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Estrogen receptor-alpha as a predictive biomarker in endometrioid endometrial cancer

Floor J. Backes [a,*], Christopher J. Walker [a], Paul J. Goodfellow [a], Erinn M. Hade [b], Garima Agarwal [a], David Mutch [c], David E. Cohn [a], Adrian A. Suarez [d]

[a] Division of Gynecology Oncology, Department of Obstetrics and Gynecology, The Ohio State University College of Medicine, The James Comprehensive Cancer Center, Columbus, OH, United States
[b] Center for Biostatistics, Department of Biomedical Informatics, The Ohio State University College of Medicine, Columbus, OH, United States
[c] Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Washington University School of Medicine, Siteman Cancer Center, St Louis, MO, United States
[d] Department of Pathology, The Ohio State University College of Medicine, Columbus, OH, United States

HIGHLIGHTS

- Patients with ERα negative endometrial cancer are more often diagnosed with higher grade and advanced stage disease.
- Mutations in *ESR1* might explain why some women with low BMI (without unopposed estrogen exposure) develop endometrial cancer.
- Lymph node involvement is more common in patients with lack of ERα expression.

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ABSTRACT

Background. We sought to validate the prognostic significance of estrogen receptor alpha (ERα) expression and to investigate the relationship between *ESR1* mutation status and outcomes in a large cohort of patients with endometrial cancer. We also investigated the predictive value of ERα for lymph node involvement in a large surgically staged cohort.

Methods. A tumor microarray (TMA) was constructed including only pure endometrioid adenocarcinomas, stained with ER50 monoclonal antibody, and assessed using digital image analysis. For mutation analysis, somatic DNA was extracted and sequenced for *ESR1* gene hotspot regions. Differences in patient and tumor characteristics, recurrence and survival between ERα positive and negative, mutated and wild-type tumors were evaluated.

Results. Sixty (18.6%) tumors were negative for ERα. Absence of ERα was significantly associated with stage and grade, but not with disease-free or overall survival. ERα was a strong predictor of lymph node involvement (RR: 2.37, 95% CI: 1.12–5.02). Nineteen of 1034 tumors (1.8%) had an *ESR1* hotspot mutation; twelve in hotspot 537Y, four in 538D and three in 536 L. Patients with an *ESR1* mutation had a significantly lower BMI, but were comparable in age, stage and grade, and progression-free survival.

Conclusion. Patients with ERα negative endometrioid endometrial cancer are more often diagnosed with higher grade and advanced stage disease. Lymph node involvement is more common with lack of ERα expression, and may be able to help triage which patients should undergo lymphadenectomy. Mutations in *ESR1* might explain why some low risk women with low BMI develop endometrial cancer.

1. Introduction

Endometrial cancer is the 4th most common cancer in women and the 7th most common cause of cancer death in the US [1,2]. It accounts for twice the number of deaths as cervical cancer and approximately half the number of deaths as ovarian cancer; still endometrial cancer remains an understudied disease [1,2].

Hysterectomy and bilateral salpingo-oophorectomy is the cornerstone of endometrial cancer treatment. Further surgical treatment (i.e. need for lymph node dissection) and adjuvant therapy for endometrial cancer continues to be the subject of international debate and ongoing clinical trials. Unlike other tumor types, e.g. breast cancer [3–6], there are currently no predictive and/or prognostic molecular markers used in the routine clinical management of endometrial cancer. Our aim was to identify a biomarker that, if validated, could be easily implemented into clinical care to help identify which patients could benefit from or

* Corresponding author at: Division of Gynecologic Oncology, Department of Obstetrics & Gynecology, , Ohio State University, M210 Starling Loving, 320 W 10th Avenue, Columbus, OH 43210, United States.
E-mail address: Floor.Backes@osumc.edu (F.J. Backes).

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could be spared adjuvant therapy, or even lymph node dissection, in order to minimize risks associated with potentially unnecessary treatment.

Unopposed estrogen exposure is a well-known major risk factor for endometrial cancer. Recent data has suggested that estrogen receptor alpha (ERα, gene symbol *ESR1*), which is involved in epithelial-mesenchymal transition (EMT), could be a promising prognostic marker [7–9]. Wik et al. studied several cohorts of endometrial cancer that had a variety of histologic subtypes and different stage tumors. Absence of ERα expression (assessed by immunohistochemistry) was seen in 21% of 239 endometrioid cases and was associated with reduced survival [9]. The reported hazard ratio was 3.5 (95% CI 1.2–3.7, $p < 0.001$) when adjusted for age, grade, and stage, suggesting ERα may be a very powerful prognostic marker [9]. However, not all of the patients investigated in the multivariate analysis of endometrioid tumors were surgically staged and thus could have included occult advanced cancers, which confer a worse prognosis, although the true significance of the finding remains to be determined. Wik and colleagues also found that low ERα expression was associated with EMT and *PIK3CA* alterations, which may have implications for the choice of adjuvant therapy and targeted agents, raising the possibility that ERα expression could be both prognostic and predictive in endometrial cancer [9]. In addition, query of The Cancer Genome Atlas (TCGA), demonstrated a strong association between *ESR1* mutation status and progression free survival.

Thus, the primary objective of this study was to evaluate the prognostic and predictive significance of ERα protein expression using a novel digital image analysis approach, and *ESR1* hotspot mutations in a cohort of patients with endometrial cancer who have undergone comprehensive surgical staging. Exploratory analyses were undertaken to investigate the association between ERα and lymph node status.

2. Methods

2.1. Immunohistochemistry

2.1.1. Patient cohort

Approval for this study was obtained from the Cancer Institutional Review Board. Patients treated at the Ohio State University Medical Center between 2007 and 2012 who were diagnosed and treated for endometrial cancer were identified (cohort 1). Only pure endometrioid adenocarcinomas grade 1–3 at any stage were included (other histology or mixed types were excluded). Patients were comprehensively surgically staged with full pelvic and aortic lymph node dissection (unless aortic lymphadenectomy was not deemed safe). Patient records were retrospectively reviewed for clinical and pathologic characteristics, treatment information, and dates of recurrence, death and last follow up. A tumor micro-array (TMA) was constructed by identifying well preserved areas with the highest tumor cellularity on hematoxylin and eosin–stained slides. A single 1 mm core from each patient donor block was mounted into a recipient paraffin block using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD).

2.1.2. ERα expression

Slides from the TMA were cut at 4 μm. Immunoperoxidase staining was carried out as previously described [10] on the Dako Cytomation Autostainer (Copenhagen, Denmark). For ERα, the ER50 monoclonal antibody (M 7047 at 1:50 dilution) was used. There are no clinical guidelines on fixation of endometrial cancers for IHC; however, formalin fixation times for most of these specimens have been estimated to fall within the 6–72 h, which has been determined to be ideal for ERα IHC of breast cancer specimens [11]. Digital image analysis [using Tissue Studio 3.5 software (Definiens®, Munich, Germany)] was performed on ERα IHC slides scanned at 20× using ScanScope XT by Aperio (Vista, California). The Tissue Studio 3.5 software uses a context-based, relational analysis of the component pixels in digital slide images to differentiate between types of tissue (adenocarcinoma vs stroma); identify and count IHC-stained nuclei; quantify the intensity and completeness of IHC staining. An algorithm was developed using a dozen selections of ERα-stained nuclei and adjacent tissue (Fig. 1). Sections were chosen to give a representative selection of tissue morphologies and stain intensities. An image analysis technician in cooperation with one of the researchers (AAS) designated areas as adenocarcinoma vs stroma. The software then used these designations to recognize similar values in other tissue specimens and assign them to the correct category. Morphologic assignments by the software for each slide were reviewed by the image analysis technician and the researcher (AAS) to confirm their accuracy, and minor adjustments were made as needed. Adenocarcinoma was designated as the region of interest and tissue in this category was subjected to the detect nuclei (positive/negative) algorithm. Detection parameters were customized to researcher (AAS) specifications. Within the region of interest, the software counted positive and negative-staining nuclei, and counted high and low-staining positive nuclei. Totals and percentages were calculated for each category.

ERα expression was recorded as the percentage of stained cells, and classified by dichotomizing IHC expression regardless of intensity as present (≥1% staining cells) or absent (<1%) per ASCO/College of American Pathologists Guidelines [12]. To confirm accurate interpretation by digital analysis software cores with <5% ERα expression were reviewed by a pathologist (AAS) and visually scored as ERα-positive or -negative.

2.2. ESR1 mutations

2.2.1. DNA isolation

Formalin fixed paraffin-embedded (FFPE) archival tissue was obtained from cohort 1 (OSU). An H&E stained section for each case was reviewed by a pathologist and marked to identify regions of tumor. Tissue from three adjacent unstained sections were macro-dissected based on the marked H&E. Somatic DNA was isolated using the MaxWell 16 FFPE Tissue LEV DNA Purification Kit (Promega Corp) and quantitated using the Quant-iT PicoHreen dsDNA assay kit (Thermo Fisher Scientific).

Cohort 2 included patients treated for endometrioid endometrial cancer at Washington University (St. Louis, MO) between 1991 and 2010. DNA had previously been extracted from flash-frozen tissues [13–16] and cases had been deidentified. Mutation and clinical data for Cohort 3 was obtained using TCGA database [17] selecting for endometrioid adenocarcinoma.

2.2.2. ESR1 mutation screening

We performed amplicon-based sequencing on cohorts 1 and 2. Mutation calls for cohort 3 were obtained from TCGA data matrix. For cohort 1, approximately 10 ng of DNA was used to amplify a 221 bp fragment in exon 8 of the *ESR1* gene which contained the Y537 and D538 hot spot region. PCR amplification was performed using standard conditions and the Amplitaq Gold Master mix (Thermo Fisher Scientific) with the following primers; forward-gctcgggttggctctaaagt, reverse-ATGAAGTAGAGCCCGCAGTG. PCR products were directly sequenced using an ABI 3730 DNA Analyzer and the BigDyeTerm v3.1 cycle sequencing kit (Thermo Fisher). Sequencing files were analyzed using the DNAStar SeqMan Pro software (DNASTAR Inc.). Cohort 2 was amplified using the following primers: forward-GCTCCCATCCTAAAGTGGGT CTTTAA, reverse-TGTGGGAGCCAGGGAGCTCTCAGAC.

2.3. Statistical methods

Patient demographics are summarized via descriptive statistics. Comparisons of these characteristics by ERα status are made via a two-sample *t*-test for continuous covariates or by chi-squared (or Fisher's exact) tests for categorical covariates. The influence of ERα as a prognostic marker of survival (recurrence free survival (RFS) and overall survival (OS)) was evaluated by the method of Kaplan and