [39] [40]. However, these esophageal cell lines expressed the necessary co-regulators for AR signalling, as they exhibited AR transactivation activity following either transient transfection or stable transduction with the AR gene. We further showed that FKBP5, HMOX1, FBXO32, WNT5A, VEGFA and KLK3 were androgen-responsive genes in the OE33-AR cell line following treatment with DHT.

This is the largest study of AR expression in EAC and it shows that in most patients tumor epithelial cells express AR. This is the first study to show AR to be functional in the majority, but not all, cases of EAC, as defined by nuclear localisation and expression of the androgen responsive gene FKBP5. Significantly, it was sufficiently powered to show that AR and the androgen-responsive gene FKBP5 were independently associated with decreased overall survival. The correlation between nuclear localisation of AR and expression of FKBP5 in our cohort of EACs and the correlations between the expression of androgen-responsive genes in an independent cohort of patients, suggests that AR is functional in at least the majority of tumors. It further suggests that AR, FKBP5 or other androgen responsive-genes influence survival. These findings raise the possibility of novel therapeutic options for EAC, such as the use of drugs which target AR signaling, or the androgen-responsive genes.

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