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Molecular Changes in Endometriosis-Associated Ovarian Clear Cell Carcinoma

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Abstract

BACKGROUND—Endometriosis is frequently associated with and thought of having propensity to develop into ovarian clear cell carcinoma (OCCC), although the molecular transformation mechanism is not completely understood.

METHODS—We employed immunohistochemical (IHC) staining for marker expression along the potential progression continuum. Expression profiling of microdissected endometriotic and OCCC cells from patient-matched formalin-fixed, paraffin-embedded samples was performed to explore the carcinogenic pathways. Function of novel biomarkers was confirmed by knockdown experiments.

RESULTS—PTEN was significantly lost in both endometriosis and invasive tumor tissues, while estrogen receptor (ER) expression was lost in OCCC relative to endometriosis. XRCC5, PTCH2,

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

eEF1A2, and PPP1R14B were significantly overexpressed in OCCC and associated endometriosis, but not in benign endometriosis ($p \leq 0.004$). Knockdown experiments with *XRCC5* and *PTCH2* in a clear cell cancer cell line resulted in significant growth inhibition. There was also significant silencing of a panel of target genes with histone H3 lysine 27 trimethylation, a signature of polycomb chromatin-remodeling complex in OCCC. IHC confirmed the loss of expression of one such polycomb target gene, the serous ovarian cancer lineage marker WT1 in OCCC, while endometriotic tissues showed significant co-expression of WT1 and ER.

CONCLUSIONS—Loss of PTEN expression is proposed as an early and permissive event in endometriosis development, while the loss of ER and polycomb-mediated transcriptional reprogramming for pluripotency may play an important role in the ultimate transformation process. Our study provides new evidence to redefine the pathogenic program for lineage-specific transformation of endometriosis to OCCC.

Keywords

Ovarian clear cell carcinoma; endometriosis; gene expression; epigenetic reprogramming

INTRODUCTION

Endometriosis is a common gynecologic disorder that affects 5–20% of women of reproductive age [1]. It is classically defined as the presence of endometrial glands and stroma outside of the uterine cavity and musculature [2]. Although it is a benign lesion, it does share several characteristics with invasive cancer. Like cancer, endometriosis has the capacity to both invade locally and spread. In addition, endometriosis can attach to, invade and damage affected tissues [3]. Although infrequent, endometriosis has also been shown to have the ability to undergo malignant transformation [3]. In 1925, Sampson was the first to describe a case of malignant transformation of endometriosis to an ovarian carcinoma [4]. Several studies have since indicated that women with endometriosis carry an increased risk of developing epithelial ovarian cancer (EOC), especially of the clear cell and endometrioid histologic sub-types [5, 6].

Ovarian clear cell carcinoma (OCCC) represents 12% of cases of EOC in Western countries [7]. The treatment of patients with OCCC is particularly challenging, as this histologic sub-type is notoriously insensitive to conventional platinum- or taxane-based chemotherapy. Although survival for localized OCCC is generally favorable, with response rates to first-line chemotherapy estimated between 22 to 55%, the responses to subsequent treatments are uniformly low and the prognosis remains poor for higher stage disease [8].

While the molecular events underlying OCCC development remain largely unknown at this time, a substantial amount of evidence supports the concept that OCCC develops in a step-wise fashion from endometriosis, likely through an intermediate, atypical endometriotic lesion [9, 10]. Contributing to this theory, have been reports of both loss and gain of immunoexpression shared between OCCC and neighboring endometriosis [10, 11]. Recent studies have contributed additional novel markers to the immuno-phenotype of OCCC [10, 12]. Most notably, mutations of the *ARID1A* gene and loss of expression of the gene product, a SWI/SNF chromatin-remodeling complex factor and a proposed tumor

suppressor, represent some of the most common genetic alterations identified thus far in OCCC [10].

Recent technological advancements have enabled the ability to identify additional disease markers and to evaluate the molecular events during the development of human cancers by acquiring genomic and gene expression profiles from formalin-fixed, paraffin-embedded (FFPE) tissues, once regarded as being unsuitable for profiling applications due to fragmented and chemically modified nucleic acids [13]. We present herewith results of a complete study starting with immunohistochemistry (IHC) of endometriosis and malignant ovarian neoplasms utilizing traditional and contemporary markers and then gene expression profiling to reveal potential novel disease markers emblematic of the underlying molecular events in the potential progression of endometriosis to OCCC. For gene expression profiling, patient-matched cases that included a primary OCCC, endometriosis directly adjacent to the primary OCCC and histologically benign endometriosis located at an area distant from the primary OCCC were used.

MATERIALS AND METHODS

Clinical Specimens

Archival specimens were collected and archived under protocols approved by Brigham and Women's Hospital Institutional Review Board. Commercially available ovarian tissue microarrays (OVC1021, Pantomics, CA, USA) were added for initial immunohistochemical (IHC) screening.

Immunohistochemistry and Laser Capture Microdissection

Standard immunohistochemistry (IHC) with microwave in 0.1 M citrate buffer (pH 6.0) as the antigen retrieval method was performed on FFPE sections using reagents from Vector Laboratories, Inc (Burlingame, CA, USA) as described before [14]. Antibodies used in this study are listed in Supplementary Table S1. Immunohistochemical staining was evaluated by two independent gynecologic pathologists using a quantitative scoring system. Cell staining intensity was scored along a scale from 0 (negative) to 3 (strongly positive). The area of cell staining was scored along a scale from 0 (negative staining) to 3 (100% of the cells exhibited staining). The final immunohistochemical score represented the product of the averaged intensity and the area scores.

For Laser Capture Microdissection, metallic slides with polyethylene terephthalate (PET) membrane (Leica Microsystems Inc, IL, USA) were pre-coated with 0.1% poly-L-lysine (Sigma-Aldrich, MO, USA) and together with the tissue sections (10–11 μm) were incubated at 60°C for 2 hrs. IHC was performed using ER primary antibody to highlight endometriosis (distant and adjacent) tissues. Laser Capture Microdissection was performed using a Leica AS LMD laser microdissection system (Leica Microsystems, IL, USA) according to the manufacturer's instruction.

RNA Isolation, microarray hybridization and data analysis

Total RNA was isolated from cells using the total RNA isolation protocol from the NuGEN's Ovation FFPE WTA system (NuGEN, CA, USA). 2 U of DNase was added/μg nucleic acid to digest any contaminating genomic DNA, and phenol/chloroform (Sigma-Aldrich, MO, USA) purified. 100 ng of each RNA sample was used for target labeling by a two-round amplification protocol, and 3.5μg of fragmented labeled RNA of each sample was hybridized to the Human Gene 1.1 ST Array on an Affymetrix GeneAtlas Fluidic station (Affymetrix, CA, USA). Gene expression data were normalized, background-corrected, and log2-transformed using Expression Console (Affymetrix, CA, USA). Differentially expressed genes were identified using Significance Analysis of Microarrays (SAM) with false discovery rate (FDR) <0.05 and fold change >2, as well as the Bioconductor package Linear Models for Microarray Data (LIMMA). Differentially expressed genes were searched for important signaling networks using Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis computational program [15].

Knockdown expression of biomarkers and cell growth study

Mission® siRNAs targeting PTCH2 (SASI_Hs01_00148675 and SASI_Hs01_00148676) and Ku86 (SASI_Hs01_00099411 and SASI_Hs01_00099412) and Universal Negative Control siRNA (SIC001) were purchased from Sigma-Aldrich (MO, USA). The siRNAs were transfected separately into the ovarian clear-cell cancer cell line RMG1 using Lipofectamine 2000 transfection reagent (Life Technologies, NY, USA). Knockdown of biomarker expression was confirmed by Western blot analysis.

Cell growth study was performed using methylthiazol tetrazolium (MTT) solution (5 mg/mL in PBS, Sigma-Aldrich, St. Louis, MO). Absorbance at 562 nm was determined on an ELx800 absorbance microplate reader (Bio-Tek, Winooski, VT). The growth assay was performed in triplicates and repeated twice.

Statistical Analysis

All calculations were performed with MINITAB statistical software (Minitab, State College, PA) unless otherwise indicated. ANOVA was used to compare the mean IHC scores between benign and malignant paraffin sections and between different tumor histologies with post hoc Tukey's multiple comparisons test to examine significant differences between groups. If the equal population variances assumption was not met, the non-parametric Kruskal-Wallis test was performed to compare the results obtained by ANOVA. For cell growth assay, Significance of differences was determined using 2-tailed T-Test. A *P*-value of less than 0.05 was considered statistically significant for all tests.

RESULTS

Immunophenotypes of 5 traditional and contemporary Markers in Unmatched Samples

A panel of normal ovarian, endometriotic and invasive ovarian tumor samples (n=175) were subjected to IHC staining for hKIM-1, HNF-1β, ARID1A, ER, and PTEN, markers that were suggested for OCCC [10–12]. ANOVA analysis results are shown in Figure 1 and accompanying Supplementary Tables S2–S6 with detailed significant information.

Significant overexpression of tumor biomarkers hKIM-1 ($p<0.0001$) and HNF-1β ($p=0.01$) was found in invasive ovarian cancer as compared to both normal ovarian and endometriosis tissues. However, when the analysis was performed with invasive ovarian tumors subdivided into different histologic subtypes, only HNF-1β showed specific significant overexpression in OCCC, whereas overexpression of hKIM-1 was found in all four histologic subtypes relative to normal ovarian tissues. In contrast to the tumor biomarkers, ANOVA and post hoc analyses revealed significantly lower expression of ARID1A ($p<0.0001$) and PTEN ($p=0.0004$) in invasive ovarian cancer than either normal ovarian or endometriosis tissues. The only difference between these two biomarkers is that there was no significant difference in ARID1A expression between normal ovarian and endometriosis samples, whereas both endometriosis and invasive ovarian tumors showed significantly reduced expression of PTEN than normal ovaries. This difference still holds when different histologic subtypes of invasive ovarian tumors were under consideration. ARID1A expression was significantly lower for OCCC, endometrioid and mucinous ovarian cancer when compared to both normal ovarian and endometriosis tissues ($p<0.0001$), whereas low PTEN expression was found in endometriosis in addition to OCCC, endometrioid and mucinous ovarian cancer. PTEN expression was also significantly lower for endometriosis samples when compared to serous ovarian cancer. Of all the invasive ovarian cancer sub-types, OCCC had the lowest level of PTEN expression (Table S5).

Unlike the other 4 biomarkers, ER staining ($p=0.1323$) did not show significant differences when normal ovarian tissues, endometriosis, and invasive ovarian tumors were compared (Figure 1 and Table S6). However, the expression was remarkably lower for OCCC and mucinous ovarian tumors in histologic subtype analyses ($p<0.0001$, Table S6).

Immunohistochemistry of 5 Markers in Patient-Matched Cases

To evaluate the timing of gain or loss of the marker expression during the progression from endometriosis to OCCC, seven sets of patient-matched cases were subjected to IHC staining. The results of the matched samples (Figure 2) showed that both ARID1A ($p=0.495$) and PTEN expression ($p=0.14$) were not significantly different between the matched endometriosis tissues and clear cell primary tumors. Expression of the other three biomarkers was significantly different ($p\leq0.003$) between primary clear cell tumors and the matched endometriosis tissues. Post hoc analyses showed that in primary OCCC, both hKIM-1 and HNF-1β expression was significantly higher, whereas ER expression was significantly lower compared with both adjacent and histologically benign distant endometriosis. Representative staining of the biomarkers in the three tissue entities is shown in Figure S1.

Gene Expression Profiling of OCCC and matched endometriotic cells

To compare the molecular differences between primary OCCC cells and the associated endometriotic cells, we procured distant and adjacent endometriotic cells and the primary cancer cells from FFPE samples of the four matched cases that had enough materials for Laser-capture microdissection (LCM). The endometriotic cell populations were immunostained for ER prior to LCM to highlight the endometriotic cells (Figure S2). Total RNA was isolated from the microdissected cell populations and gene expression profiling

was performed on Affymetrix Human Gene 1.1 ST Arrays. Analysis of the expression profiles using Significance Analysis of Microarrays (SAM) and Linear Models for Microarray Data (LIMMA) revealed 112 significant differentially expressed genes (Figure 3A and Table S7). Some genes such as *GPX3* and *eEF1A2* have been reported being elevated in ovarian tumors including OCCC [16, 17]. Submission of the gene list for enrichment analysis using Ingenuity Pathway Analysis (IPA) platform demonstrated that most of these genes are involved in cell-to-cell signaling and cell morphology functions (Figure 3B).

eEF1A2, PPP1R14B, PTCH2, and XRCC5 were overexpressed in OCCC and associating endometriosis and knockdown of expression slowed OCCC cell growth

To validate the microarray profiling results, IHC was performed on the matched cases and benign endometriosis samples for four selected differentially expressed biomarkers (eEF1A2, PPP1R14B, PTCH2, and XRCC5) based on their potentially important functions in cell signaling and drug response. eEF1A2 (p=0.004) and the other three biomarkers (p<0.001) were overexpressed in OCCC compared to benign endometriosis (Table 1). Tumor-associated endometriotic tissues also showed higher expression than benign endometriosis and post hoc Tukey's analysis showed that for both XRCC5 and PPP1R14B, the differences were statistically significance. Representative images of the IHC staining are presented in Figure 4A.

To explore the potential function of the new biomarkers in cell growth, we used small interfering RNAs to knock down the expression of PTCH2 and XRCC5, respectively, in a human OCCC cell line, RMG1 (Figure 4B). The knockdown cancer cell lines showed significant decrease of cell growth compared with RMG1 cells transfected with control siRNA.

Gene Set Enrichment Analysis revealed genome-wide silencing of polycomb target genes and WT1 in OCCC

To elucidate the underlying mechanisms by which OCCC is developed from endometriosis, the whole transcriptomes of the matched samples were analyzed using Gene Set Enrichment Analysis (GSEA) [15]. The analysis results (Table S8 and Figure S3) showed that endometriotic cells are enriched with gene sets for olfactory signaling pathway, GPCR downstream signaling and ion-channel activity, whereas OCCC cells are enriched with gene sets associated with several types of human cancer, nuclear structures and RNA metabolic processes. However, the most striking gene set enrichments were histone H3 lysine 27 trimethylation (H3K27me3) genes (Table S8 and highlighted in Figure 5A) derived from several studies on embryonic stem (ES) cells and poorly differentiated breast cancer [18, 19]. These gene sets were expressed in endometriotic cells but mostly silenced in OCCC cells. We noted the presence of ovarian serous histotype tumor marker Wilms' tumor protein 1 (WT1) in the H3K27me3 target gene list (Figure 5B). IHC study showed that WT1 was highly expressed in endometriosis and its expression was significantly correlated with ER (Pearson correlation coefficient = 0.785, p=0.001). In comparison, WT1 expression was significantly diminished in OCCC (p<0.001, Figures 5C and D).

We have also compared our OCCC expression profiles to the OCCC expression profile relative to normal ovaries reported by Stany *et al.* [20] using GSEA. The most common gene sets shared by the two studies are involved in RNA metabolic processes (Table S9). Furthermore, when the analysis was performed using transcription factor Motif Gene Set Collection, similar enrichment of target gene promoters containing transcription factor motifs for Elk1 and YY1 was found.

DISCUSSION

The first part of current study displays the immunohistochemical pattern of expression of selected markers and, more importantly, speculates as to the timing of the molecular changes during the transformation progression from endometriosis to OCCC. We found low ARID1A expression among our OCCC samples and the expression within the primary OCCC was not significantly different from adjacent and distant endometriosis (p=0.495). These results suggest that loss of ARID1A expression was frequently seen in the endometriosis adjacent to the primary malignant site. Moreover, our results also suggest that loss of ARID1A is an early event in the carcinogenic pathway and occurs as early as histologically benign endometriosis regardless of features of “atypical endometriosis”.

The expression of another tumor marker hKIM-1 in OCCC was significantly greater than normal ovaries and endometriosis. However, there were no significant differences in the expression among all the histotypes of invasive ovarian cancer. The classical marker, HNF-1β, meanwhile, showed more specific increase of expression in OCCC and serous tumors. HNF-1β showed significantly increased levels in the primary OCCC compared to matched endometriosis, suggesting that overexpression of this tumor biomarker is a relatively late carcinogenic event.

Meanwhile, ER showed significant downregulation in OCCC compared with normal ovaries, endometriosis, and other subtypes of ovarian tumors. In the matched samples, high ER expression remained in both distant and adjacent endometriotic lesions while the expression was lost within the primary OCCC, suggesting a late carcinogenic event. In contrast, loss of PTEN expression was found in almost every endometriosis case in unmatched and matched samples. Taken together, we suggest that while endometriosis samples with early loss of PTEN expression is not sufficient per se to progress to malignant transformation, they are nevertheless permissive to subsequent genetic insults for malignancy development. An analogous example is endometrial hyperplasia, in which *Pten* mutations were frequent with and without atypia [21], two entities with considerably different potential for malignant transformation.

The second part of the current study was the implementation of gene expression profiling of patient-matched sets to identify novel biomarkers and new insights into the transformation mechanisms. We found that ER was the best marker to highlight endometriotic cells for microdissection. Four novel differentially expressed biomarkers were selected for further studies. eEF1A2 has been implicated as an oncogene in ovarian pathogenesis [16]; PTCH2 is involved in hedgehog signaling and it modulates tumorigenesis in the presence of *Ptch1* haploinsufficiency in medulloblastoma and other tumors [22]; PPP1R14B is an inhibitor of

protein-phosphatase 1 and is required for cell migration and retraction [23]; XRCC5 is one subunit of the Ku ATP-dependent DNA helicase heterodimer for double-strand break rejoining repair and was found upregulated in ovarian cancer triggered in response to genotoxic stress [24]. Besides overexpression of these four novel biomarkers in OCCC, IHC also showed that they were overexpressed in endometriosis associated with OCCC as compared to endometriosis in patients without cancer. Apparently, the OCCC-associated endometriosis tissues already harbor aberrant gene expression prior to the visible onset of neoplasia. Knockdown experiments showed that OCCC cells with specific knockdown of either PTCH2 or XRCC5 demonstrated significant slower growth. Hedgehog activation is known to be important for cell proliferation [22]; XRCC5 was also reported to contribute to proliferation through NF-kappa B activation in gastric cancer cells [25]. These novel proteins might have a functional role in OCCC pathogenesis and will be studied later.

GSEA analysis of the expression profiles yielded several interesting findings (Figure S3). First, a large set of olfactory signaling receptor genes were expressed in endometriotic cells but not in OCCC. Low expression of these genes in OCCC might be due to their uniformly late replication timing, as suggested by a recent analysis study of whole-exome sequence data from 27 tumor types [26]. The second finding is the enrichment of endometriotic cells with ion-channel activity genes. These ion channel proteins might be responsive to inflammation and potentially contribute to the chronic pelvic pain in endometriosis patients [27, 28]. Another finding is that although we did not find many common OCCC-enriched gene sets shared between our study and the study by Stany *et al.*, probably because of the difference in the reference cell type, the consistent presence of RNA processing OCCC-enriched gene sets in Tables S8 and S9 implicates the important role of RNA metabolism in OCCC pathogenesis. In a recent study to catalogue 33 novel cancer genes that are significantly mutated in 21 tumor types, three genes (*PCBP1*, *QKI*, and *RPL5*) are associated with RNA processing and metabolism [29]. Evidence is accumulating to suggest that RNA metabolic processes regulate many tumorigenic and metastatic processes including DNA damage response [30], epithelial-mesenchymal transition [31], and the viability of cancer stem cells [32].

Importantly, there was an extensive enrichment of gene sets with H3K27me3 histone modification in endometriotic cells that were silenced in OCCC. H3K27me3 is a chromatin silencing mark and a signature for gene silencing by polycomb group (PcG) complexes [33]. These target gene sets were found expressed in specific lineages of normal differentiated cells but were silenced in embryonic stem cells [18, 34] and pluripotent cells [19]. The international collaborative Project Grandiose study of somatic cell reprogramming has shown an increase of H3K27me3 silencing mark during cell transition to pluripotent state [35]. In essence, the PcG-regulated gene sets have to remain repressed to maintain pluripotency and are poised for activation during lineage cell differentiation [34]. WT1 was identified in these gene sets and its increased expression in endometriosis as compared to OCCC was confirmed by IHC. WT1 is an interesting regulator of mesenchyme maintenance and has an essential function in tissue repair and regeneration [36]. WT1 is also a lineage-specifying marker for serous ovarian cancer [37]. Hence, the expression of these extensively large lineage-specifying gene sets in endometriosis but silenced in OCCC suggests an

epigenetic reprogramming mechanism that transforms the differentiated endometriotic cells to a pluripotent state in OCCC. To this end, many studies have suggested that OCCC is particularly enriched with subpopulations of cancer stem cells [38, 39]. Since reprogramming to a pluripotent state involves increased activity of defined transcription factors, our GSEA analysis using Motif gene set collection revealed enrichment of Elk1 and YY1 target genes in OCCC (Table S9). Elk1 and YY1 may play a significant role in the polycomb-based epigenetic reprogramming.

In conclusion, we have reported the temporal changes of known and novel biomarkers in endometriosis-associated OCCC. More importantly, for the first time, our profiling analyses expand on a paradigm that a polycomb-based reprogramming and the associated transcription factors for pluripotency as the critical carcinogenic events involved in the development of OCCC from the intermediary endometriosis lesions. There are two interesting points that are worth further investigation: First, similar to ER-negative OCCC, the PcG-regulated gene sets were found silenced in ER-negative breast tumors [18]. Hence, estrogen signaling may affect the PcG function. Second, it is known that there is epigenetic antagonism between polycomb and SWI/SNF chromatin remodeling complexes in stem cell programming and oncogenesis [40]. ARID1A is a subunit of SWI/SNF complex that has frequent loss of function in OCCC. Hence, a better understanding of interactions between these two antagonizing epigenetic complexes could prove important for reducing the risk of OCCC malignancy in endometriosis patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Loss of PTEN expression is proposed as an early event in endometriosis development
- Loss of ER expression is proposed as a late event during tumorigenesis
- XRCC5, PTCH2, eEF1A2, and PPP1R14B are upregulated during cancer development
- Silencing of polycomb target genes in ovarian clear cell cancer cells
- Pluripotency may reprogram endometriotic cells to oncogenic path

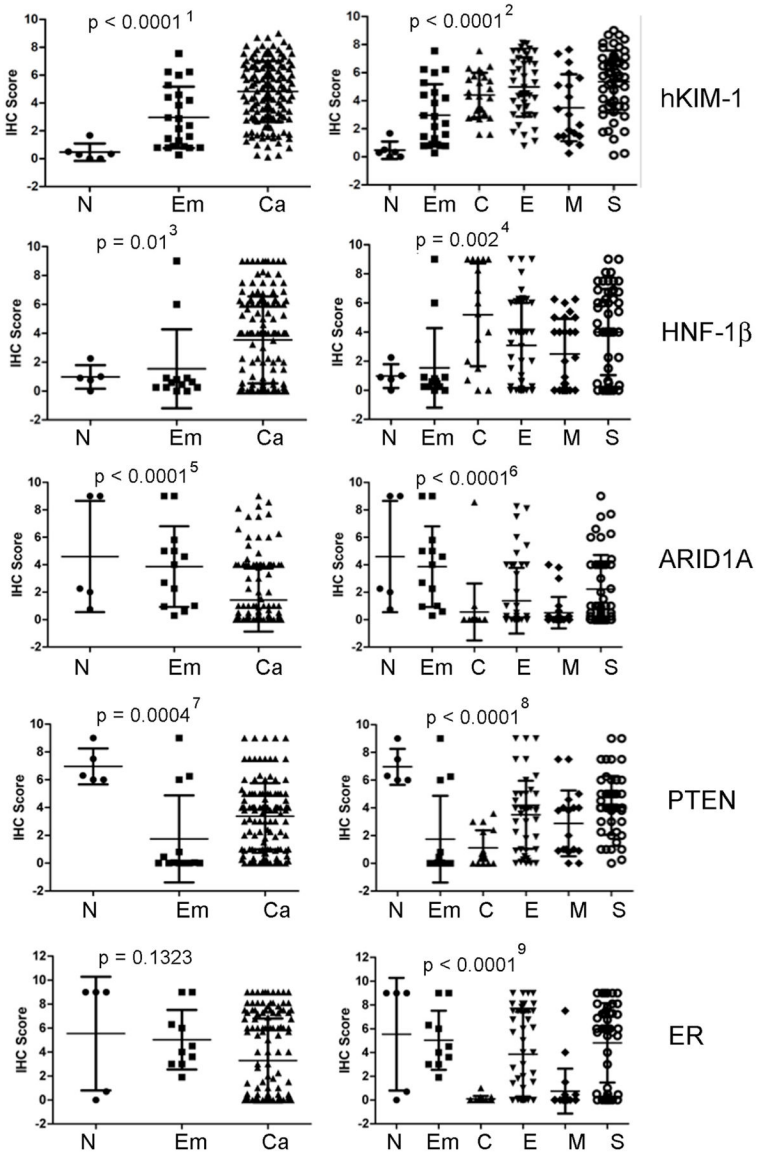


Figure 1.

Immunohistochemical staining of hKIM-1, HNF-1β, ARID1A, PTEN and ER in unmatched tissue types: N, normal ovarian; Em, endometriosis; Ca, invasive cancer; C, clear cell subtype; E, endometrioid subtype; M, mucinous subtype; and S, serous subtype of invasive cancer.

Notes for Post hoc analysis:

1. Significant differences for: normal ovarian vs. endometriosis and invasive cancer; endometriosis vs. invasive cancer.
2. Significant differences for: normal ovarian vs. clear cell; normal ovarian vs. endometrioid; normal ovarian vs. mucinous; normal ovarian vs. serous; endometriosis vs. endometrioid; endometriosis vs. serous; mucinous vs. serous.

3. Significant differences for: normal ovarian vs. invasive cancer; endometriosis vs. invasive cancer.
4. Significant differences for: normal ovarian vs. clear cell; endometriosis vs. clear cell; clear cell vs. mucinous.
5. Significant differences for: normal ovarian vs. invasive cancer; endometriosis vs. invasive cancer.
6. Significant differences for: normal ovarian vs. clear cell; normal ovarian vs. endometrioid; normal ovarian vs. mucinous; endometriosis vs. clear cell; endometriosis vs. endometrioid; endometriosis vs. mucinous; mucinous vs. serous.
7. Significant differences for: normal ovarian vs. endometriosis; normal ovarian vs. invasive cancer.
8. Significant differences for: normal ovarian vs. endometriosis; normal ovarian vs. clear cell; normal ovarian vs. endometrioid; normal ovarian vs. mucinous; endometriosis vs. serous; clear cell vs. endometrioid; clear cell vs. serous.
9. Significant differences for: normal ovarian vs. clear cell; normal ovarian vs. mucinous; endometriosis vs. clear cell; endometriosis vs. mucinous; clear cell vs. endometrioid; clear cell vs. serous; endometrioid vs. mucinous; mucinous vs. serous.

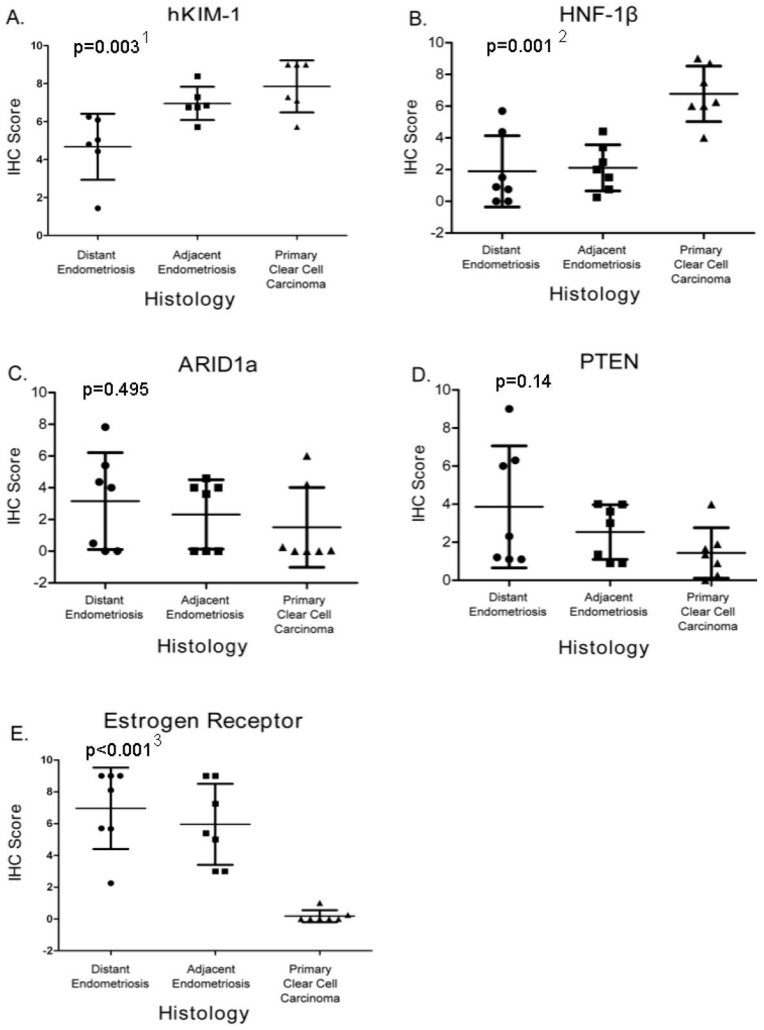


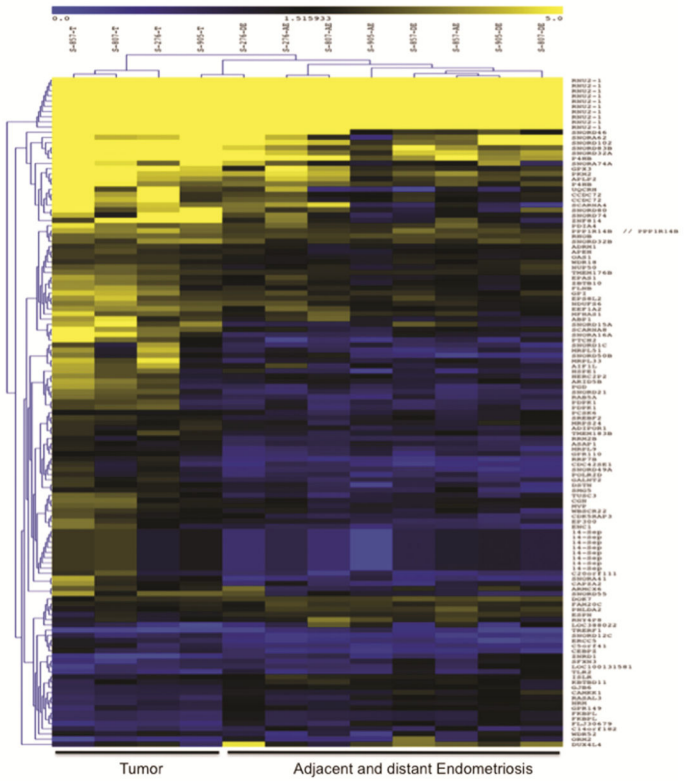
Figure 2.

Immunohistochemical staining of hKIM-1, HNF-1β, ARID1A, PTEN and ER in matched tissue types.

Notes for Post hoc analysis:

1. Significant differences for: distant endometriosis vs. primary OCCC; adjacent endometriosis vs. distant endometriosis.
2. Significant differences for: primary OCCC vs. adjacent and distant endometriosis.
3. Significant differences for: primary OCCC vs. adjacent and distant endometriosis.

A.



B.

ID	Score	Focus Molecules	Top Functions
1	48	22	Antigen Presentation, Cell-To-Cell Signaling and Interaction, Hematological System Development
2	38	18	Cell Morphology, Embryonic Development, Organ Development
3	27	14	Nucleic Acid Metabolism, Small Molecule Biochemistry, Cell Morphology
4	20	11	Infectious Disease, Renal and Urological Disease, Hematological System Development and Function
5	19	11	Carbohydrate Metabolism, Lipid Metabolism, Small Molecule Biochemistry

Figure 3.

(A) Heat map for the significant differentially expressed genes between OCCC tumors and associated endometriotic cells.

(B) Top ranking enriched pathways revealed by IPA analysis of the significant differentially expressed genes.

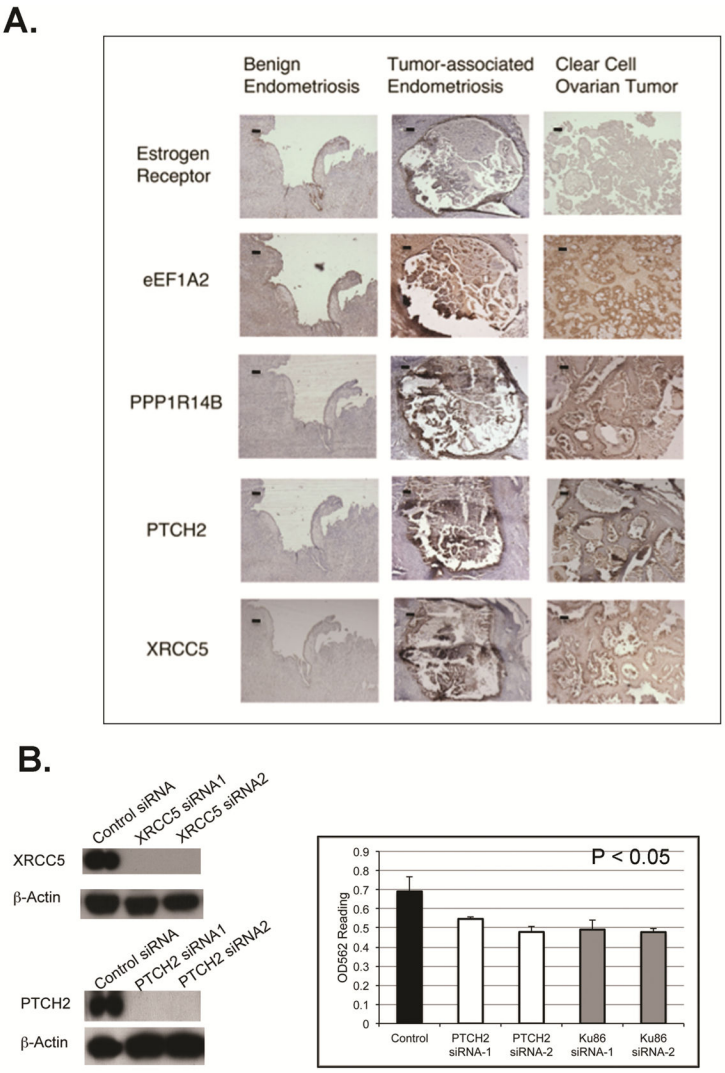
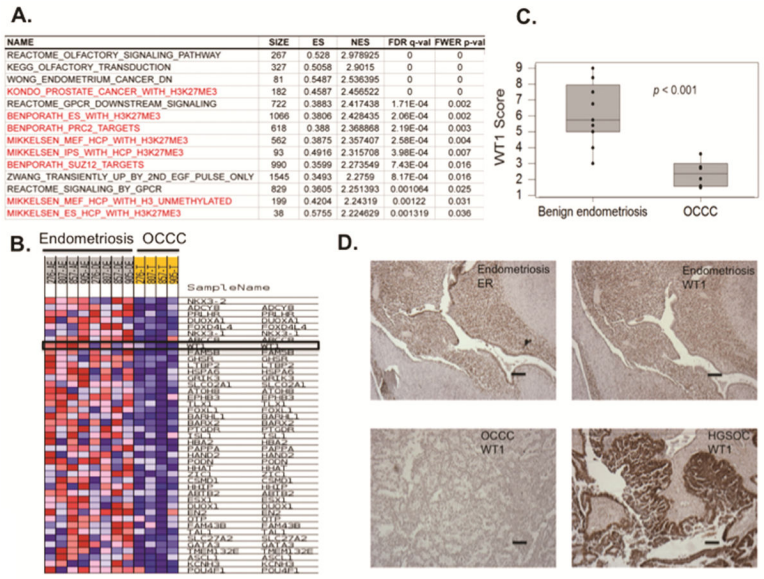


Figure 4.

(A) Representative immunohistochemical staining images of ER, eEF1A2, PPP1R14B, PTCH2, and XRCC5 in benign endometriosis, tumor-associated endometriosis, and primary OCCC. Scale bars represent 50 μm.

(B) Western blot analysis to show the knockdown of XRCC5 and PTCH2 expression and results of the MTT assay to compare the cell growth.



NAME	SIZE	ES	NES	FDR q-val	FWER p-val
REACTOME_OLFACTORY_SIGNALING_PATHWAY	267	0.528	2.978925	0	0
KEGG_OLFACTORY_TRANSDUCTION	327	0.5058	2.9015	0	0
WONG_ENDOMETRIUM_CANCER_DN	81	0.5487	2.536395	0	0
KONDO_PROSTATE_CANCER_WITH_H3K27ME3	182	0.4587	2.456522	0	0
REACTOME_GPCR_DOWNSTREAM_SIGNALING	722	0.3883	2.417438	1.71E-04	0.002
BENPORATH_ES_WITH_H3K27ME3	1066	0.3806	2.428435	2.06E-04	0.002
BENPORATH_PRC2_TARGETS	618	0.388	2.368868	2.19E-04	0.003
MIKKELSEN_MEF_HCP_WITH_H3K27ME3	562	0.3875	2.357407	2.58E-04	0.004
MIKKELSEN_IPS_WITH_HCP_H3K27ME3	93	0.4916	2.315708	3.98E-04	0.007
BENPORATH_SUZ12_TARGETS	990	0.3599	2.273549	7.43E-04	0.016
ZWANG_TRANSIENTLY_UP_BY_2ND_EGF_PULSE_ONLY	1545	0.3493	2.2759	8.17E-04	0.016
REACTOME_SIGNALING_BY_GPCR	829	0.3605	2.251393	0.001064	0.025
MIKKELSEN_MEF_HCP_WITH_H3_UNMETHYLATED	199	0.4204	2.24319	0.00122	0.031
MIKKELSEN_ES_HCP_WITH_H3K27ME3	38	0.5755	2.224629	0.001319	0.036

Figure 5.

(A) Gene sets enriched in endometriotic cells showing the gene sets for H3K27me3 target genes (in red).

(B) Partial heat map showing the top ranking genes of the PRC2 Target gene set from (A). The position of WT1 in the heat map is boxed.

(C) Boxplot to show the WT1 immunohistochemical scores of endometriosis samples vs. OCCC samples.

(D) Representative immunohistochemical images of ER and WT1 staining in endometriosis, WT1 staining in an OCCC sample. The WT1 staining in a high-grade serous ovarian carcinoma (HGSOC) sample is included as reference. Scale bars represent 50 μm.

Table 1

Expression of four novel biomarkers in benign endometriosis, clear cell ovarian carcinoma and tumor-associated endometriosis.

Biomarker	Tissue type	Num. of cases	Mean of scores	St Dev	*P*-value
eEF1A2					0.004
	Benign Endometriosis	13	4.281	1.89	
	Tumor-associated Endometriosis	4	6.863	1.492	
	Clear cell Ovarian Tumor	6	7.242	1.501	
PPP1R14B[*]					<0.001
	Benign Endometriosis	12	1.204	1.269	
	Tumor-associated Endometriosis	9	4.711	1.209	
	Clear cell Ovarian Tumor	11	6.845	1.563	
PTCH2					<0.001
	Benign Endometriosis	11	1.165	1.176	
	Tumor-associated Endometriosis	9	5.500	1.156	
	Clear cell Ovarian Tumor	9	5.972	2.193	
XRCC5[*]					<0.001
	Benign Endometriosis	13	0.696	0.919	
	Tumor-associated Endometriosis	9	4.818	1.501	
	Clear cell Ovarian Tumor	11	6.955	2.396	

All biomarkers showed significant differences between OCCC and benign endometriosis and between OCCC and tumor-associating endometriosis in post hoc analyses.

[*]Significant differences between benign endometriosis and tumor-associating endometriosis.