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Downregulation of FOXO1 mRNA levels predicts treatment failure in patients with endometrial pathology conservatively managed with progestin-containing intrauterine devices

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Abstract

Objective—To examine hormone receptor expression levels and downstream gene activation in pre-treatment and post-treatment biopsies in a cohort of patients with endometrial pathology who were being conservatively managed with a progestin-containing intrauterine device (IUD). A molecular signature of treatment failure is proposed.

Methods—A retrospective analysis of pre- and post-treatment biopsy specimens from 10 women treated with progestin-containing IUD for complex atypical hyperplasia (CAH) or grade 1 endometrioid adenocarcinoma was performed. Expression of estrogen receptor (ER), progesterone receptor (PR) and PR target genes was examined by immunohistochemistry (IHC) and quantitative RT-PCR.

Results—The mean treatment duration was 14.3 months. Four CAH patients had stable disease or regressed after treatment, and four progressed to endometrioid adenocarcinoma. Both patients with an initial diagnosis of endometrioid adenocarcinoma regressed to CAH or no disease. In general, hormone receptor levels diminished post-treatment compared to pre-treatment biopsies; however, we noted unexpected higher expression of the B isoform of PR (PRB) as well as ER in

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Authors' contributions

H.D.R., M.J.C., and K.K.L. conceptualized the hypotheses and designed the research strategy; M.J.G. provided specimens; H.D.R., M.J.C., E.J.D., Y.Z., S.Y., J.-M.S., M.M., and D.D. performed the experiments; H.D.R., E.J.D., and K.W.T. performed the statistical analysis; H.D.R., Y.Z., M.S., and E.C.S. performed blinded histologic analyses, H.D.R., M.J.C., E.J.D., Y.Z., S.Y., K.W.T., S.Y., D.D., and K.K.L. analyzed data; and H.D.R., K.W.T., and K.K.L. wrote the paper. All authors read and approved the final manuscript.

Competing interests

K.W.T. and D.D. are co-owners of Immortagen, L.L.C. All other authors declare that they have no competing interests.

those patients who progressed to frank cancer. There was a trend towards a non-nuclear cytoplasmic location of PRB in these patients. Importantly, the differentiating impact of PR signaling, as determined by the expression of the progestin-controlled tumor suppressor FOXO1, was lost in individuals who progressed on therapy.

Conclusions—FOXO1 mRNA levels may serve as a biomarker for response to therapy and an indicator of PR function in patients being conservatively managed with a progestin-containing IUD.

Keywords

progestin-containing intrauterine devices; progesterone receptor; FOXO1; endometrial hyperplasia; endometrial cancer

Introduction

Endometrial cancer is the most common gynecologic malignancy in women, with 54,870 estimated new cases in the US in 2015 [1]. The majority of patients with endometrial cancer have Type 1 disease, which arises primarily through excess estrogen unopposed by progesterone [2]. Patients with endometrial hyperplasia, or increased glandular proliferation, are at increased risk of developing endometrial cancer [3]. Hyperplasia is most often caused by unopposed estrogen stimulation and occurs in the following four histologic categories: 1) simple hyperplasia; 2) simple hyperplasia with atypia; 3) complex hyperplasia (CH); and 4) complex hyperplasia with atypia (also complex atypical hyperplasia, CAH) [4].

Approximately 25% of patients with endometrial hyperplasia with atypia will develop endometrial cancer if left untreated [3]. Indeed, one study reported that 43% of cases had a concomitant diagnosis of carcinoma when hysterectomy was performed within 12 weeks of initial biopsy [5]. However, the molecular mechanisms underlying the progression from atypical hyperplasia to endometrioid adenocarcinoma remain incompletely understood.

Current guidelines suggest that patients diagnosed with endometrial cancer or the precursor CAH should be managed by hysterectomy. However, for patients who are poor surgical candidates due to medical comorbidities or those that still desire child bearing, hormonal therapy with progesterone has been suggested as an alternative treatment for CAH or low-grade endometrioid adenocarcinoma [6–14]. For example, one study demonstrated a 65% complete response, 23% with complete response followed by recurrence, and only 28% with persistent or progressive disease following progestin treatment [15]. Another study suggested that management with a progestin-containing intrauterine device (IUD) may be superior to oral progestins (94.8% vs. 84.0% regression rate) [16].

Despite the relatively high response rates of CAH and endometrial endometrioid adenocarcinoma patients to progestin-based therapy, some patients eventually relapse or develop progressive disease. Therefore, it is important to identify which patients are at a higher risk of treatment failure. Patient characteristics such as body mass index and histopathologic features such as glandular cellularity have been correlated with response to progestin therapy [15, 17]. For endometrial cancer, expression of the hormone receptors estrogen receptor (ER) and progesterone receptor (PR) have been positively correlated with

response [18–21]. However, identification of molecular biomarkers for progression from CAH to endometrial cancer has proven more challenging, with some studies providing conflicting results [15, 22]. The objective for this study was to examine hormone receptor expression levels and downstream gene activity in pre-treatment and post-treatment biopsies in a cohort of patients with CAH or grade 1 endometrioid adenocarcinoma who were being conservatively managed with a progestin-containing IUD. These data shed light on the molecular signature of therapeutic response vs. failure to conservative management in such patients.

Materials and Methods

See Supplementary Methods for detailed descriptions of methods.

Human Subjects

The study was reviewed and approved by the University of Iowa Institutional Review Board (IRB, ID#201211782). Retrospective chart review identified 10 patients with abnormal endometrial biopsy findings that were managed with a progestin-containing IUD (Mirena IUD) at the University of Iowa Hospitals and Clinics (Figure 1). The sample set included 8 cases with complex atypical hyperplasia (CAH) and 2 cases of grade 1 endometrial endometrioid adenocarcinoma pre-IUD insertion. All subjects had been evaluated with an endometrial biopsy via dilatation and curettage before IUD placement. Biopsies were also available for repeat sampling. This study utilized the last retrieved biopsy sample. The duration between the initial biopsy and the latest biopsy ranged from 6.0–29.6 months, with a mean interval of 14.3 months. “No progression” was defined as regression to normal (N=3), complex hyperplasia (N=1) or stable disease (N=1). Three patients progressed from CAH to endometrioid adenocarcinoma. One CAH patient had benign endometrium at the final biopsy, but subsequently developed a mixed grade 3 endometrioid/undifferentiated adenocarcinoma of the uterus. She was diagnosed with a metastatic axillary lymph node from a core biopsy that subsequently prompted a hysterectomy. This patient was thus included in the “Progression” group in this study for a total of four “Progression” patients. Patient characteristics for all 10 patients are provided in Table 1.

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) endometrial biopsy and hysterectomy tissue sections were assessed for expression of hormone receptors and FOXO1 via immunohistochemistry (IHC) and quantitated as described [23, 24] by three investigators blinded to sample identity.

Quantitative RT-PCR

Quantitative RT-PCR was performed on available pre- and post-IUD insertion biopsy samples (N=9) as previously described [25].

Statistical Analysis

IHC data were analyzed using paired t-test. For quantitative RT-PCR studies, fold change and statistical significance were determined using the $2^{-\Delta\Delta Ct}$ method [26, 27] and standard t-

test, respectively, with unequal variances [28]. A p value < 0.05 was considered statistically significant.

Results

Treatment with a progestin-containing IUD is associated with a decrease in ER, PR and PR isoform B levels

Study design and patient characteristics are provided in Figure 1 and Table 1, respectively. We first examined hormone receptor expression by IHC in paired endometrial biopsies obtained at the time of initial diagnosis (“pre-IUD insertion”) and the last patient visit (“post-IUD insertion”). The mean interval between IUD placement and last clinic visit was 14.3 months. Of the 10 patients that were included in the study, most demonstrated a significant reduction in PR protein expression post-IUD insertion as compared to pre-insertion (Figure 2A–C). Levels of the progesterone receptor isoform B (PRB) were also markedly decreased in post-treatment biopsies (Figure 2A–C), consistent with a previous report demonstrating downregulation in response to progestin-based therapy [20]. Pearson correlation analysis demonstrated that post-IUD insertion expression of ER significantly correlated with PRB (Figure 2D), in line with the reported relationship between hormone receptors in endometrial tumors [29].

Analysis of hormone levels in patients who progressed vs. those that did not progress on therapy

We next asked how hormone receptor levels change in patients who progressed vs. those who did not. “No progression” was defined as stable disease (N=1) or regression to normal or complex hyperplasia (N=3; N=1, respectively). In the “No progression” group, we detected a significant decrease in levels of ER, PR and PRB in the post-IUD insertion biopsies as compared to pre-IUD insertion (Figure 3A), indicating that progestin-containing IUD mediates canonical ligand-mediated receptor downregulation. However, ER and PRB levels in the post-IUD insertion biopsies were significantly higher in the “Progression” group as compared to the “No progression” group (Figure 3B–D). These data suggest that the expected ligand-mediated downregulation of PR [25] is lost in patients with CAH that progress to endometrioid adenocarcinoma. It was next important to determine whether the increase in PRB expression correlated with enhanced or loss of expression of the expected progestin-controlled genes associated with endometrial differentiation.

Gene expression changes associated with progestin-containing IUD

Progesterone promotes differentiation of the uterine endothelium through PR-mediated transcription of several pro-differentiation genes, including amphiregulin (*AREG*), progesterone-associated endometrial protein (*PAEP*, also termed “glycodelin”), and FOXO1 [21]. Of the PR target genes, mRNA expression of FOXO1 was significantly decreased by 28.9-fold in post-IUD insertion biopsies in the “Progression” vs. the “No progression” group (Figure 4A). FOXO1 mRNA levels were downregulated in response to treatment in patients who progressed, whereas FOXO1 was upregulated in patients who did not progress (Figure 4B). FOXO1 is an important tumor suppressor in the endometrium that is controlled by progesterone in the normal setting. This gene contains a well-documented progesterone

response element and is a primary transcriptional target of PR [21]. Therefore, these data demonstrate that the unexpected upregulation of PRB levels in patients who progressed did not result in the enhancement of endometrial differentiation. On the contrary, the transcriptional activity of PRB in these patients was diminished. Studies were extended to determine if the decrease in FOXO1 mRNA levels corresponds with a change in protein expression, but we did not observe appreciable differences in total or nuclear FOXO1 levels (Figure 4C–E), consistent with the observation that, as a PR-controlled gene, FOXO1 message as opposed to protein levels are the most sensitive and specific readout of PR activity.

Nuclear distribution of hormone receptor levels

Studies in endometrial cancer cell lines and tumor specimens have demonstrated differential localization of the PR isoforms PRA and PRB [29, 30]. We next evaluated the relative distribution of hormone receptors by IHC. Post-IUD biopsies had a significant decrease in nuclear PR and PRB as compared to pre-IUD biopsies (Figure 5A–C). We also analyzed cytoplasmic expression of PRB since only this isoform is shuttled from the cytoplasm to the nucleus in response to ligand [30] and found a trend towards enhanced post-treatment cytoplasmic PRB in those patients who progressed ($p=0.183$, Figure 5D, E).

Discussion

Our objective in this study was to determine how hormone receptor levels and activity change in CAH and early stage endometrial cancer patients being conservatively managed with a progestin-containing IUD, with the ultimate goal of using hormone receptor expression or activity as a surrogate to identify patients at risk for progression. In general, PR and PRB levels decrease in response to progestin-IUD therapy, as has been previously demonstrated in the non-therapeutic study of the histologic impact of medroxyprogesterone acetate in patients scheduled for hysterectomy for endometrial cancer (GOG 211) [20]. The mechanism underlying short term hormone receptor down-regulation in response to ligand has been well documented: ligand causes receptor phosphorylation – the necessary precursor for transcriptional activation – while at the same time serving as a signal for receptor ubiquitination and destruction in the proteasome. In theory, receptor transcriptional activity requires rapid recycling on and off DNA in order to promote maximal gene expression. Hence, the initial loss of PR in response to hormonal therapy is expected and is actually a potential marker for drug activity [25]. However, in the patients who progressed, the magnitude of the expected decrease in PR was blunted, and there was a trend towards enhanced non-nuclear (cytoplasmic) PRB. Accompanying these changes was the loss of expression of the PR-dependent gene FOXO1 in cases that progressed. We propose that these cases have lost ligand-mediated gene expression, evidenced by both downregulation of FOXO1 mRNA as well as ligand-dependent receptor downregulation, which contributes to progression to cancer.

For patients who wish to preserve fertility or for whom surgical treatment is not an option, the use of progesterone-based therapy is a reasonable alternative. However, patient response remains varied, with those diagnosed with endometrial hyperplasia having a better outcome

compared to those with grade 1 endometrial cancer [31, 32]. Moreover, recurrence occurs in about half (54%) of patients with CAH or grade 1 endometrial cancer that initially responded to treatment [33]. In our small study of a cohort of 10 patients, six patients regressed or had stable disease.

In this study, the availability of pre- and post-treatment biopsy specimens provided an opportunity to examine how extended exposure to progestin impacts hormone receptor expression. Based on the GOG 211 study [20], we originally hypothesized that we would observe a significant loss in hormone receptor expression in response to therapy. However, we unexpectedly found that, in patients who progressed on therapy, the magnitude of the decrease in hormone receptor levels in post-treatment biopsies was significantly blunted. Thus, we concluded that our original hypothesis was too simplistic, and we must consider the differences in short-term (natural) vs. long-term (pathological) downregulation of hormone receptors. In a separate study, we examined the different mechanisms underlying short-term vs. long-term PR downregulation using a panel of endometrial cancer cells as models [25]. Whereas the well-differentiated endometrial cancer cell line ECC1 demonstrated canonical ligand-mediated PR downregulation in response to progesterone, we found that PR is downregulated through epigenetic silencing in the moderately-differentiated Ishikawa line and the poorly-differentiated Hec50co line. Depending on the mechanism of downregulation, a histone deacetylase inhibitor or a hypomethylating agent effectively restored functional PR expression (including expression of PR target genes) and thereby sensitivity to progestin therapy [25]. Therefore, we put forth the concept both PR expression and activity should be analyzed in patients being managed with a progestin-containing IUD. Our data suggest that FOXO1 mRNA levels are one such marker for PR activity and may serve as an indicator of treatment failure. In these cases, the patient should receive more frequent screening to monitor PR and FOXO1 levels in addition to histologic characteristics, and potentially be considered for surgical management. Future studies are necessary to understand why tumors have lost the ability to transcribe FOXO1 despite residual PR expression. Another future line of investigation is to determine the mechanisms and consequences of PRB cytoplasmic localization, which may reveal novel approaches to restore sensitization to progesterone.

FOXO1 is a transcription factor that is negatively regulated by PI3K/Akt signaling [34]. Phosphorylation of FOXO1 by Akt results in cytoplasmic retention, thereby abrogating transcriptional activity. Others have reported that FOXO1 expression is induced by treatment with progestin, resulting in growth inhibition in endometrial epithelial cells [35]. The downregulation of FOXO1 is therefore consistent with progression, potentially through loss of growth inhibition.

The ultimate goal is to identify a pre-treatment biomarker for patients who are unlikely to respond to therapy. Reports have been controversial with regards to the utility of hormone receptor expression to predict response to progestin-based therapy. We did not observe any significant difference in pre-treatment ER, PR, or PRB levels in patients who progressed vs. those that did not. However, Upson et al reported that PRB over-expression confers a decreased risk of stable disease or progression by 90% in patients with atypical hyperplasia [22], while Gunderson et al. reported that ER and PR expression did not predict for

treatment response to progesterone therapy [15]. In GOG211 which examined ER, PR, and Bcl-2, lower pre-treatment levels of the proliferative indicator Ki-67 were the only pre-treatment predictor of histologic response [20]. Towards identifying a biomarker for response, we also screened patients who progressed to cancer for mutations in genes commonly altered in endometrial cancer [2] but did not detect mutations in p53, KRAS, PTEN, or PI3 kinase (data not shown).

Data presented herein were collected at the initial biopsy and the last available biopsies, with a median interval of 14.3 months. Most patients are routinely biopsied every 3–6 months within the first 2 years of diagnosis. Others have reported regression rates of 72.7–100%, with optimum results observed after 6 months of hormonal therapy [36]. A lack of response at the first post-IUD insertion biopsy is generally considered treatment failure [33]. Our study was designed to capture the maximum effect of the treatment in patients who progressed vs. those that did not. It should be recognized that this project only included a small sample size. There is also the possibility that the patients that were sampled already had a small focus of endometrial cancer even though the initial sampling results showed benign pathology. However there is no other way to confirm this other than by performing a hysterectomy. Regardless of these limitations, this study does provide insight into the effect of progestin-containing IUD on hormone receptor levels and activity. Due to the complex regulation of hormone receptor expression and activity, both up and down in response to ligand, a downstream readout of PR transcriptional activity is necessary to fully evaluate therapeutic response. Data from our pre- vs. post-IUD samples in patients who progressed relative to those that did not suggest that FOXO1 mRNA levels should be considered as a biomarker for response in patients being conservatively managed with a progestin-containing IUD. Future efforts should include a larger prospective study to confirm the key observation of loss of FOXO1 in patients who progress on therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- We performed a retrospective analysis of hormone receptor expression and activity in patients managed with progestin-containing IUD for endometrial pathologies.
- The PR target gene FOXO1 was significantly downregulated in patients who progressed on therapy.
- FOXO1 mRNA levels may be an indicator of PR function and therefore response to progestin-based therapy.

Figure 1. Study Design

CAH, complex atypical hyperplasia; CH, complex hyperplasia; EA, endometrioid adenocarcinoma.

Figure 2. Effect of progestin – containing intrauterine device (IUD) on hormone receptor protein levels in endometrial biopsies

A. Representative IHC images in endometrial biopsy samples before (*Pre*) and after (*Post*) insertion of the IUD. Modified H-scores (glandular) take into account the percentage of positive glandular cells and the staining intensity. The percent (%) nuclear staining indicates the percentage of nuclei that stained positive for the receptor. **B.** Scatter plots of hormone receptor levels in endometrial biopsies before and after treatment using paired data.

** $p < 0.01$ vs. pre-IUD insertion. **C.** Average modified H-score values based on data in panel

(B). D. Pearson correlation analysis of the relationship between expression of PR (left panel) and PRB (right panel) with ER in post-IUD insertion biopsies.

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Figure 3. Hormone receptor protein expression in endometrial biopsies from patients that progressed vs. patients that regressed or had stable disease after conservative management with progestin – containing IUD

A, B. Modified H-scores (glandular) by IHC in patients that either had stable disease/regressed (“No progression,” **A**) or progressed (“Progression,” **B**). Hormone receptor levels (modified H-scores) pre- and post-IUD insertion are presented as scatter plots using paired data. * $p < 0.05$, ** $p < 0.01$ vs. pre-IUD insertion. **C, D.** Average modified H-score (glandular) values pre-IUD insertion (**C**) or post-IUD insertion (**D**) based on data in panels **A, B**.

Figure 4. Downregulation of FOXO1 in patients who progressed after conservative management with progestin – containing IUD

A. mRNA expression of hormone receptors and PR target genes was determined by RT-qPCR, normalized to 18S, and data displayed as the fold change in the “Progression” group relative to the “No progression” group. Comparisons of normalized expression values (Ct) employed the conventional Ct fold change method. **B.** mRNA levels of FOXO1 in post-relative to pre-IUD insertion biopsies. **C.** Representative IHC images of FOXO1 staining in endometrial biopsies **D, E.** Average modified FOXO1 H-score (glandular) values (**D**) and %

nuclear FOXO1 staining (**E**). In **A** and **B**, N=5 for “No progression” and N=2 for “Progression” in the pre-IUD insertion biopsies; N=4 for “No progression” and N=4 “Progression” in the post-IUD insertion biopsies. $\ddagger\ddagger$ $p = 0.002$ vs. “No progression” in **A**; $\ddagger\ddagger$ $p = 0.006$ vs. pre-treatment levels in **B**. In **C** and **D**, N=6 for “No progression” and N=4 for “Progression”; paired pre-and post-IUD insertion samples were available and analyzed for all subjects.

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Figure 5. Nuclear and cytoplasmic distribution of hormone receptors in endometrial biopsies from patients that progressed vs. patients did not progress on therapy

A. Average % nuclear staining. See Figure 2A for representative images. N=10; paired pre- and post-IUD insertion samples were available and analyzed for all subjects. **B, C.** Average % nuclear hormone receptor staining pre-IUD insertion (**B**) or post-IUD insertion (**C**). **D.** Scatter plot of cytoplasmic PRB levels in endometrial biopsies before (*Pre*) and after treatment (*Post*) using paired data. Modified H-score (cytoplasmic) take into account the percentage of glandular cells with positive cytoplasmic staining and the staining intensity.

p=0.07 vs. pre-IUD insertion. **E.** Average modified H-score (cytoplasmic) values post-IUD insertion. p=0.183 vs. “No Progression.” In **B**, **C**, and **E**, N=6 for “No progression” and N=4 for “Progression” groups.

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Table 1

Patient characteristics

Patient #	Age at Diagnosis	BMI	Race	Initial biopsy result	Interval between IUD placement and last clinic visit (mos)	Final biopsy result	Underwent hysterectomy
1	63	58.4	Caucasian	CAH	13.5	EA	Yes
2	52	56.6	Caucasian	CAH	15.0	CH	Yes
3	40	38.9	Hispanic	CAH	9.0	CAH	No
4	63	55.6	Caucasian	CAH	29.6	EA	Yes
5	63	58.9	Caucasian	CAH	13.3	Normal Endometrium	No
6	46	62.9	Caucasian	CAH	50.2	Normal Endometrium	No
7	28	46.5	Hispanic	EA	27.8	CAH	No
8	55	63.8	Caucasian	CAH	28.7	CH	No
9	33	48.4	Caucasian	CAH	6.0	EA	Yes
10	58	72.2	Caucasian	EA	8.6	Normal Endometrium	No

Abbreviations: CH: complex hyperplasia; CAH: complex atypical hyperplasia; EA: endometrioid adenocarcinoma