Identification of Novel Candidate Biomarkers of Epithelial Ovarian Cancer by Profiling the Secretomes of Three-Dimensional Genetic Models of Ovarian Carcinogenesis

Kate Lawrenson[1], Paulette Mhawech-Fauceglia[2], Jenny Worthington[3], Tassja J Spindler[1], Darragh O'Brien[3], Janet M Lee[1], Georgia Spain[3], Maryam Sharifian[2], Guisong Wang[4], Kathleen M Darcy[4], Tanja Pejovic[5], Heidi Sowter[6], John F Timms[3], Simon A Gayther[1#]

Affiliations

Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California, USA

Departments of Medicine and Pathology, Keck School of Medicine, University of Southern California, Los Angeles, California, USA

Cancer Proteomics Group, Institute for Women's Health, University College London, London, UK

Women's Health Integrated Research Center at Inova Health System, Gynecologic Cancer Center of Excellence, Annandale, Virginia, USA

Department of Obstetrics and Gynecology, Oregon Health & Science University, Portland, Oregon, USA

Biological and Forensic Science Department, University of Derby, Derby, UK

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Corresponding author: # Simon A Gayther, gayther@usc.edu. Department of Preventive Medicine, Keck School of Medicine, University of Southern California, 1450 Biggy Street, Los Angeles, California, USA. Phone: 323 442 8112. Fax: 323 442 7995

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Novelty and Impact Statement

Early detection of epithelial ovarian cancer (EOC) remains a substantial clinical challenge. We leveraged *in vitro* three-dimensional models to identify biomarkers secreted by ovarian cells at the earliest stages of neoplastic transformation. In an analysis of >200 primary tumours, 3 of the biomarkers identified were associated with high tumour grade in early-stage EOCs. We also identified novel prognostic biomarkers. This report demonstrates the value of transformation models for discovery of candidate tumour biomarkers.

Abstract

Epithelial ovarian cancer is still considered the most lethal gynecological malignancy and improved early detection of ovarian cancer is crucial to improving patient prognoses. To address this need, we tested whether candidate EOC biomarkers can be identified using three-dimensional *in vitro* models. We quantified changes in the abundance of secreted proteins in a 3D genetic model of early-stage EOC, generated by expressing *CMYC* and $KRAS^{G12V}$ in *TERT*-immortalized normal ovarian epithelial cells. Cellular proteins were labeled in live cells using stable isotopic amino acid analogues, and secreted proteins identified and quantified using liquid chromatography-tandem mass spectrometry. 37 and 55 proteins were differentially expressed by *CMYC* and *CMYC+*$KRAS^{G12V}$ expressing cells respectively (P<0.05; >2-fold). We evaluated expression of the top candidate biomarkers in ~210 primary EOCs: CHI3L1 and FKBP4 are both expressed by >96% of primary EOCs, and FASN and API5 are expressed by 86% and 75% of cases. High expression of CHI3L1 and FKBP4 was associated with worse patient survival (P=0.042 and P=0.002 respectively). Expression of LGALS3BP was positively associated with recurrence (P=0.0001) and suboptimal debulking (P=0.018) suggesting that these proteins may be novel prognostic biomarkers. Furthermore, within early stage tumours (I/II), high expression of API5, CHI3L1 and FASN was associated with high tumour grade (P=$3x10^{-4}$, P=0.016, P=0.010, respectively). We show *in vitro* cell biology models of early-stage cancer development can be used to identify novel candidate biomarkers for disease, and report the identification of proteins that represent novel potential candidate diagnostic and prognostic biomarkers for this highly lethal disease.

Introduction

Despite recent advances in surgery and chemotherapy, epithelial ovarian cancer (EOC) remains the most lethal gynecologic malignancy, mainly due to the absence of specific symptoms and a lack of effective screening tools. The majority of patients present with advanced stage disease where the 5-year survival rate is only 27%. For advanced stage tumours, recurrence rates are over 60 per cent, and 20 per cent of these patients respond poorly to platinum-based chemotherapy. Therefore, detecting patients with early EOC continues to be an urgent clinical need and it remains a clinical priority to detect high-grade serous EOC early during disease development.

Given the difficulties accessing the ovary for biopsy and the rapid rate at which the most aggressive EOC subtypes progress, the identification of clinical biomarkers detectable in the blood would represent a significant advance for the identification of patients with EOC. Currently the most widely used ovarian cancer biomarker is serum CA125, and this marker is particularly good at detecting disease recurrence. A second marker, HE4, was approved in 2009 for monitoring ovarian cancer progression, and can be elevated in EOC patients in the absence of CA125. However both CA125 and HE4 show poor specificity for ovarian cancer and can be elevated in other cancer types, such as endometrial cancer [1-3], or benign conditions such as endometriosis. Moreover neither marker can reliably detect early-stage EOC. It is clear that improved diagnostic tools are urgently needed, particularly for diagnosing ovarian cancer in premenopausal women [4]. One of the major challenges in diagnosing ovarian cancer is that the associated symptoms are broad and non-specific to this disease. In cases of suspected EOC, levels of serum CA125, often together with HE4, are measured and a pelvic ultrasound performed. In the United States, a Risk of Malignancy Algorithm (ROMA) score is then calculated, which also takes into consideration menopausal status and serum CA125 plus HE4 levels. Women with suspected malignancies are immediately referred to a specialist multidisciplinary team for treatment, but in the majority of cases, women diagnosed with sporadic EOC only become symptomatic once disease has spread throughout the peritoneum. Consequently most cases of EOC are diagnosed at an advanced stage.

Ideally, women over the age of 40 or with a family history of breast and ovarian cancer who are at the greatest risk of developing EOC would regularly be screened for an early-stage EOC biomarker and late-stage detection would be a rare occurrence. Unfortunately there is currently no known biomarker that is sensitive or

specific enough to be used to screen for early-stage EOC. The discovery and development of reliable biomarkers for early-stage ovarian cancer have no doubt been hindered by the scarcity of tissue and sera specimens from these patients. Some of the challenges of performing biomarker discovery using human serum specimens include variations due to sample processing, inter-patient variability and histological heterogeneity. Alternative approaches to biomarker discovery involve the analysis of *in vitro* or *in vivo* models. Some good *in vivo* models have recently been developed for certain ovarian cancer histotypes [5-9] and xenograft models have been shown to be a valuable part of biomarker development for breast cancer [10]. *In vitro* genetic models can also be developed, and in our laboratory we have developed three-dimensional models of early-stage ovarian cancers that mimic the molecular profiles of human ovarian cancers [11, 12]. In this study we sought to use a sensitive quantitative mass spectrometry approach to analyze proteins differentially expressed and secreted in our models of early-stage ovarian cancer, coupled with a comprehensive analysis of candidate biomarker expression in a large series of primary human ovarian cancer specimens. We demonstrate that using *in vitro* genetic models of ovarian cancer is a highly effective approach to the discovery of much needed candidate biomarkers for EOC.

Results

Analysis of the Secretome of Early-Stage Models of Epithelial Ovarian Cancer

Overexpression of *CMYC* and deregulation of the MAPK pathway (usually due to activating mutations in *KRAS*) are common events across all EOC histotypes (Figure 1a), with *CMYC* amplification/overexpression occurring in 39-67% of cases, and MAPK pathway activation in 40-67% of cases [13-20]. We developed models of early ovarian cancer by stably overexpressing *CMYC* and $KRAS^{G12V}$ in two phenotypically normal *TERT*-immortalised ovarian surface epithelial cell lines derived from two different patients (IOSE11 and IOSE19). Overexpression of *CMYC* alone ($IOSE11^{CMYC}$ and $IOSE19^{CMYC}$, collectively termed $IOSE^{CMYC}$) was sufficient to induce anchorage-independent growth and reduced rates of apoptosis. Subsequent expression of $KRAS^{G12V}$ ($IOSE11^{CMYC.KRAS}$ and $IOSE19^{CMYC.KRAS}$, collectively termed $IOSE^{CMYC.KRAS}$) resulted in increased cellular invasion. When grown as three-dimensional (3D) cultures, the marker expression, histology and transcriptomic profiles of these transformation models reflected those of human tumours [11, 21].

To identify novel candidate biomarkers of EOC, we labeled the transformation models with both heavy and light amino acids to enable us to perform stable isotope labeling by amino acids in cell culture (SILAC). The labeled IOSE, $IOSE^{CMYC}$ and $IOSE^{CMYC.KRAS}$ cell lines were cultured as 3D spheroids and secreted proteins harvested. Quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to accurately quantify changes in the abundance of secreted proteins between the normal and transformed cell lines. A total of 1,241 protein groups were identified by LC-MS/MS, with 2,633 protein quantifications determined across reciprocally labeled duplicates of the three sample groups (IOSE, $IOSE^{CMYC}$ and $IOSE^{CMYC.KRAS}$) which were each equal pools of two separate clones (see Supplementary Table 1 for complete dataset). When fold-change (>2 or <0.5-fold) and *P* value ($P<0.05$) cut-offs were applied, 32 proteins were up-regulated and 5 proteins were down-regulated in the $IOSE^{CMYC}$ models, while 54 proteins were up-regulated and 1 down-regulated in the $IOSE^{CMYC.KRAS}$ models, compared to parental IOSE cells (Supplementary Table 2).

Gene ontology (GO) analyses of proteins specific to the $IOSE^{CMYC}$ models revealed significant enrichment of proteins involved in extracellular matrix reorganization ($P=0.012$) and hypoxia ($P=0.020$) (Figure 1b), whereas terms specific to CMYC and $KRAS^{G12V}$ expression were predominantly associated with RNA metabolism and splicing (Figure 1c). Inosine monophosphate (IMP) and purine processing were significantly enriched in the list

of proteins commonly changing in both the IOSE^{CMYC} and the IOSE$^{CMYC.KRAS}$ models (Figure 1d). In the analysis of proteins commonly changing in both models, the most significantly enriched cellular compartment term was "proteinaceous extracellular matrix" (*P*=0.035); as expected our methodology enriched for proteins expressed in the extracellular milieu.

13 gene products were significantly changing >2-fold in both the IOSE^{CMYC} and IOSE$^{CMYC.KRAS}$ models: ADSl, API5, CHI3L1, CPE, FASN, FKBP4, LGALS3BP, NDRG1, PFAS, RCC2, SORD, TIMP1 and U2AF2. Proteins were prioritized based on subcellular location, and, using gene expression data from The Cancer Genome Atlas (TCGA), on directionality of changes in expression in our data and primary ovarian tumours (Table 1 and Figure 2). Using this approach we prioritized additional proteins that were unique to each gene: PCOLCE was highly up-regulated in the *KRAS^{G12V}* model, and PARP1, CSF1, POSTN and DPP4 were differentially expressed in the *CMYC* model. We were able to validate the findings of the MS-based analysis for API5, CHI3L1, CPE, FASN, FKBP4, NDRG1, PFAS, SORD, POSTN, PARP1, PCOLCE and DPP4 by immunoblotting the individual clones of the test samples (Figure 2a and c). CHI3L1, FASN, SORD, API5, FKBP4 and PFAS were all reproducibly up-regulated in IOSE^{CMYC} and IOSE$^{CMYC.KRAS}$ models, detected both in secreted and total cell lysates (Figure 2a and Table 1). Results for LGALS3BP, RCC2 and CSF1 were equivocal (data not shown).

Tissue Microarray Analysis of Novel EOC Biomarkers

Antibodies for 8 proteins (API5, CHI3L1, FASN, FKBP4, LGALS3BP, PCOLCE, PFAS and SORD) were suitable for immunohistochemistry in primary tissues; five of these (API5, CHI3L1, FASN, FKBP4, and LGALS3BP) showed strong expression in at least half of a test set of 6-8 primary ovarian tumour tissues (Table 1). We expanded the analysis of these five proteins, by evaluating their expression in ~210 primary EOCs. Immunohistochemical staining was performed in primary EOC tissues organized in two tissue microarrays (TMAs) that included 101 early stages (stage I/II) tumours of mixed histologies (summarized in Supplementary Table 3). Examples of the staining can be found in Figure 3. CHI3L1 and FKBP4 were both expressed by 96% of all EOCs overall, FASN by 86%, API5 by 75% and LGALS3BP by 56% of tumours. We also evaluated expression of API5, CHI3L1, FASN, FKBP4, and LGALS3BP in 2 histologically normal ovaries; API5, FASN and LGALS3BP showed no expression in normal ovarian tissues, while CHI3L1 and FKBP4

showed weak expression in 1/2 and 2/2 cases respectively. Analysis of NDRG1 was also performed; NDRG1 was expressed in 47% of early stage and 36% of late stage ovarian cancers. A detailed analysis of this potential biomarker can be found in the report of Pejovic *et al* (manuscript in preparation).

We tested for associations between biomarker expression and clinico-pathological characteristics, comparing tumours exhibiting negative protein staining (0) to all tumours with evidence of positive staining (weak staining, 1; moderate staining, 2 and strong staining, 3). We also compared negative and weakly expressing tumours (0,1) with moderate and strongly stained tumours (2,3) (Supplementary Tables 4-9). Expression of all 5 candidates was significantly associated with high tumour grade in at least one analysis (P<0.05, Fisher's exact test). When we tested for associations with tumour stage, API5 expression was positively associated with tumour stage with stage IV tumours showing the highest expression (P=0.009). We also found evidence of associations with stage for LGALS3BP (P<0.0001), which was expressed in 43 percent of stage I/II tumours and 62 percent of stage III/IV tumours, and for FASN (P=0.046).

Given the urgent need to diagnose high-grade tumours at an early stage, we analysed marker expression in early-stage tumours specifically (stage I/II). Each biomarker was expressed in early-stage tumours; FASN, FKBP4 and CHI3L1 were expressed in greater than 91 per cent of early-stage cases. In early-stage tumours (I/II), expression of API5, CHI3L1 and FASN was associated with high tumour grade (FIGO G2 and G3) $P=3\text{x}10^{-4}$, P=0.016, P=0.010, respectively, Table 2). Expression of API5, FKBP4 and LGALS3BP was positively associated with tumour recurrence (P=0.010, P=0.029 and $P=1.0\text{x}10^{-4}$) and positive expression of LGALS3BP was associated with optimal debulking status (P=0.018) as well as patient age (P=0.005).

For some of the markers, expression was associated with histological subtype. API5 expression was heterogeneous across histotypes ($P=6.0\text{x}10^{-4}$, ANOVA), with serous and clear cell tumours showing the highest frequencies of expression. CHI3L1 expression was lower in mucinous EOCs compared to other histotypes (P=0.019) and LGALS3BP expression was highest in serous and mucinous tumours (P=0.019). FASN and FKBP4 expression showed no reproducible associations with histotype.

Kaplan-Meier survival analyses with log-rank testing were performed to test for associations between biomarker expression and survival. Positive expression of CHI3L1 and FKBP4 was associated with significantly worse overall survival (P=0.002 and P=0.042, respectively) (Figure 4). Finally, we performed

survival analyses for the top 5 biomarkers using gene expression data for ~780 serous ovarian cancers, adjusted for age, stage and debulking status; higher expression of *FASN* and *LGALS3BP* was associated with improved overall survival (P=0.024 and P=0.001 respectively, Cox regression analyses, Wald statistics) (Supplementary Table 10).

Discussion

Ovarian cancers are highly lethal malignancies, and it is clear that substantial reductions in patient mortality could be achieved by earlier detection. Given the difficulties accessing the ovary for biopsy, and the rapid rate at which some EOCs progress, a blood-based biomarker would be the ideal modality for epithelial ovarian cancer (EOC) screening. While CA125 is an excellent biomarker for monitoring disease recurrence, in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening trial of over 28,000 women, screening with CA125 had no effect on ovarian cancer mortality rates [22] and so it is clear that novel biomarkers for early stage detection are urgently needed. This study aimed to address this need by using gene-specific 3D cell biology models of ovarian cancer for the discovery of novel candidate diagnostic biomarkers, followed by validation in a large series of >200 primary EOC tissues. We discovered a number of promising candidate biomarkers, including API5, CHI3L1, FKBP4, FASN and LGALS3BP. The most commonly expressed biomarker in our study was FKBP4, which was expressed by 96.1% of EOCs, with moderate and strong staining in 82.8% of cases. Moderate or high FKBP4 expression was also associated with a poorer prognosis. FKBP4 has not previously been implicated in EOC development, but this protein may have a role in regulating the response to steroid hormones, exposure to which significantly modulates a woman's risk of EOC. FKBP4 is a co-chaperone known to be involved in steroid hormone receptor signaling, including that of the progesterone receptor [23] and expression of FKBP4 is vital for uterine receptivity required for embryo implantation [24]. While FKBP4 has not been extensively studied in cancer, a recent study correlated FKBP4 expression with resistance to doxorubicin and docetaxel in breast cancer [25].

FASN was also frequently expressed in EOC specimens. FASN has also not previously been reported as a diagnostic biomarker for EOC, but it is known to be overexpressed in many cancers and has been proposed as a therapeutic target in ovarian cancer and other malignancies [26-29]. FASN expression is positively correlated with ER and PR expression in endometrial cancer [27] and has been implicated in ERBB receptor signaling in ovarian cancer [28]. FASN is involved in the synthesis of long chain saturated fatty acids, which are components of lipid rafts on the cell membrane that form sites of transmembrane protein localization. Treatment with a FASN inhibitor inhibited cell growth *in vitro* and *in vivo*, reduced PI3K signaling, and induced the apoptosis of chemoresistant ovarian cancer cells [28, 29]. Not only is FASN up-regulated in our model of EOC development

derived from ovarian surface epithelial cells, but FASN is also up-regulated in early EOC precursor lesions in the fallopian tube, which are also cells of origin for a subset of ovarian cancers, further evidence to suggest that FASN may be a reliable biomarker of early-stage EOC [30].

Of the biomarkers identified, CHI3L1 has been most widely studied as an EOC biomarker [31-39] and it was first postulated as an early-stage EOC biomarker a decade ago [34]. In our study, CHI3L1 was expressed in the majority of EOC cases, with 66% of early-stage tumours showing moderate or strong expression. CHI3L1 is a secreted glycoprotein that can be detected in the blood, and in EOC patients plasma levels of CHI3L1 positively correlate with tumour grade [37] and previous studies find that CHI3L1 appears to offer no advantage over CA125 as a screening biomarker for detection of early-stage EOC [32]. In our study CHI3L1 was strongly associated with a poorer prognosis, and high serum CHI3L1 is reportedly an independent negative prognostic factor in EOC patients [33, 36], so while CHI3L1 is unlikely to be a useful diagnostic biomarker, it may be a useful prognostic tool.

While LGALS3BP was expressed at the lowest frequency (56%) of the biomarkers identified, this protein is nonetheless of interest as a cancer biomarker. Functional data suggest that LGALS3BP is a multi-functional protein with a role in centrosome biology [40] and the promotion of oncogenic signaling via integrins [41]. LGALS3BP has shown promise as a biomarker of early neuroblastoma and neuroblastoma relapse and can be readily measured in blood sera by ELISAs [42]. LGALS3BP expression occurs in multiple cancer types, which may suggest it may not be an optimal biomarker for EOC due to a lack of specificity, but that this protein may be generally involved in tumourigenesis and may therefore represent a novel therapeutic target. LGALS3BP demonstrated strong associations with clinical parameters; positive staining of LGALS3BP protein was strongly associated with tumour recurrence and optimal debulking, suggesting a biological role for LGALS3BP in promoting aggressive tumour phenotypes. Whilst we did not detect an association with LGALS3BP protein and survival in the Kaplan-Meier survival analyses, we did note an association between positive LGALS3BP expression and worse survival that was nearing statistical significance (P=0.075). Further analyses into the functional role of LGALS3BP in models of advanced EOC are clearly warranted.

As part of this study we also performed survival analyses for the top 5 candidate biomarkers, based on protein expression in ~200 EOC cases, and gene expression in ~800 serous ovarian cancer cases. We identified

CHI3L1, FKBP3, *FASN* and *LGALS3BP* as novel prognostic biomarkers. These gene expression and protein analyses identified different results, which is likely due to the differences in sample size between the two analyses, and the relatively low concordance between gene expression and protein abundance. However both RNA and protein biomarkers can be useful clinical biomarkers and it is clear that the prognostic markers we identified warrant further study as prognostic tools or therapeutic targets.

Additional candidates that we identified but did not follow-up in more detail also warrant further study, such as CSF1, PARP1, PCOLCE, THBS1 and TIMP1. Notably, of these, PARP1 is known to be commonly expressed in EOC [43] and is an effective anti-cancer therapy target, but as yet, has not been evaluated as a blood-borne biomarker for EOC. Other candidate markers we identified are less well characterized. For example, API5 is an anti-apoptotic protein and has not previously been reported as a biomarker, but was strongly, positively associated with tumour grade in early-stage EOCs, which may suggest a role for this protein in the development of apoptosis resistance during tumourigenesis in high-grade ovarian cancer.

We used a model that is based on deregulation of the MAPK/PI3K pathways and overexpression of *CMYC.* This study also provides insight into novel functions for *CMYC* and $KRAS^{G12V}$ in ovarian cancer biology. Most notably, in cells expressing $KRAS^{G12V}$ we detected an enrichment of proteins involved in RNA splicing and RNA processing. While MAPK signaling has been previously implicated in splicing of single RNA molecules [44], these data suggest that mutant KRAS expression may affect splicing on a global level, through interactions with splicing factors such as SF3A1 and SF3B1. The receptor tyrosine kinase-MAPK pathway is altered in 40% of EOCs [15] and *CMYC* in up to 67 percent of cases overall, while 8q24, the *CMYC* locus, is associated with genetic susceptibility to EOC in the general population [45]. Although the biomarkers we identified were found to be relevant to the majority of EOCs, not all EOCs harbor alterations in these pathways, and MAPK/PI3K signaling is more commonly deregulated in certain histological subtypes *versus* others. Moreover, it is now clear that ovarian cancer subtypes arise from different cellular precursors, including fallopian tube secretory epithelial cells [5, 46] and endometriosis epithelial cells [9, 47]. Additional models may be developed using these cell types, with subtype-specific alterations such as *p53, PAX8 and BRCA1* loss to mimic in the development of high-grade serous ovarian cancer, or *HNF1B* overexpression to model the early development of clear cell ovarian cancer. Analysis of the secretome of a series of histotype-specific early-stage models could be used to

develop a panel of diagnostic biomarkers that would be expected to show greater sensitivity and specificity than any one biomarker alone. One caveat with this approach is that our hypothesis is based on the assumption that early-stage detection can prevent advanced-stage disease. This is strongly supported by clinical evidence that patients with high-grade serous cancer detected at stage I/II have better overall survival than patients with high-grade serous ovarian cancer detected at stage III/IV [48]. The results of large-scale ongoing screening trials, particularly the UKCTOCS study [49], will therefore be of significant relevance to biomarker discovery studies for EOC; this trial will report on whether early-stage detection can improve mortality, an assumption that has yet to be definitively demonstrated for EOC, but is key to the successful translation of early-stage biomarkers into the clinical setting.

To explicitly demonstrate the clinical potential of these biomarkers, the next stage for this research will be to test the abundance of these candidate biomarkers in the blood sera of women preceding a diagnosis of ovarian cancer. Four of the top five biomarkers identified were expressed in the majority of EOCs, three of which (API5, CHI3L1 and FASN) were associated with higher tumour grade in early-stage tumours. Early-stage high-grade tumours are a group of malignancies that most urgently need early detection, suggesting follow-up of API5, CHI3L1 and FASN in patient sera specimens should be prioritized. In addition, high expression of these 3 proteins (CHI3L1, FKBP4, and LGALS3BP) was associated with worse disease outcome, making them potential prognostic factors. While this study demonstrates a proof-of-principle, ultimately it is hoped that these biomarkers could lead to much needed earlier detection of ovarian cancer.

Materials and Methods

Cell Culture and SILAC Labeling

Immortalized ovarian surface epithelial cell lines (IOSE11, IOSE19) and those overexpressing *CMYC* ($IOSE11^{CMYC}$, $IOSE19^{CMYC}$) and expressing mutant *KRAS* ($IOSE11^{CMYC.KRAS}$, $IOSE19^{CMYC.KRAS}$) have been previously described [11, 21]. Cell lines were routinely tested for *Mycoplasma* contamination and profiled by STR typing at the University of Arizona Genomics Core facility for authentication. Cells were transferred into SILAC DMEM (Pierce) with 15% dialyzed fetal bovine serum, suitable for SILAC (Sigma). Heavy or light arginine and lysine (Pierce) were added to the cell culture media such that the two clones of each cell line were reciprocally labelled. Culture media were also supplemented with 0.5 µg/mL hydrocortisone, 5 µg/mL insulin (both Sigma) and 10 ng/mL epidermal growth factor (Invitrogen); media for the *CMYC* and *CMYC+KRAS*^{G12V} overexpressing cell lines was additionally supplemented with 3 µg/mL blasticidin-S hydrochloride (Sigma) or 125 or 1000 µg/mL G418 (for IOSE11 and IOSE19 models, respectively) to maintain selection. Cells were cultured for >6 passages before use. Total cell lysates were prepared and the labeling efficiency tested by GeLC-MS/MS as described below. Labeling efficiency was determined to be >97% for all 6 sample types and arginine to proline conversion was determined to be <6% in all cases.

Preparation of Secreted Proteins

Plates for three-dimensional culturing were prepared by twice coating P100 dishes with polyHEMA (Sigma) dissolved in 95% ethanol (VWR). Coated plates were washed for 5 mins in PBS before use. $50x10^6$ cells were plated onto polyHEMA-coated plates for 48 hours to induce spheroid formation. To serum starve, spheroids were washed three times with PBS and placed in serum-free media supplemented with heavy or light amino acids for 24 hrs. Conditioned media were then harvested, supplemented with protease inhibitor cocktail (Sigma) and concentrated using polyethersulfone membrane Vivaspin® 20 columns (Vivaproducts) with a 10K molecular weight cut-off. Protein concentrations were then determined using a Bradford assay (Pierce).

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Equal amounts of protein from the two clones of each model cell line were mixed according to heavy or light labeling status, and then mixed with an equal amount of oppositely labeled parental cell line pool. Thus, the

light CMYC and light CMYC+KRAS^{G12V} pools were each compared with the heavy IOSE pool and vice versa generating 4 samples for analysis. 50 µg protein of each was then separated on a 15 cm 10% SDS-PAGE gel and stained with InstantBlue Coomassie stain (Expedeon). Lanes were sliced into 43 pieces. Proteins were reduced with dithiothreitol, alkylated with iodoacetamide, digested with trypsin and the resulting peptides extracted, all according to our standard procedures [50]. Extracted peptides were then analyzed in an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) coupled to an Ultimate 3000 chromatography system (Dionex). Reversed-phase chromatographic separation was carried out on a 75 µm i.d. X 250 mm Acclaim PepMap100 C18 Nanocolumn, 3 µm bead size, 100 Å pore size (Dionex; #164261) with a linear gradient of 10–50% solvent B (99.9% ACN/0.1% FA). The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap MS and iontrap MS/MS acquisition. Survey full scan MS spectra (from *m/z* 390 to 1700) were acquired in the Orbitrap with a resolution of 60,000 at *m/z* 400 and an automatic gain control target value of $5x10^5$ ions. The six most intense ions were selected for CID with fragmentation ions detected in the LTQ ion trap. Target ions that had been selected for MS/MS were dynamically excluded for 60 s. For accurate mass measurement, the lock mass option was enabled using the polydimethylcyclosiloxane ion *m/z* 455.120025) as an internal calibrant. For peptide identification, protein group assembly and relative quantification, raw data files produced in Xcalibur software (Thermo Scientific) were processed using MaxQuant version 1.3.0.5 (Max Planck Institute of Biochemistry) and searched against the human UniProtKB database (release 2012_09 of 03-Oct-12: 538,010 entries). The database was also concatenated with reversed copies of all sequences, whereby each lysine and arginine residue was swapped with the preceding amino acid for determination of FDR. Searches were performed using the following parameters; MS tolerance was set to ±10 ppm and MS/MS tolerance to 0.8 Da, minimum peptide length was set to 7 amino acids and 2 missed cleavages were allowed. Carbamidomethylation of cysteines was set as a fixed modification, whilst methionine oxidation and protein N-terminal acetylation were set as variable modifications. MS/MS spectra, pre-determined to result from heavy-labelled peptides were submitted to the database with the Lys6 and Arg10 labels set as additional fixed modifications, whilst un-determined spectra were searched separately with the labels set as variable modifications. Identified peptides and proteins were filtered with an FDR of 1%. Whenever the set of identified peptides in one protein was equal to or contained the set of peptides identified in another, these two proteins were joined as a protein group. Shared peptides remained in all groups where they

were identified, but were most parsimoniously associated with the protein group containing the highest number of peptides (razor peptides). Proteins were required to contain at least two peptides, of which one was group unique. Peptide ratios were calculated as the median of all evidences of a SILAC peptide pair and were normalized separately for lysine and arginine-labeled peptides and for each LC-MS/MS run. Protein ratios were calculated as the median of normalized razor and unique peptides and a minimum of 3 ratio counts were required for quantification. Protein groups were imported into Perseus (Max Planck Institute of Biochemistry) and the significance of up/down-regulated changes in protein expression was determined. Proteins were accepted as being significantly up/down-regulated with a significance B value of <0.05. Significance B was corrected for multiple-hypothesis testing with a Benjamini-Hochberg FDR.

Tissue Microarray Construction, Staining and Analysis

We created a TMA of 55 early-stage ovarian carcinomas at the University of Southern California (USC). Blocks of early-stage ovarian tumour specimens were retrieved from the archives at USC+County Hospital. Hematoxylin and eosin (H&E) stained tumour sections were examined to identify morphologically representative regions. Core biopsies (1 mm diameter) were punched from each tumour and transferred to a receiver paraffin block. One section of the TMA was stained with H&E to verify the presence of the tumour. The ovarian cancer population-based tissue microarray from Oregon Health & Science University has been previously described [43]. For immunohistochemical (IHC) staining, sections (4μm) were deparaffinized with xylene, then washed with ethanol. Sections were incubated with 3% H_2O_2 for 10 minutes and blocked for 30 minutes using a serum-free protein block (Dakocytomation). Sections were treated with an EDTA buffer saline solution, microwaved for 20 minutes, and then incubated with primary antibodies (see Supplementary Table 9) for 1 hour at room temperature. As a chromogen the diaminobenzidine complex was used. Two pathologists (PMF, MS) scored marker expression using a double-head microscope. Intensity of staining was scored as 0, negative; 1 low; 2, moderate, and 3, strong. This evaluation was performed twice, with a one-month period intervening and there any discordant scoring was reviewed by both pathologists and a consensus reached. We tested for differences in marker expression in association with patient characteristics and clinical parameters using Fisher's Exact and Chi Squared Tests. Kaplein-Meier survival estimates using log-rank testing were

determined for each marker after stratifying tumours into negative/low expression (0,1) versus moderate/high (2,3) expression.

IRB Approval

All patient samples used in this study were collected with informed patient consent and all protocols were preformed with approval from the institutional review boards at the University of Southern California, University College London or Oregon Health & Science University.

Survival Analyses Using Gene Expression Microarray Data

The methods for the gene expression survival analyses have been previously described [51]. Briefly, the CuratedOvarianData [52] were downloaded and data extracted for serous ovarian cancer cases, profiled using Affymetrix U133 arrays, where survival outcomes were known. Datasets were combined using the Combat method, and Cox regression analyses performed to test for associations between expression of candidate genes and overall survival. Analyses were performed in 'R' using the Bioconductor package (www.bioconductor.org).

Figure and Table Legends

Figure 1. Discovery of candidate biomarkers using genetic models of EOC and gene ontology analyses of identified proteins. (a) Frequency of CMYC amplification/overexpression, KRAS mutation and MAPK alteration in EOC, stratified by histological subtype. † Frequency of MAPK alterations were calculated as cumulative frequencies of KRAS, BRAF, HER2, EGFR and EGFR mutation/overexpression (where available) using the references cited. HGSOC, high-grade serous ovarian cancer; EC, endometrioid ovarian cancer; CCOC clear cell ovarian cancer; MOC, mucinous ovarian cancer. (b-d) DAVID (david.abcc.ncifcrf.gov) was used to perform gene ontology analyses of proteins uniquely changing in (b) $IOSE^{CMYC}$ models or (c) $IOSE^{CMYC+KRAS}$ models. Genes commonly changing following both CMYC and CMYC+KRAS expression are shown (d).

Figure 2. Immunoblotting and analysis of gene expression for the top candidate biomarkers. (a) Candidate biomarkers (Table 2) were validated by Western blotting. Western blotting for LGALS3BP, CSF1 and RCC2 was also performed, but yielded inconclusive results (data not shown). (b) Analysis of gene expression for candidate biomarkers using TCGA gene expression microarray data. Statistically significant differences in gene expression (tumour versus normal) are indicated in bold (P<0.05).

Figure 3. Immunohistochemistry of the top 5 candidate novel EOC biomarkers. Examples of negative (0), weak (1), moderate (2) and strong (3) staining are shown. LGALS3BP did not show different grades of expression and so was scored as negative (0) or positive (1). Brightfield microscopy, brown color indicates positive staining, samples were counterstained with eosin (blue).

Figure 4. Survival Analyses. Moderate or strong expression of (a) CHI3L1 and (b) FKBP4 is associated with significantly worse overall survival. Kaplan-Meier survival curves.

Table 1. Top candidate biomarkers. We selected proteins that were commonly changing in the $IOSE^{CMYC}$ and $IOSE^{CMYC+KRAS}$ models for further follow-up; 13 genes were significantly changing over 2-fold in both sets of models, all of which were up-regulated. Four of the top candidates are known MYC targets (FASN, FKBP4, NDRG1 and SORD) [53]. Candidates were prioritized based on subcellular location and gene expression in The Cancer Genome Atlas (TCGA) gene expression microarray dataset of 489 high-grade serous ovarian cancers and 8 normal fallopian tubes. † Categories for subcellular location: 0, cytosolic with no evidence of secretion; 1,

membrane bound; 2, secreted. [#] Gene expression in TCGA is opposite to direction in change of protein abundance in SILAC experiment and so these candidates were excluded from further analyses. [$] U2AF1 and ADSL are widely/ubiquitously expressed respectively, and so were excluded from downstream analyses. Validation by Western blotting was performed on the secreted protein lysates used for biomarker discovery, as well as in independent preparations of total cell lysates (TCLs) from the models cultured in 2D and 3D. Immunohistochemistry was performed on whole sections of primary ovarian cancer tissues. [%] IHC for NDRG1 was performed as part of a larger study, focused on detailed analysis of NDRG1 (Pejovic *et al*, manuscript in preparation).

Table 2. Associations between biomarker expression and tumour grade in early stage (I/II) EOCs.

Statistically significant associations are shown in bold (Fishers Exact Tests).

Supplementary Table Legends

Supplemental Table 1. Full LC-MS/MS dataset showing protein identifications and quantification in the four comparisons.

Supplemental Table 2. Proteins differentially secreted in IOSE^{CMYC} and IOSE$^{CMYC+KRAS}$ models. Results from reciprocal labeling experiments are shown, where fold-change was statistically significant in at least one experiment (P<0.05, bold font) and directionality shows the same trend in both experiments.

Supplemental Table 3. Patient population characteristics for the tissue microarrays used in this study

Supplemental Table 4. Analysis of API Expression and Associations with Clinical Parameters. Statistically significant associations are shown in bold (Fishers Exact and Chi Squared Tests).

Supplemental Table 5. Analysis of FASN Expression and Associations with Clinical Parameters. Statistically significant associations are shown in bold (Fishers Exact and Chi Squared Tests).

Supplemental Table 6. Analysis of FKBP4 Expression and Associations with Clinical Parameters. Statistically significant associations are shown in bold (Fishers Exact and Chi Squared Tests).

Supplemental Table 7. Analysis of CHI3L1 Expression and Associations with Clinical Parameters. Statistically significant associations are shown in bold (Fishers Exact and Chi Squared Tests).

Supplemental Table 8. Analysis of LGALS3BP Expression and Associations with Clinical Parameters. Statistically significant associations are shown in bold (Fishers Exact and Chi Squared Tests).

Supplemental Table 9. Antibodies used for western blotting and immunohistochemistry; suppliers and dilutions used.

Supplemental Table 10. Survival analyses. Expression of each gene was analyzed as a continuous variable, and adjusted for age at diagnosis, stage and debulking status.

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Novel EOC biomarkers identified using 3D *in vitro* models

Table 1

						Average Fold-change		Validation (Western)			Immunohistochemistry	
No	Models	Protein	Subcellular Location[†]	TCGA P Value	TCGA Direction (Tumours v Normal)	IOSE v CMYC	IOSE v CMYC+ KRAS	In Discovery Samples	In Independent TCL (2D)	In Independent TCL (3D)	% Expressing Tumours	N
1	CMYC & $KRAS^{G12V}$	CHI3L1	2	0.723	NA	11.94	14.37	Yes	No	No/Yes	100	4
2	CMYC & $KRAS^{G12V}$	LGALS3BP	2	0.013	Up	2.54	6.03	No	No	No	50	4
3	CMYC & $KRAS^{G12V}$	TIMP1[#]	2	0.027	Down	2.62	4.06	NA	NA	NA	NA	NA
4	CMYC & $KRAS^{G12V}$	CPE	1/2	0.750	NA	8.05	7.40	Yes	No	No	100	4
5	CMYC & $KRAS^{G12V}$	U2AF2[$]	0	0.003	Up	3.32	4.69	NA	NA	NA	NA	NA
6	CMYC & $KRAS^{G12V}$	KRT1[#]	1	0.001	Down	2.09	3.08	NA	NA	NA	NA	NA
7	CMYC & $KRAS^{G12V}$	NDRG1[%]	0/1	0.005	Up	4.08	3.61	Yes	Yes	NA	NA	NA
8	CMYC & $KRAS^{G12V}$	RCC2	0	$2.92x10^{-7}$	Up	3.54	4.20	No	No	No	NA	NA
9	CMYC & $KRAS^{G12V}$	FASN	0	0.008	Up	3.94	3.82	Yes/No	Yes	Yes	100	6
10	CMYC & $KRAS^{G12V}$	SORD	0/1	0.993	NA	3.53	4.28	Yes	Yes	Yes	0	6
11	CMYC & $KRAS^{G12V}$	API5	0	$1.41x10^{-5}$	Up	3.09	3.15	Yes	Yes	No	83.3	5
12	CMYC & $KRAS^{G12V}$	FKBP4	0	0.001	Up	3.93	3.07	Yes	Yes	Yes	100	6
13	CMYC & $KRAS^{G12V}$	ADSL[$]	0	0.990	NA	2.78	2.95	NA	NA	NA	NA	NA
14	CMYC & $KRAS^{G12V}$	PFAS	0	0.302	NA	4.20	3.45	Yes	Yes	No	66.6	6
15	$KRAS^{G12V}$ only	PCOLCE	2	0.899	NA	-	63.92	Yes	NA	NA	NA	NA
16	CMYC only	PARP1	0	$2.22x10^{-8}$	Up	2.46	-	Yes	NA	NA	NA	NA
17	CMYC only	CSF1	2	0.034	Down	6.45	-	No	NA	NA	NA	NA
18	CMYC only	POSTN	2	0.034	Down	0.40	-	Yes	NA	NA	NA	NA
19	CMYC only	DPP4	2	0.239	NA	0.15	-	Yes	NA	NA	NA	NA

Table 2

		Negative versus all positive expression			Negative/weak versus moderate/strong expression		
Marker	**Grade Grouping**	**0**	**1,2,3**	***P*-value**	**0,1**	**2,3**	***P*-value**
API5	FIGO 1	12	15		24	3	
	FIGO 2/3	18	49	0.142	32	35	**3.00E-04**
FASN	FIGO 1	7	20		12	15	
	FIGO 2/3	4	65	**0.010**	15	54	**0.042**
FKBP4	FIGO 1	1	25		9	17	
	FIGO 2/3	5	58	0.667	11	52	0.097
CHI3L1	FIGO 1	5	21		12	14	
	FIGO 2/3	2	67	**0.016**	20	49	0.146
LGALS3BP	FIGO 1	19	8		NA	NA	
	FIGO 2/3	39	29	0.351	NA	NA	NA

Figure 1

(a)

Gene/ Pathway	Frequency of Alteration [Reference]			
	HGSOC	EC	CCOC	MOC
CMYC	39% [13]	43% [14]	67% [14]	63% [14]
KRAS	15% [15]	9% [16]	25% [16]	44% [17]
MAPK†	40% [15]	~67% [16,18]	~55% [16,19]	>67% [13,20]

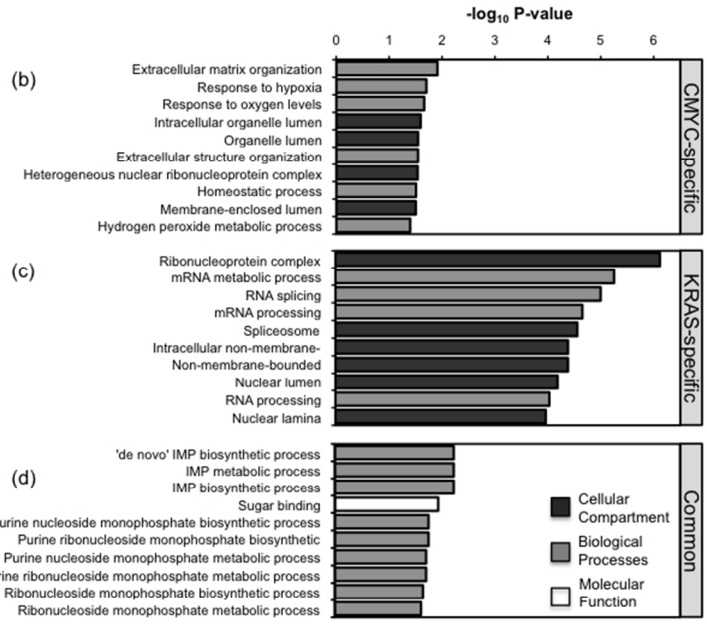

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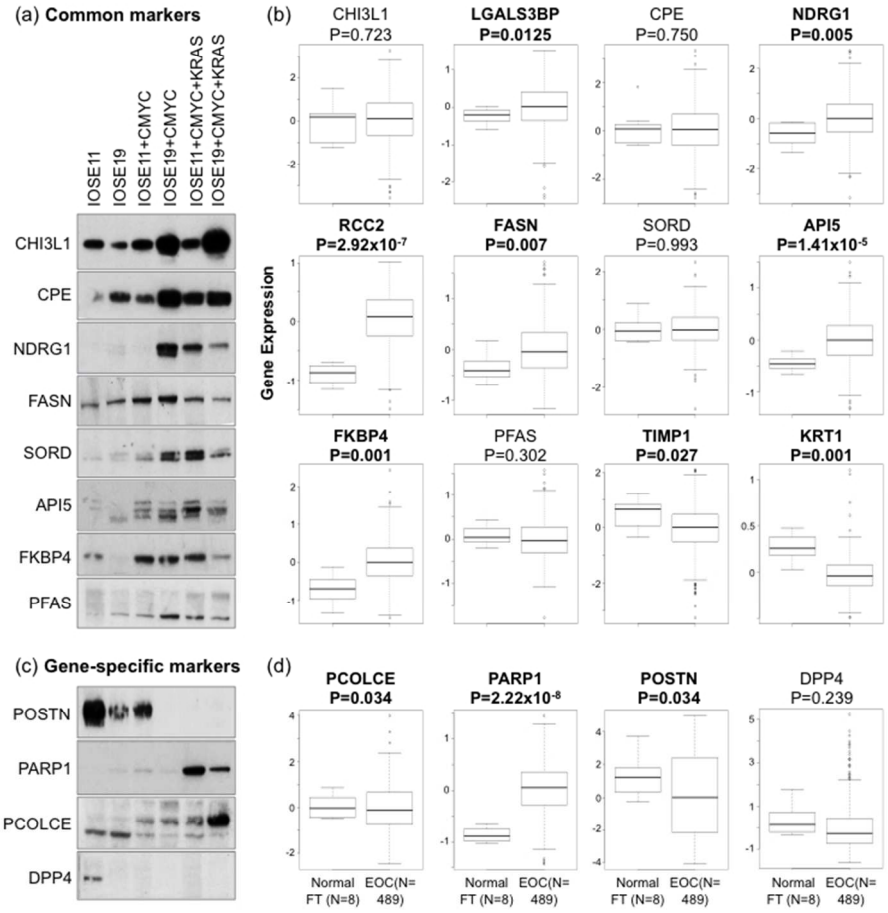
Figure 2



264x352mm (72 x 72 DPI)

Figure 3

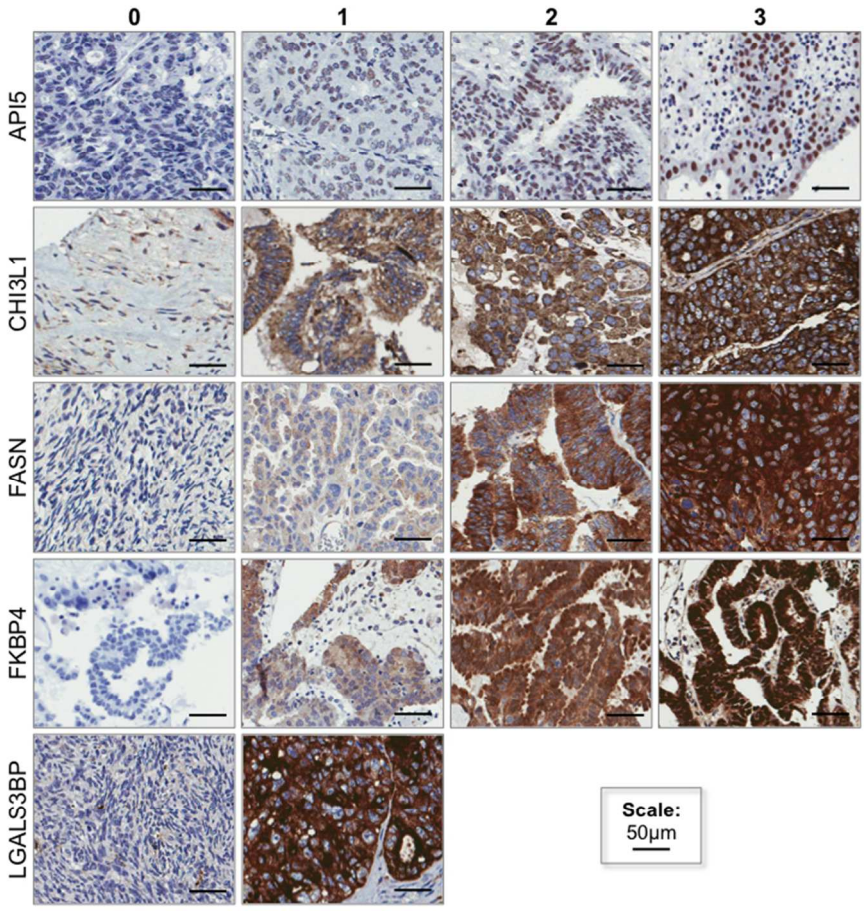


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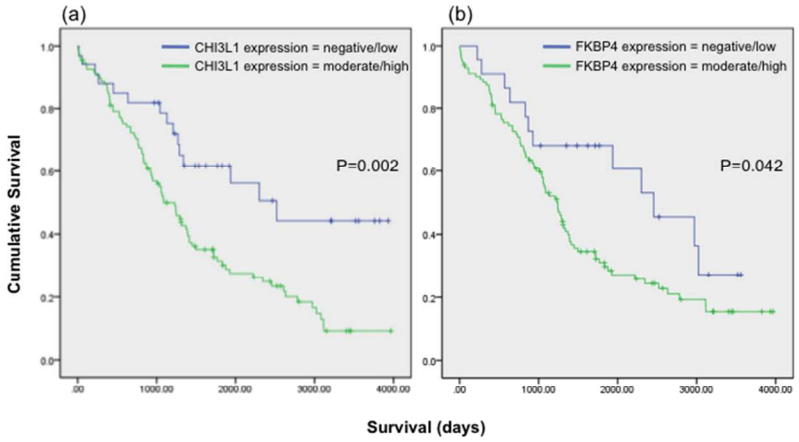
Figure 4



264x352mm (72 x 72 DPI)