IL6-STAT3-HIF SIGNALLING AND THERAPEUTIC RESPONSE TO THE ANGIOGENESIS INHIBITOR, SUNITINIB, IN OVARIAN CLEAR CELL CANCER.

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TRANSLATIONAL RELEVANCE: Advanced stage OCCA is associated with poor patient outcome, with low response rates to platinum-taxane based regimens. We observed distinctly different gene expression and copy number patterns in OCCA compared with HGSC. Collectively these findings argue for novel therapeutic approaches for OCCA. Our findings highlight MET and IL6. Whilst MET is gained in both HGSC and OCCA, the focal nature of MET gain and consistent over expression in OCCA are suggestive of a driver mutation. Our findings indicate that OCCA patients should be specifically included in current clinical trials of anti-IL6 antibodies, and that over expression of IL6 and PTHLH may contribute to thrombosis and hypercalcemia of malignancy commonly seen in OCCA. The impressive clinical responses of two patients to sunitinib are consistent with molecular parallels between renal clear cell cancer and OCCA, and the over expression of HIF2A and HIF1A in OCCA observed in this study.
ABSTRACT

Purpose: Ovarian clear cell adenocarcinoma (OCCA) is an uncommon histotype that is generally refractory to platinum-based chemotherapy. We analyse here the most comprehensive gene expression and copy number datasets to date, to identify potential therapeutic targets of OCCA.

Experimental Design: Gene expression and DNA copy number were performed using primary human OCCA tumour samples, and findings confirmed by immunohistochemistry on tissue microarrays. Circulating IL6 levels were measured in serum from patients with OCCA or high-grade serous cancers and related to progression-free and overall survival. Two patients were treated with sunitinib and their therapeutic responses measured clinically and by positron emission tomography.

Results: We find specific over-expression of the IL6, STAT3 and HIF pathway in OCCA tumours compared with high-grade serous cancers. Expression of PTHLH and high levels of circulating IL6 in OCCA patients may explain the frequent occurrence of hypercalcemia of malignancy and thromboembolic events in OCCA. We describe amplification of several RTK, most notably MET, suggesting other potential therapeutic targets. We report sustained clinical and functional imaging responses in two OCCA patients with chemotherapy-resistant disease who were treated with sunitinib, showing significant parallels with renal clear cell cancer.

Conclusion: Our findings highlight important therapeutic targets in OCCA, suggest that more extensive clinical trials with sunitinib in OCCA are warranted, and provide significant impetus to the growing realisation that OCCA is molecularly and clinically distinct to other forms of ovarian cancer.
INTRODUCTION

Ovarian clear cell adenocarcinoma (OCCA) is a histological subtype of ovarian cancer that is characterized by a particularly poor response rate to current chemotherapy regimens. The occurrence of OCCA is associated with co-existent endometriosis (1). OCCA are generally refractory to platinum-based therapy, with a response rate of only 11-15% (2). Given the poor response rates there is a need to develop novel clinical approaches to OCCA and this rests on first gaining insight into the biology of the disease. Investigation of OCCA has been neglected in favour of the more common HGSC, and most studies only include a small number of OCCA samples as part of a larger series of ovarian tumours (3-6).

Relatively little is known of the signalling pathways that drive OCCA. Hepatocyte Nuclear Factor 1 beta (HNF1B) was discovered as a biomarker of OCCA (7). HNF1B appears to be a lineage specific marker that is expressed in pre-neoplastic lesions and may play a role in apoptotic escape. A gene expression pattern typical of OCCA has recently been derived from OCCA cell lines and involves genes associated with oxidative stress, glyconeogenesis, and MAPK and cytokine activation (8).

Independent karyotypic and array-based genome-wide measures of DNA copy number have identified gain at 17q23-25 (9-11) as being associated with over-expression of PPM1D, a protein phosphatase that regulates the stress-induced ERK kinase, p38.

To date no published genomic studies have involved characterization of a large number of tumours using high-resolution contemporary gene expression and DNA copy number platforms. Here we explore the genomic features of a cohort of 59 OCCA, demonstrating amplification and over expression of multiple cytokine and growth factor receptors and signalling components.

RESULTS

Clear cell ovarian cancer accounts for less than 10% of invasive ovarian cancer diagnoses in Western countries and it was therefore necessary to access a large patient cohort to obtain substantial numbers of samples. Patients were obtained from the Australian Ovarian Cancer Study (AOCS), a population-based cohort of over 1800 women accrued from 2002 to 2006 (12). Details of the clinical characteristics of the AOCS OCCA patient cohort and genomic analyses performed are provided in Table 1, Supplementary Tables 1 and 2.

Over-expression and amplification of cytokine and receptor tyrosine kinase signalling.
Affymetrix U133 2.0 GeneChip arrays were used to obtain comprehensive gene expression signatures for 31 OCCA samples, which were compared with data generated previously by our group on over 200 HGSC and endometrioid cancers(12) and human ovarian surface epithelium (HOSE)(13). Unsupervised clustering of the top ~9500 most variant genes easily segregated OCCA from other ovarian cancer histotypes (Figure 1A). A large co-regulated cluster of genes was highly expressed in OCCA that included the immunohistochemical biomarker of OCCA HNF1B (TCF2)(9), the transcriptional targets of HNF1B, PKHD1(14) and P450 xenobiotic metabolism genes CYP2C9, CYP2C19, and CYP2C18(15), the prolactin receptor (PRLR), and UDP Glycosyl transferases involved in fatty acid and steroid metabolism (Figure 1B). Immunohistochemical studies have demonstrated that immune infiltration is less prevalent in OCCA compared with HGSC(16), and this was reflected in the low level expression of immune markers (Figure 1B).

We noted highly specific up-regulation of PTHLH (PTHRP, Figure 1C; Supplementary Figure 1), a gene whose expression is commonly associated with hypercalcemia of malignancy (HCM)(17), and stanniocalcin-1 (STC1, Figure 1C), a protein hormone that regulates calcium/phosphate homeostasis and is up-regulated by IL6 in response to hypoxia(18). Strong and specific over-expression of IL6 was observed (p < 0.001, Figure 1C and Supplementary Figure 1). IL6 is known to regulate PTHLH expression(19) and to be strongly pro-angiogenic in ovarian cancer(20). IL6 expression was tightly correlated with both PTHLH (PTHRP, p<0.0001) and HIF2Α (EPAS1, p<0.0001; Supplementary Figure 1). Ontology analysis showed an enrichment of genes associated with coagulation, inflammation-mediated cytokine signalling, and hypoxia and oxidative stress (Supplementary Table 3). Expression of proliferative markers and cell cycle associated genes was reduced in OCCA compared with HGSC. The gene expression profile was concordant with a previously described OCCA cell line derived signature (8) (Supplementary Figure 2), indicating that the epithelial fraction of OCCA tumours rather than stromal infiltration dominates the gene expression profile.

DNA copy number data was generated using Affymetrix SNP6.0 arrays (Supplementary Table 1). Regions of copy number change were both smaller and less frequent in OCCA than HGSC (Fisher Exact, p < 0.05). Whilst some amplifications and deletions are shared between HGSC and OCCA (eg. 8q24 involving MYC), a distinct pattern of chromosomal aberration was observed in OCCA samples (Figure 2A). By contrast, the pattern of copy number change was highly consistent with that obtained from an independent set of 18 Japanese OCCA samples (Figure 2B), indicating that the variation observed between HGSC and OCCA cancers represented a meaningful difference between the biology.
of the two tumour types. A frequency plot of copy number change shows numerous regions of gain and loss associated with genes involved in oncogenic signalling (Figure 2C) including gains associated with several RTK including ERBB2 (13/39 samples), ERBB3 (8/39) and MET (5/39). Gain of MET is of particular interest as it was focal and corresponded to the peak of amplification (Figure 2D and 2E). Approximately 50% of OCCA over-expressed MET mRNA >8-fold in OCCA compared with HGSC (p<0.0001, Figure 1C and Supplementary Figure 1), indicating amplification-dependent and independent pathways of MET expression in OCCA. Specific over-expression of the MET ligand, HGF, was observed in OCCA compared with HGSC tumours (p<0.004, Supplementary Figure 1). Changes were also found in intracellular signalling components, including gain of STAT3 (14/39), IL6R and SHC1 (13/39), src family kinase LYN (29/39), PPM1D (12/39) and heterozygous loss of the STAT3 phosphatase PTPRD. Minimal regions of chromosomal change involving at least four samples identified 76 regions of gain and 30 regions of loss (Figure 2C, lower panel; Supplementary Table 4). A gene ontology analysis of genes associated with minimal regions showed enrichment of genes related to cytokine and stress related pathways (Supplementary Table 5) including JAK/STAT signalling (p<0.001).

**IL6 and downstream signalling proteins are strongly over-expressed in OCCA.**

Figure 1C shows that IL6 over expression is a consistent feature of OCCA. IL6 signals via STAT3(21) to Hypoxia Induced Factor (HIF)(22), a key hypoxia-induced transcription factor controlling angiogenesis that controls VEGF-A expression. Consistent with our array data, IHC analysis of primary tumours showed high-level nuclear expression of phospho-STAT3 (p=0.0008; Fisher exact) and HIF1A (p< 0.0001; Fisher exact) in the epithelial fraction of the tumour (Figure 3). Specific staining of the src-family kinase LYN was also found in OCCA samples (p<0.0001; Figure 3). Staining was further validated in an independent cohort of OCCA and HGSC samples from Gynaecologic Tissue Bank at Vancouver General Hospital (Supplementary Methods). In the Vancouver cohort, HIF1A staining in OCCA was also higher than in HGSC (p<0.0001) and pSTAT3 was of borderline significance (p=0.0678). Strong IL6 protein expression was seen in the epithelial fraction of OCCA tumours (Figure 4A). However, in contrast to the mRNA data, differential expression of IL6 in OCCA compared with HGSC was not readily apparent, likely due to the secretion of IL6 from cells. High levels of secreted IL6 were detected in the media of OCCA cultures (Figure 4B). Significantly higher levels of circulating IL6 were found in sera collected from OCCA compared with HGSC patients (p<0.01, Figure 4C). Whilst the level of IL6 in HGSC was associated with increasing tumour stage, IL6 concentrations in OCCA patients were independent of extent of disease (Figure 4D). Increased serum
IL6 was associated with reduced progression-free and overall survival in OCCA patients in a univariate analysis (Supplementary Figure 5). Although there too few samples to fit a multivariate model, we note that IL6 levels were not associated with FIGO stage in OCCA tumours but were so in HGSC patients (Figure 4D), suggesting that the prognostic significance of IL6 in OCCA may be independent of stage.

Recent clinical data suggest that low stage OCCA has a better outcome than equivalently staged HGSC (23). We therefore performed a supervised analysis of gene expression and DNA copy number against progression-free, overall survival and tumour stage (Supplementary Methods). Although a number of genes and regions were obtained, no genes or regions of chromosomal aberration were statistically significantly associated with these clinical parameters after correcting for multiple testing (Supplementary Table 6 and 7, respectively). Mutation in ARID1A has been reported in ~46% of OCCA (24). ARID1A mutation status was determined for 24 AOCS OCCA samples in the study by Wiegand et al(24). Consistent with the notion that wild-type tumours are disrupted in the SWI/SNF pathway by mechanisms independent of ARID1A mutation(24), we found no difference in gene expression between tumours with or without mutation (Supplementary Table 8).

**Sunitinib response in chemotherapy-resistant OCCA patients.**

The profound up-regulation of HIF1A and EPAS1 (HIF2A) we observed in OCCA and the molecular parallels with renal clear cell cancer suggest that patients might benefit from treatment with sunitinib, a potent inhibitor of several tyrosine kinases including VEGFR, PDGFR, KIT that has significant activity in renal clear cell cancer(25) and approved for clinical use. This is supported by our experience in two patients who were treated with sunitinib and response assessed by monitoring of CA125 levels and with serial F-18 fluordeoxyglucose (18-FDG) positron emission tomography (PET). Both patients had progressed within 6 months of initial platinum and paclitaxel chemotherapy and had not responded to liposomal doxorubicin. Patient 1 had widespread painful enlarged lymph nodes as well as subcutaneous tumours, and was initially treated with 50 mg daily of sunitinib for 4 weeks followed by a two-week break. She showed a rapid response as judged by falling CA125 levels (Figure 5A) and decreased uptake of 18-FDG (Figure 5B) as well a decrease in size of measurable tumours. However, her CA125 levels rose and nodal disease worsened during the off-treatment phase. After two further cycles she was changed to continuous sunitinib 37.5 mg daily. There was an ongoing decrease in CA125 levels, the size and number of involved lymph nodes and subcutaneous tumours, and an overall clinical improvement. She maintained a clinical response for 20 months, at which time several nodal deposits...
progressed and sunitinib treatment ceased. Patient 2 had an ovarian carcinosarcoma with predominantly clear cell differentiation with a large pelvic mass, multiple bulky liver metastases and peritoneal nodules. She was also treated with 50mg daily of sunitinib using the 4 weeks on, 2 weeks off schedule. She had significant improvement in symptoms including reduced pain and fatigue, decreased CA125 levels, and improvement on serial PET-CT scans (Supplementary Figure 4). The sunitinib dose was reduced to 37.5 mg from cycle three due to diarrhoea, nausea and hand-foot syndrome. Clinically and radiologically, her disease remained stable for the next three cycles. However, CA125 levels began to fluctuate with a rise during the two-week off-treatment phase followed by a decline in the four week on-treatment phase. She developed a Mallory-Weiss tear during the sixth cycle following vomiting that was probably related to sunitinib, and treatment was withheld for three weeks. Despite reintroduction of the sunitinib, disease progression occurred and she died 11 months after commencing sunitinib treatment.
DISCUSSION

OCCA is an uncommon ovarian cancer histotype and consequently, with the exception of a recent study involving a large series of cell lines(8), individual high-resolution genomic studies have involved small numbers of samples of typically less than 10 primary tumours(3-6). By drawing on a very large population cohort (AOCS), as well as independent sample sets in Japan and Canada for validation, we have provided the first large, high-resolution gene expression and DNA copy number datasets for OCCA.

The contrast between the genomic features of OCCA and HGSC was striking, with OCCA tumours showing a distinct pattern of gene expression, and a more limited and largely different pattern of chromosomal change. HGSCs most likely arise from secretory cells in the distal fallopian tube(26), are characterised by lack of TP53 mutation(27) and frequent mutation in BRCA1 or BRCA2 pathway(28). These molecular events are much less frequent in OCCA, which has a different pattern of mutations, involving for example activating mutations in PIK3CA and loss of PTEN(29). The progenitor cell for OCCA is unknown, however, the well-established association of OCCA with endometriosis(1, 30-32), recent observations linking molecular changes in atypical endometriosis to those found in co-existing clear cell carcinoma(33), including ARID1a mutations present OCCA and adjacent atypical endometriotic legions (24) strongly implicate metaplastic endometrial cells as a source of these tumours. It should be noted that endometriosis is not always reported in OCCA patients and specific molecular differences between OCCA cases presenting with (atypical) endometriotic lesions or without evidence of endometriosis has not been thoroughly investigated. In any case, apparent differences in progenitor cells and distinct molecular features of OCCA and HGSC underscore the conclusion that these diseases are largely unrelated and require different approaches to therapeutic intervention.

In breast cancer, some tumours are characterised by loss of homologous recombination and chromosomal instability, whereas others are more karyotypically normal and are characterised by features of oncogene addiction (34). HGSC and OCCA provide a parallel with breast cancer in, which loss of HR repair is a key feature of HGSC and de-regulation of cytokine signalling pathways appear central to OCCA. Gain was observed in several RTK, most notably MET, which is validated therapeutic target in range of solid cancers(35). MET gain appeared to be highly focal in OCCA and this together with specific over expression of MET in OCCA samples suggests that MET may be an important therapeutic target in a subset of OCCA samples. The over-expression of its ligand, HGF, supports this notion. A number of genes and chromosomal regions were associated with time-to-relapse
and overall survival, however, none were significant after correcting for multiple testing. Given the relatively small number of samples evaluated against a large number of genes and regions, the failure to maintain an association with outcome is not surprising and these loci deserve further attention in larger studies. Previously gained at 17q23-25 encompassing PPM1D, has been associated with outcome and was observed in 12/39 cases. In our series of cases, the peak region of amplification as assessed by GISTIC analysis (36) mapped adjacent to PPM1D (Supplementary Figure 3) suggesting that it may not be the driver of amplification at this locus in our series of patients.

We noted strong and specific up-regulation of IL6 in OCCA compared with HGSC, and an association of serum IL6 levels with progression-free and overall survival. IL6 has tumor-promoting actions on both malignant and stromal cells in a range of experimental cancer models(21, 37). IL6 is expressed by myeloid cells in colitis-associated cancer(37) where it stimulates the proliferation of premalignant epithelial cells and protects them from cell death. We observed strong over-expression of IL6 in OCCA in the epithelial fraction of the tumour and IL6 mRNA and protein was strongly and specifically over-expressed in OCCA compared with HGSC cell lines. IL6 expression in solid cancers is also associated with immune cell infiltration, however OCCA have lower levels of immune cell infiltration, including macrophages, compared with HGSC(16). What may be the role of IL6 in OCCA? IL6 has been shown to be a downstream effector of oncogenic ras (38) and has been implicated in several human cancers including multiple myeloma (reviewed in (39)). IL6 exists in a cytokine network with TNF, CXCL12 and IL8, important for the growth of HGSC(40). IL6 is elevated in women with endometriosis(41, 42), a precursor of OCCA, and is over expressed in endometriotic samples compared with eutopic endometrium (43). Therefore de-regulation of IL6 expression appears to be an early event in the development of OCCA. IL6 signals via STAT3 and activates expression of downstream genes including PTHLH and HIF1A(22, 39, 44). We observed strong expression of activated pSTAT3 in tumours and cell lines and nuclear HIF1A in TMA, consistent with autocrine activation of the pathway. Whether PTHLP and HIF1A are direct targets of the IL6 pathway in OCCA as in other cell types remains to be tested. We note that the expression of IL6 and HIF2A (EPAS1) are particularly closely correlated, supporting a causal association. HIF2A is a pro-angiogenic transcription factor that appears to be is more important than HIF1A for promoting VEGF expression in renal clear cell cancer(45), a solid tumour type that is molecularly related to OCCA(6). The regulation of HIF2A is poorly defined, however, it is a transcriptional target of Oncostatin M, a member of the IL6 family(46).
The prominent expression of IL6, PTHLH, and HIF proteins may explain important clinicopathological features of OCCA. Intense PTHLH expression was a consistent feature of OCCA. Over-expression of PTHLP induces hypercalcemia of malignancy, a condition that is more commonly associated with OCCA than any other ovarian histotype (47). Thromboembolic events are also more common in OCCA than other types of ovarian cancer (48, 49), and IL6 overexpression is associated with increased risk of thrombosis (50). Anti-IL6 antibodies are in clinical development and a Phase II trial in ovarian cancer has been recently completed (F. Balkwill personal communication). The pronounced expression of IL6 in OCCA suggests that this histotype may deserve special attention in the development of anti-IL6 therapy. In addition, the high levels of circulating IL6 observed in many patients may have implications for dosing schedules if effective treatment of OCCA is to be attempted.

Consistent with strong up-regulation of HIF1A and HIF2A in OCCA, and the previously demonstrated molecular similarity of OCCA and renal clear cell cancers (RCCC) (6), we observed a favourable clinical response in two patients treated with sunitinib, which is approved for the treatment of RCCC. Prior to commencing sunitinib, both patients had progressive disease that was refractory to conventional chemotherapy. Sunitinib targets multiple RTK (25) expressed in tumour and stromal cells, although it has been suggested that collapse of the vascular network at concentrations lower than required to directly kill the epithelial fraction may be most important (51). How sunitinib acts in OCCA is unclear, but given the pronounced expression of HIF1A and HIF2A it is likely that inhibition of VEGF signalling is important. Previous completed trials of anti-angiogenic agents in ovarian cancer have been done in populations with predominantly HGSC (52-55). Sunitinib is currently being tested in a Phase II clinical trial of OCCA expected to complete accrual in late 2012. The responses observed in the two patients treated, together with data obtained from our genomic studies, indicate that a molecularly targeted approach to OCCA is likely to provide an improvement over the current limited response rates and poor prognosis observed with the standard platinum-taxane chemotherapy.
METHODS

Patient Cohorts

OCCA samples were drawn from the Australian Ovarian Cancer Study (AOCS), a population-based cohort of over 1800 women with ovarian cancer recruited between 2002 and 2006(14). Patients for this project were ascertained from AOCS initially using abstracted pathology reports, followed by review of diagnostic hematoxylin and eosin stained sections from tissue collected at primary surgery by a panel of anatomical pathologists to confirm an OCCA diagnosis. Samples from a cohort of primary OCCA patients was subjected to partially overlapping genomic, immunohistochemical (IHC) and serum IL6 measurements. Summary clinical characteristics of this OCCA cohort and a control set of HGSC samples for IHC are provided in Table 1. Details of the analyses performed with individual AOCS samples are provided in Supplementary Table 1. For validation studies, single nucleotide polymorphism (SNP) microarray data was obtained from 18 Japanese OCCA (Supplementary Table 2). Validation of IHC data was performed using 246 ovarian tumour samples (29 OCCA, 217 HGSC) obtained from the British Columbia Cancer Agency (Supplementary Table 2). Two patients with platinum resistant OCCA were treated with the small molecule inhibitor sunitinib, which was obtained from Pfizer. Patient consent, sample collection, and project design were approved by Institutional Review Boards at the contributing institutions.

Sample Processing

DNA and RNA was isolated from serial sections (9-12 x 100 µm) of snap-frozen tissue embedded in optimal cutting temperature compound (OCT) and sectioned using a cryomicrotome. Flanking 5 µm sections stained with hematoxylin and eosin were used to estimate the proportion of tumour cells present in the specimen. Whole tumour sections were used for all gene expression studies and for DNA copy number analysis all cases were microdissected (except for 5 cases where percentage tumour nuclei exceeded 90%) prior to DNA extraction. RNA and DNA extraction and quantification was performed as described previously (56).

Microarray analyses

Affymetrix U133 Plus 2.0 and SNP 6.0 oligonucleotide arrays were used to measure global changes in gene expression and DNA copy number. Gene expression and DNA copy number data is made available on NCBI Gene Expression Omnibus [GEO submission pending]. Analysis of gene expression data was essentially as described previously(12, 56). DNA copy number analysis was essentially as described previously(57). Additional information on normalization, filtering, hierarchical clustering,
differential gene expression statistical analysis, gene ontology, and copy number analyses can be found in Supplementary Methods.

**Tissue Microarray and Immunohistochemistry**

An Advanced Tissue Arrayer (Chemicon International) was used to obtain 0.6 mm cores from representative formalin-fixed paraffin embedded tissues and construct tissue microarrays (TMA) in an agarose matrix as previously described (12). Immunohistochemistry (IHC) was performed using 3 μm sections that were dewaxed, rehydrated and stained using an Dako Autostainer with Envision+ amplification (Dako). Staining was visualized with diaminobenzadine (DAB). Antibodies were HIF1A (Novus, NB100-479 at 1:1500), phospho-STAT3 (Cell Signalling Technologies, D3A7 at 1:50), LYN (Cell Signalling Technologies, C13F9 at 1:200) and IL6. Two sample sets were used to generate TMAs for IHC analysis: One set of 40 OCCCAs specimens was obtained from AOCS, of which 22 were also used for gene expression analysis and 29 were also used DNA copy number analysis (Supplementary Table 1). A second set of samples was obtained from the OvCaRe group at the British Columbia Cancer Agency and was completely independent of the genomic analysis (Supplementary Table 2). Results for both TMAs were scored by an anatomical pathologist (C.B.G). Statistical comparisons of protein expression in OCCA and serous cancers were made using the AOCS and Canadian samples independently. In both cases scoring systems were binarized into high and low groups during statistical analysis (see figure legends).

**Cell culture**

Ovarian cell lines were obtained from public repositories (ATCC: TOV112D, TOV21G and ES2; NCI: SKOV3 and IGROV1; HSRRB: RMG1, RIKEN: JHOC5, JHOC7, JHOC9). HAC2 was kindly provided by Dr Aikou Okamoto (Japan) and OVHS1 from Dr Ian Campbell (Australia). All cell lines were maintained in RPMI1640 with 10% FBS in a humidified incubator at 37°C with 5% CO2. Experiments were conducted with sub-confluent cells in log phase growth unless otherwise noted.

**Measurement of circulating IL6 levels in conditioned media and serum**

Cells were plated at $3 \times 10^5$ cells/well in a 6 well plate, cell culture supernatants removed after 48 hours of culture and cytokine concentrations measured using Quantikine® ELISA kits following manufacturer’s instructions (R&D Systems). OCCA and HGSC serum samples were obtained from the AOCS cohort, relying only on patients where serum samples were collected immediately pre-
surgically. Clotted blood samples were collected in plain tubes, allowed to clot, serum collected by centrifugation at 2500rpm or 1300 x g for 10 min and stored in 1 ml aliquots at -80°C until processed. Serum IL6 concentrations were estimated using electrochemiluminescence (ECL) detection with Meso Scale Discovery (MSD®, Gaithersburg, MD) assays and performed according to the manufacturer’s protocol. After defrosting at 4°C, samples were centrifuged briefly at 2000 x g for 1-2 min at 4°C. The calibrator standards, patient and normal healthy controls were incubated on MSD® single-plex IL6 microplates (MSD® cat no. K151AKB-2). Plates were washed and read using SECTOR Imager 2400 software (MSD®). Statistical significance of difference between OCCA and HGSC serum samples was calculated using a Wilcoxon Rank Sum Test. A Kaplan-Meier survival curve was plotted and a log-rank statistic computed to test the significance of serum IL6 levels versus patient outcome.
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Figure Legends.

Figure 1: Gene expression of ovarian clear cell adenocarcinomas. (A) Dendrogram of agglomerative unsupervised clustering including OCCA samples, human ovarian surface epithelium (HOSE), serous and endometrioid ovarian carcinomas (“Tothill molecular subtypes” of HGSC), low-grade/borderline serous carcinomas and low-grade endometrioid carcinomas. OCCA samples are distinctly separated from the other groups of ovarian tumours. (B) An expression heatmap of human ovarian surface epithelium (HOSE), molecular subtypes of serous and endometrioid carcinomas, and OCCA. Samples are ordered by subtype class, demonstrating discrete clusters of co-regulated genes. A co-regulated gene cluster highly specific to OCCA samples including specific biomarkers of OCCA such as HNF1B and a number of its transcriptional targets is clearly visible. Molecular subtypes of serous and endometrioid carcinomas defined by Tothill et al. (NCBI GEO Accession# GSE12172) are labelled and used for comparison in (A) and (B), as per color legend, as are HOSE samples from Bonome et al. (data donated by Dr. Michael Birrer). (C) Zoomed image of selected genes from the larger heatmap whose expression is tightly correlated with the OCCA subtype. Serous and clear cell histological types are shown.

Figure 2: Patterns of DNA copy number changes in OCCA. Comparison of DNA copy number change in (A) OCCA versus HGSC or (B) OCCA derived from the AOCS cohort versus at independent set of Japanese OCCA tumours. Differential regions of gain (red) or loss (blue) were assessed at the cytoband level and significance values calculated after correcting for multiple testing. OCCA and HGSC show distinctly different patterns of chromosomal change compared with OCCA derived from independent datasets. (C) Frequency (0-100%) of copy number changes across the genome and annotated with genes of interest associated with peak regions of gain (red) or loss (blue). (D) Frequency plot of chromosome 7 associated with the MET gene (red line) for HGSC and OCCA tumours. E) Histogram depicting the distribution of amplified regions of different lengths (megabases) associated with the MET locus for individual HGSC and OCCA tumours. The frequency plot and histogram show that MET gain is associated with focal regions of gain in OCCA compared with being more commonly included in a broad region of copy number gain in HGSC.

Figure 3: Immunohistochemical (IHC) staining supports activation of a central IL6-STAT3-HIF1A pathway in OCCA with potential involvement of the src-family kinase LYN. Typical high-level IHC positivity is shown in OCCA samples for (A) nuclear HIF1A, (B) the src-family kinase LYN, and (C) phopho-STAT3 (Y705). Mosaic plots summarizing IHC results of OCCA and HGSC samples are
shown in lower panels (D, E, & F), both the vertical (score) and horizontal (cohort) dimensions are proportional to the number of samples is each class. A thin column at the right of each mosaic plot represents the score proportions for all samples. High and low staining groups within serous and clear cell tumours were binarized for analysis: high staining was > 2 for HIF1a and pSTAT3, > 0 for LYN. For all three markers tested OCCA samples showed a higher proportion of high-level positive staining (p<0.001; see also supplementary methods).

**Figure 4:** IL6 is strongly expressed in OCCA. A) Immunohistochemical staining of IL6 in a representative OCCA sample, with strong cytoplasmic expression apparent in the epithelial fraction of the tumour. B) OCCA cell lines show high levels of secreted IL6 compared with other ovarian cancer lines. C) Serum samples collected pre-surgically show that OCCA patients have higher levels of circulating IL6 compared with HGSC patients. D) Serum IL6 levels are associated with increasing tumour stage in HGSC but not OCCA patients.

**Figure 5:** Response to sunitinib in OCCA A) CA125 levels over a 60 month period in a patient initially treated with chemotherapy followed by sunitinib. 1: Fall in CA125 following surgery and commencement of carboplatin and taxane treatment. 2: Commencement of treatment with letrozole. 3: Commencement of treatment with liposomal doxorubicin. 4: Commencement of sunitinib 50 mg daily for 4 weeks, followed by a two-week break. 5: Resumption of sunitinib (50 mg daily, 4 weeks, 2 week break). 6: Commencement of continuous sunitinib (37.5 mg daily). Open arrows indicate when PET scans depicted in B) were taken. B) 18-FDG PET images before and after treatment with sunitinib. Arrows indicate 18-FDG uptake in left cervical nodes and a subcutaneous tumour in right chest wall prior to sunitinib.


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Table 1. Sample cohort used for genomic and immunohistochemical analyses.

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*Although Silverberg grading criteria were applied to many OCCA tumor during the pathological review process all OCCA tumours are considered “high-grade” by convention.
Clinical Cancer Research

IL6-STAT3-HIF SIGNALLING AND THERAPEUTIC RESPONSE TO THE ANGIOGENESIS INHIBITOR, SUNITINIB, IN OVARIAN CLEAR CELL CANCER

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