Identification of Novel Therapeutic Targets in Microdissected Clear Cell Ovarian Cancers

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Abstract

Clear cell ovarian cancer is an epithelial ovarian cancer histotype that is less responsive to chemotherapy and carries poorer prognosis than serous and endometrioid histotypes. Despite this, patients with these tumors are treated in a similar fashion as all other ovarian cancers. Previous genomic analysis has suggested that clear cell cancers represent a unique tumor subtype. Here we generated the first whole genome expression profiling using epithelial component of clear cell ovarian cancers and normal ovarian surface specimens isolated by laser capture microdissection. All the arrays were analyzed using BRB ArrayTools and PathwayStudio software to identify the signaling pathways. Identified pathways validated using serous, clear cell carcinoma cell lines and RNAi technology. In vivo validations carried out using an orthotopic mouse model and liposomal encapsulated siRNA. Patient-derived clear cell and serous ovarian tumors were grafted under the renal capsule of NOD-SCID mice to evaluate the therapeutic potential of the identified pathway. We identified major activated pathways in clear cell involving in hypoxic cell growth, angiogenesis, and glucose metabolism not seen in other histotypes. Knockdown of key genes in these pathways sensitized clear cell ovarian cancer cell lines to hypoxia/glucose deprivation. In vivo experiments using patient derived tumors demonstrate that clear cell tumors are exquisitely sensitive to antiangiogenesis therapy (i.e. sunitinib) compared with serous tumors. We generated a histotype specific, gene signature associated with clear cell ovarian cancer which identifies important activated pathways critical for their clinicopathological characteristics. These results provide a rational basis for a radically different treatment for ovarian clear cell patients.

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Introduction

Clear cell ovarian cancer (CCOC) was originally described as a mesonephroma ovarii in 1939 by Schiller due to its similar appearance to renal cell carcinoma [1]. Further studies since that time, has provided evidence that these tumors are of ovarian origin [2,3,4,5,6]. CCOC represents 4–14% of all epithelial ovarian cancers and its clinical behavior differs from that of the other epithelial histotypes [4,6,7]. Patients with stage I CCOC have a 27% risk of recurrence [8] with a five year survival rates for of 60% compared with 80%, for serous tumors [8]. Patients with late stage disease also have a poorer prognosis when compared to patients with advanced stage serous ovarian cancer [8]. This likely reflects CCOC’s lower rate of response to the traditional platinum/taxane-based chemotherapy, reported to be between 11–45% during first-line treatment [8,9]. CCOC patients also have higher rates of thromboembolic events when compared to patients with other epithelial ovarian cancer histotypes [10].
Histologically, CCOC cells are “clear” due to the high cytoplasmic glycogen content that is an artifact of H & E staining [11,12]. CCOC has been found to have ultrastructural similarity to clear cell carcinoma of the vagina and endometrium. This ultrastructural similarity carries over to genetic similarity as well. Zorn et al. found similar gene expression profiles of clear cell tumors of the ovary, endometrium, and kidney with use of an 11,000 probeset array [13]. This genetic overlap, however, did not extend to the comparison of serous and endometrioid histotypes of ovarian and endometrial origin. Another gene expression profile of CCOC using a 7,129 probe set array demonstrated it to be very distinct from the other histotypes [14]. These studies suggest that there are similar pathways that lead to the clear cell histotype regardless of the origin of origin.

In this study, we present the results of the first whole genome expression profiling of microdissected CCOC samples. Gene ontology and pathway analysis identified major activated pathways involved in hypoxic cell growth, angiogenesis, and glucose metabolism. We hypothesized that these pathways might provide a mechanism for the aggressive clinical nature of CCOC. We demonstrate that clear cell cancer cell lines survive better than serous cell lines under hypoxia and glucose deprived conditions and this is in part due to these activated pathways involving HIF1α and enolase. In vivo experiments using patient tissue demonstrate that clear cell tumor xenografts are exquisitely sensitive to antiangiogenesis therapy (sunitinib) compared with serous tumors. Combination therapy of sunitinib and RNAi to HIF1α and enolase demonstrates synergistic anti tumor activity. These results provide a rational basis for specific therapy in these patients.

Materials and Methods

Ethics Statement

Ovarian cancer tissue specimens were obtained with informed written consent from patients undergoing bilateral salpingoophorectomy at Vancouver General Hospital following a protocol approved by the University of British Columbia Clinical Research Ethics Board, Canada. Specimens used for profiling were collected under the protocols approved by the institutional review boards of the Brigham and Women’s Hospital (Boston, MA, USA) and were obtained with informed written consent from the patients. Animal care and experiments were carried out in accordance with the guidelines and approval by the University of British Columbia - British Columbia Cancer Agency Research Ethics Board (UBC BCCA REB) (Number: H04-60131) and M.D. Anderson Cancer Center Institutional Animal Care and Use Committee, USA (IACUC Number: 12-02-18233).

Tissue specimens, microdissection, RNA isolation and amplification

Ten clear cell ovarian cancer specimens were obtained from the primary tumors of previously untreated ovarian cancer patients at the Brigham and Women’s Hospital (Boston, MA). A set of 10 normal ovarian surface epithelium (OSE) cytobrushing specimens was also obtained from the normal ovaries of patients at the time of surgery for benign indications. Frozen sections (7 μm) were affixed onto FRAME slides (Leica, Wetzlar, Germany), fixed in 70% alcohol for 30 seconds, stained by 1% methyl green, rinsed in deionized water, and air-dried. Microdissection was performed using a MD LMD laser microdissecting microscope (Leica). Tumor cells (~5,000) were dissected for each case. RNA was isolated, extracted, and purified as previously described [15]. In order to generate sufficient cRNA for microarray analysis, a two-cycle amplification protocol (Affymetrix) was utilized that has been previously described [16].

Microarray analysis

All array data is Minimum Information About a Microarray Experiment (MIAME) compliant and the raw data has been deposited in a MIAME compliant database (GEO, Accession Number: GSE29430).

Data Normalization. Global normalization at a target value of 500 was applied to all 20 of the arrays under consideration using Gene Chip Operating Software (Affymetrix). Normalized data were uploaded into the National Cancer Institute’s Microarray Analysis Database (mAdb) for quality control screening and collation prior to downstream analyses (http://nciarray.nci.nih.gov/index.shtml), Biometric Research Branch (BRB) ArrayTools version 3.2.2 software developed by Drs. Richard Simon and Amy Peng Lam of the Biometrics Research Branch of the National Cancer Institute was used to filter and complete the statistical analysis of the array data. BRB-ArrayTools is a multifunctional Excel add-in that contains utilities for processing and analyzing microarray data using the R version 2.0.1 environment (Development Core Team, 2004). Hybridization control probe sets and probe sets scored as absent at α1 = 0.05 or marginal [M] at α2 = 20.065 were excluded. In addition, only those transcripts present in greater than 50% of the arrays and displaying a variance in the top 50th percentile were evaluated.

Class comparison, gene ontology, and pathway analysis. A multivariate permutation test in BRB ArrayTools was utilized to identify differentially expressed genes. A list of probesets containing <10% false positives at a confidence of 95% was obtained after a total of 2,000 permutations. Differential expression was considered significant at a p-value of <0.001. A random variance t test and global assessment was performed as previously described [16].

In order to identify particular functional categories of genes that were highly enriched in CCOC, we identified gene ontology (GO) categories that were statistically significant among the list of differentially regulated genes. A Hotelling T-square test was performed to identify significant GO categories. In order to identify signaling pathways involved in CCOC, the gene list that was generated from the microarray analysis was imported into PathwayStudio software (Ariadne Inc, Rockville, MD).

Quantitative RT-PCR

Quantitative real-time PCR was performed for the 10 clear cell ovarian cancer samples and the 10 OSE samples. 50 ng of the double-amplified product was used from all 20 samples using primer sets specific for 12 selected genes and the housekeeping genes GAPDH, GUSB, and cyclophilin. An iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used in conjunction with the One-Step qRT-PCR with SYBR Green kit (Invitrogen Life Technologies, Inc., Carlsbad, CA) according to previously described cycling conditions [17]. To calculate the relative expression for each gene, the 2DDCT method was used averaging the Ct values for the three housekeeping genes.

Cell lines and culture conditions

The human clear cell ovarian cancer cell lines ES-2, TOV21G, and RMG1, and the serous cell lines OVCA 420 and OVCA 432 were maintained in a 1:1 mixture of medium 199 (Invitrogen Life Technologies, Inc. Carlsbad, CA) and medium 105 (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine. The human serous ovarian cancer cell lines SK-OV-3 and OVCAR-3 were maintained in RPMI 1640 (Invitrogen...
Life Technologies) supplemented with 10% FBS and 1% L-glutamine. CAOV3 and OVCA 429 cells were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% FBS and 1% L-glutamine. ES-2, TOV21G, SKOV3, OVCAR3 CAOV3 and RMG1 cell lines were purchased from American Type Culture Collection and Japanese Collection of Research Bioresources. OVCAR 420, OVCAR 432 and OVCA 429 were obtained from the Laboratory of Gynecologic Oncology at Brigham and Women’s Hospital [18].

**Hypoxia/Glucose deprivation conditions**

Cells from clear cell and serous ovarian origins were plated and placed in the conditions of normoxia with DMEM, 10% FBS, 1% L-glutamine or hypoxia with glucose-free DMEM, 10% FBS, 1% L-glutamine (Invitrogen Life Technologies). Hypoxia was produced by flushing an incubator (Thermo Forma Series II, Thermo Fischer Scientific, Inc., Waltham, MA) with nitrogen to achieve a mixture of 1% O₂, 5% CO₂, and 94% nitrogen. The incubator was maintained at 37°C.

**Cell proliferation assays**

The cancer cell lines were seeded in 96-well plates in 8 replicates (ES-2, TOV21G, OVCA 420, SK-OV-3, OVCAR-3, OVCAR-420, OVCAR-429: 5 × 10² cells per well, CAOV3, OVCAR-432, RMG1: 1 × 10⁵ cells/well) and placed in either conditions of normoxia with media containing normal glucose (NN) or hypoxia/glucose deprivation (HG) for 24, 48, and 72 hours. After these periods, relative numbers of viable cells were measured using the fluorometric, resazurin-based Cell Titer Blue assay (Promega, Madison, WI) according to the manufacturer’s instructions at 560ₐ/590ₑₘ nm in a Victor3 multi-label counter (PerkinElmer, Germany). Doubling times for each cell line under each condition were calculated using Prism 4.02 Software (Graph Pad, San Diego, USA). Fold change in doubling time of hypoxia/glucose deprivation compared to normal conditions was calculated and compared in the conditions of normoxia and hypoxia/glucose deprivation.

**Cell cycling assay**

The cell cycle status of the ES-2 and OVCA 420 cells were compared in the conditions of normoxia and hypoxia/glucose deprivation by flow cytometry. Briefly, 7.5 × 10⁴ ES2 cells and 2.0 × 10⁶ OVCA420 cells were seeded in 60 mm² plates in triplicate and allowed to incubate overnight. The media was changed on the next day, and the cells were placed under the above conditions. After 48 hours of incubation (normoxia at 37°C or hypoxia/glucose deprivation at 37°C), the media and adherent cells were collected and centrifuged at 1500 × g for 5 minutes. The pellet was washed in 2 ml phosphate-buffered saline (PBS) and centrifuged at 1500 × g for 5 minutes. The cells were resuspended in 200 μl of cold PBS. 2 ml of ice-cold 70% ethanol was added, and the cells were incubated on ice for 30 minutes to permeabilize. The cells were then centrifuged at 1500 × g for 10 minutes. The supernatant was decanted and 900 μl of room temperature PBS was used to resuspend the cells. 100 μl of RNase A (10 mg/ml, Worthington Biochemical Corp., Lakewood, NJ) and 10 μl of propidium iodide (1 mg/ml, Sigma) were added, and the tubes were then incubated at 37°C for 30 minutes, avoiding light. DNA contents were determined by flow cytometry (FACSCalibur, Becton, Dickinson, and Company, Franklin Lakes, NJ) and the histograms of DNA contents were analyzed using FlowJo 7.2 (Tree Star, Inc., Ashland, OR) to characterize the population fractions in each phase of the cell cycle.

**Caspase-3 assay**

The direct measurements of caspase 3 activity were made using a caspase-3 fluorometric-kit (Invitrogen Life Technologies). Briefly, 2.0 × 10⁵ OVCA420 cells were seeded in 60 mm² plates and allowed to incubate overnight. On Day 0, the media was replaced and the plates were then placed in the conditions of normoxia/normal glucose or hypoxia/glucose deprivation. Cells were collected at 24, 48, and 72 hours. The cells were collected, pelleted, resuspended in 50 μl of chilled Cell Lysis Buffer, and incubated on ice for 10 minutes. Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). 50 μl of 2 x Reaction Buffer and 10 mM DTT were added to each 50 μl aliquot of cell lysate. 5 μl of 1 mM DEVD-AFC substrate was then added to each sample while avoiding the light. The samples were then incubated at 37°C for 1 hour in the dark. MEF cells (60 mm plate, 80% confluent) treated with 10 μl cycloheximide and 30 ng TNFα were used as a positive control. Fluorescence was then assessed in a Victor3 multi-label counter (PerkinElmer, Germany) with 405ₑₚ/555ₑₘ nm filters. The fold increase in Casase-3 activity was determined by relative fluorescence per μg protein.

**Necrosis assay**

In order to assay for the presence of necrosis, the CytoTox-ONE™ homogeneous membrane integrity assay was used as recommended by the manufacturer (Promega). This assay measures the release of LDH from cells with damaged membranes. Briefly, 5 × 10⁵ OVCA420 cells were plated in a 96 well plate in triplicate. The media was changed the next day, and the cells were placed in the conditions of either normal oxygen/normal glucose or hypoxia/glucose deprivation. 48 hours later, the plates were removed from the incubator and equilibrated to 22°C. In order to generate a Maximum LDH Release Control, 2 μl of lysis solution was added to the control wells placed in normal oxygen/normal glucose. 100 μl of CytoTox-ONE™ Reagent was added to each well. After 10 minutes of incubation at 22°C, 50 μl of Stop Solution was added to each well. The plates were shaken for 10 seconds. From each well, 100 μl was transferred to an opaque plate and fluorescence was recorded at 560ₑₚ/535ₑₘ nm in a Victor3 multi-label counter (PerkinElmer, Germany). After subtracting the culture medium background, the percent cytotoxicity was calculated by dividing the experimental fluorescence by the Maximum LDH Release fluorescence.

**Treatment of cell lines with siRNA oligonucleotides**

Knockdown of HIF1 α and Enolase 1 was performed using siRNA oligonucleotides (Qiagen, Inc). A reverse transfection protocol was performed using Oligofectamine (Invitrogen Life Technologies, Inc) as recommended by the manufacturer in a 96-well plate format. For each well, 50 nM of siRNA and 0.5 μl Oligofectamine transfection reagent was diluted in 50 μl of serum-free DMEM and allowed to incubate at room temperature for 30 minutes. ES-2 cells, TOV-21G cells, and RAG-1 cells were seeded in a 96-well plate at 1.0 × 10⁴ cells, 2.0 × 10⁴ cells, and 5.0 × 10⁴ cells per well, respectively. Scrambled siRNA was used as a negative control. 48 hours after transfection, the media was replaced with glucose-free DMEM, and cells were placed under the conditions of hypoxia. Growth was assessed at 0 and 24 hours by Cell Titer Blue assay (Promega). The proliferation assays for ES2 and TOV21G cell lines were performed using 75 nM siRNA for 24, 48 and 72 hours after transfection.
In vivo reduction at ENO1 or HIF1α using siRNA-DOPC with or without sunitinib inhibits COCC tumor progression in athymic nude mice

Female athymic nude mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). ES2 cells were trypsinized, washed and resuspended in Hank’s balanced salt solution (Gibco, Carlsbad, CA) and injected intraperitoneal into mice (1×10⁶ cells/mouse). In order to down-regulate Enolase and HIF1α in vivo, the respective siRNA was employed. Non-targeting, nonspecific sequence 5'-ATT GCT CCG AAC GTC CCA CGT-3' and HIF-1α 5'-CAG TGG TCA CCA TTA A3'. These target specific siRNAs were purchased from Sigma-Aldrich and prepared as previously described [19,20]. The lyophilized DOPC incorporated siRNA was hydrated with PBS and injected intraperitoneal twice weekly following our previously published protocols [21] at 3.0 mg siRNA/200 mL as a control. The same volume of PBS injected intraperitoneal was used for subsequent CD31 staining.

SU11248/sunitinib malate (Sutent, Pfizer) was suspended in carboxymethylcellulose vehicle formulation, containing carboxymethylcellulose sodium (0.5% wt/vol), NaCl (1.8% wt/vol), Tween 80 (0.4% wt/vol), benzyl alcohol (0.95% wt/vol), and reverse osmosis deionized water (added to final volume) and adjusted to pH 6.0 as previously described [22]. Sunitinib structure and activity have previously been reported [23]. Drug aliquots were prepared once weekly and kept in the dark at 4°C. Mice were treated with 40 mg/kg in 200 μL vehicle by gavage once daily. Daily gavage with vehicle alone was used as a control. Seven days after ES2 cell injection, mice were randomly divided and treated with control, Enolase or HIF1α siRNA-DOPC ± sunitinib (n = 10/group). Treatment was continued for 3 weeks, at which point, all mice in the experiment were sacrificed and necropsied, and tumors were harvested. Tumor weight and nodule count were recorded. Tumor tissue was frozen in optimal cutting temperature (OCT) media to prepare frozen slides for immunohistochemical staining for CD31.

Immunohistochemical staining for CD31

Immunohistochemical staining for CD31 antigen was performed on frozen slides to evaluate tumor microvessel density (MVD). Slides were fixed in cold acetone for 10 minutes. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol and nonspecific epitopes were blocked using 5% normal horse serum and 1% normal goat serum. Slides were then incubated with anti-mouse CD31 (1:800 dilution, Pharmingen San Diego, CA) at 4 degrees overnight. After washing with PBS, the appropriate HRP-conjugated secondary antibody in blocking solution was added for 1 hour at room temperature. Slides were developed with 3, 3'-diaminobenzidine (DAB) chromogen (In-vitrogen, Carlsbad, CA) and counterstained with Gill No. 3 hematoxylin (Sigma-Aldrich, St. Louis, MO). MVD was calculated by viewing 10 representative 200× fields per slide in each treatment group and counting the number of microvessels per field. A microvessel was defined as an open lumen with at least one CD31-positive cell immediately adjacent to it.

Differential effect of sunitinib on transplantable patient-derived ovarian cancer tumors

Six-to-eight week old female NOD-SCID mice were bred by the BC Cancer Research Centre Animal Resource Centre, BC Cancer Agency, Vancouver, Canada. Mice were housed under specific, pathogen-free conditions in sterile filter-top cages in high efficiency particulate air-filtered ventilated racks, and received sterile rodent chow and water. Ovarian cancer tissue specimens were obtained with informed consent from patients undergoing bilateral salpingoophorectomy at Vancouver General Hospital. Briefly, to develop transplantable cancer tissue lines, fresh tumor tissue was cut into small pieces and grafted into the subrenal capsule site of female NOD-SCID mice for subsequent serial transplantation and characterization as previously described [24,25]. A panel of ovarian cancer tissue xenograft models, i.e., three serous carcinoma tissue lines (LTL237, 247 and 259) and one clear cell carcinoma tissue line (LTL175) (http://livingtumorlab.com/PDC_Ovarian.html), was used.

For Sunitinib efficacy studies, 96 pieces of tissue (4×2×1 mm³) from xenografts of each established tumor tissue line were grafted under renal capsules of 24 female NOD-SCID mice, as previously described [25]. When the implants were well-established, reaching an average volume of about 20 to 50 mm³ [24,25], the animals were sorted into four groups (6 mice/group; 2 grafts per kidney). Treatment assignments were to sunitinib or inactive vehicle (negative control). Sunitinib was administered as a 0.5% carboxymethyl cellulose suspension using a dosage of 40 mg/kg body weight (orally; once daily, for two weeks) found efficacious for a variety of mouse xenograft models, as described elsewhere [26]. The mice were provided with food and water ad libitum and monitored daily for changes in general health and signs of stress, including body weight loss, diarrhea, changes in food/water intake, appearance (hunched posture, sunken eye, labored breathing) and behavior (lethargy). Effects on tumor growth were assessed by measurement of tumor volume at necropsy using calipers and the formula: volume (mm³) = 0.52×length×width×height (in mm), as previously described [25] and by histochemical analysis of tumor tissue sections (see below).

Measurement of tyrosine phosphorylation of VEGFR2 and PDGFRβ in xenografts via Western blotting

Within 2 h of the last administration of sunitinib in the above efficacy studies, xenografts from treated and control mice were snap frozen in liquid nitrogen and stored at −80°C for subsequent use. Lysates were prepared by homogenization of tissues with cold lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 10% v/v glycerol, 1% Triton X-100, 1% sodium orthovanadate, 2 mM NaF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 2 μg/mL pepstatin-A) as described elsewhere [26]. For immunoprecipitation, protein amounts were adjusted to 500 μg. Proteins were precleared with protein A-Sepharose (Cat. No. 16-125, Upstate Biotechnology Inc., Lake Placid, NY) for 15 min at 4°C. Supernatants (1 mL) were incubated with 4 μg rabbit anti-VEGFR2 (Cat. No. 07-716) or anti-PDGFRβ (Cat. No. 05-825) antibodies (Upstate Biotechnology Inc.) for 90 min at 4°C and, following addition of 25 μL 50% (v/v) Protein A-Sepharose, further incubated for 60 min at 4°C. Antigen-antibody-bead complexes were washed at least three times with lysis buffer, followed by addition of 25 μL loading buffer and boiling for 1 min for protein elution. Proteins were separated by 5% SDS-PAGE gel and then transferred onto PVDF membrane for detection of phosphorylated tyrosine using a mouse monoclonal antibody to phosphotyrosine (Cat. No. sc-7020, Santa Cruz Biotechnology Inc.). Membranes were subsequently stripped and reprobed for detection of total VEGFR2 and PDGFRβ using the same antibody preparations used for immunoprecipitation.

Apoptosis detection

Paraffin-embedded tissue sections (5 μm thick) were examined by TUNEL assay (ApopTag® Apoptosis Detection Kit, Chemicon,
Temecula, CA) as previously described (26). Briefly, sections were incubated for 15 min with 20 \( \mu \)g/mL proteinase K at room temperature and then thoroughly washed in distilled water. DNA fragments produced by the apoptotic process were tagged with digoxigenin nucleotides via a 60 min incubation at 37°C with terminal deoxynucleotidyl transferase (TdT) in a humidity chamber. The sections were then rinsed and incubated for 30 min at room temperature with anti-digoxigenin conjugated with fluorescein. After counterstaining with 4′,6-diamidino-2-phenylindole (DAPI), the slides were examined for the percentage of fluorescein-tagged cells using a Zeiss Axioplan-2 fluorescence microscope.

Histology, immunohistochemistry, and microvessel density estimation
Preparation of paraffin-embedded tissue sections, their staining and immunohistochemical analyses were carried out as previously described (23, 24). Anti-von Willebrand factor VIII antibody (Cat. No. A0082, DAKO Diagnostics Canada Inc.; 1:200) was used for identification of microvessels. All tissue sections were lightly counterstained with 5% (w/v) Harris hematoxylin (H&E). Control sections were processed in parallel with rabbit non-immune IgG (Dako, Carpinteria, CA) used at the same concentrations as the primary antibodies. Microvessel density, i.e., the number of blood vessels per \( \times 400 \) microscopic field, was determined via microscopic analysis of von Willebrand factor VIII-stained tissue sections.

Statistical analysis
Data are expressed as means \( \pm \) S.E.M. ANOVA was used to compare multiple group mean values. Shaffer statistics and Student’s t-test were used for analyzing any difference between two groups. Comparison of the percentage of cells in different cell cycle phases was performed with two-way ANOVA with use of
Results

Whole genome expression profiles of microdissected CCOC identifies differentially expressed genes

The gene expression patterns of RNA isolated from the epithelial component of 10 clear cell ovarian cancer specimens isolated by laser capture microdissection were compared to similarly isolated RNA from 10 normal ovarian surface epithelium specimens using Affymetrix U133 plus 2 arrays. After normalization and initial analysis, 16,013 informative probesets passed filtering criteria. Using a multivariate permutation test providing 95% confidence that the number of false discoveries did not exceed 10%, 3,288 probesets for 2,559 genes were found to be differentially regulated, defined by a 1.5-fold or greater difference in expression with a statistical significance of p<0.001. Graphic representation of this differential gene expression can be seen in Figure 1a. To validate the microarray data, 12 genes that were differentially expressed were randomly selected for qRT-PCR analysis. Ten of the 12 genes were differentially expressed on qRT-PCR, giving an overall microarray validation rate of 83% (Figure 1b, c and Table S1).

Identification of activated pathways in CCOC

In order to identify activated pathways present within CCOC, functional categories of differentially expressed genes were identified using gene ontology (GO) analysis. Statistically significant categories demonstrate a large number of genes involved in carbohydrate metabolism, glucose metabolism, glycolysis, and blood vessel development (Table 1). The 3,288 probesets and their associated relative expression data when compared to OSE were imported into PathwayStudio 6.0 software. Pathways involved in angiogenesis, coagulation, glucose metabolism, cell proliferation, and cell motility were evident (Figure 1d). For instance, HSPCA, which has been shown to stabilize HIF1α [27], was found to be over-expressed. Genes regulated by HIF1α involving glycolysis (ENO1 [27] and SLC2A1 [28]) and angiogenesis (PGF [29], VEGF [30], and FLTI [31]) were identified. Both PROS1 and F3 were found to be dysregulated. F3 is up-regulated by FLTI1 [32] and CP [33]. NOTCH1, which was found to be over-expressed, has been shown to up-regulate both VEGF [34] and SLC2A1 [35], contributing to both the angiogenesis and glycolysis pathways. Table 2 provides a more detailed description of these genes.

CCOC are resistant to hypoxia/glucose deprivation induced necrotic death

The presence of a dominant activated pathway involving angiogenesis and glycolysis in ovarian clear cell tumors suggested that these cells have developed mechanisms to survive in low oxygen and glucose conditions. This provides survival signals for clear cell tumors under conditions where other tumors might die. We tested the growth of ovarian cancer cell lines of papillary serous and clear cell origin under the conditions of normoxia/normal glucose (NN) compared to hypoxia (1% O2) and glucose deprivation (HG). Deprivation of oxygen and glucose had a minimal effect on the growth of clear cell cell lines (ES2 and TOV21G) when compared to serous cell lines (OVCA-420 and OVCAR-429) which were essentially completely growth inhibited (Figure 2a). Further we compared the doubling times of the cell lines and the fold change in doubling time between the conditions of NN and HG were calculated. The bar graph demonstrates a statistically significant difference when the fold change in doubling times is averaged by histotype, showing that the three clear cell ovarian cancer cell lines were less affected by hypoxia/glucose deprivation than the six serous cell lines (Figure 2b, p = 0.0037). These results highlight the importance of hypoxia-related and glycolysis pathways in clear cell ovarian cancer.

A cell cycle analysis of serous cell lines after 48 hours in the conditions of HG demonstrated a significant increase in the G1 phase as well as a decrease in S phase under the HG conditions. However, only OVCA-420 demonstrated a more global effect of HG with a significant increase in both G2/M phase (Figure S1). In addition to cell cycle arrest, we observed a large portion of OVCA-420 cells were detached after 48–72 hours of HG conditions, which was not seen in ES-2 clear cell cell lines. Trypan blue exclusion assay was performed on both cell lines which confirmed that 40% of cells under HG were dead for OVCA-420 and ES2 cell lines were resistant to HG conditions (Figure 2c). To determine the mechanism of cellular death, a proliferation assay was performed under treatment with Z-VAD-FMK to see if cell death was prevented. No decrease in cell death was noted suggesting that apoptosis was not a major contributor to cellular death (Figure 2d).

This was supported by the lack of caspase activity after 24, 48, and 72 hours (Figure S2). A cellular necrosis assay was performed on OVCA-420 cells grown in hypoxia/glucose deprivation and showed a 100% increase in necrosis (Figure 2e).

Table 1. Gene Ontology categories found to have a statistically significant higher number of genes than expected by chance.

| GO Category                        | No. genes | p value |
|------------------------------------|-----------|---------|
| Cytoskeleton                        | 76        | <1e-07  |
| Cell cycle                          | 39        | <1e-07  |
| DNA metabolism                      | 37        | <1e-07  |
| Carbohydrate metabolism             | 27        | <1e-07  |
| Cell motility                       | 17        | <1e-07  |
| Blood coagulation                   | 12        | <1e-07  |
| Glucose metabolism                  | 10        | <1e-07  |
| Cell growth                         | 10        | <1e-07  |
| Glycolysis                          | 8         | <1e-07  |
| DNA repair                          | 8         | <1e-07  |
| Blood vessel development            | 5         | <1e-07  |
| Microtubule cytoskeleton organization and biogenesis | 5 | <1e-07 |

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siRNA targeting HIF1α or ENO1 sensitizes CCOC cell lines to HG conditions

The microarray results and the in vitro FG assays suggested that clear cell cancers were protected from the growth inhibiting effects of these stresses by the activation of specific pathways. To validate this mechanistically we “knocked-down” key genes in these pathways by using siRNA technology. Three clear cell cancer lines (ES-2, TOV21G, and RMG1) were transfected with siRNA targeting HIF1α (hypoxia) and ENO1 (glycolysis). Reduction of the expression of both genes at these cell lines (Figure 3a) demonstrated significant growth inhibition to HG conditions compared to control transfected cells. Both ES-2 and TOV21G cells demonstrated statistically significant growth inhibition when transfected with siRNA targeting ENO1.
The degree of knockdown efficiency of ENO1 correlated with the amount of growth inhibition; while RMG1 demonstrated growth inhibition with knockdown of ENO1, it was the least efficient of the three clear cell cell lines and this amount of growth inhibition was not statistically significant (p = 0.12). Serous ovarian cancer cell lines OVCA420 and OVCA429 were growth inhibited in hypoxia (1% O2) and glucose deprivation (HG) conditions and the transfection with siRNA targeting HIF1α and ENO1 has limited effect on further growth inhibition compared to the control transfected cells (Figure 3c and d). Further the Knockdown of HIF1α and Enolase 1 was performed on clear cell cell lines ES-2 and TOV21G using siRNA. Forty eight hours after transfection, the proliferation assays were carried out for hypoxia/glucose deprivation for another 24, 48, and 72 hour. This experiment supports that HIF1α and Enolase 1 expression are important for the ability of clear cell ovarian cells to grow efficiently in hypoxic/low glucose conditions (Figure 3e and f).

**Table 2. Pathway genes.**

| Entrez gene ID | Gene      | Description                                           | Fold change | Function                                      |
|---------------|-----------|-------------------------------------------------------|-------------|-----------------------------------------------|
| 133           | ADM       | Adrenomedullin                                        | 4.37        | Angiogenesis, Cell proliferation and invasion.|
| 966           | CDS9      | CD59, Complement regulatory protein                   | –2.47       | Inhibitor of complement membrane attack complex (MAC) action |
| 1356          | CP        | Ceruloplasmin                                         | 13.78       | Copper homeostasis                             |
| 1398          | CRK       | v-crk sarcoma virus CT10 oncogene homolog (avian)     | 1.86        | Cell proliferation, focal adhesion Cell motility |
| 7852          | CXCR4     | Chemokine (C-X-C motif) receptor 4                    | 4.42        | Cell invasion and motility                     |
| 2023          | ENO1      | Enolase 1 (alpha)                                     | 2.21        | Glycolysis                                     |
| 2026          | ENO2      | Enolase 2(gamma, neuronal)                             | 2.39        | Glycolysis                                     |
| 2152          | F3        | Coagulation factor III (thromboplastin, tissue factor)| 4.71        | Thrombosis                                     |
| 2321          | FLT1      | fms-related tyrosine kinase 1                         | 5.63        | VEGF receptor, angiogenesis                    |
| 3091          | HIF1A     | Hypoxia-inducible factor 1, alpha subunit             | 2.64        | Promoter for genes involved in angiogenesis and glycolysis. |
| 3098          | HK1       | Hexokinase 1                                          | 4.18        | Glycolysis                                     |
| 3099          | HK2       | Hexokinase 2                                          | 5.85        | Glycolysis                                     |
| 3320          | HSPCA     | Heat shock protein 90kDa alpha, class A member 1      | 2.874       | Protein binding                                |
| 3624          | INHBA     | Inhibin, beta A                                       | 4.16        | Angiogenesis                                   |
| 182           | JAG1      | Jagged 1 (Alagille syndrome)                          | 2.61        | Notch ligand, cell proliferation               |
| 4193          | MDM2      | Mdm2, transformed 3T3 cell double minute 2             | 2.92        | Oncogene                                       |
| 4851          | NOTCH1    | Notch homolog 1, translocation-associated (Drosophilia)| 8.6        | Cell fate decisions                            |
| 5160          | PDHA1     | Pyruvate dehydrogenase (lipoamide) alpha 1            | 2.09        | Glycolysis                                     |
| 5214          | PKP       | Phosphofructokinase, platelet                          | 4.3         | Glycolysis                                     |
| 5228          | PGF       | Placental growth factor                               | 3.35        | Angiogenesis                                   |
| 5335          | PLCG1     | Phospholipase C, gamma 1                              | 3.07        | Cell motility                                  |
| 10544         | PROCR     | Protein C receptor, endothelial (EPCR)                | –14.93      | Binds activated protein C, inhibiting blood coagulation |
| 5627          | PROS1     | Protein S (alpha)                                     | –5.1        | Prevents coagulation and stimulates fibrinolysis |
| 5728          | PTEN      | Phosphatase and tensin homolog (mutated in multiple advanced cancers 1) | –2.43      | Tumor suppressor                               |
| 6392          | SDHD      | Succinate dehydrogenase complex, subunit D, integral membrane protein | –1.82      | HIF1α degradation                             |
| 6513          | SLC2A1    | Solute carrier family 2 (facilitated glucose transporter), member 1 | 3.76       | Glucose transport                              |
| 7980          | TFP2      | Tissue factor pathway inhibitor 2                     | –2.47       | Inhibits tissue factor                         |
| 7056          | THBD      | Thrombomodulin                                        | –4.44       | Activates protein C, inhibiting blood coagulation |
| 7078          | TIMP3     | TIMP metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory) | –5.35      | Inhibitor of matrix metalloproteinases        |
| 7422          | VEGF      | Vascular endothelial growth factor                    | 2.71        | Angiogenesis                                   |

Differentially expressed genes identified in the clear cell microarray involved in coagulation, angiogenesis, cell proliferation, cell motility, and glucose metabolism (average fold change $\geq1.5$; P $<0.001$). doi:10.1371/journal.pone.0021121.t002
hypothesize that inhibition of angiogenesis should have potent effects on clear cell ovarian cancer growth. To test this, we evaluated the therapeutic potential of sunitinib in clear cell ovarian cancer. Sunitinib is a small molecule inhibitor of receptor tyrosine kinases (TKIs) such as VEGFR (vascular endothelial growth factor receptor) and PDGFR (platelet-derived growth factor receptor) tyrosine kinases. While we could have used other TKIs, sunitinib was easily available and in preclinical studies. It was found to exhibit robust antitumor and antiangiogenic activity in a variety of cancer models. Inhibition by sunitinib of VEGF and PDGF signaling pathways appears to be particularly critical for tumor-induced angiogenesis. Recently, sunitinib has been approved by the FDA for clinical therapy of renal clear cell carcinoma and gastro-intestinal stromal tumors. To functionally validate the pathways in vivo, we applied siRNA technology along with antiangiogenic agents in athymic nude mice orthotopic models. The ES2 cell line was used since it reproducibly produced large tumors over a short period of time. Tumor weight was found to be significantly reduced in ENO1 and HIF1α siRNA treated mice with respect to its control group (Figure 4a). Likewise treatment of the mice with antiangiogenic agent (sunitinib) produced large reduction in tumor weight. Combination of siRNA and antiangiogenic agents produced

![Figure 2. Clear cell ovarian cancer cell lines were more resistant to hypoxia/glucose deprivation than serous ovarian cancer cell lines.](image)
synergistic activity. A similar trend was observed for tumor nodule
and tumor microvessel density estimations (Figure 4b,c).

Growth of patient-derived CCOC tissue xenografts
sensitive to sunitinib

As cancer models based on xenografts of cultured cancer cell
lines in general do not adequately represent the disease as it
present in humans, we used patient derived clear cell and serous
ovarian cancer tumors grafted under the renal capsule of NOD-
SCID mice to mimic the clinically relevant cancers. This
method allows high tissue perfusion and potentially rapid
development of graft microvasculature. More importantly, the
ovarian cancer tissues can be grown and serially transplanted
under renal capsules of NOD-SCID mice, with minimal
histological and genetic changes, and with retention of
sensitivity to cytotoxic chemotherapy. These patient-derived
cancer tissue lines are therefore very similar to the original
cancer specimens and, as such, their xenografts in NOD-SCID mice
provide ovarian cancer models that closely resemble the
patients’ malignancies. To further validate our findings we
tested patient tumor explants directly in NOD-SCID mice. As
shown in Figure 5a, treatment with sunitinib markedly inhibited
growth of the LTL175 clear cell cancer (P<0.01), whereas it
had essentially no effect on the growth of the serous carcinoma
lines LTL237, 247 and 259 (stable tumor size). To confirm that
the sunitinib had receptor tyrosine kinase inhibitory activity in
vivo, lysates of LTL247 and LTL175 xenografts were treated for
2 weeks with sunitinib or vehicle (control) and were examined
by western blotting for amounts of phosphorylated tyrosine
residues on VEGFR2 and PDGFRβ proteins. As shown in
Figure 5b and 5c, there was active tyrosine phosphorylation of
both VEGFR2 and PDGFRβ in the two tumors. The 2-week
treatment with sunitinib reduced tyrosine phosphorylation of
VEGFR2 and PDGFRβ in both the serous carcinoma (LTL247)
and CCOC (LTL175) tissues to very low levels. Our genomic
data supports a model where clear cell cancers of the ovary have
activated pathways involving angiogenesis. We propose that this
explains, in part, their clinical aggressive behavior and rapid
tumor growth. As such, we hypothesize their growth would be
exquisitely sensitive to antiangiogenesis agents. Serous tumors
appear to have minimal activation of this pathway and as such
should be somewhat insensitive to these agents. The result of
this experiment demonstrates that there is sufficient sunitinib to inhibit the target but the serous tumors are less dependent on these pathways than clear cell tumors. This experiment confirms this but it is critical to ensure this is not the result of lack of drug effect on its target.

Differential effect of sunitinib on tumor mass and cell morphology/viability of CCOC xenografts correlate with microvessel density

Subrenal capsule LTL175 xenografts treated for two weeks with vehicle (controls) showed markedly enlarged tumor masses (Figure 5a) which, as indicated by H&E staining of tissue sections, consisted of viable cancer cells (Figure 5d.a). In contrast, sunitinib-treated tumors showed much lower tumor mass and lower cell numbers (Figure 5d.b) than controls. Since sunitinib has anti-angiogenic activity [26], we investigated the effect of the 2-week treatment with sunitinib on the microvessel densities of LTL237, 247, 259 and 175 tumors by microscopic analysis of von Willebrand factor VIII-stained tumor sections. Whereas sunitinib had no significant effect on the microvessel densities of the tumors of the three serous carcinoma tissue lines, it led to markedly lower (<66%; P<0.01) microvessel density of the LTL175 clear cell carcinoma tumors (Figure 5e).

Effect of sunitinib on apoptosis of LTL175 tumors

Immunohistochemical analysis of TUNEL-stained tissue sections showed that LTL175 ovarian clear cell carcinoma xenografts, treated for 2 weeks with sunitinib, had a substantially higher number of apoptotic cells than control xenografts (Figure 5f). On average, the number of apoptotic cells in a 400× microscopic field was approximately 2-fold higher in the sunitinib-treated tumor tissue (P<0.05) (Figure 5g).

Discussion

This is the first report of whole genome expression profiling of microdissected CCOC specimens. We carried out a direct comparison of our gene list with a publically available datasets of clinical samples (GSE6008) [36]. GSE6008 contains expression profiling for 8 clear cell carcinomas and 4 individual normal ovary samples using Affymetrix HG_U133A array. Robust Multichip Average (RMA) analysis identified 3365 genes using a two sample T-test (p<0.001). Comparison of this gene list with our CCOC gene list identified 731 common genes. Pearson's Chi-squared test with Yates' continuity correction identified the overlap between the two sets of genes (731 genes) is significant and not due to chance (p-value<0.001). These 731 genes correspond to 29% of our CCOC gene list. We were not expecting a large overlap in these datasets because we generated our CCOC gene list from the laser capture microdissected epithelial component of clear cell ovarian cancers. These are pure populations of epithelial cells without the contamination of stromal components. In another study, Yamaguchi et al derived an ovarian clear cell carcinoma gene signature using cell lines and clinical samples expression analysis [37]. They have identified a 437 probe sets corresponding to 320 genes as the CCOC signature. We have found 76 genes from this 320 genes overlap to our gene signature and which was highly significant (p-value<0.001). Of note, the CCOC signature reported by Yamaguchi includes molecular networks of hypoxia-inducible factor 1 (HIF1α) which is reported in our study.

Comparison of the CCOC microarray gene list with a similarly generated gene list from microdissected serous ovarian tumors [38], identified that 73% of genes in the list were unique to CCOC. These unique genes likely explain the clinico-pathologic properties of CCOC. For instance, a large number of genes
involved in coagulation were also found to be dysregulated, which may explain why patients with CCOC have been found to have a higher incidence of thromboembolic events when compared to patients with other epithelial ovarian cancers [10]. F3 is one such gene [32], and is a major coagulation initiator. F3 is up-regulated by both FLT1 and CP [32,33]. Furthermore, F3 is inhibited by TFPI2 [39], which is down-regulated in our microarray. Other down-regulated genes that are inhibitors of coagulation include THBD, PROCR, CD59, and PROS1 [40,41].

More importantly, analysis of the CCOC data set revealed major activated pathways involved in angiogenesis and glycolysis. Both VEGF and its receptor, FLT1, were found to be up-regulated, as well as INHBA. INHBA is a member of the transforming growth factor-β superfamily that has been found to be up-regulated by VEGF [42]. Furthermore, it has been found to be an autocrine factor involved in tubulogenic morphogenesis by up-regulating VEGF and FLT1 [42,43]. ADM is involved in angiogenesis and is transcriptionally up-regulated by HIF1α [44].

Figure 5. Effect of sunitinib on the growth of patient-derived CCOC tissue xenografts. (a) Effect of a two-week treatment with sunitinib on growth of subrenal capsule xenografts in NOD-SCID mice (6 mice/group; 2 grafts per kidney) of transplantable serous (LTL237, 247 and 259) and clear cell (LTL175) ovarian carcinoma tissue lines derived from patients’ cancers. Growth of the xenografts is expressed as % tumor volume determined at necropsy by measurement with calipers. Data are presented as means ± S.E.M. (b, c) Effect of sunitinib on VEGFR2 and PDGFRβ tyrosine phosphorylation as shown by Western blot analysis. Subrenal capsule xenografts of ovarian LTL247 serous carcinoma tissue and LTL175 clear cell carcinoma tissue in mice, treated for 2 weeks with sunitinib or vehicle (control), were lysed and processed for Western blot analysis of VEGFR2 (b) and PDGFRβ (c) tyrosine residues. The results are representative of 3 experiments. (d) Representative H&E-stained tumor sections of control (d.a), and sunitinib-treated (d.b) LTL175 tissue. (e) Effect of sunitinib on microvessel density of subrenal capsule xenografts in NOD-SCID mice of serous (LTL237, 247, 259) and clear cell (LTL175) ovarian carcinoma tissue lines. Data are presented as the average number of blood vessels per ×400 microscopic field ± S.E.M. (f) Effect of sunitinib on apoptosis in LTL175 xenografts in NOD-SCID mice treated with sunitinib for 2 weeks. Representative tissue sections of control (f. a) and sunitinib-treated (f. c) TUNEL-stained tumor tissue and counterstained with DAPI (f. b, control; f. d, sunitinib-treated); (g), percentage of apoptotic cells determined via microscopic analysis using a 400 × microscopic field; data presented as means ± S.E.M.

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The dysregulation of genes involved in angiogenesis is likely multifactorial, and our pathway analysis reveals cross-over with other pathways. NOTCH1 has been shown to up-regulate VEGF and SLC2A1 [34,35]. The VEGF receptor, FLT1, has been shown to phosphorylate CRK and PLCG1, both of which are involved in cell motility[45]. Our results suggest one important protein in this pathway is HIF1α. The microarray suggests several mechanisms for activation of the HIF1α pathway in CCOC. MDM2 was found to be over-expressed, and it has been found to up-regulate HIF1α expression [46]. HSPCA stabilizes HIF1α protein, avoiding degradation via a VHL-independent pathway [47], and this gene was found to be over-expressed as well. SDHD is involved in HIF1α degradation [46], and was found to be under-expressed.

Multiple genes within glucose metabolism are up-regulated in CCOC. SLC2A1 is involved in glucose transport, and HK1/HK2 and ENO1/ENO2 are involved in glycolysis. All are known to be transcriptionally up-regulated by HIF1α [27,28,48]. HIF1α has been found to help protect cells against apoptosis, and this effect is mediated through the regulation of glucose transporters and glycolysis enzymes [28]. In vivo studies evaluating the tumouricidal effects of HIF1α disruption have demonstrated a greater correlation with glucose metabolism disruption [49,50]. Overexpression of ENO1 and HIF1α in CCOC also demonstrates a clinical link to CCOC's association with endometriosis. Up to 50% of patients with endometriosis have been found to have autoantibodies to enolase [51,52], possibly implying an immune response to overproduction of enolase. We demonstrated overexpression of ENO1 in both CCOC tumors and associated endometriotic lesions (Figure S3). Furthermore, recent literature has reported elevated HIF1α mRNA and protein levels in ectopic endometrial implants [53].

The relevant importance of these activated pathways are demonstrated in our in vitro and in vivo model systems. There is a major phenotypic difference between clear cell and serous cell lines in their rate of proliferation under in vitro conditions of HG. Knockdown of genes in either pathway, sensitizes the clear cell lines to HG conditions. This experiment suggests three major points. First, this suggests that a mechanism for the poorer prognosis for clear cell cancers when compared to the serous histotype, may be the ability of the cells to survive in the environment with limited oxygen and nutrients. Clear cell cancer cells were less affected by these stressful conditions when compared to serous cell lines. Second, disruption of the either angiogenesis or glycolytic pathways sensitizes these cells to these conditions, implying that they play a role in providing these cells with a survival advantage. Third, these pathways may serve as therapeutic targets.

The in vivo importance of the angiogenesis and glycolytic pathway for tumor growth was seen by systemic usage of small molecule inhibitors or small interfering RNA (siRNA) in nude mice. Since the microarray revealed mechanisms for activation of pathways of angiogenesis, we evaluated the antitumor activity of sunitinib. Sunitinib (SU11248; sutfent), an antiangiogenic drug, was effective at inhibiting cellular proliferation and in combination with HIF1α and enolase siRNA's indicated a synergistic antitumor activity, with a reduction in tumor nodule and tumor microvessel density.

To provide an additional level of validation, patient derived clear cell and serous ovarian cancer tumors were grafted under the renal capsule of NOD-SCID mice to mimic the clinically relevant cancers. This method allows high tissue perfusion and potentially rapid development of graft microvasculature. More importantly, the ovarian cancer tissues can be grown and serially transplanted under renal capsules of NOD-SCID mice, with minimal histological and genetic changes, and with retention of sensitivity to cytotoxic chemotherapy. These patient-derived cancer tissue lines are therefore very similar to the original cancer specimens and, as such, their xenografts in NOD-SCID mice provide ovarian cancer models that closely resemble the patients' malignancies [24,25]. Sunitinib markedly reduced the growth and the microvessel density of the clear cell carcinoma xenografts, it did not significantly affect the tumor volume nor the microvessel density of any of the three serous carcinomas.

Based on our microarray and results of knockdown and patient derived tumor xenografts experiments, angiogenesis and glycolysis pathways appear to be important in the survival and progression of CCOC. The marked effect of sunitinib on the growth and viability of the clear cell xenografts as distinct from the serous carcinoma xenografts indicates that sunitinib is potentially useful for targeted therapy of CCOC. Our studies with combination therapy of sunitinib and RNAi demonstrate synergistic anti tumor activity in CCOC and these results provide a rational basis for specific therapy in these patients. Drugs targeting angiogenesis (antibodies or TKIs) along with ones which inhibit tumor metabolism (mTor inhibitors) would be one possible choice.

Supporting Information

Figure S1 Cell Cycling analysis of OVCA420 (serous) and ES-2 (clear cell). After 48 hours of normal oxygen/normal glucose (NN) and hypoxia/glucose deprivation (HG), a cell cycling analysis was performed, demonstrating a significant increase in the both G2/M phase in only the OVCA420 cell line. (DOC)

Figure S2 Caspase assay for OVCA420. Caspase-3 activity was determined by relative fluorescence per μg protein (see methods section for details). (DOC)

Figure S3 Immunohistochemical staining for ENO1. Both endometriotic lesions (left) and associated clear cell ovarian tumors (right) strongly stain for enolase 1 (ENO1). (DOC)

Table S1 qRT-PCR analysis of randomly selected 12 genes and corresponding primer sequences. * Student T-test P-value. (DOC)

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Author Contributions

Conceived and designed the experiments: SM AKS MJB. Analyzed the data: MPS VV LO DB MJB. Wrote the paper: SM DB AKS MJB. Performed the microarray experiments and in vitro experiments: MPS VV LO MJB. Performed siRNA in vivo experiments: RLS PV-M GL-B AKS. Performed sunitinib studies in patient-derived tumor tissue xenografts: HX TK YW JNM PWG DMM CBG DGH SLE Y-ZW. Provided tumor tissue samples: SM HX TK YW JNM PWG DMM CBG DGH SLE Y-ZW. Provided reagents/materials/analysis tools: SM AKS MJB. Prepared figures and drafted the manuscript: MPS VV MJB.
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