Lack of Estrogen Receptor-α Is Associated with Epithelial–Mesenchymal Transition and PI3K Alterations in Endometrial Carcinoma

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Abstract

**Purpose:** We hypothesized that estrogen receptor-α (ER-α) status in endometrial carcinomas, associated with poor prognosis, is reflected in transcriptional signatures suggesting targets for new therapy.

**Experimental Design:** Endometrial carcinoma samples in a primary investigation cohort (n = 76) and three independent validation cohorts (n = 155/286/111) were analyzed through integrated molecular profiling. Biomarkers were assessed by immunohistochemistry (IHC), DNA oligonucleotide microarray, quantitative PCR (qPCR), single-nucleotide polymorphism (SNP) array, and Sanger sequencing in the cohorts, annotated for comprehensive histopathologic and clinical data, including follow-up.

**Results:** ER-α immunohistochemical staining was strongly associated with mRNA expression of the receptor gene (ESR1) and patient survival (both P < 0.001). ER-α negativity associated with activation of genes involved in Wnt-, Sonic Hedgehog-, and TGF-β signaling in the investigation cohort, indicating epithelial–mesenchymal transition (EMT). The association between low ER-α and EMT was validated in three independent datasets. Furthermore, phosphoinositide 3-kinase (PI3K) and mTOR inhibitors were among the top-ranked drug signatures negatively correlated with the ER-α-negative tumors. Low ER-α was significantly associated with PIK3CA amplifications but not mutations. Also, low ER-α was correlated to high expression of Stat3, a marker associated with PTEN loss, and a high PI3K activation signature.

**Conclusion:** Lack of ER-α in endometrial cancer is associated with EMT and reduced survival. We present a rationale for investigating ER-α’s potential to predict response to PI3K/mTOR inhibitors in clinical trials and also suggest EMT inhibitors to ER-α-negative endometrial carcinomas. Clin Cancer Res; 19(5); 1094–105. ©2012 AACR.

Introduction

Endometrial cancer is the most common gynecologic malignancy in developed countries and accounts yearly for 50,000 deaths worldwide (1). Although 3 quarters of these cancers are treated at an early stage, 15% to 20% recur (2). The most common basis for determining risk of recurrent disease is the categorization into type I and II endometrial carcinoma (3). Type I tumors are most frequent, character-
E-cadherin, P-cadherin, trial carcinomas, alterations of EMT-related markers such as aggressive tumors in different organs (12, 13). In endometrial carcinomas, alterations of EMT-related markers for epithelial-mesenchymal transition (EMT). Drug signature analyses suggest testing of PI3K/mTOR inhibitors in ER-α–negative tumors in particular. Subsequently, we also find ER-α–negative tumors to be associated with several potential measures for phosphoinositide 3-kinase (PI3K) pathways activation. This study confirms ER-α as a strong and robust prognostic marker in endometrial carcinoma and provides a rationale for clinical trials exploring the potential of ER-α as predictive marker for response to inhibitors of the PI3K pathway and EMT.

Translational Relevance
Estrogen receptor-α (ER-α) is shown to be a strong prognostic marker and potentially predictive for response to hormonal treatment in endometrial carcinoma, although, contrasting breast cancer, not yet systematically implemented for tailoring therapy to patients with endometrial cancer. In one investigation series and three independent validation sets (in total 628 patients), we identify and validate an association between ER-α–negative endometrial carcinomas and markers for epithelial-mesenchymal transition (EMT). Drug signature analyses suggest testing of PI3K/mTOR inhibitors in ER-α–negative tumors in particular. Subsequently, we also find ER-α–negative tumors to be associated with several potential measures for phosphoinositide 3-kinase (PI3K) pathways activation. This study confirms ER-α as a strong and robust prognostic marker in endometrial carcinoma and provides a rationale for clinical trials exploring the potential of ER-α as predictive marker for response to inhibitors of the PI3K pathway and EMT.

Low ER-α Associates with EMT and PI3K Alterations in Endometrial Carcinoma

intravasate, circulate, extravasate, and resist apoptosis (9–11). Markers of EMT are known to be associated with more aggressive tumors in different organs (12, 13). In endometrial carcinomas, alterations of EMT-related markers such as E-cadherin, P-cadherin, α-, and β-catenin, have been associated with metastatic disease and reduced survival (14–16). The EMT process is suggested to be a potential target for new cancer therapies (17, 18). Importantly, data linking EMT to drug resistance have been reported, indicating that targeting EMT may be relevant for cancer treatment (18).

In the present study, we hypothesized that ER-α–negative endometrial carcinomas are associated with an aggressive clinicopathologic phenotype and are likely to be distinguished from ER-α–positive tumors by underlying genetic alterations and differences in gene expression, related to key processes in cancer development and progression. On the basis of data from 4 independent patient series, we report for the first time that ER-α–negative endometrial carcinomas are associated with increased EMT. Also, inhibitors of the PI3K/mTOR pathways are suggested as relevant for the treatment of ER-α–negative endometrial cancers in particular.

Patients and Methods

Patients and follow-up
Four independent endometrial carcinoma patient cohorts (including both endometrioid and nonepithelioid histologic subtypes) were studied to explore the associations between hormone receptor status, prognosis and alterations in transcriptional signatures identifying molecular phenotypes, and potential targets for new therapy (Supplementary Fig S1): (i) a primary investigation cohort of 76 snap-frozen, prospectively collected endometrial carcinomas with high tumor purity was studied by DNA oligonucleotide microarray for mRNA expression levels, and single-nucleotide polymorphism (SNP) array and Sanger sequencing for detection of PIK3CA amplification and PIK3CA mutations, respectively. (ii) A prospective validation cohort of 155 snap-frozen endometrial cancers was subjected to quantitative PCR (qPCR) validation of candidate markers picturing findings in the primary investigation set. PIK3CA Sanger sequencing was also applied for tumors in this series. Both prospective series were collected sequentially, taking into account tumor purity (see later). (iii) A retrospective population-based validation cohort of 286 patients with formalin-fixed paraffin-embedded (FFPE) tumor tissue was used for clinical validation. Protein expression of candidate markers and vascular invasion was assessed by immunohistochemistry (IHC), as previously reported (5, 15, 19, 20). For all these cohorts, biopsies were sampled from primary tumors in hysterectomy specimens. (iv) Finally, publically available external gene expression array data from 111 patients were investigated for external validation (Supplementary Table S1).

Patients in series 1 through 3 (Supplementary Table S2) were treated at the Department of Obstetrics and Gynecology, Section of Gynecological Cancer, Haukeland University Hospital (Bergen, Norway), a referral hospital for patients in Hordaland County, representing approximately 10% of the Norwegian population and with a similar incidence rate and prognosis as the total Norwegian population of endometrial cancers (15).

Comparison of clinicopathologic variables for the different cohorts is presented in Supplementary Table S3. The primary investigation set largely reflects the general population of endometrial cancers from the region, whereas the prospective validation series and the external dataset are more enriched for aggressive cancer subtypes. The prospective validation series (1981–90) was also subjected to formal histopathologic revision for adequate subtyping and grading in previous studies (15, 20).

Oligonucleotide DNA microarray analyses
DNA was extracted from snap-frozen, surgically removed tumor biopsies from clinically well-defined endometrial carcinomas with more than 80% neoplastic tissue for the vast majority (only 4 cases, 3 endometrioid, and 1 serous carcinoma had tumor purity of 50%). The RNA was obtained according to standard protocols using the RNeasy Mini Kit protocol (Qiagen) and hybridized to Agilent Whole Human Genome Microarrays 44k (Cat.no. G4112F), according to manufacturer’s instructions (www. agilent.com). Arrays were scanned using the Agilent Microarray Scanner Bundle. Microarray signal intensities were determined using BRB-ArrayTools (http://linus.nci.nih. gov/BRB-ArrayTools/). Mean spot signal data were used as intensity measure. The expression data were normalized, using median over entire array. The DNA oligonucleotide arrays were conducted around the same time, in the same laboratory, by the same personnel, and in 1 batch.

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Clin Cancer Res; 19(5) March 1, 2013 1095

Published OnlineFirst January 14, 2013; DOI: 10.1158/1078-0432.CCR-12-3039

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Genes differentially expressed between ER-α-positive and -negative tumors were considered significantly differentially expressed if False Discovery Rate (FDR) < 0.05; the test was conducted by Class comparison analysis in BRB Array Tools (21). Global hierarchical clustering was conducted using weighted average linkage and Pearson correlation as similarity measures. Gene set enrichment analyses (GSEA: www.broadinstitute.org/gsea) and BRB Array Tools were used to explore pathways and transcription factor gene sets differentially expressed between ER-α-positive and -negative tumors (22, 23). Signature scores for gene sets in GSEA were created by using a pathways activation score as implemented in the GSEA application for R (www.broadinstitute.org/gsea).

Connectivity map
The correlation between global gene expression pattern and potential new therapeutic strategies for ER-α–negative tumors was assessed in the primary investigation cohort and validated in the external gene expression dataset, using the drug signatures database Connectivity Map (24). The drug signatures in Connectivity Map are based on genes differentially expressed between cell lines treated with various drugs/compounds compared with the corresponding untreated cell lines. As such, the database drug signatures represent gene expression alterations presumed to reflect drug effects. Genes differentially expressed (FDR < 0.05) between subsets with high and low ER-α protein and ESR1 mRNA expression levels were included in the signatures that were the basis for the analyses in Connectivity Map.

Quantitative PCR
cDNA was synthesized from 1 μg RNA from 155 tumor samples in the prospective validation series, using the High capacity RNA to cDNA kit (Applied Biosystems Inc.). mRNA expression of ESR1 and a selection of cell adhesion-/EMT markers were determined using the TaqMan gene expression assays ESR1-Hs00174860_m1 (ER-α), CDH1-Hs01013958_m1 (E-cadherin), CDH2-Hs00983056_m1 (N-cadherin), CDH3-Hs00999915_m1 (P-cadherin), CTNNB1-Hs00170025_m1 (β-catenin), CTNNNA2-Hs00189285_m1 (α-catenin), and CTNNN1-Hs00931670_m1 (p120 catenin). For mRNA expression of ER-α, Stathmin, and cell adhesion-/EMT markers (E-cadherin, P-cadherin, β-catenin, and p120 catenin), as well as evaluated for vascular invasion and myometrial infiltration, as previously described (5, 15, 19, 20, 28), ER-α IHC data were available for all cases in the primary investigation set, for 153 cases in the prospective validation series and for 266 cases in the retrospective validation cohort.

Validation in external gene expression dataset
Publicly available gene expression data corresponding to tumors resected from endometrial carcinoma tissue were obtained from the Expression Project for Oncology (expO: http://expo.intgen.org/geo/home.do). Gene Expression Omnibus (GEO) accession numbers and information on clinicopathologic data for these 111 tumors are listed in Supplementary Table S1. The GEO datasets were run at Affymetrix U133+2 arrays. For these datasets, individual probes were sequence-matched against Acierview (NCBI) to construct transcript level probe sets (19). The 2 different datasets were not merged as the GEO dataset was used for validation of findings in the primary investigation series. Calibration to get comparable expression levels was therefore not necessary.

DNA analyses
Genomic DNA was extracted from surgically dissected, fresh frozen primary tumors. SNP array and Sanger sequencing of PIK3CA (exon 9 and 20) was conducted for 230 and 215 cases, respectively, as previously described (19, 29), overlapping with the primary investigation set and the prospective validation series.

Statistics
Data were analyzed using PASW 18 (Predictive Analytics Software, version 18.0, IBM). P values of less than 0.05 were considered statistically significant except for the oligonucleotide microarray analyses (see earlier). Categories were compared using Pearson χ² or Fisher exact test when appropriate. Spearman correlation coefficient was reported for bivariate correlation between continuous variables. Mann–Whitney U test was used for analysis of continuous variables between categories. Univariate survival analyses of time to death due to endometrial carcinoma (disease-specific survival) and time to recurrence for patients without metastases at time of diagnosis (recurrence-free survival) were conducted using the Kaplan–Meier method (log-rank test). Entry date was the time of primary surgery. Patients who died from other causes were censored at the date of death in
the analyses of disease-specific survival. Variables included in the Cox proportional hazards regression analyses were examined by log–log plot before incorporated in the regression models. When categorizing continuous variables (e.g., mRNA expression values of genes, gene signature scores, IHC score indices, etc.) cutoff points were based on median or quartile values, considering also the frequency distribution for each marker. Groups with similar survival were merged. For gene expression data (gene signatures and single genes), upper quartile defined “high expression”. ER-α-negative cases in the primary investigation and retrospective validation series included cases with staining index (SI) 0. In the prospective validation series, low ER-α included staining index ≤ 2 cases.

Estimation of sample size was done by $x^2$ test using software East4, 2005 Cytel Software Corp. To reach 90% power detecting a 30% difference in 5-year survival (90% for patients with markers within reference range vs. 60% with pathologic markers) at a 5% level of significance, 65 patients were needed assuming a positive to negative ratio of the markers of 1:3.

Results

Lack of ER-α is associated with aggressive endometrial cancer

ER-α protein and ESR1 mRNA expression estimated by microarray and qPCR were significantly associated in the primary investigation set (Fig. 1A and B). The strong correlation between mRNA and protein expression was confirmed in the prospective validation cohort (Table 1). Low ER-α protein and ESR1 mRNA expression and transcriptional clusters containing the majority of ER-α-negative cases were significantly correlated with an aggressive clinicopathologic phenotype and poor survival in all patient cohorts studied (Table 1; Fig. 1C and D; Supplementary Fig. S2). Low ESR1 mRNA was associated with reduced survival in the primary investigation set (multivariate analyses, adjusted for age, FIGO stage, histologic type, and histologic grade; HR, 1.4; 95% CI, 1.05–1.8; $P = 0.02$). In the prospective validation set a significant interaction between ESR1 mRNA and FIGO stage ($P = 0.03$) was detected. The association between ESR1 mRNA levels and survival was therefore further assessed separately for FIGO I/II and FIGO III/IV tumors.
Table 1. Clinicopathologic variables in relation to low ER-α (by IHC or mRNA expression)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Primary investigation cohort (n = 76)</th>
<th>Prospective validation cohort (n = 155)</th>
<th>Retrospective validation cohort (n = 236)</th>
<th>External data set (n = 111)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>IHC ER-α-neg. mRNA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Low ESR1</td>
<td>IHC ER-α-neg. mRNA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Low ESR1</td>
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<tr>
<td>Age&lt;sup&gt;a&lt;/sup&gt;, y</td>
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<td>1.0</td>
<td>0.1</td>
<td>17 (21)</td>
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<tr>
<td></td>
<td>&gt;65</td>
<td>9 (25)</td>
<td>1.0</td>
<td>18 (25)</td>
<td>19 (16)</td>
</tr>
<tr>
<td>FIGO stage</td>
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<td>12 (18)</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>16 (50)</td>
</tr>
<tr>
<td></td>
<td>III/IV</td>
<td>7 (64)</td>
<td>1.0</td>
<td>21 (43)</td>
<td>26 (51)</td>
</tr>
<tr>
<td>Histologic type</td>
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<td>14 (21)</td>
<td>0.04</td>
<td>&lt;0.001</td>
<td>17 (13)</td>
</tr>
<tr>
<td></td>
<td>NE&lt;sup&gt;2&lt;/sup&gt;</td>
<td>5 (56)</td>
<td>1.0</td>
<td>18 (69)</td>
<td>9 (9)</td>
</tr>
<tr>
<td>Histologic grade</td>
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<td>0.003</td>
<td>&lt;0.001</td>
<td>9 (9)</td>
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<td></td>
<td>3</td>
<td>10 (50)</td>
<td>1.0</td>
<td>26 (51)</td>
<td>28 (51)</td>
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<td>ER-α-negative (IHC)</td>
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<td>&lt;0.001</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Poor survival&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>0.01</td>
<td>No data</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: E, Endometrioid; NE, Nonendometrioid; neg, negative.
<sup>a</sup>Part of the data in the prospective validation cohort reported previously (5), now with extended follow-up time.
<sup>b</sup>mRNA expression determined by DNA oligonucleotide microarray.
<sup>c</sup>mRNA expression determined by qPCR.
<sup>d</sup>Chi-square and Fisher exact tests when appropriate.
<sup>e</sup>Mann–Whitney U test.
<sup>f</sup>Median age, prospective validation series, 64 years.
<sup>g</sup>Carcinosarcomas, serous, clear cell, and undifferentiated subtypes.
<sup>h</sup>Kaplan–Meier survival analyses; log-rank test. Missing data: Primary investigation set, grade: n = 1, prospective validation series; grade; n = 1; ER-α status (IHC); n = 2. Retrospective validation series, ER-α status: n = 20, FIGO stage: n = 1.

(adjusted for age, histologic grade, and histologic type). A borderline statistically significant association between low ESR1 mRNA and reduced survival was seen for FIGO I/II cases (HR, 2.9; 95% CI, 0.9–9.2; P = 0.07) but not in the stratified analysis of the small subset (n = 33) of more aggressive FIGO III/IV cases (P = 0.7). Furthermore, an independent association with survival for ER-α protein expression was confirmed in multivariate survival analyses for the subgroups of endometrioid tumors, adjusting for age, FIGO stage, and histologic grade (Supplementary Table S4), aiding in the identification of high-risk patients within this clinically challenging patient group expected to have a good prognosis. Also for the whole cohort, ER-α status maintained prognostic impact adjusted for the same variables and histologic subtype (footnotes, Supplementary Table S4).

Taken together, this clearly supports that ER-α protein and ESR1 mRNA expressions are strongly correlated, robust, and reproducible measures for identification of aggressive endometrial carcinomas.

**Integrated analyses associates lack of ER-α with epithelial–mesenchymal transition**

To explore potential biologic processes contributing to the aggressive phenotype of ER-α-negative endometrial carcinomas, we examined transcriptional differences between ER-α-positive and -negative tumors. In the primary investigation cohort, supervised analyses of the oligonucleotide DNA microarray expression data revealed 303 unique genes differentially expressed between ER-α-positive and -negative tumors; expression of 60 unique genes were significantly higher in ER-α-negative as compared with ER-α-positive tumors. Six of these genes were previously shown to be linked to signaling pathways and processes related to cancer invasion (Supplementary Table S5A), including SERPINE-1, suggested to be a marker for TGF-β signaling (30).

Clustering of the mRNA expression (microarray data) of ESR1 and a selection of EMT-related transcription factors (SNAI1, SNAI2, TWIST1, ZEB1, and ZEB2) indicated an association between low ESR1 expression and high expression of the EMT transcription factors (Supplementary Fig. S2C). Furthermore, in GSEA, gene sets indicating activation of Sonic Hedgehog (SHH; ref. 31), Wnt (32), and TGF-β signaling (33) were found among the most significantly enriched gene sets in ER-α-negative compared with -positive tumors (FDR < 0.25). Downregulation of MTAS, potentially leading to increased expression of the transcription factor Snail (ref. 34; www.biocarta.com), was also
found in ER-α-negative tumors. From the literature, these pathways are known to be involved in EMT activation (10, 34). To assess the gene expression signatures’ correlation to survival and to visualize the associations between gene signatures and ER-α expression, signature scores were generated for each gene set, based on expression of the genes defining the pathways (for details see http://www.broad-institute.org/gsea/msigdb). High SHH-, TGF-β, and Wnt-signature scores were significantly correlated with ER-α-negative tumors (P < 0.001; P = 0.001; P = 0.007, respectively) and survival (P < 0.001; P < 0.001; P = 0.009, respectively; Fig. 2A–D). Also for the endometrioid subgroup of tumors, high SHH-, TGF-β, and Wnt-signature scores were significantly correlated with ER-α-negative cancer (P < 0.001, P = 0.007, P = 0.01, respectively) and survival (P = 0.004, P = 0.02, and P = 0.04, respectively). In transcription factors gene sets analyses (primary investigation cohort) based on ER-α protein status, several transcription factors related to EMT activation and TGF-β signaling were identified (Supplementary Table S5B), further supporting the association between ER-α and EMT in endometrial carcinoma. In sum, these data indicate an association between ER-α-negative endometrial cancer and EMT, and the association with survival further supports biologic relevance of the signatures assessed. In contrast, in the ER-α-positive tumors, 2 pathways related to estrogen and ER activity were among the top-ranked gene sets enriched in the ER-α-positive tumors (GSEA, FDR < 0.05); a Biocarta pathway related to ER activity (“Pelp1 Modulation of Estrogen Receptor Activity”), and a gene set generated from identified estrogen-regulated genes from breast cancer tumors and cell lines (35).

For validation of the associations in tumors of low ER-α expression, at first we examined a publicly available external DNA microarray dataset for associations between low ESR1 mRNA expression and the gene sets identified from the primary investigation set. ESR1 expression was inversely correlated with the SHH-, TGF-β, and Wnt-signature scores (P < 0.001, r = −0.46; P < 0.001, r = −0.37; and P = 0.002, r = −0.30, respectively) and correlated with the MTA3 signature score (P < 0.001, r = 0.60), also supporting the association between ER-α-negative endometrial cancer and EMT identified in our primary investigation set. This association was further validated in the prospectively collected cohort of 155 snap-frozen primary tumor specimens. Expression levels for cell adhesion-/EMT markers were estimated by qPCR. Tumor mRNA ESR1 expression was significantly correlated with E-cadherin, catenin p120, α-catenin and β-catenin mRNA levels (all P < 0.001). ER-α protein expression was correlated to the same markers (all P ≤ 0.02), and also negatively correlated to N-cadherin mRNA expression (P = 0.035). Furthermore, ER-α protein expression was significantly associated with high mRNA expression of the suggested TGF-β signaling marker SERPINE-1 (− PAI-1), both in the primary investigation set (P < 0.002; Fig. 2E) and prospective validation series (P < 0.004). SERPINE-1 mRNA expression was also associated with high TGF-β signature score (Fig. 2F).

Finally, in the additional independent, retrospective population-based cohort of 286 endometrial carcinomas, ER-α-negative tumor status confirmed to be significantly correlated to pathologic protein expression of the EMT markers E-cadherin, β-catenin, and catenin p120, the EMT-related P-cadherin (36) as well as invasive properties reflected by vascular invasion and deep myometrial infiltration (Table 2).

A signature for EMT is associated with prognosis in endometrial cancer

In the prospective validation cohort, we generated an EMT gene expression signature by combining a panel of cell adhesion markers with known involvement in EMT (see Patients and Methods), and we explored the signature’s relation to prognosis in endometrial cancer. High EMT signature score (upper quartile of the score) was strongly associated with poor survival (Fig 2G) and recurrence-free survival (P = 0.002), also for the subsets of endometrioid cases only (P = 0.006).

To further assess the prognostic value of the EMT signature score compared with the mRNA levels of single EMT markers, we included separately the EMT signature score and markers, one at a time, in Cox multivariate analyses together with age, FIGO stage, histologic subtype, and histologic grade. Here, the EMT score had independent prognostic impact with HR = 1.4 (95% CI, 1.02–1.7; P = 0.005), contrasting the individual EMT markers, of which none had significant independent prognostic impact.

The EMT signature score was negatively correlated to ER-α (P < 0.001) and ESR1 mRNA expression (Fig. 2H). In Cox survival analyses, including EMT score and ER-α expression, both markers had independent prognostic impact on disease-specific survival with HR = 2.6 (95% CI, 1.03–6.6; P = 0.04) and 5.0 (95% CI, 1.9–12.8; P = 0.001), respectively. In sum, this validates that EMT, measured by an EMT gene expression signature score, is associated with ER-α-negative tumors and adds prognostic information in addition to ER-α status in endometrial carcinoma.

Integrated analyses identify PI3K and mTOR inhibitors as potential drugs for patients with ER-α-negative tumors

Connectivity Map version 02 (24) was queried for drug signatures negatively correlated to the gene expression signature of ER-α-negative tumors. The gene lists acquired from the class comparison analyses based on ER-α protein status were analyzed. Among 1,309 small molecules represented in Connectivity Map, 1 mTOR and 2 PI3K inhibitors as well as a retinoic acid receptor agonist were the 4 top-ranked compounds with signatures most significantly negatively correlated with ER-α-negative tumors, as listed in Table 3. In the external endometrial cancer dataset, we confirmed PI3K and mTOR inhibitors to be the top-ranked compounds significantly negatively correlated to the signature defined by low ESR1 mRNA expression (Table 3). These data support that targeting the PI3K-AKT-mTOR pathway, may be particularly relevant in ER-α-negative endometrial cancers.
Figure 2. Correlations between ER-α status, markers for EMT, and outcome in endometrial carcinomas. Primary investigation series: the signature scores of 2 of the gene sets suggesting the presence of EMT and being enriched in ER-α-negative tumors (identified in GSEA, FDR < 0.25), were significantly associated with ER-α IHC expression (A and C) and disease-specific survival (B and D). SERPINE-1, a suggested marker for TGF-β signaling, relates to ER-α IHC expression (E), and is also associated with a TGF-β signature score (F).

Prospective validation series: the level for an EMT mRNA signature is a strong predictor of disease-specific survival (G) and is significantly associated with ESR1 mRNA expression (H). Red and green color for high and low ER-α IHC expression, respectively (F and H). Survival curves are estimated by the Kaplan–Meier method according to level of the signature scores. For each category the number of cases is given followed by the number of endometrial carcinoma deaths. All the P values are significant (<0.01) also after Bonferroni correction.
We further explored the association between low ER-α expression and potential measures for PI3K activation: PIK3CA mutations and amplifications, PIK3CA, PTEN, and AKT1 mRNA levels, Stathmin protein expression, a suggested surrogate marker for PTEN loss and PI3K activation (19, 37), and a publicly available PI3K activation score (38). Low ER-α expression was significantly associated with amplifications of the 3q26 region, harboring PIK3CA (P = 0.02; Fig. 3A), and also with high mRNA expression of the PI3K signaling family members PIK3CA and AKT1 as well as low PTEN expression (P = 0.05, P = 0.01 and P = 0.005, respectively). ER-α-low tumors were not significantly associated with PIK3CA mutation status (P = 0.8; Fig. 3B), neither with any statistical trend for the association when investigating the correlation with exon 9 and 20 separately (P = 0.6 and P = 0.9, respectively). However, high PI3K activation score was negatively correlated to ESR1 mRNA expression (P = 0.001, r_s = −0.4).

In line with this, a high PI3K score was found to be correlated with high SHH- and Wnt-signature scores (P < 0.001, r_s = −0.5 and P < 0.001, r_s = −0.4, respectively) and negatively correlated with MT3A signaling (P = 0.01, r_s = −0.3) but not with the TGF-β signature score. Also, low ER-α protein expression was significantly associated with high Stathmin protein expression (P = 0.03). High Stathmin expression was also associated with markers for EMT as well as deep myometrial growth, vascular invasion (Table 2), and high EMT signature score (P = 0.003). However, although our results are based only on statistical associations, the findings from this study suggest a potential for targeting PI3K/mTOR in ER-α-negative endometrial carcinomas and suggest presence of EMT in tumors with PI3K pathways alterations.

**Discussion**

Current clinical decision making in the treatment of endometrial carcinomas mainly relies on surgical FIGO stage, histologic subtype, and histologic grade. This risk-stratification is suboptimal and may lead to over- and under-treatment. The distinction between type I and II endometrial cancers is clinically important, although with considerable morphologic and molecular overlap (4). Improved identification of patients at risk of recurrences and poor outcome is needed, and molecular markers may be helpful in this setting. We wanted to apply a population-based approach in the present study, aiming to associate ER-α status with potential targets for new therapy, across all histologic subtypes and by applying state of the art risk categories for treatment stratification including surgical FIGO 2009 stage, histologic subtyping, and histologic grading.
Here, we confirm a strong prognostic impact of ER-α in endometrial cancer, for several independent patient cohorts, as well as in subgroups of endometrioid cases. The prognostic importance of ER-α was confirmed in multivariate survival analyses including clinicopathologic factors. This may potentially aid in the identification of patients at risk of poor outcome within the particularly challenging patient groups expected to have a good prognosis based on standard markers applied in the clinic. We clearly confirm the role of ER-α as a strong prognostic marker in endometrial cancer, as indicated for around 30 years (5, 6, 39, 40). Still, implementation of this biomarker for tailoring systemic therapy has been slow (4, 8). Importantly, we found that ER-α–negative tumors are associated with EMT and PI3K pathway alterations, providing potential targets for treatment of patients with this subgroup of endometrial carcinoma.

The transcriptional differences between ER-α high and low cases are explored by high-throughput analyses, known to be associated with a risk of false discovery. To adjust for potential errors related to multiple testing, validations of the findings in independent patient series and with alternative methods are recommended. Our conclusions are strengthened by the use of several independent datasets to validate both the association between ER-α and EMT, and the association between low ER-α and potential measures for PI3K activation.

ER-α has been suggested as a predictive marker for response to hormonal therapy in endometrial carcinoma (7). Thigpen and colleagues found significantly better response to medroxyprogesterone in patients with ER-α and progesterone receptor (PR)-positive compared with -negative tumors (41). Still, a Cochrane Review from

Table 3. Drug signatures negatively correlated to ER-α-negative endometrial cancer

<table>
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<tr>
<th>Rank</th>
<th>Name of compound</th>
<th>Known target/action</th>
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<th>P^b</th>
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<td>Sirolimus</td>
<td>mTOR inhibitor</td>
<td>44</td>
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<td>LY-294002</td>
<td>PI3K inhibitor</td>
<td>61</td>
<td>&lt;0.00001</td>
</tr>
<tr>
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<td>Retinoic acid receptor agonist</td>
<td>22</td>
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</tr>
<tr>
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<td>Wortmannin</td>
<td>PI3K inhibitor</td>
<td>18</td>
<td>0.00022</td>
</tr>
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<td>PI3K inhibitor</td>
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<td>3</td>
<td>LY-294002</td>
<td>PI3K inhibitor</td>
<td>61</td>
<td>0.00004</td>
</tr>
</tbody>
</table>

^a^ N = number of instances in which the compounds were tested in the Connectivity map.

^b^ The expression changes from the compounds tested were scored according to the ER-α/ESR1 expression signatures and the P value for each compound represents the distribution of this score in the N instances as compared with the distribution of these scores among all compounds tested, using a permutation test (24).
Low ER-α Associates with EMT and PI3K Alterations in Endometrial Carcinoma

2010 on hormonal treatment in advanced endometrial cancer concludes that in sum, there is insufficient evidence that hormonal treatment improves the survival for patients with advanced or recurrent endometrial cancer. However, the lack of stratification according to hormone receptor status represents a significant problem when evaluating therapy response (8). Our findings suggest that treatment protocols systematically implementing ER-α levels may have a yet unexplored potential in endometrial carcinoma.

The current study suggests the presence of EMT as an important feature in ER-α–negative endometrial carcinomas. ER-α loss has previously been associated with increased expression of Snail, an E-cadherin repressor, in these tumors (42), and a functional link between ER-α loss and EMT activation has not been found in endometrial cancer cell line studies, although in cell lines from other cancer types (34, 43). Functional studies have also linked loss of ER-α, PI3K signaling, and activation of STAT3 to EMT activation through TGF-β signaling (44). This, together with our finding of increased expression of a TGF-β signaling marker, SERPINE-1 (30), in ER-α–negative tumors suggest that TGF-β is involved in the EMT process in ER-α–negative endometrial carcinomas. A recent study identifying TGF-β as a key factor for invasion in endometrial cancer adds support to this (45). Further studies of functional implications of both TGF-β signaling and ESR1 loss in relation to EMT and PI3K activation in endometrial carcinoma would be highly relevant.

Individual markers of EMT have been reported to have prognostic impact in endometrial carcinomas and other cancer types (15, 46), and estimation of cadherin E to P- and E to N-switches has indicated additional prognostic impact in various cancers (15, 47). EMT is activated through different signaling pathways (9, 10, 13), and an EMT signature may better reflect the EMT process than individual markers (11). The strong prognostic impact of the EMT gene expression signature score in our dataset is in line with this, supporting the importance of EMT in aggressive endometrial carcinomas (16). Also, the EMT process is hypothesized as a potential target for therapy in cancer (18). Therapy targeting TGF-β and other EMT-related pathways are currently being evaluated in cancers (17, 18), and TGF-β and N-cadherin inhibitors are presently in phase II studies (www. clinicaltrials.gov, August 2012). Given the observed association between low ER-α expression and EMT, the potential role of EMT inhibiting therapies should also be explored for patients with ER-α–negative endometrial carcinomas.

The possibilities for targeted therapy in endometrial carcinoma are limited (4) and in particular for ER-α–negative tumors. PI3K and mTOR inhibitors are today in phase I/II trials in advanced endometrial cancer (www. clinicaltrials.org, August 2012), based on molecular changes reported in aggressive endometrial carcinomas (4, 19). Data from a clinical phase II trial of mTOR inhibition are promising (48). Although the number of compounds examined in Connectivity Map is limited (~1,300), and other relevant drugs not included may thus be undetected in the present study, our data add knowledge by suggesting that PI3K/mTOR inhibitors may be particularly relevant for ER-α–negative endometrial carcinomas, and an association with EMT is also indicated. PTEN loss of function and PIK3CA mutations seem to be more frequent in type I cancers, whereas type II cancers are characterized by ER-α loss, PIK3CA amplification, and P3K signaling activation (4, 19, 49). Our finding that amplification of the PIK3CA region and not PIK3CA mutations are associated with ER-α loss seems to be in line with this. Recent studies on other cancer types support that PIK3 signaling activation may be related also to PIK3CA amplifications (50–52). Taken together, this suggests a potential for PI3K inhibitors for ER-α–negative tumors in particular. Still, subgroups within ER-α–positive tumors with PI3K alterations or PTEN loss may also be relevant for such treatment.

Our results are partially based on indirect measures for drug effects, through assessment of associations with effects showed in functional studies. Further mechanistic studies of PI3K/mTOR and EMT inhibition related to ER-α levels will be relevant to elucidate the potential of ER-α as a predictive marker for response to these inhibitors. Assessing ER-α in pretreatment samples from patients included in clinical trials with these inhibitors is also a relevant ‘next step’ before conduction of randomized controlled trials with stratification according to ER-α status to further define predictive markers for the response to PI3K inhibitors in endometrial cancer.

Conclusion

By integrated profiling of endometrial carcinomas, we found that ER-α–negative tumors are associated with the process of EMT as well as PI3K activation, and the 2 seems to be related. Our study provides a rational for clinical trials exploring the effects of PI3K/mTOR and EMT inhibitors in endometrial carcinoma, and in particular assessing the potential of ER-α to predict therapy response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Wik, M.B. Ræder, C. Krakstad, J. Trovik, E. Birkeland, E.A. Hoivik, M. Mannelqvist, I.M. Stefansson, A.M. Oyan, K.H. Kalland, L.A. Akslen, H.B. Salvesen

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Acknowledgments

The authors thank Bendik Nordanger, Gerd Lillian Hallseth, Britt Edvardsen, Mari Kyliene Halle, Pal Christian Njølstad, and Erlend Njølstad for technical assistance, and the biostatisticians Roy M. Nilsen and Kjell Petersen for advice about statistical analyses and microarray analyses, respectively.
Grant Support
Helse Vest, Research Council of Norway and The Norwegian Cancer Society, Harald Andersens legat supported this study.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 23, 2012; revised December 6, 2012; accepted December 18, 2012. published OnlineFirst January 14, 2013.

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