Introduction

Reviewer 1 - Significance: PI's group identified the sum of IHC protein levels of genes POSTN, CXCL14 and pSmad 2/3 to triage tumors into high-and-low-risk groups for suboptimal debulking. In spite of this, clinically meaningful survival was not achieved. **Response:** These markers were identified retrospectively in sub-optimally debulked tumors and therefore they did not determine the specific treatment strategy or influence patient survival. Through retrospective analysis we have now confirmed that they also correlate with poor survival, a hallmark of suboptimally debulked tumors (figure added to grant). We now propose to validate these markers pre-operatively in an independent set of tumors and generate preclinical data targeting EOC with specific anti TGF-β treatment. **Approach:** PI failed to justify how the biomarkers would benefit EOC patients. Preclinical experiments are poorly designed. Data shows that "debulking" induces hyperactivation of the TGF-β pathway resulting in tumor spread, but in preclinical experiment's debulking is not done. PI only proposes inhibition of TGF-β signaling. **Response:** We have clarified this issue in the grant. The dubulking procedure itself does not induce hyperactivation of the TGF-β signaling, but we believe that such signaling is endogenously hyperactive in those tumors that cannot be optimally debulked. The new biomarker panel allows preoperative diagnosis of these tumors so that these specific patients can be stratified for a better therapeutic approach. These patients do not benefit from upfront aggressive debulking and would be better treated with neoadjuvant therapy with interval debulking including chemotherapy that targets the pathways inducing the dissemination process of these tumors. The goal of this grant is to generate preclinical data using currently available agents for rapid clinical translation such as TGF-β inhibitors. Other targets from the biomarker panel will also be tested and we have discussed this in the alternative procedures. We have also added an experiment comparing the outcome of NCAT-IDS versus upfront debulking in our preclinical mice models.

Reviewer 2- Investigators: The nature of the interaction between investigators is poorly described. **Response:** We have a subcontract with our computational scientist collaborator, Dr. Wang, who will supervise our post-doctoral fellow in the data analysis during optimization of the debulking signature. Dr. Wang has the statistical tools and knowledge to perform such analysis; a skill beyond the competencies of our lab. We have also 3 unpaid collaborators: 1) Dr. Del Carmen who will provide consultancy on the relevance of the project development from the surgeon's perspective, 2) Dr, Huntsman who wil provide us with samples and clinical data of the TMA, 3) as requested by reviewer 3 we have also added the consultancy of a pathologist specialized in ovarian cancer. **Innovation:** The approach focuses on the TGF-β which is a usual suspect in tumors. Similar experiments on this signal transduction pathway may have been performed unsuccessfully in the past. **Response:** To our knowledge we are the only group characterizing the role of TGF-β specifically in suboptimally debulked EOC and the effect of inhibiting TGF-β as proposed herein has not been tested in the context of ovarian cancer. The concept of using a TGF-β driven pathway to stratify patients for treatment and then use inhibitors of this pathway to improve the treatment of EOC patients is highly novel. **Approach: 1)** It is not clear how this study is integrated in other studies of the principal investigator. Some sense of how important this pathway is in comparison with other pathways considered would help assessing the impact of the study. **2)** A better description of alternative approaches would give greater confidence in the likelihood of the study success. **Response:** Our lab has focused on translating the genomics of ovarian cancer into more effective clinical management of their disease. We have projects characterizing the genomes of early stage cancers and the features of long term survivors. This project fits perfectly into our efforts as it utilizes the laboratory's expertise in genomics, biology, and bioinformatics, but is the only project focused on the up-front treatment of ovarian cancer. We expect this to effectively integrate into the other targets we are working on, most of which are important later in the course of the disease. We have explained this in the grant.

Reviewer 3 Overall: Unclear relevance of the mouse models chose in Aim 2, the absence of comparative pathology or expert in mouse modeling, and the less defined focus of Aim 2. More specific stratification of tumors is required and should include methylation profiles, assessment of HR deficiency or other histopathological subtypes. **Response:** We have rewritten the aim to focus it better. Our goal is to: 1) validate our hypothesis that the TGF-β pathway is responsible for the biology of tumors that cannot be optimally debulked, and 2) demonstrate that targeting this pathway can improve the treatment of these tumors. Our lab has extensive experience in ovarian cancer animal models and we believe that PDXs are the most clinically relevant model for preclinical testing. We also use a syngeneic mouse model to evaluate the effect immune component on our therapeutics. Dr. Tambouret, who is a surgical pathologist specialized in gynecologic oncology, has agreed to review our preclinical tumor models to make sure they are clinically relevant. Due to the limited budget, we have chosen to focus on high grade serous histology, but agree with the reviewer that in the future additional histopathologic subtypes should be investigated. We also discuss the value of additional genomics profiling in the alternative approaches of Aim 1.

Specific Aims

The current standard of care for epithelial ovarian cancer (EOC) includes debulking surgery followed by adjuvant chemotherapy. Resection of residual disease is associated with a better prognosis. However, optimal debulking cannot be in approximately 20% of the patients. These patients undergo extensive surgery, complex post-operative recovery, delay to subsequent chemotherapy and ultimately present with unfavorable quality-of-life indexes and poor prognosis. A randomized phase III trial showed that neoadjuvant chemotherapy with interval debulking surgery (NACT-IDS) provides equivalent survival to primary debulked patients and increases quality of life indexes in some patients. This study has emphasized the need to stratify patients based on these 2 approaches. **We hypothesize that suboptimally debulked EOC has a distinct genomic pattern that can be used for pre-operative diagnosis of these tumors and provide targeted therapies for this subset of EOC patients.**

To test this hypothesis we used a meta-analytic signature development approach and leveraged 1,525 publicly available expression profiles to retrospectively investigate the potential correlation between the transcriptome of the primary tumor and the outcome of debulking surgery. By utilizing 8 carefully curated microarray datasets to train and validate, we have developed a 198-gene signature predictive of suboptimal debulking surgery. Gene ontology analysis of our “debulking signature” indicated activation of TGF-β signaling pathway and overexpression of genes related to this pathway and regulating dissemination of cancer cells as well as resistance to chemotherapy.

The goal of this project is to develop a validated genomic signature which can be developed into clinical diagnosis, and perform pre-clinical studies testing whether targeting one of the most enriched pathway of this signature, TGF-β, is effective.

If successful, our data not only will provide a new stratification of EOCs based on a molecular signature that predicts the outcome of debulking surgery, but will also provide **a novel pre-operative diagnostic tool** that will assist the surgeons to manage these tumors and **new molecular therapies which target sub optimally debulked tumors.** Patients with this signature may be more suitable for neoadjuvant therapy involving traditional taxol/platinum chemotherapy combined with inhibition of TGF-β and/or other targets of the signature.

Specific Aim 1: To validate a gene expression signature predicting the outcome of debulking surgery that can be used as a pre-operative diagnostic tool.

We propose a two tier validation study using carefully annotated and pathologically reviewed EOC specimens from two independent large cohorts. We will validate the predictive powers of the 198 genes by analyzing their RNA levels in 1,536 specimens through a custom-built Nanostring platform. This study will leverage the approved NIH/RC4 grant (1RC4CA156551) of the PI to obtain 1,536 samples from the Gynecologic Oncology Group protocol 218 (GOG-218). We will then select compacted 10 to 20 gene signatures and test whether these smaller, i.e. commercially viable, signatures maintain a predictive power greater than 80%. The best performing signature(s) will then be used for immunohistochemistry analysis of its encoded proteins using an independent cohort of 1,307-sample, formalin fixed paraffin embedded (FFPE) tissue microarray (TMA) from the British Columbia Cancer Agency. We expect to develop a clinically applicable, biomarker panel predictive of surgical success with accuracy over 90%.

Specific Aim 2: Preclinical testing of new approaches for targeted therapy of suboptimal debulked tumors. Our previously developed “debulking signature” suggests hyperactivation of the TGF-β pathway resulting in tumor spread and chemoresistance. In this aim, we will evaluate whether inhibition of this pathway decreases the disseminating potential of ovarian cancer cells and improves surgical outcome of intraperitoneal tumors. Specifically, we will determine the capacity of a small molecule inhibitors of the TGF-β signaling, LY2157299, to inhibit the disseminating potential of ovarian cancer cells and improve management of ovarian cancer models established in mice. LY2157299 was well-tolerated and effective anticancer drugs in preclinical and Phase I / II clinical studies. If successful, these studies will provide indication for a new targeted therapy that may be applied in a neo-adjuvant setting to increase the rate of optimal debulking, or used as adjuvant therapy to improve prognosis of sub-optimally debulked patients.

Innovation

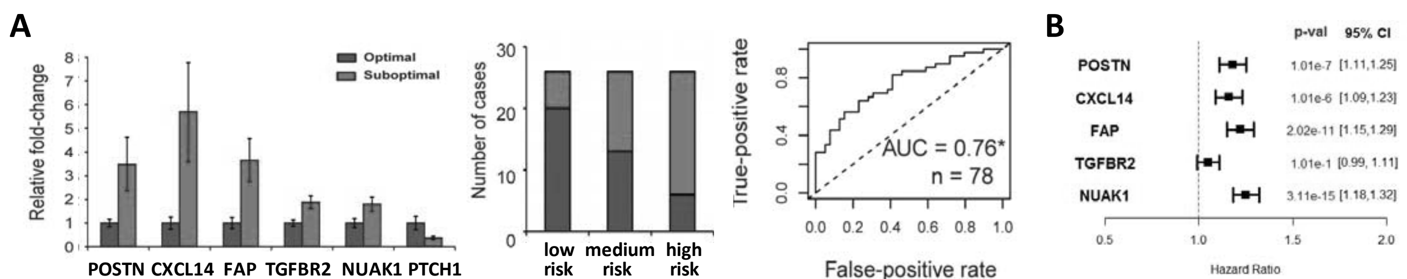
This study brings innovation in the management of suboptimal debulked EOC as it provides: 1) the first genomic signature which can preoperatively identify women who would benefit from up-front surgery and 2) the establishment of new molecular therapeutic approaches targeting sub optimally debulked tumors.

Research Strategy

A. Significance

A1. Prediction of suboptimal cytoreduction of ovarian cancer is an unmet clinical need: Epithelial ovarian cancer (EOC) is the leading cause of gynecologic cancer related death in the US. Over 70% of the patients are not diagnosed until the development of advanced disease (Stage III or IV) with the tumor spread beyond the pelvis to the abdomen and lymph node or outside of the peritoneal cavity. The standard clinical management for advanced stage EOC includes aggressive cytoreductive (debulking) surgery followed by adjuvant chemotherapy. Debulking surgery improves patient outcome when tumors can be optimally debulked (less than 1cm of residual tumor) (1, 2). However, optimal cytoreduction cannot be achieved in 20% of patients even with radical procedures such as splenectomy or diaphragmatic resection. These patients have complicated and prolonged postoperative recovery, respond poorly to adjuvant chemotherapy and usually die within 3 years (3). A randomized phase III trial demonstrated that neoadjuvant chemotherapy with interval debulking surgery (NACT-IDS) is as effective as up front debulking surgery with adjuvant chemotherapy (4). Patients receiving NACT-IDS suffer less intra- and post-operative complications; have decreased surgery-associated morbidity and increased quality-of-life indexes. However, NACT-IDS did not improve patients' overall or progression-free survival, due to the potential decrease of survival for specific patients whose optimal debulking was delayed (5, 6). We believe that preoperative identification of tumors that would not benefit from upfront debulking surgery would allow targeted application of NACT-IDS only to patients with such tumors and ultimately result in improved survival. Unfortunately, current diagnostic strategies are not reliable for preoperative prediction of optimal debulking (7, 8). While it is clear that surgical ability is critical to achieve optimal debulking, it remains unresolved whether the contribution of successful debulking surgery to prognosis is due to (i) solely the physical removal of tumor cells or (ii) also the intrinsic biology of tumor, reflecting less aggressiveness and dissemination (9). **Identification of biomarkers and pathways that contribute to the difficulty in debulking remains an unmet clinical need. Our goal is to validate and optimize a molecular signature that distinguishes sub-optimal from optimal debulked EOCs**. This signature will be used as a clinical tool to preoperatively identify patients who benefit most from primary debulking or NACT-IDS and to establish a therapy that targets those tumors that cannot be optimally debulked. It will specifically improve the outcome of upfront therapy; additional stratification using other biomarkers and targeted therapeutic approaches can be further integrated during later course of EOC treatment.

A2. Development of a molecular signature predicting suboptimal debulking: High-throughput genomic analysis identifies predictive signatures with potential clinical utility, but predictive signatures are difficult to validate and translate to clinical applications due to: 1) small sample size, 2) unaudited and unreliable clinical annotation, 3) laboratory-specific biases, and 4) training performed on non-representative cohorts (10). Meta-analysis data curation allows analyzing expression profiles obtained from multiple studies that, taken together, form a combined repository of thousands clinically annotated samples, i.e. a robust database to identify and validate clinically useful gene signatures (11). Our laboratory has developed a *curatedOvarianData* database that provides standardized gene expression and clinical data for 2,970 EOC patients from 23 studies spanning 11 gene expression platforms (URL: http:// bcb.dfci.harvard.edu/ovariancancer) (12). This work facilitates biomarker discovery through a meta-analysis framework that limits the impact of cohort-specific biases and combines the statistical powers of numerous studies.



(C) The prediction accuracy of the multivariate model in which the 3 IHC validated genes were equally weighted similar as in Fig. 1A. **Fig. 1: Validation of selected genes associated with debulking status. (A)** qRT-PCR in the Bonome validation data (n=78). Shown are the observed fold-changes for the selected 6 genes with significantly overexpression in suboptimal tumors and the prediction accuracy of a multivariate model. Samples were stratified into groups of high and low risk for suboptimal surgery based on the tertiles of the multivariate risk score related to the qRT-PCR value of each gene. The accuracy of the multivariate risk prediction is further shown with a ROC curve. **(B)** Meta-analysis of the prognostic impact of selected genes. Shown are the hazard

Our curatedOvarianData database contains 8 datasets with available surgical outcome information. These datasets involve many separate medical institutions and surgical techniques, ensuring a broad cross section of clinical approaches. Through integration of all 8 transcriptional profiling datasets, totaling 1,261 high-grade, late-stage EOCs (including 442 samples from the TCGA dataset), we have identified a novel signature consisting of 198 genes that distinguishes tumors that can be optimally debulked from those that allow only a suboptimal cytoreduction. (13). Utilizing “leave-one (dataset)-out” cross-validation, we have shown the ability of our “debulking signature” to correctly discriminate optimal and suboptimal debulked tumors in 7 of 8 datasets (13). Gene Ontology and pathway enrichment analyses of our “debulking signature”, has indicated hyperactivation of the TGF-β/Smad signaling as the most powerful selective feature of suboptimally debulked

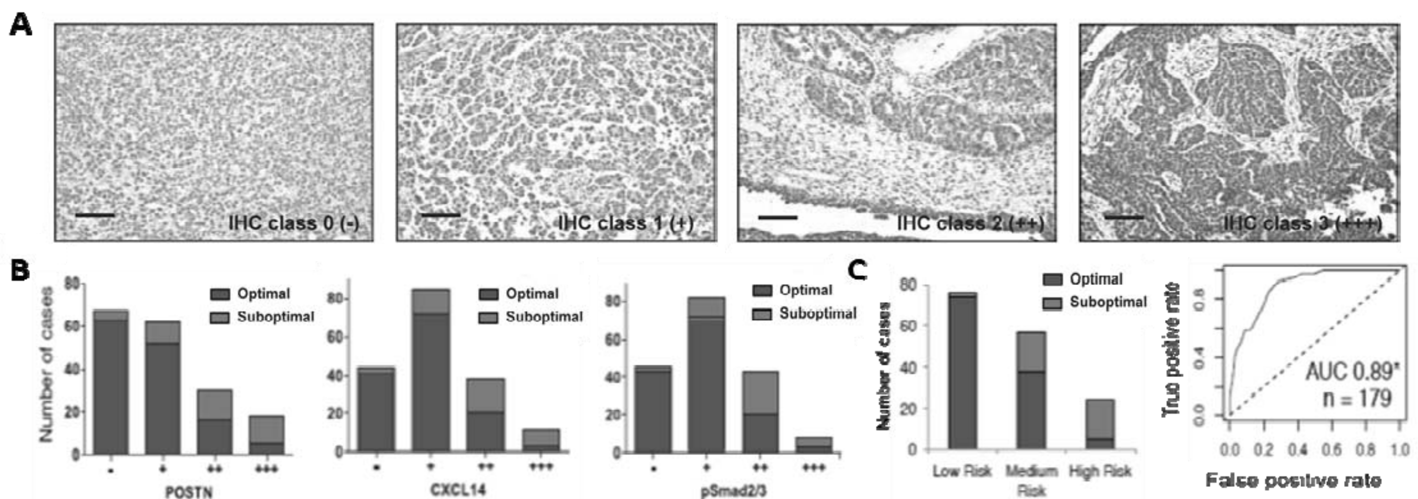


Fig. 2: Validation of selected genes associated with debulking status by IHC. (A) IHC staining of phospho-Smad2/3 as a surrogate marker of TGF-β pathway activation in the validation tissue microarray. Examples of different staining intensity were shown. **(B)** The frequency of optimal and suboptimal tumors stratified by the IHC grade of POSTN, CXCL14 and phospho-Smad2/3.

tumors (p=0.004, by Pathway studio 7.1, Ariadne Genomics) (13).

To independently validate our debulking signature we have tested expression of 7 selected genes with known biological role in ovarian tumorigenesis by qRT-PCR using an independent cohort of stage III and IV tumors consisting of 78 samples (14) excluded from the meta-analysis. Of the 7 genes tested, 6 were significantly associated with surgery outcome ($p<0.05$, Student’s t-test, Fig. 1A). We then applied a multivariable risk prediction analysis through a receiver operating characteristic (ROC) and validated the predictive power of expression levels of these 6 genes (AUC of 0.76, 95% CI 0.66-0.87, Fig. 1A). Meta-analysis of 15 transcriptomic datasets totaling 2271 demonstrated negative prognostic impact of POSTN, CVCL14, FAP, TGFBR2 and NUAK1, suggesting that genes of the ‘debulking signature’ confer the poor overall survival associated with suboptimal debulking (Fig. 1B). Furthermore, protein levels of two signature genes (POSTN and CXCL14) and of Smad2/3 (a surrogate marker of TGF-β pathway activation) were analyzed by immunohistochemistry (IHC) in an independent cohort of 179 patients, which confirmed association of these protein markers with suboptimally debulked tumors (Fig. 2). The sum of IHC intensities for these three proteins provided a tool that classified 92.8% of samples in the high and low risk groups for suboptimal debulking, with an AUC of 0.89 (95% CI 0.84-0.93). These data indicate that if all other factors that influence EOC debulking such as the surgeon, patient frailty and blood loss are equal, we can observe a genomic difference between optimally and suboptimally debulked tumors. This distinct genomic pattern of suboptimally debulked tumors leads to a different biologic behavior that requires a distinct/targeted therapeutic approach.

A3. Characterization of biologic behavior of sub-optimally debulked tumors: Analysis of the biological function of the transcriptional network of the debulking signature indicates up-regulation of genes that belong to the TGF-β pathway and support tumor dissemination through: 1) epithelial-mesenchymal transition (EMT) and metastasis (*MMP2, PLAU, SERPINE1, TIMP3, POSTN, VCAN, FN1, TGFBI, SPARC, LOX,* and *CYR61*) and 2) angiogenesis (*VCAN, POSTN, CNY61* and *LOX*). These data suggest that sub-optimally debulked tumors are prone to increased intraperitoneal dissemination which impedes optimal debulking of the tumor and allows rapid spread of residual tumor foci following the debulking surgery. The latter phenomenon may account, among others, for the decreased survival observed in suboptimally debulked tumors. Indeed, among patients with complete chemoresponse, those with sub-optimal debulking have poorer prognosis than patients with

optimal debulking (15-17). In addition, two meta-analyses observed an association between TGF-β inducted extra-cellular matrix (ECM) remodeling during EMT and platinum resistance in EOC (18, 19), and another study has linked TGF-β signaling to resistance of paclitaxel in breast cancer (20). To understand whether the TGF-β signaling could induce chemo-resistance in EOC, we compared the expression profiles of 442 TCGA high-grade, late stage EOCs and performed a correlative analysis between hyperactivation of the TGF-β signaling and the magnitude of response to platinum/taxol chemotherapy. For genes with significant higher expression in incomplete chemo-response, the top hit Gene Ontology term by Fisher's test was GO:0030198 (extracellular matrix organization, $p=2.70\times10^{-8}$). Most of the 44 differentially expressed genes in this GO term are positively regulated by TGF-β signaling, such as *BMP4, FAP*, *COMP*, *TGFBI*, *MMP2*, *CTGF*, *PDGFRA*, *LUM*, *POSTN*, *SPARC*, *LOX*, *PAI-1* and *VCAN* (Figure 3). A similar result was also obtained using a Kolmogorov-Smirnov (K-S) like test, which evaluates the contribution of each gene in the GO term by analyzing its p-value in the corresponding differential gene expression analysis (for GO:0030198, $p= 2.80\times10^{-5}$ classic model; p= 0.00156 elimination model). Moreover, when using a differential gene expression analysis model adjusted for "debulking status" (n=412), the GO term GO:0030198 with the above TGF-β responsive genes still remains significant (p=0.021) in the K-S model, suggesting TGF-β signaling may confer chemoresistance in EOC. These data further support the testing of TGF-β inhibition as a targeted therapy for suboptimally debulked tumors; inhibition of the TGF-β signaling not only will decrease disseminated growth of residual post-surgical lesions, but will also sensitize these tumors to chemotherapy.

A4. Tumor suppressive effect by TGF-β inhibition: We have screened a panel of human and murine EOC cell lines for their TGF-β responsiveness through a CAGA12-luciferase reporter with Smad-binding element (21). Both immortalized ovarian surface epithelial cells, HIO-80 and HIO-120 presented robust TGF-β responsiveness yet undetectable endogenous TGF-β activity. Ligand induced TGF-β signaling was also prevalent in 80% of the 19 EOC cell lines, with 10 (52.6%) EOC cell lines presenting endogenous TGF-β activity, which can be further inhibited by LY2157299 (Fig. 4A), a small molecule inhibitor which targets TGFBR1 and abrogates TGF-β induced canonical and non-canonical signaling (Fig. 4B). Conversely, administration of LY2157299 abrogates endogenous and TGF-β induced expression of biomarkers in the "debulking signature", migration and invasion activities as well as the ability to adhere to mesothelial monolayer, a prerequisite step for the establishment of metastases (Fig. 4C-E). Functional disruption of TGF-β receptors by CRISPR technology delivers similar tumor-suppressive effects in SKOV3 cells, including *in vivo* inhibition of xenograft growth in immunocompromised mice (Fig. 4F-G).

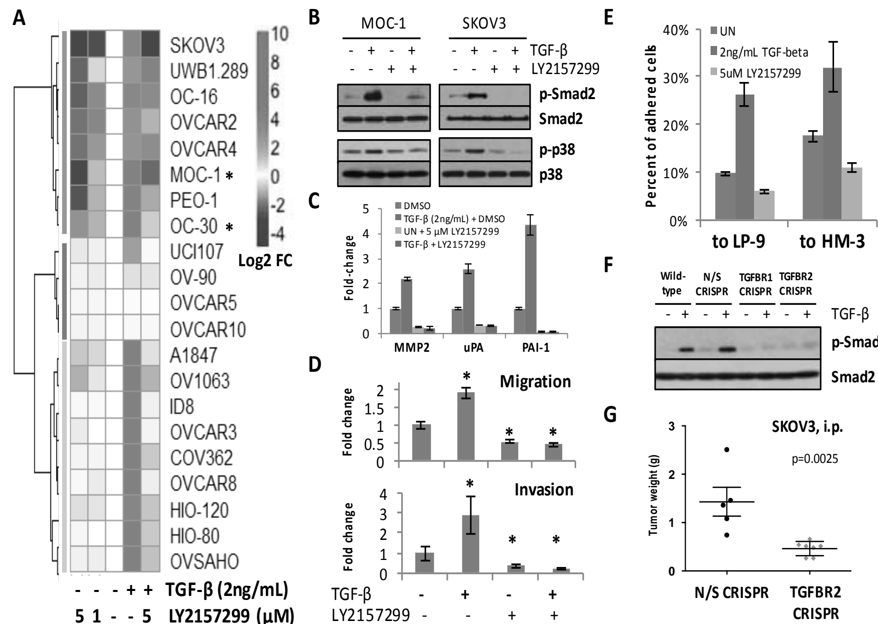


Figure 4. Anti-tumor effect of TGF-β inhibition on ovarian cancer cells. (A) $CAGA_{12}$-luciferase assay to screen TGF-β responsiveness in 21 ovarian cell lines upon 48h treatment with TGF-β (2ng/mL) and LY2157299 (5 μM). Unsupervised clustering of Log2 fold-change revealed three distinct clusters: baseline TGF-β activity (dark orange bar), exogenous TGF-β responsive (light orange bar), and TGF-β irresponsive (blue bar). **(B)** LY2157299 abrogates TGF-β induced canonical (by p-Smad2) and non-canonical (by p-p38) signals. **(C-E)** *In vitro* Inhibitory effect of LY2157299 on SKOV3 cells: **(C)** Expression of MMP2, uPA/PLAU and PAI-1 which constitute the "debulking signature"; **(D)** Boyden chamber (0% to 2% FBS) based migration and invasion; **(E)** Adhesion to mesothelial monolayer. SKOV3 cells were primed with TGF-β and LY2157299 for 48h prior to plating onto confluent monolayer of LP-9 or HM-3 cells. **(F-G)** TGFBR2 functional disruption by CRISPR (**F**, by pSmad2 activation) inhibits the *in vivo* tumorigenicity of SKOV3 cells **(G)**.

We hypothesize that suboptimally debulked EOC has a distinct genomic pattern that can be used for pre-operative diagnosis of these tumors and provide targeted therapies for this subset of EOC patients. The goal of this project is to develop a validated genomic signature which can be developed into clinical diagnosis, and perform pre-clinical studies testing whether targeting one of the most enriched pathway of this signature, TGF-β, is effective. If successful, our data will provide the basis for the development of **a pre-operative diagnostic tool** that will assist the surgeons in managing these tumors. Patients with this signature

may be more suitable for neoadjuvant therapy involving taxol/platinum chemotherapy combined with inhibition of the TGF-β pathways followed by interval debulking surgery.

B. Innovation: This study brings innovation in the management of suboptimal debulked EOC as it provides: 1) the first genomic signature that can preoperatively identify women who would not benefit from up-front surgery and 2) the establishment of new molecular therapeutic approaches targeting sub optimally debulked tumors.

C. Approach

C1.To validate a gene expression signature predicting the outcome of debulking surgery that can be used as a pre-operative diagnostic tool.

C1.1.Research design and rationale: To develop a clinically useful signature for tumor debulking it is important to: 1) perform a prospective validation using uniformly treated and fully annotated samples obtained with standard procedures from clinical trials, and 2) compact the current 200-gene signature to a more manageable one composed of 10-20 genes. We aim to obtain a panel of biomarkers that (i) is commercially viable, (ii) has 90% sensitivity and specificity (by ROC curve, Fig. 2C) and can thus be utilized as a preoperative predictive tool of surgical outcome, and (iii) serves as therapeutic target to facilitate management of tumors that predict to be suboptimally debulked.

C.1.2. RNA NanoString analysis: Under the recently approved NIH/RC4 study of the PI (1RC4CA156551-01), total RNA from 1536 pathologically reviewed FFPE specimens were collected from the GOG 0218 trial. All specimens are fully annotated, 41% are suboptimal debulked stage III; 27% are optimally debulked stage III and 32% are stage IV (maximum effort of debulking, but considered suboptimal debulked). Other patient characteristics include tumor grade, histology, age, patient overall survival, progression free survival, response to chemotherapy and patient quality-of-life. We will subject these FFPE RNA samples to a customized NanoString® platform to validate the expression level of the previously developed 198-gene “debulking signature”. NanoString quantifies the expression levels of 800 genes, using 100ng total FFPE RNA as input, with precision superior to qPCR as it eliminates the amplification step required for microarray hybridization and thus the potential bias from non-linear amplification [19-21]. As proof-of-principle, we completed a pilot test of 10 FFPE RNA specimens. Two independent RNA extractions (technical duplicates) from one specimen were subjected to the platform. NanoString technology demonstrated to have a detection limit of targeted RNA at concentration of 0.5fM in the hybridization (equivalent to approximately 1 RNA copy per mammalian cell, >98% call rate for all probes on the platform) as well as high reproducibility between the two technical duplicates, even when the RNA input was lowered to 30 ng (Fig. 4).

Univariate and multivariate risk prediction model (Fig. 1) will be established based on the expression level of each gene adjusted to the tumor stage, which negatively affects optimal surgery. We will use the GOG tumors to evaluate the predictive power of selected signatures on the likelihood of optimal debulking. We will compare the predictive value of our 198-gene signature with the one of different sets of compact 20-gene signatures. The 20-gene signatures will be designed by selecting genes from the 198-genes debulking signature using the following priorities: (i) genes with top ranked expression profiles as determined by the meta-analysis, (ii) genes coding for proteins inducing dissemination and/or cisplatin resistance, and (iii) genes coding for factors for which exist antibodies for IHC analysis. Selection of differentially expressed genes will be done by removing genes with low fold-changes (< 1.3 in suboptimal vs. optimal) and ranking the remaining genes by univariate p-value. We will calculate the risk score for each signature using the following equation: $s = \sum_i w_i x_1 / \sum_i |w_i|$ where w is the weight +1 or –1 (upregulated or downregulated gene, respectivley), and x is the normalized gene expression level. The scores are used to construct the ROC curves and compute the areas that fall under the curves (AUC) with 95% confidence intervals. Signatures with an AUC of at least 0.8 will be selected and compared to the POSTN gene alone to ensure added advantage of additional genes. To assess whether alternative methods can improve classification accuracy, statistical learning approaches such as support vector machine and tree-based methods will be evaluated for their performance in predicting debulking status, using genes from the 198-gene signature as well as the 20-gene compact signatures.

C1.3. Protein IHC analysis: To further validate the relevant genes composing our proposed 20-gene signature(s) we will analyze the protein levels of markers in our signatures by IHC. To validate activity of the TGF-β signaling we will also include its surrogate phosphorylated marker pSmad2/3 (Ser465, Fig. 2B & C). To guarantee horizontal reproducibility of our signature, protein analysis will be done on an independent 1307-sample TMA of late stage EOCs constructed by our collaborators at the British Columbia Cancer Agency

(Canada), which contains one retrospective cohort with 612 optimally debulked and 250 suboptimally debulked samples (22) and one prospective cohort of 445 samples with known status of residual disease. In addition to the comprehensive information of the patient characters, the expression level of 21 and tissue-based biomarkers (CA125, CRABP-II, EpCam, ER, F-Spondin, HE4, IGF2, K-Cadherin, Ki-67, KISS1, Matriptase, Mesothelin, MIF, MMP7, p21, p53, PAX8, PR, SLPI, TROP2, WT1) are also available for this TMA. IHC evaluation will be performed blindly. A weighed score will be obtained for each sample by multiplying the intensity of the staining and the percentage of positive cells as previously described (23, 24). Statistical analysis will be performed same as for the NanoString studies using the weighed IHC scores as variables.

C1.4. Expected results and alternatives: We expect that the NanoString validation of our debulking signature will reduce the number of candidate genes and provide a more selective signature predictive of surgery outcome with an accuracy of more than 80%.The thresholds parameters for gene selection (fold-changes and univariate p values) were set empirically as a starting point and may be adjusted to decrease the total number of signature genes. Based on our experience (13), we expect that the 20-gene signature will generate an AUC larger than 0.5 and outperform the single genes in the signature to predict surgical outcome. The gene list can be further optimized by looking for single genes with large AUCs or by increasing the number of genes in the signature. Through IHC analysis we will develop a clinically applicable, biomarker predictive panel with accuracy over 90%. We may obtain some false negative results due to technical difficulties that may be encountered in protein analysis (i.e. degraded proteins, masked antigens, lack of available antibodies). However, the goal is not to obtain the largest possible number of markers, but on the contrary to identify a small number of markers (3-10) with high prediction value (i.e. whose expression is detected in a large number of suboptimally debulked tumors). Our preliminary data suggest that we may already have 2 valid biomarkers, however, if further validation will not support sufficient predictive power, additional stratification of the tumors will be performed using methylation profiles and analysis of HR deficiency; both are hallmarks of EOC.

C2.Preclinical testing of new approaches for targeted therapy of suboptimal debulked tumors

C2.1.Rationale: Our preliminary data indicate that hyperactivation of the TGF-β pathway confers a highly-disseminative phenotype impeding optimal debulking (Fig. 1,2 & 4) and produces chemoresistance which is prevalent in suboptimally debulked EOCs (Fig. 3). These observations make TGF-β a promising therapeutic target against suboptimal debulked EOC. While several TGF-β targeting approaches have emerged, including LY2157299 (Eli Lilly) (25, 26) and Fresolimumab (Sanofi) (27, 28) which have entered into Phase II oncology clinical trials, their potential anti-tumor efficacy has never been tested in ovarian cancer (29, 30). We aim to test TGF-β inhibition as a novel targeted therapy to be applied in a neo-adjuvant setting and increase the rate of optimal debulking, or used as adjuvant therapy to improve prognosis of sub-optimally debulked patients.

C2.2.Tumor Models and TGF-β inhibitors: Orthotopic murine xenograft models will be used to test the anti-tumor efficacy of TGF-β inhibition against EOC. *In vivo* models have the advantages to interrogate the tumor-host interaction that may modulate the dissemination and chemoresistance of EOC cells (31). This is especially important for a pleiotropic pathway like TGF-β, which can reshape the host microenvironment to niches fostering both primary lesions and metastases (32, 33). Several TGF-β responsive genes in the "debulking signature" such as *POSTN*, *FAP* and *LOX* are markers of desmoplastic activation of tumor-associated fibroblasts (TAFs), which may induce the extracellular matrix to form a physical barrier that reduces the bioavailability of therapeutic reagents while also providing pro-survival signaling to the tumor cells. Moreover, TGF-β signaling has also been characterized for its immunosuppressive property (32, 34). Thus, to maximize the clinical relevance this project will utilize: **1)** A patient derived xenograft (PDX) model generated from EOC cells deriving from the ascitic fluid of patients affected by recurrent, platinum/taxane resistant serous EOC that was sub-optimally debulked (OC-16 or OC-30, Fig. 4A). The PDXs were generated and are maintained using immuno-compromised (SCID, SHO) mice. **2)** The mouse syngeneic model (MOC-1) derived from a spontaneous EOC originated in $Pten^{-/-}/Tp53^{(R172H)}$ double-inactivated mice (35). Both the PDX and the murine ovarian cancer cell line have been confirmed for the Müllerian epithelial origin (PAX8 and Cytokeratin 7 positive) and EOC genetic identity (DNA fingerprint and NGS based CNV analysis). While the PDXs allow testing sensitivity to TGF-β inhibitors of cancer cells that preserve the genomic background of patients' tumors, the syngeneic model allows evaluating the contribution of potential immune mechanisms in the specific treatment. All models were characterized for baseline TGF-β activity (Fig. 4A and 4B).

Three approaches will be used for TGF-β inhibition. Systematic TGF-β targeting in xenograft bearing mice will be achieved by LY2157299 and Fresolimumab. LY2157299 is a small molecule inhibitor of TGF-β receptor I (TGFBR1) that blocks canonical TGF-β signaling (25, 26) and non-canonical signals relayed from the upstream TGFBR2 to modulate the canonical signaling (Fig. 4B). Fresolimumab is a humanized antibody

neutralizing all three TGF-β ligands; an equivalent clone **1D11** able to sequester TGF-β1/2/3 from both human and murine origin will be used in our murine models (27, 28). We further propose a third approach by generating a TGFBR2-disrupted derivative through CRISPR technology (Fig. 4F-G, $TGFBR2^{CRISPR}$). Compared to systematic TGF-β inhibition, which nullifies the TGF-β signaling in both tumor and stroma components, $TGFBR2^{CRISPR}$ still preserves intact TGF-β signaling in the stroma. The derived data will therefore highlight the biological significance of microenvironmental TGF-β signaling on tumor progression and provide the rationale to extend systematic TGF-β inhibition to EOC of which the epithelial component is defective of TGF-β signaling.

C2.3.Research Design: EOC cells will be labeled with firefly luciferase through lentiviral transduction to facilitate real-time, non-invasive bioluminescent imaging (BLI) of *in vivo* tumor growth and distribution. TGFBR2-null derivatives will also be generated by CRISPR from the labeled cell lines. Orthotopic intraperitoneal (IP) and intrabursal (IB) xenografts will be established in SCID mice (for patient derived EOC cells) or immunocompetent C57BL/6 mice (for murine EOC cells). The IB model allows localized tumor establishment and mimics the dissemination of ovarian cancer from a primary lesion, whereas the IP model causes formation of multiple tumor lesions disseminated throughout the peritoneum, thus mimicking patients' advanced stage cancer at time of diagnosis. We calculate a minimal size of 8 based on the *in vivo* data from SKOV3-$TGFBR2^{CRISPR}$ (Fig. 4F-G), to have 80% chance of observing a 50% difference in mean tumor volume between groups at significance of 0.05. Treatment will be started after the establishment of the xenografts.

For the **IB model** which interrogates the anti-disseminative effect of TGF-β inhibition, four study groups will be designed: 1) wild-type cells without treatment (control); 2) $TGFBR2^{CRISPR}$ group; 3) LY2157299 group (100 mg/kg, p.o. daily) and 4) Fresolimumab 1D11 (100 mg/kg, IP daily). Inhibition of tumor dissemination will be monitored through BLI imaging (total BLI reading minus BLI from the primary tumor defined as the signal from the right dorsal flank area) as well as pathological examination of tumors after sacrifice. For the **IP model** which interrogates the chemo-sensitizing effect of TGF-β inhibition, mice receiving one of the above four anti-TGF-β treatment (n=24 each for untreated, $TGFBR2^{CRISPR}$, LY2157299 and Fresolimumab 1D11) will be further randomized into 3 groups (n=8 each) to receive concurrent cytotoxic treatment of 1') vehicle; 2') cisplatin (2mg/kg, i.p. every 3d); and 3') paclitaxel (10mg/kg, i.v. weekly). Tumor burden by BLI imaging or gross weight after sacrifice will be used as read-out of potential sensitization of cisplatin/paclitaxel by TGF-β inhibition. We will perform IHC to delineate potential mechanisms involved in anti-TGF-β mediated tumor inhibition. The biochemical efficacy of TGF-β inhibitory reagent and cytotoxic reagents will be evaluated by IHC staining of phosphor-Smad2/3 and cleaved PARP (or cleaved cytokeratin-18) respectively. IHC will also be performed on both primary xenograft and metastases to check the levels of biomarkers for proliferation (Ki-67), angiogenesis (CD31), and desmoplastic stromal/TAF activation (FAP or α-SMA). Intratumoral infiltration of CTLs and tumor-radical immune cells will also be investigated by CD8 and Granzyme B respectively.

Finally the IP model will be used to mimic efficacy of NACT-IDS compared to upfront debulking surgery. Specifically, mice with disseminated IP tumors will be divided in 2 cohorts: 1) mice receiving pharmaceutical TGF-β inhibition before being subject to debulking surgery and further treated with cisplatin after the surgery, 2) mice undergoing upfront debulking surgery, followed by chemotherapy, without prior TGF-β inhibition. Tumor recurrence and growth following the debulking surgery will be monitored by BLI in both cases until animals are sacrificed. Progression-free and overall survival will be compared between the 2 groups.

C2.3.Expected results and alternatives: All the *in vivo* techniques described herein are standard procedures used in our laboratory (23, 24). We do not expect any difficulties in growing tumors as these models were previously tested in our lab for tumor growth. We expect that TGF-β inhibition will decrease tumor dissemination, and increase tumor sensitivity to cisplatin/taxol, which ultimately will lead to prolonged survival. We also expect phenotypical modulation of stroma such as decreased desmoplastic TAF activity, normalized angiogenesis and increased CTL density. Both LY2157299 and 1D11 were previously tested in animal models of other tumors, thus we do not expect difficulty inhibiting the TGF-β signaling with these reagents, however penetration of these drugs in disseminated models of EOC was never tested. If the drugs will not be able to penetrate effectively our tumor models, we expect however to observe some effects with the use of tumor cells edited through CRISPR-mediated TGF-β inhibition. However, this mechanism of TGF-β inhibition does not take in consideration the effect of stromal TGF-b activity. To overcome this problem, we may consider using a combination of CRISPR-edited cells and pharmaceutical inhibition. We chose to test inhibition of TGF-β as this pathway was top ranked in our debulking signature and a potentially good target to proof our hypothesis that biomarkers from the debulking signature that can be used as specific targets for suboptimally debulked tumors in neoadjuvant therapy. Other potential targets include integrin signaling (Cilengitide) or BMP signaling (Dovitinib) and can be tested individually or in combination with TGF-β inhibitors.

Authentication of Key Biological and/or Chemical Resources

Tumor models: For this project we will use two types of tumor models established in mice: 1) immortalized ovarian cancer cells derived from tumors originated in immunocompetent $Pten^{-/-}$/$P53^{(R172H)}$ double-mutant C57BL/6 mice from which the murine ovarian cancer cell line MOC-1 was derived, 2) patients derived xenografts (PDXs) generated by our lab from patients that were sub-optimally debulked and that present activation of the TGF-β signaling. All our models have been confirmed for the Müllerian epithelial origin (PAX8 and Cytokeratin 7 positive), genetic identity (through DNA fingerprint and NGS based CNV analysis) and tumorigenicity in mice.

We do not expect significant changes in these models throughout the short period of this award as we will be using frozen, low passage aliquots of the murine cell line and frozen, single use only, PDX samples collected and tested after the first growth passage in mice. However, our models can be authenticated through immunohistochemistry analysis of PAX8 and Cytokeratin 7 and CNV analysis.

We will use only commercially available mice purchased from Charles River laboratories. These include: C57 black 6 mice to grow the murine tumor model, and SCID SHO mice to grow the PDXs.

Immunohistochemistry: For the immunohistochemistry analysis we will use commercially available antibodies. These include: 1) the antibody targeting the phosphorylation marker pSmad2/3 (as a surrogate marker of TGF-β pathway activation) that was used in our preliminary results (Cell Signaling), and 2) additional antibodies that will be used as determined from the results of the experiments described in Aim 1.

Gene expression (NanoString) analysis: we will use customized probes designed and produced by NanoString Technologies for each gene of the debulking signature that will be validated in Aim 1.

TGF-β inhibition Systematic TGF-β targeting in xenograft bearing mice will be achieved by LY2157299 (Eli Lilly& Co.) and Fresolimumab (Genzyme). LY2157299 is a small molecule inhibitor of TGF-β receptor I (TGFBR1). Fresolimumab is a humanized antibody neutralizing all three TGF-β ligands. Both these drugs have been used in clinical trials for other cancers and will be provided by the respective companies. For adaptation into murine system, an equivalent clone **1D11** which is capable of sequestering TGF-β1/2/3 from both human and murine origin will be used herein and provided by Genzyme.

TGF-β will be also inhibited by editing the celsl through the CRISPR system including a plasmid expressing CAS9 to be used in combination with a Guide RNA plasmid. Both these plasmids are commercially available from AddGene. The TGF-β-specific oligos were designed in house and cloned into Guide RNA plasmid. The sequence of these oligos will be made public upon publishing the results.