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Justin W. Gorski
University of Kentucky, justin.gorski@gmail.com
Author ORCID Identifier:
https://orcid.org/0000-0002-4272-0183
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Justin W. Gorski, Student

Dr. Jill M. Kolesar, Major Professor

Dr. Claire Clark, Director of Graduate Studies

UTILIZING PATIENT-DERIVED EPITHELIAL OVARIAN CANCER TUMOR ORGANOIDS TO PREDICT CARBOPLATIN RESISTANCE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Justin Wayne Gorski
Lexington, Kentucky
Director: Dr. Jill M. Kolesar, Professor of Pharmacy
Lexington, Kentucky
2021

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https://orcid.org/0000-0002-4272-0183

ABSTRACT OF DISSERTATION

UTILIZING PATIENT-DERIVED EPITHELIAL OVARIAN CANCER TUMOR ORGANOIDS TO PREDICT CARBOPLATIN RESISTANCE

The development of patient-derived tumor organoids (TOs) from epithelial ovarian cancer tumor obtained at the time of primary or interval debulking surgery has the potential to play an important role in precision medicine.

Here, we utilize TOs to test front-line chemotherapy sensitivity and to investigate genomic drivers of carboplatin resistance. We developed six high grade serous epithelial ovarian cancer tumor organoids from tissue obtained during debulking surgery (2 neoadjuvant carboplatin exposed, 4 chemo-naïve). Each organoid line was screened for sensitivity to carboplatin at four different doses (100, 10, 1 and 0.1μM). Cell viability curves and resultant EC50 values were determined after normalizing to untreated negative controls.

One organoid line, UK1254, was predicted to be resistant to carboplatin based on EC50 value (50.2 μM) above clinically achievable Cmax. UK1254 had a significantly shorter progression free survival (PFS) than the rest of the subjects (P=0.0253) and was treated as a platinum-resistant recurrence. Subsequent gene expression analysis revealed extensively interconnected differentially expressed pathways related to NF-kB, cellular differentiation (PRDM6 activation) and linkage of B-cell receptor signaling to the PI3K-Akt signaling pathway (PI3KAP1 activation).

This study demonstrates that patient derived tumor organoids can be developed from patients at the time of primary or interval debulking surgery and possibly can be used to predict clinical platinum sensitivity status or to investigate drivers of carboplatin resistance.

KEYWORDS: Ovarian Cancer; Tumor Organoids; Chemotherapy Resistance; Carboplatin; Integrated Genetic Analysis

Justin Wayne Gorski

(Name of Student)

09/23/2021

Date

UTILIZING PATIENT-DERIVED EPITHELIAL OVARIAN CANCER TUMOR ORGANOIDS TO PREDICT CARBOPLATIN RESISTANCE

By
Justin Wayne Gorski

Dr. Jill M. Kolesar
Director of Dissertation

Dr. Claire Clark
Director of Graduate Studies

09/23/2021
Date

DEDICATION

To my late Nana, Jessie R. Seege (née Wadrzyk).
Your strength in the face of the loss of your daughters will not be forgotten.

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The following dissertation, while an individual work, benefited from the insights and direction of several people. First, my Dissertation Chair, Jill M. Kolesar, PharmD, MS exemplifies the high-quality scholarship to which I aspire. This work would not have been possible without her ever-present focus on mentorship and dedication to the education of the next generation of scientists. Next, I would like to thank B. Mark Evers, MD for acquiring funding from the National Institute of Health which helped finance this project. This work would not have been possible with his leap of faith to bring me on to his T32 grant as the first postdoctoral fellow from the Division of Gynecologic Oncology. Next, I wish to thank the complete Dissertation Committee, and outside reader, respectively: Frederick R. Ueland, MD, Christine Fillmore Brainson, PhD, Thomas H. Kelly, PhD, Chi Wang, PhD, and Nathan Vanderford, PhD. Each individual provided insights that guided and challenged my thinking, substantially improving the finished product. In particular, I would like to acknowledge Dr. Ueland for his trust, belief in my abilities and ever-present wisdom. My path to becoming a gynecologic oncologist would not have been possible without his foresight and mentorship.

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CHAPTER 1. INTRODUCTION

1.1 Background

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancy in the United States. In 2021, it is estimated that 21,410 women will be diagnosed with ovarian cancer and that it will be responsible for 13,770 deaths in the U.S. [1]. The high mortality rate is primarily due to the predominance of late stage detection and the high rate of recurrence due to chemotherapy resistance.

The current standard of care treatment for advanced stage disease includes surgical debulking, with a goal of removing all macroscopic disease (R0 cytoreduction), in combination with platinum-based neoadjuvant or adjuvant chemotherapy [2]. Despite this aggressive front-line treatment more than 80% of patients will recur [3]. Historically, the platinum free interval (PFI; time from the last dose of front-line adjuvant platinum-based chemotherapy to the detection of recurrence) is used to classify patients into platinum sensitive, resistant and refractory groups [4]. These groups have considerably different clinical outcomes. "Platinum sensitive" individuals (PFI ≥6 months) comprise approximately 75% of recurrences and have a median overall survival (OS) of 24-36 months. Conversely, 15% of patients are "platinum resistant" (PFI <6months) with a median OS of only 9-12 months. Disease in "platinum refractory" individuals progresses during treatment and makes up about 10% of recurrences. This group suffers the worst outcome with a median OS of 3-5 months [5-6]. For the clinician, the choice of treatment in the platinum resistant or refractory setting is difficult. Response rates to non-platinum based cytotoxic chemotherapy are similar and overall quite poor: topotecan (20%),

gemcitabine (19%), liposomal doxorubicin (26%), oral etoposide (27%), docetaxel (22%), weekly paclitaxel (21%) [2].

1.2 Role of Chemosensitivity Assays

Despite the dramatic difference in outcomes between platinum sensitive, resistant and refractory groups there is not a validated method to predict clinical response to platinum-based chemotherapy and all individuals receive the same up-front therapy. Chemosensitivity assays using patient derived tumor cells have been increasingly explored to satisfy this unmet clinical need. In fact, some National Comprehensive Cancer Network (NCCN) centers employ the use of chemosensitivity assays to guide management in the face of recurrence when there are multiple equivalent chemotherapy options available [2]. Early investigations primarily using extreme drug resistance assays [7-8] or phenotypic drug response assays [9] which were initially promising but have failed to produce sufficient evidence of efficacy to change the standard of care or warrant reimbursement [10]. However, patient derived tumor organoid (TO) chemosensitivity assays have recently emerged as a more accurate model of in vivo tumor biology [11] and have shown promise to predict clinical chemotherapy response in vitro [12].

1.3 Primary and Secondary Objectives

Here, we developed and validated six patient derived epithelial ovarian cancer TO lines which were subsequently screened for sensitivity to front line standard of care chemotherapeutic agents. TO genetic sequencing was used to identify genomic determinants of carboplatin resistance. Our primary objective was to assess the ability of

TOs to predict clinical outcomes to initial chemotherapy. Secondary objectives included identification of an integrated genomic signature of platinum resistance in EOC.

CHAPTER 2. MATERIALS AND METHODS

2.1 Research Subjects

Women with suspected or histologically confirmed epithelial ovarian with a plan to undergo cytoreductive surgery were eligible for study inclusion. All patients who were potentially eligible were approached for enrollment by trained clinical research staff during their pre-operative clinic visit which occurred one to four weeks before the scheduled debulking surgery. Written informed consent was obtained from all subjects and they were enrolled in the Total Cancer Care Protocol: A Lifetime Partnership with Patients Who Have or May be at Risk of Cancer (MCC 17-MTB-01, UK IRB #44224). Clinical outcome data was collected prospectively, deidentified and correlated with TO chemosensitivity assay and genomic data by an honest broker. Disease assessment were performed per routine clinical practice by the treating provider to assess progression free survival (PFS). Patient outcomes were followed until all patients demonstrated clinical evidence of recurrence or progression as defined by RECIST version 1.1 criteria [13]. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Kentucky.

2.2 Tumor Organoid (TO) Development and Validation

Fresh ovarian tumor tissue was obtained from patients at the time of debulking surgery, dissociated into a single ovarian cancer cell suspension, and established in Matrigel® Growth Factor Reduced Basement Membrane Matrix (Corning) *in vitro* using factor defined media [14-15]. TOs were passaged at least two times to eliminate stromal

cells by digesting the Matrigel® matrix with trypsin-EDTA/TrypLE followed by gentle mechanical dissociation. Once ovarian cancer TOs were established, the TOs were fixed, representative sections were H&E stained and compared with primary tumor by a board certified pathologist.

2.3 Chemosensitivity Screens

Established TOs were enzymatically dissociated into single cells, and plated in 384 well plates. The cells were cultured for 72 hours prior to administration of carboplatin at five different doses (0, 100, 10, 1 and 0.1μM). After culturing for an additional 72 hours, organoids were incubated with Hoescht nuclear counterstain and imaged on a spinning disc confocal high content imager. After imaging was completed, viability was measured by employing the MTS assay (Promega). Raw data were generated in triplicate, and the average cell viability for each drug concentration was determined after normalizing values to untreated negative controls. Cell viability curves were generated, and EC50 values were determined.

2.4 Sequencing Methods

The Tempus xT next generation targeted oncology sequencing assay was utilized to perform a gene mutation and expression analysis for all six generated TO cell lines. TO total nucleic acid was extracted and digested by proteinase K. RNA was purified from the total nucleic acid by DNase-I digestion. DNA and RNA sequencing was performed as previously described [16]. Briefly, 100ng of DNA for each TO sample was mechanically sheared to an average size of 200 base pairs (bp) using a Covaris ultrasonicator. DNA

libraries were prepared using the KAPA Hyper Prep Kit, hybridized to the xT probe set, and amplified with the KAPA HiFi HotStart ReadyMix. 100ng of RNA for each tumor sample was heat fragmented in the presence of magnesium to an average size of 200 bp. Library preps were hybridized with the IDT xGEN Exome Research Panel and target recovery was performed using Streptavidin-coated beads, followed by amplification with the KAPA HiFi Library Amplification Kit. The amplified target-captured DNA tumor libraries were sequenced to an average unique on target depth of 500x on an Illumina HiSeq 4000. Samples were further assessed for uniformity with each sample required to have 95% of all targeted bp sequenced to a minimum depth of 300x [17].

2.5 Gene Mutation and Gene Expression Bioinformatic Analysis

For somatic mutation analysis, an oncoplot was generated based on the maftools [18] package to visualize non-silent somatic mutations in DNA repair genes. For gene expression analysis, genes that were unexpressed or lowly expressed in all samples (no sample with counts per million mapped reads (CPM) > 1) were excluded from analysis. The differential expression analysis of the carboplatin-resistant versus carboplatin-sensitive groups was performed using the edgeR package [19]. Significantly differentially expressed genes were identified based on a threshold of false discovery rate (FDR) < 5% and annotated for gene ontology terms. A volcano plot was generated for results visualization. All these analyses were performed using R 4.0.3. The pathway enrichment and network analysis were performed using Qiagen's Ingenuity Pathway Analysis (IPA) system for the core analysis of the RNA sequencing data and overlaid with the Global Molecular Network Overlay in the IPA knowledge base.

2.6 Statistical Analysis

The classification of each TO cell line as carboplatin-sensitive or carboplatin-resistant was based on the comparison of the carboplatin EC50 value to the clinically achievable plasma concentration of carboplatin. Resistant cell lines were defined as having a carboplatin EC50 above the plasma Cmax of carboplatin [20]. Sensitive TO cell lines had a carboplatin EC50 within achievable plasma concentrations. One sample t-test was used to compare carboplatin-resistant and pooled carboplatin-sensitive EC50 values using GraphPad Prism 8. The Kaplan–Meier method was used to estimate PFS curves for platinum-sensitive and carboplatin-resistant patients. The PFS curves were compared via the log-rank test using R 4.0.3. A $p < 0.05$ was considered as statistically significant.

CHAPTER 3. RESULTS

3.1 Subject Demographic and Treatment Characteristics

The tumor samples used to generate the TO lines were derived from a relatively homogenous population. All subjects had histologically proven advanced-stage epithelial ovarian or fallopian tube cancer. Histologic subtype was exclusively high-grade serous. Primary disease sites were localized to the ovary (75%) and fallopian tubes (25%). One TO line (UK1393) was generated from a metastatic implant in the omentum, but all others were developed from the primary site of disease (Table 1).

Table 1. Demographic characteristics of included subjects.

ID	Age	TNM Stage	FIGO Stage	Primary Site	Histology	Grade
UK1236	48	ypT3cN0M1	IIIC	Ovary	Serous	3
UK1254	49	ypT3cNX	IIIC	Ovary	Serous	3
UK1267	55	T2bN0	IIB	Fallopian Tube	Serous	3
UK1393	46	T3cNX	IIIC	Ovary[1]	Serous	3
UK2238	58	T3aN1b	IIIA	Fallopian Tube	Serous	3
UK2326	62	T3cNX	IIIC	Ovary	Serous	3

[1] TO developed from metastatic omentum implant.

Treatments courses of the study subjects were also relatively homogenous. All participants were treated with a platinum and taxane doublet. One subject's taxane therapy (UK1236) was switched from paclitaxel to abraxane due to allergic reaction. Most subjects were chemonaïve (66.7%) at the time of debulking surgery. However, two subjects (33.3%) were exposed to three cycles of neoadjuvant chemotherapy before interval debulking surgery. Optimal cytoreduction was achieved in 50% of patients and all other debulking surgeries achieved <0.5cm of residual disease. Most patients did not receive maintenance therapy. However, one patient received olaparib and another was enrolled in a Gynecologic Oncology Group (GOG) clinic trial studying the effects of the PARP inhibitor rucaparib and immunotherapy agent nivolumab [21]. It is uncertain if the patient received study drugs or placebo (Table 2).

Table 2. Treatment courses of enrolled subjects.

ID	Residual Disease (cm)	Neoadjuvant	Adjuvant	Maintenance
UK1236	0	carboplatin & paclitaxel[1] x1 cycle; carboplatin & abraxane x 2 cycles	carboplatin & abraxane x3 cycles	None
UK1254	<0.5	carboplatin & paclitaxel x 3 cycles	carboplatin & paclitaxel x 3 cycles	GOG 3020: rucaparib v. placebo & nivolumab v. placebo
UK1267	0	None	carboplatin & paclitaxel x 6 cycles	None
UK1393	0	None	carboplatin & paclitaxel x 6 cycles; bevacizumab cycles 2-6	None
UK2238	<0.5	None	carboplatin & paclitaxel x 6 cycles	olaparib
UK2326	<0.5	None	carboplatin & paclitaxel x 6 cycles	None

[1] Carboplatin (AUC = 6) and paclitaxel (175 mg/m2) IV every 21 days was used as the standard dosing regimen.

3.2 Tumor Organoid Validation

Histologic concordance between each ovarian cancer TO cell line and its respective primary ovarian cancer tumor sample was confirmed. After the establishment of the TO cell line, a sample of it was formalin-fixed and stained using hematoxylin and eosin (H&E) (Figure 1).

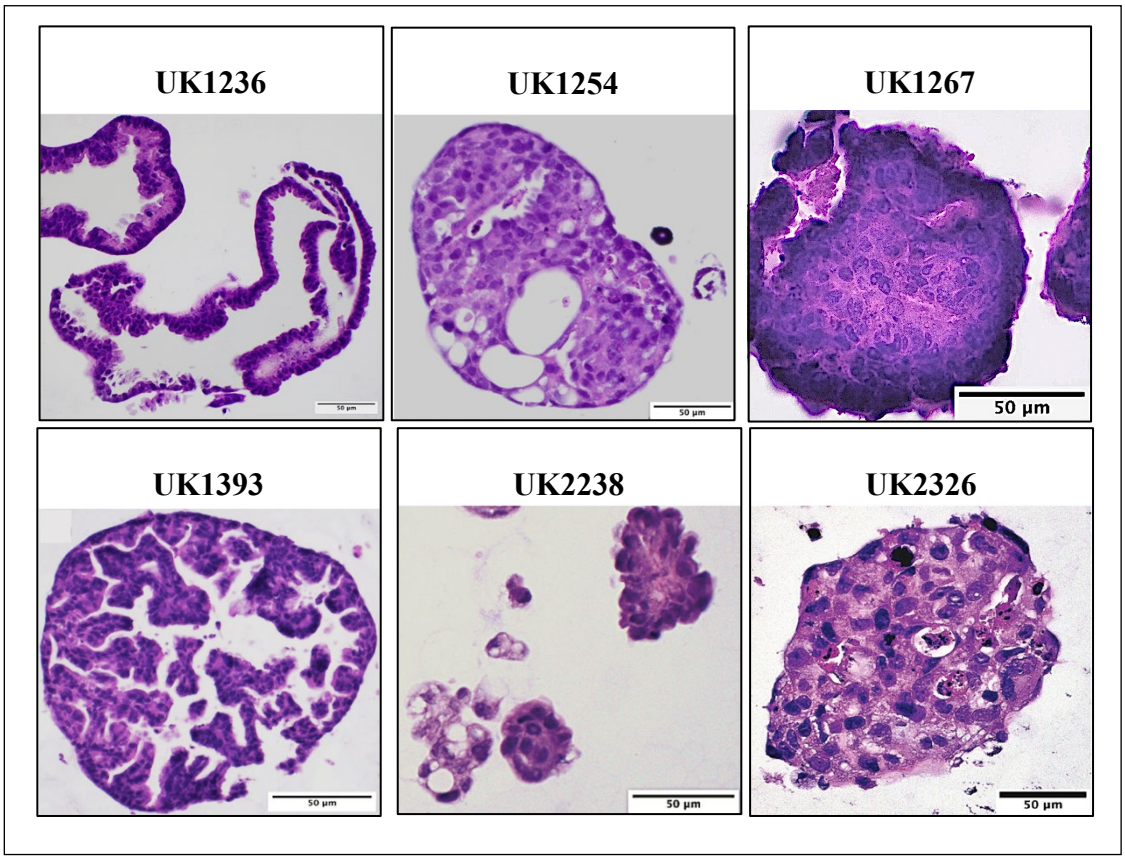


Figure 1. Hematoxylin and eosin (H&E) micrographs of established epithelial ovarian cancer tumor organoids.

Primary tumor samples for each established cell line were also formalin-fixed and H&E stained. The TO sample and respective tumor sample were compared. All TO lines were determined to be similar to their respective parental tumor samples after examination by a board-certified pathologist.

3.3 Chemosensitivity Screens

Cell viability curves and resultant EC_{50} values were determined for all generated TO lines (Table 3).

Table 3. Cell viability EC_{50} values for each TO cell line when treated with carboplatin and subject progression free survival (PFS).

ID	Carboplatin EC_{50} (µM)	PFS (days)
UK2326	0.8	398
UK1267	1.1	338
UK2238	3.3	391
UK1236	28.5	579
UK1393	44.8	445
UK1254	50.2[1]	252

[1] Above clinically achievable plasma Cmax.

The mean EC_{50} value for UK1254 exceeded achievable plasma carboplatin Cmax (50 µM) and was the highest of all TO line EC_{50} values. Conversely, all other TO lines were determined to be sensitive to carboplatin, with a significantly lower pooled cell viability EC_{50} mean value ($p = 0.018$). All carboplatin-sensitive TO cell lines demonstrated EC_{50} values within the range of achievable plasma concentrations (Figure 2).

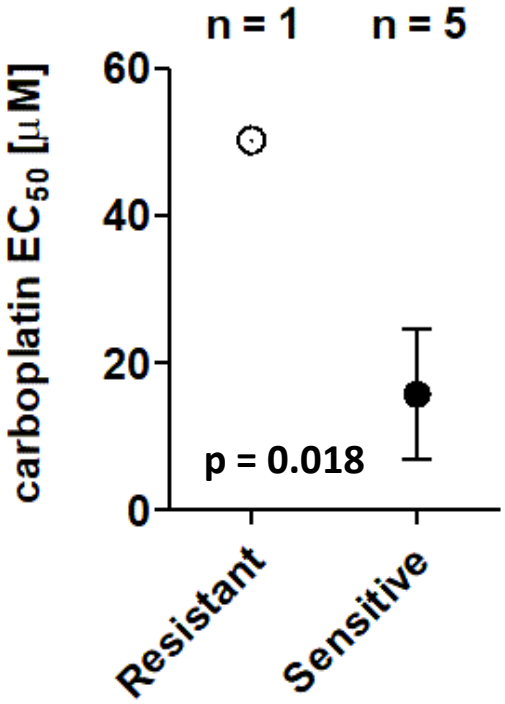


Figure 2. Scatter plot demonstrating cell viability EC_{50} mean ± SEM for the carboplatin resistant (UK1254) and pooled carboplatin sensitive TO lines when treated with carboplatin. One sample t-test was used to compare resistant and sensitive EC_{50} values (p=0.018).

3.4 Clinical Outcomes

The number of days from completion of adjuvant chemotherapy until recurrence or progression as demonstrated by RECIST criteria was used to determine each subject's progression free survival (PFS) (Table 3). UK1254 had a significantly shorter PFS than the rest of the subjects with a p = 0.025 (Figure 3).

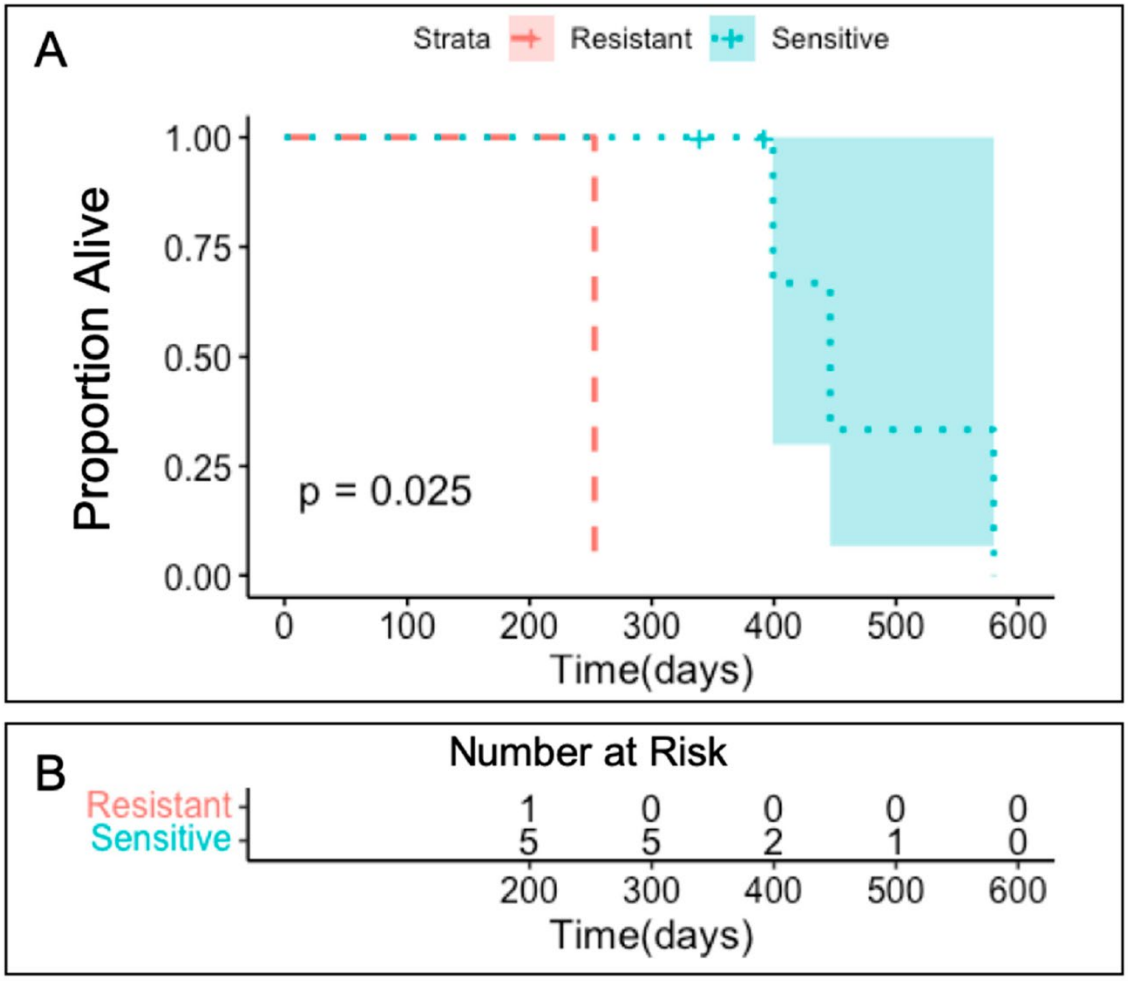


Figure 3. (A). Kaplan Meier Survival plot demonstrating progression free survival (PFS) of enrolled subjects when stratified by carboplatin TO chemosensitivity assay results. Platinum Resistant (red): UK1254. Platinum Sensitive (blue): UK1236, UK1267, UK1393, UK2238 and UK2326. (B.) Plot demonstrating number of subjects at risk for progression in platinum resistant and sensitive groups.

Thus, clinical outcomes directly correlate with TO cell viability chemosensitivity assay results.

3.5 Tumor Organoid Mutation Analysis

A limited mutation analysis was performed for all generated TO lines using the Tempus xT gene panel. Genes that were mutated in multiple cell lines and a selection of DNA repair genes were specifically interrogated to explore similarities in mutation profiles between TO cell lines for all enrolled subjects (Figure 4).

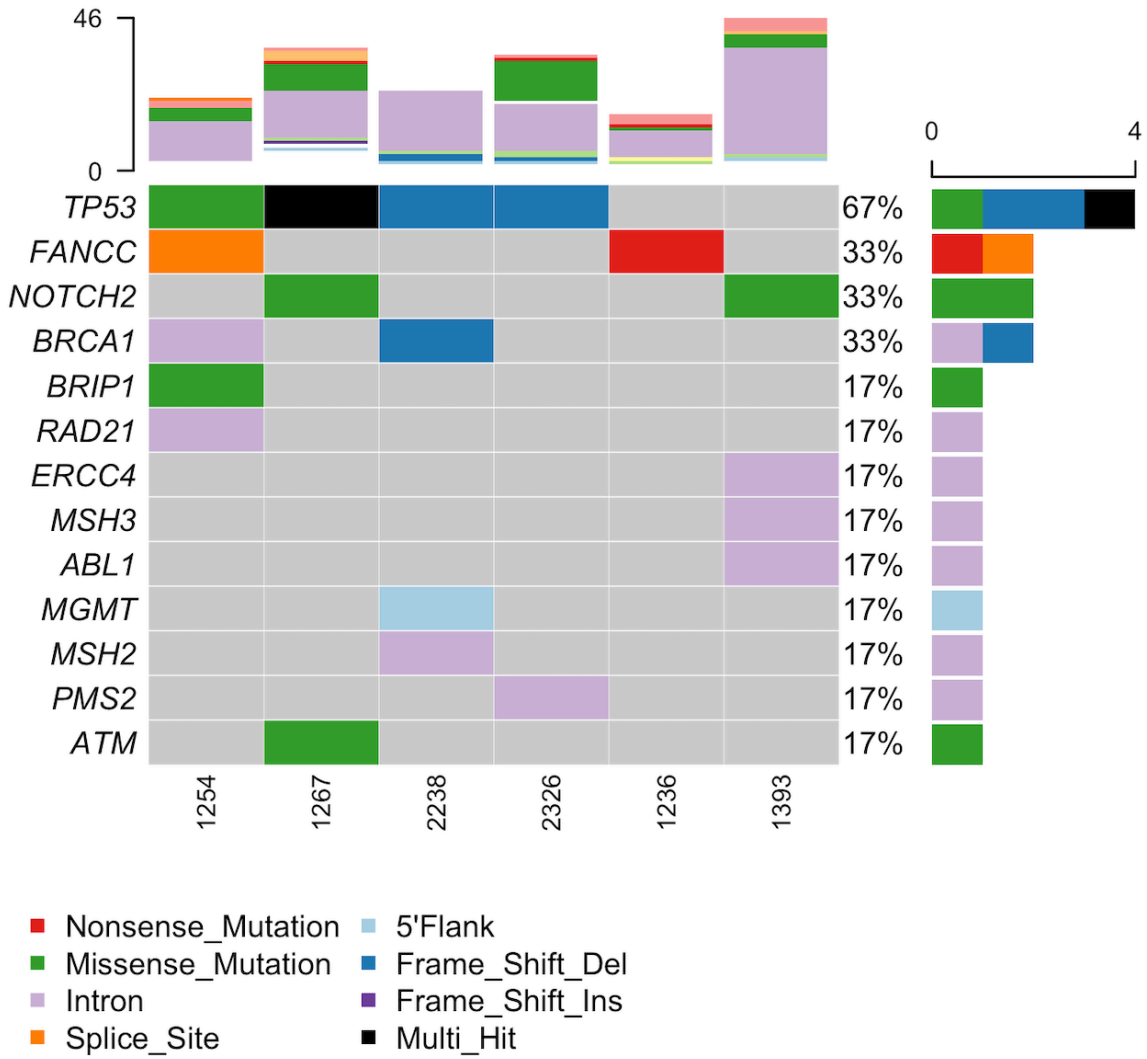


Figure 4. Oncoplot demonstrating the top mutated genes for all TO cell lines and select DNA repair genes. Carboplatin Resistant: UK1254. Carboplatin Sensitive: UK1267, UK2238, UK2326, UK1236 and UK1393.

As expected in high grade serous ovarian cancer (HGSOC), the most commonly mutated gene was TP53 (4/6, 67%). The second most commonly altered genes were FANCC (2/6, 33%) which is a critical component of the Fanconi Anemia core complex [22], and NOTCH2 (2/6, 33%) which is a key part of the Notch signaling pathway that controls normal morphological development of multicellular organisms. Genomic mutations in the DNA repair genes were mostly relegated to intron alterations but also notably include a BRCA1 frame shift deletion mutation in UK2238, BRIP1 missense mutation in UK1254 and an ATM missense mutation in UK1267.

3.6 Tumor Organoid Gene Expression Analysis

We then performed a gene expression analysis using the RNA sequencing data in Qiagen's Ingenuity Pathway Analysis (IPA) to evaluate differences in expression and pathways to better understand the mechanism of carboplatin resistance. All subjects had TO RNA sequencing data available. A total of 71 genes were significantly differentially expressed (FDR values < 0.05) between the carboplatin resistant TO and the carboplatin sensitive TOs after appropriate thresholds were applied. Genes that met significance cutoff criteria are represented in blue. Genes eliminated after thresholds were applied are represented in red (Figure 5).

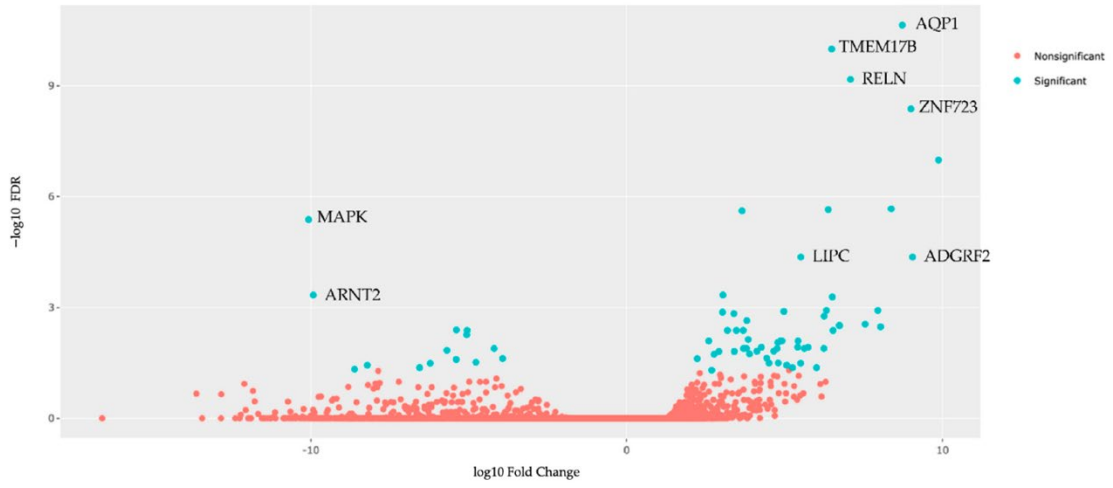


Figure 5. Volcano plot of the 71 differentially expressed genes identified when the carboplatin resistant TO line (UK1254) is compared to the carboplatin-sensitive TO lines (UK1236, UK1267, UK1393, UK2238, and UK2326).

The top upregulated and downregulated differentially expressed genes comparing the carboplatin-resistant group to the carboplatin-sensitive group are displayed in Table 4. In the top ten upregulated genes, many are involved in transmembrane transport, cellular differentiation, and immune response modulation. In the top ten downregulated genes, many are involved in regulation of cellular growth, cellular stress response, and lipid metabolism. Notably, TMEM178B is not yet linked with an established biological pathway identifier and may represent a novel finding. Note, a complete list of differentially expressed genes is available in the appendix.

Table 4. Top differentially expressed genes comparing the carboplatin-resistant group to the carboplatin-sensitive group. (A) Upregulated pathways in carboplatin-resistant TO compared to the carboplatin-sensitive TO group. (B) Downregulated pathways in carboplatin-resistant TO compared to the carboplatin-sensitive TO group.

A. **Upregulated**					
Gene	**LogFC**	**P Value**	**QValue (FDR)**	**Pathway ID**	**Pathway Description**
1. AQP1	8.722968	1.46E-15	2.26E-11	GO:0022857	Transmembrane transport activity
2. TMEM178B	6.489275	1.30E-14	1.01E-10	---	---
3. RELN	7.083244	1.29E-13	6.68E-10	GO:0030154	Cell dedifferentiation
4. ZNF723	8.998623	1.08E-12	4.20E-09	GO:0003700	DNA Binding transcription factor activity
5. HAVCR1	9.870356	3.29E-11	1.02E-07	GO:00023676	Immune system process
6. FXYD2	8.374937	8.24E-10	2.13E-06	GO:0030234	Enzyme regulator activity
7. TGM3	6.3814	1.01E-09	2.24E-06	GO:0006464	Cellular protein modification process
8. OGFRL1	3.648762	1.25E-09	2.41E-06	GO:0007165	Signal transduction
9. LIPC	5.511889	2.91E-08	4.31E-05	GO:0006629	Lipid metabolic process
10. ADGRF2	9.051376	3.06E-08	4.31E-05	GO:0007165	Signal transduction

B. Downregulated					
Gene	LogFC	P Value	QValue (FDR)	Pathway ID	Pathway Description
1. MAPK1	-10.0724	2.43E-09	4.19E-06	GO:0030154	Cell differentiation
2. ARNT2	-9.9226	3.89E-07	0.000463	GO:0006950	Response to stress
3. STRA6	-5.39401	6.80E-06	0.00405	GO:0006629	Lipid metabolic process
4. RBP1	-5.05496	8.05E-06	0.004168	GO:0006629	Lipid metabolic process
5. ANTXR1	-5.06802	1.13E-05	0.005453	GO:0007010	Cytoskeleton organization
6. LTBP1	-4.19728	3.59E-05	0.01285	GO:0006464	Cellular protein modification process
7. AXIN2	-5.6952	4.47E-05	0.01442	GO:0008283	Cell population proliferation
8. SLFN11	-3.93117	8.66E-05	0.02396	GO:0006950	Cell response to stress

9. PHACTR1	-5.39943	9.58E-05	0.025592	GO:0007010	Cytoskeleton organization
10. LYPD1	-4.77696	0.000116	0.030426	GO:0007267	Cell-cell signaling

Next, an in-depth network analysis used the RNA sequencing data to determine cell-specific pathways impacted by carboplatin resistance. Notably, leukocyte extravasation signaling, GP6 signaling, cardiac hypertrophy signaling, and PI3K signaling in B lymphocytes were predicted to be increasingly activated in the carboplatin-resistant TO, as demonstrated by pathways represented with shades of orange. Conversely, the CD40 signaling, HER-2 signaling, and MSP-RON signaling in macrophages were suspected to have decreased activation in the carboplatin-resistant phenotype and are represented in shades of blue (Figure 6A). Pathways that were differentially activated and downregulated were found to be extensively interconnected (Figure 6B).

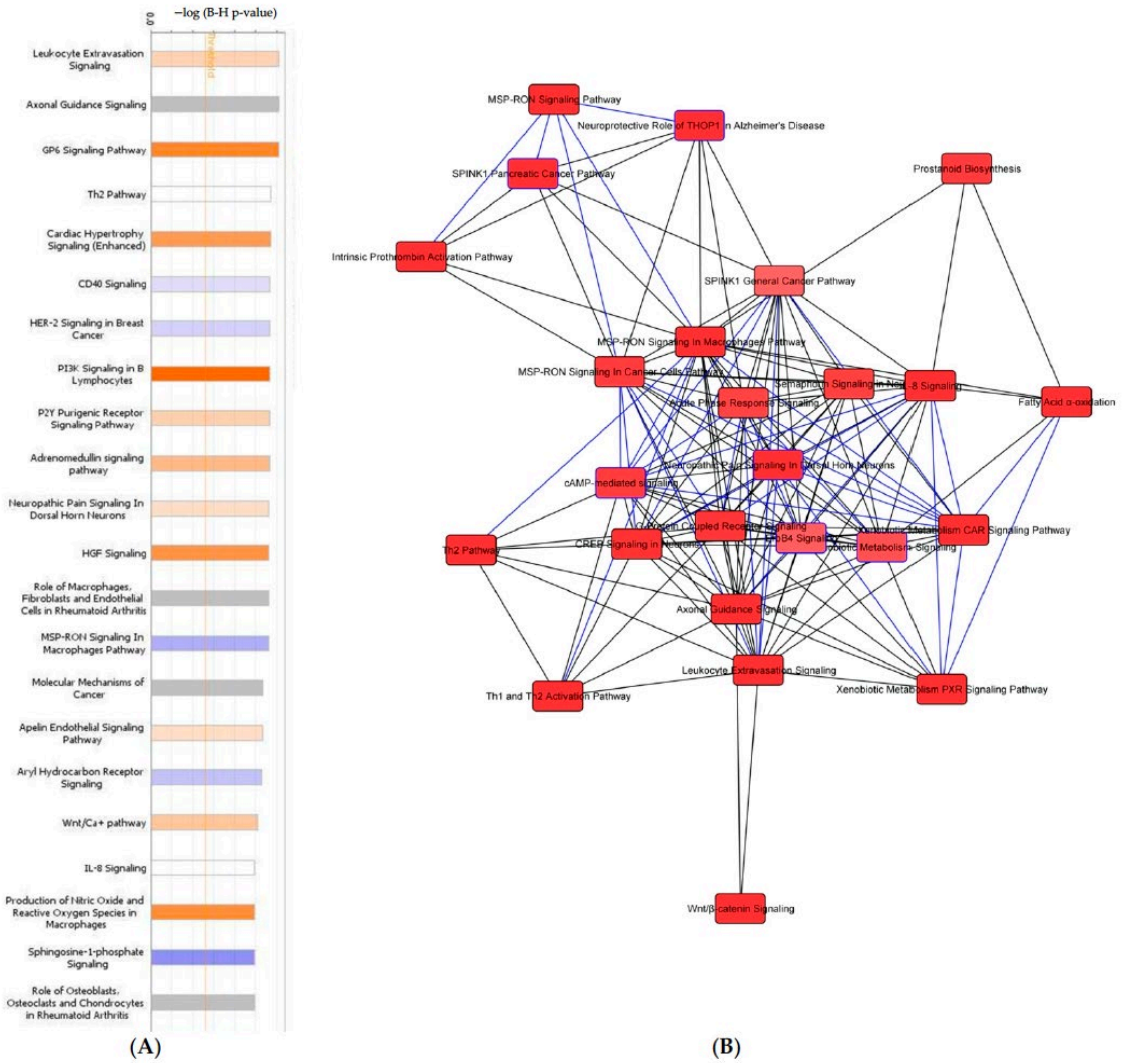
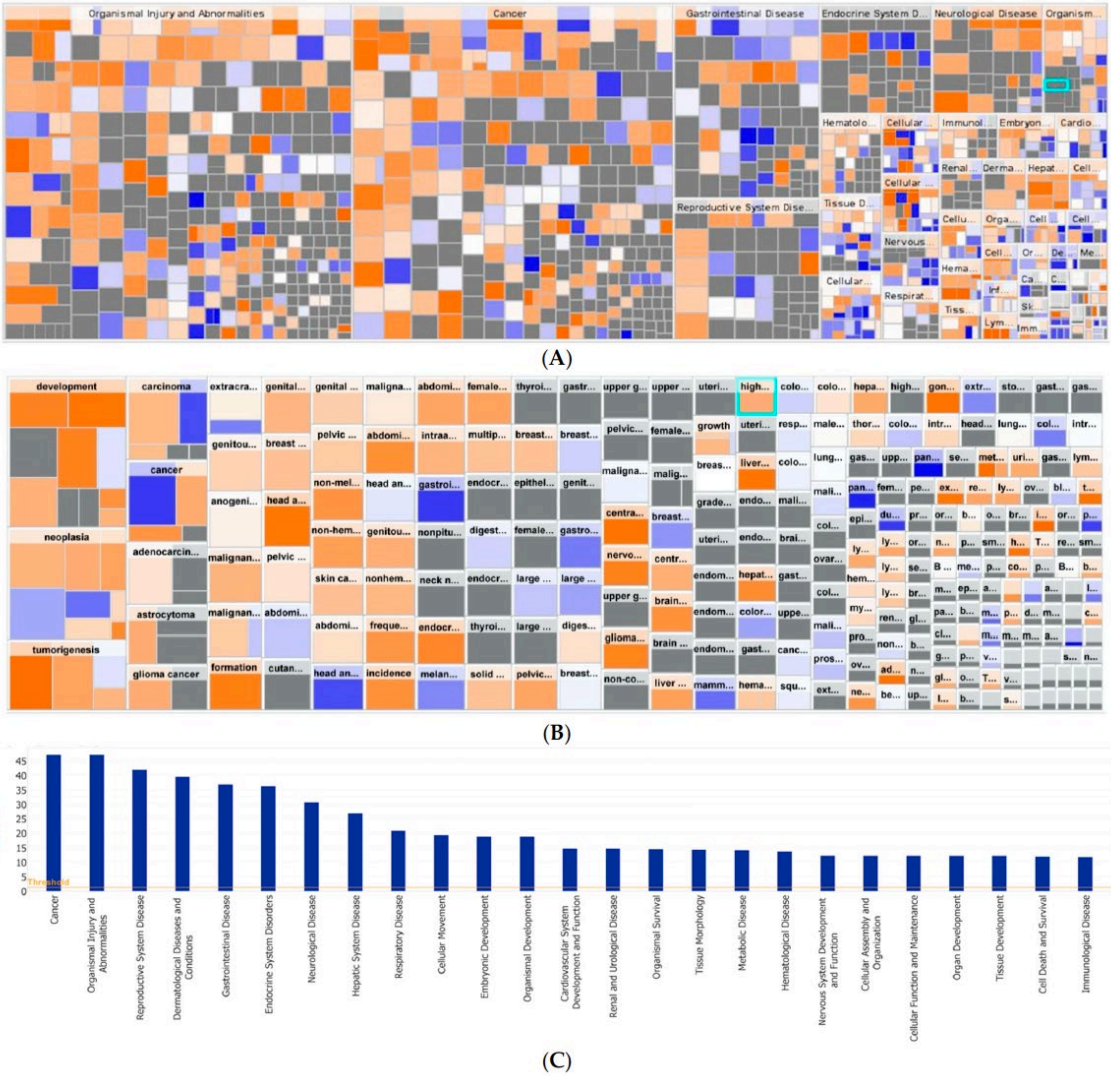


Figure 6. Differential expression analysis of carboplatin-resistant TO versus carboplatin-sensitive subjects. (A) Pathway analysis of genes differentially expressed between carboplatin-resistant and carboplatin-sensitive TOs. (B) Network analysis of the pathways differentially expressed between carboplatin-resistant and carboplatin-sensitive TOs.

To better assess the clinical applicability of the gene expression analysis, we converted the pathway analysis to a heatmap with analysis by disease and organ system (Figure 7A). The length of the box denotes the -log(p-value). The color of the boxes

correlates with the z-score, with the intensity of blue representing z ≤ 0 and the intensity of orange representing z ≥ 0. Pathways related to organismal injury and abnormalities, cancer, gastrointestinal disease, and reproductive system disease predominated. This suggests that carboplatin resistance is partly mediated by the alteration of injury-associated biological mechanisms and well-established cancer-related pathways.



(A)

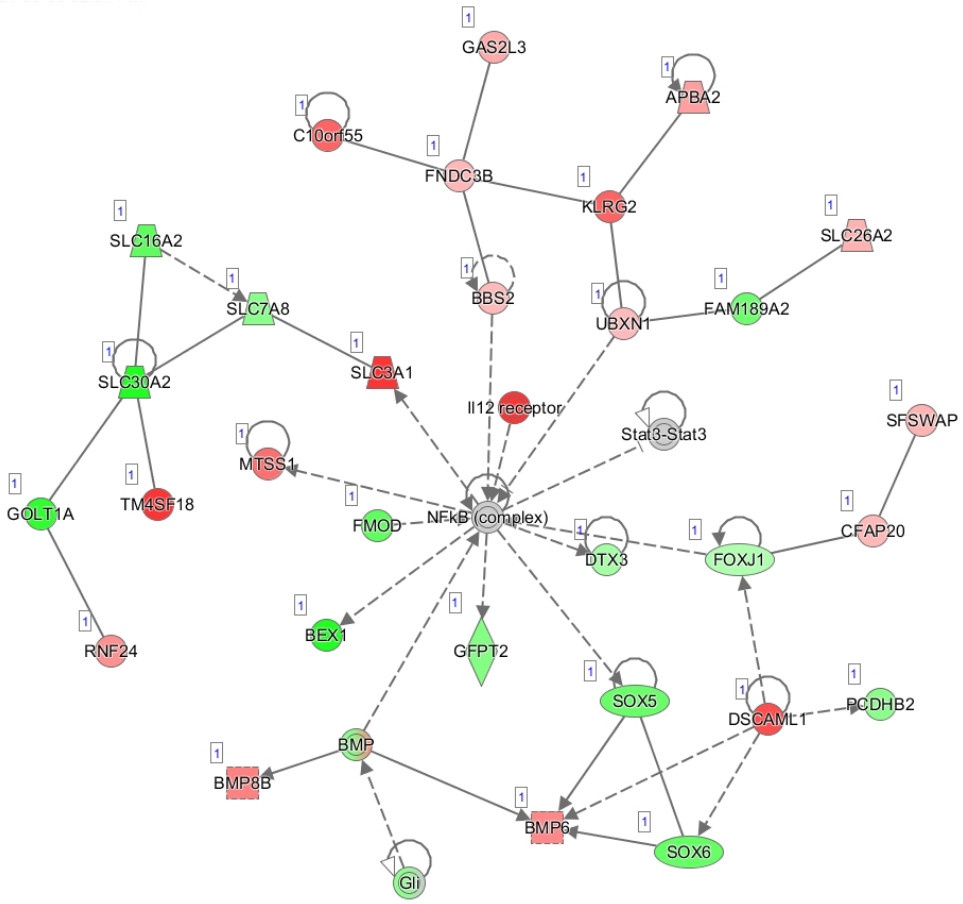
(B)

(C)

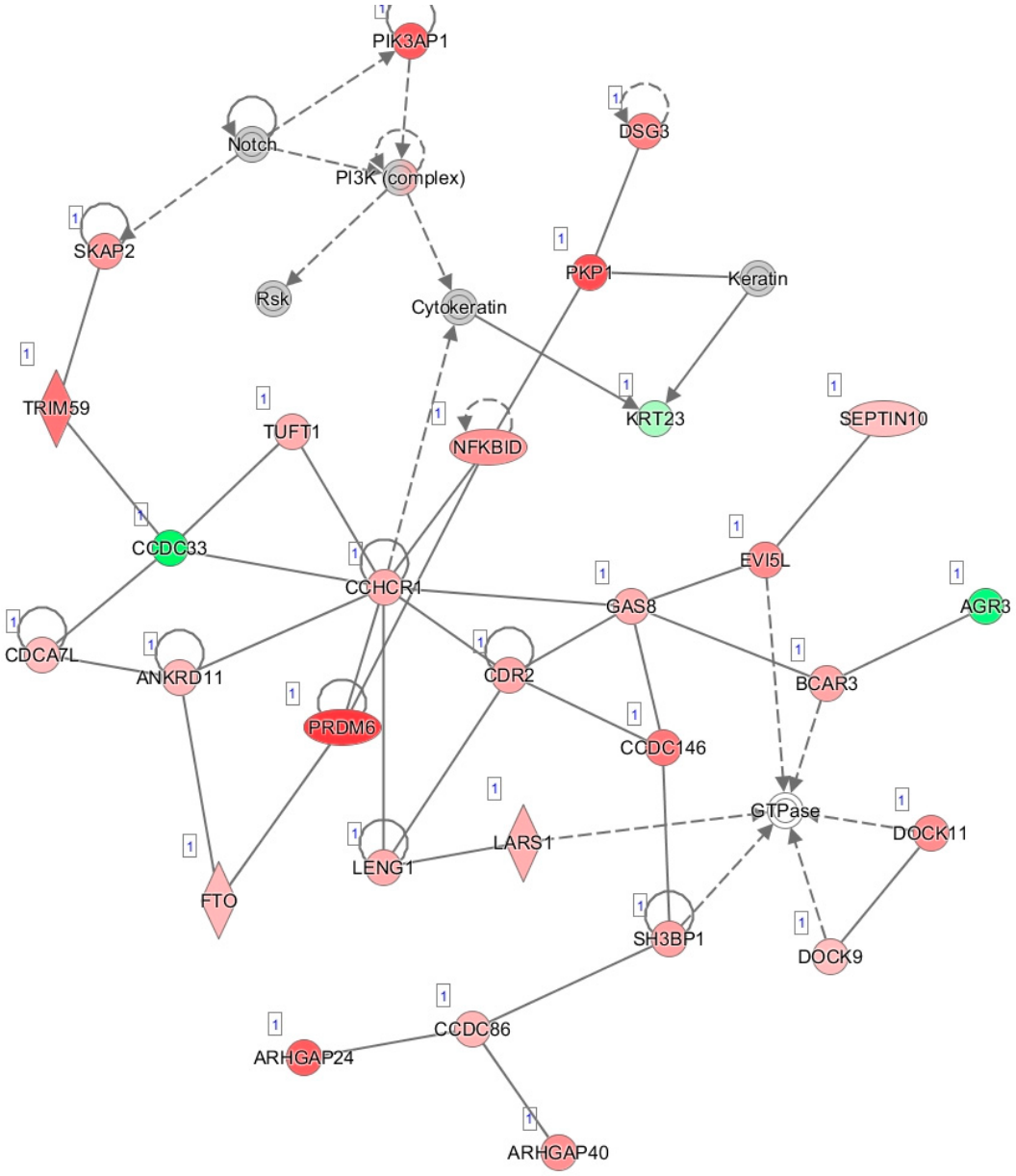
Figure 7. Gene network analysis between carboplatin-resistant and carboplatin-sensitive subjects. (A) Heatmap of the network analysis of genes differentially expressed between carboplatin-resistant and carboplatin-sensitive TOs by organ and disease system. The color and intensity of the boxes correlate with the z-score. Blue represents $z \leq 0$, and orange represents $z \geq 0$. (B) Heatmap of network analysis separated by cancer disease process. (C) Disease system pathways involved in carboplatin resistance are shown through network analysis of genes differentially expressed between carboplatin-resistant and carboplatin-sensitive TOs.

Finally, we performed network mapping using IPA with Global Network Overlay to explore the effect of carboplatin resistance on genes that were determined to be significantly altered between the carboplatin resistant TO and the carboplatin sensitive group. Upregulated expression is denoted in red with the color intensity corresponding to increased significance. Conversely, downregulated expression is notated in green with color intensity again corresponding to increased significance. Network mapping results were filtered by statistically significant p-values with expression fold changes ≥ 0. We focused on the most significantly altered gene network (Figure 8A) and the second most significantly altered gene network (Figure 8B). Exploration of the most significantly altered network map (Figure 8A) revealed an interplay between various pathways all centered around NF-kB when the carboplatin resistant TO was compared to the carboplatin sensitive TOs. The second most significantly altered network (Figure 8B) demonstrates interplay between pathways involved in cellular differentiation (PRDM6 activation) and

linkage of B-cell receptor signaling to the PI3K-Akt signaling pathway (PI3KAP1 activation) [23].



(A)


PIK3AP1
Notch
PI3K (complex)
DSG3
SKAP2
PKP1
Keratin
Rsk
Cytokeratin
TRIM59
TUFT1
NFKBID
KRT23
SEPTIN10
CCDC33
CCHCR1
EVI5L
GAS8
AGR3
CDCA7L
ANKRD11
CDR2
BCAR3
PRDM6
CCDC146
GTPase
DOCK11
LARS1
LENG1
FTO
SH3BP1
DOCK9
CCDC86
ARHGAP24
ARHGAP40

(B)

Figure 8. Network analysis of genes differentially expressed between carboplatin resistant and carboplatin sensitive TOs. Network mapping by Qiagen IPA with Global Network Overlay demonstrates the most significant gene network (A) and second most significant gene network (B).

CHAPTER 4. DISCUSSION

4.1 Overview

This prospective, observational, exploratory study demonstrates that tumor organoid (TO) development from chemo-naïve and neoadjuvant chemotherapy exposed epithelial ovarian cancer patients is both feasible and potentially predictive of clinical response to front line therapy. Commiserate with this study, other groups have successfully developed TOs from epithelial ovarian cancer patients and utilized TOs to screen for sensitivity to chemotherapeutic agents [24]. However, to our knowledge, this study is the first to report prospective correlation of carboplatin chemo-sensitivity screening with progression free survival (PFS).

4.2 Mutation Analysis Interpretation

Our mutation analysis provides insight into the genetic underpinnings driving tumorigenesis in our population. As expected in high grade serous ovarian cancer, alterations in the tumor suppressor gene TP53 was the most common mutation. The second most commonly altered gene, FANCC, is a critical component of the Fanconi Anemia core complex. A dysregulated Fanconi Anemia pathway is frequently identified in epithelial ovarian cancer due to its extensive interconnection with DNA repair pathways [25]. Missense mutations in Notch2 occurred in two of the carboplatin sensitive TO lines (UK1267 and UK1393). A wide range of cancer types have been found to overexpress

Notch2 or to exhibit Notch2 gain-of-function mutations. Overactive Notch2 signaling has been linked to the dysregulation of certain miRNAs, to tumor-associated stromal cell input, and to modulation of internal and external stimulation conditions in tumor cells which contribute to chemo and radio-resistance [26]. If the Notch2 missense mutations identified in these ovarian cancer TO cell lines renders the Notch 2 protein nonfunctional then a dysregulated Notch 2 signaling pathway may be partially responsible for the observed carboplatin sensitivity. Exploration of the mutation patterns of a selection of DNA repair genes reveals mostly intron mutations in the carboplatin sensitive cohort which likely do not impact function. However, notably in the carboplatin resistant TO cell line UK1254, a BRIP1 missense mutation was identified but the functional significance of this mutation is uncertain [27].

4.3 Gene Expression Analysis Interpretation

Although the gene panel mutation analysis provides some insight into the molecular drivers of tumor cell growth, they do not paint a complete picture of carboplatin resistance in UK1254. Our comparative gene expression analysis using TO RNA sequencing and IPA pathway analysis provides insight into the biological processes that are potentially driving chemotherapy resistance. Exploration of the most significantly altered network map (Figure 8A) revealed an interplay between various pathways all centered around NF-kB when the carboplatin resistant TO was compared to the carboplatin sensitive TOs. In addition to apoptosis threshold determination, the transcription factor NF-kB regulates multiple aspects of the innate and adaptive immune functions and serves as a pivotal

mediator of inflammatory responses [28]. It has been well established that various dysregulated signaling pathways can activate the NF-κB signaling pathway in ovarian cancer which in turn promotes chemoresistance, cancer stem cell maintenance, metastasis and immune evasion [29-31]. The second most significantly altered network (Figure 8B) demonstrates interplay between pathways involved in cellular differentiation (PRDM6 activation) and linkage of B-cell receptor signaling to the PI3K-Akt signaling pathway (PI3KAP1 activation) [23]. These functions combined may be responsible for the observed clinical and *in vitro* carboplatin resistance of UK1254. Discovery of these cellular alterations provides novel insight into the mechanism of carboplatin resistance in UK1254 and may be able to be exploited with targeted therapy.

We found that in the top ten upregulated genes, many have been linked to platinum-based chemotherapy resistance (AQP1 [32-33] and RELN [34]), to poor prognosis when exposed to platinum agents (LIPC [35] and FXYD2 [36]) or increased invasiveness (ADGRF2 [37]) when overexpressed. Notably, upregulation of TMEM178B and ZNF723 have not been directly linked to carboplatin resistance and understanding of their biological function in cancer remains limited. Interestingly, transmembrane protein 178B, the gene product of TMEM178B, has been identified as a novel downstream target of the nuclear factor kappa beta (NF-κB) ligand/phospholipase C gamma-2 signaling axis that modulates osteoclast activation [38]. NF-*κ*B is a pleiotropic transcription factor key which determines the death threshold of cancer cells after exposure to platinum drugs and inhibition of NF-*κ*B sensitizes cells to the effects of platinum-based chemotherapy [39]. Thus, overexpression of TMEM178B may produce a biological effect similar to upregulation of NF-*κ*B and warrants further investigation. In the top ten downregulated

genes, many have been linked to platinum-based chemotherapy resistance (MAPK1[40-41], SLFN11 [42] and LYPD1 [43]), poor clinical outcome (ARNT2 [44]) or oncogenesis via constitutive activation of wnt/β signaling (AXIN2 [45]) when under expressed.

4.4 Strengths and Limitations

The main strength of our study is that we were able to successfully correlate TO chemosensitivity assay results with clinical progression free survival (PFS) despite only including six subjects in this analysis and that we have identified genomic predictors of response. This is in contrast to prior ovarian organoids publications which report *in vitro* sensitivity to antineoplastics, but fail to include correlation with clinical outcome and genomic predictors of resistance. Genomic predictors of platinum resistance performed at initial surgery have the potential to guide subsequent clinical management with the advantages of convenience and speed over organoid sensitivity testing [24, 46-48]. The major limitation of our study is the small sample size. We intentionally only utilized advanced stage, high grade serous epithelial ovarian cancer specimens in an effort to create the most clinically and genetically homogenous sample possible. Although this strategy decreased the number of subjects eligible for inclusion in this study, it reinforces the clinical applicability of our results to this specific patient population with an unmet clinical need. In addition, focusing on this homogenous population, limits the generalizability of our findings to other types of ovarian cancer. An additional limitation of this study is the lack of normal tissue organoid controls. At the time of debulking surgery, some subjects

lacked normal human ovarian tissue due to complete destruction by malignancy. Thus, matched normal ovarian tissue was unavailable for culture.

4.5 Clinical Impact

Early stratification of patients into carboplatin sensitive and resistant cohorts, before clinical recurrence, may help delineate who should receive maintenance therapy with bevacizumab or a biosimilar. Furthermore, the combined use of TO chemosensitivity assay results and genomic markers of carboplatin resistance into a predictive scoring system of recurrence may provide the basis for additional cycles of cytotoxic chemotherapy beyond the traditional six. We envision that if the methodologies utilized here are applied to a larger cohort we could develop a novel epithelial ovarian cancer predictive scoring system. Similar systems such as the Oncotype DX test is currently standard of care for adjuvant chemotherapy stratification in early stage, ER+, HER2/neu negative breast cancer and intermediate risk prostate cancer. The development of an accurate scoring system that predicts an individual's front line PFS has the potential to change the standard of care for high grade serous ovarian cancer treatment and improve outcomes for thousands of patients every year.

4.6 Conclusions

Tumor organoid (TO) development from chemo-naïve and neoadjuvant chemotherapy exposed epithelial ovarian cancer patients is both feasible and potentially predictive of clinical response to front line therapy. An integrated TO mutation and gene expression analysis can be utilized to investigate molecular mechanisms of carboplatin resistance. Combination of these methods may provide the basis for development of a predictive recurrence scoring system that can be utilized to tailor maintenance and additional adjuvant therapy to individual patient needs.

CHAPTER 5. FUTURE DIRECTION

5.1 Overview

The next steps of our study will focus on validation of the carboplatin resistance signature identified in the platinum resistant tumor organoid line UK1254. We acknowledge that our study is limited by sample size and that many attempts have failed to understand the mechanism of carboplatin resistance in high grade serous epithelial ovarian cancer. Despite decades of research, no reliable clinical predictors of platinum resistance have been identified and all patients continue to receive platinum based chemotherapy in the front-line setting.

We propose that we must validate the recently discovered set of genomic drivers of platinum resistance with a large cache of ovarian cancer somatic whole exome sequencing (WES) and RNA sequencing (RNASeq) data and with a tissue microarray (TMA). Our study will mostly focus on utilization of subjects with similar histology and grade to the developed TO cell lines i.e. "Type II" epithelial ovarian cancer [49]. Unique to our study, both of these validation resources will be stratified based on subject sensitivity to carboplatin. This will allow for a homogenous and properly stratified cohort.

5.2 Specific Aims

Over the last few years we have developed a bank of tumor organoids (TO) from Total Cancer Care (TCC) consented ovarian cancer patients at the University of Kentucky Markey Cancer Center (UKMCC). Established TOs were assessed for sensitivity to

carboplatin *in vitro* which correlate to the clinical outcome measure, progression free survival (PFS). Our TO models have comprehensive clinical, demographic and genomic profiles and will serve as the "discovery set" for identifying a high grade serous ovarian cancer carboplatin resistance signature. We propose to validate our platinum resistance signature with a larger TCC/ORIEN cohort complied from eight other member institutions and with a tissue microarray (TMA) that will be created with tumor tissue from our UKMCC ovarian cancer TCC/ORIEN subjects.

Aim 1: Validate the identified platinum-resistance signature in a larger population with the inter-member Total Cancer Care/Oncology Research Information Exchange Network (TCC/ORIEN) ovarian cancer genomic database.

Hypothesis: The platinum resistance signature will be valid when tested against a large population of clinically stratified ORIEN subjects.

Aim 2: Validate the genomic platinum-resistance signature with protein expression patterns using an ovarian cancer tissue microarray.

Hypothesis: The platinum resistance signature can be reproduced with protein expression patterns.

The proposed studies will investigate the underlying tumor biology of platinum resistant ovarian cancer while controlling for various clinical factors that influence

outcomes. Validation of a platinum-resistance signature in ovarian cancer is the first step in identifying patients unlikely to respond to current front-line therapy. Early identification of inherent platinum resistance could help the treating clinician alter the chemotherapeutic regimen since these patients derive little clinical benefit from current treatment approaches.

5.3 Innovation

The next step of our study is innovative because the outcome (validated biomarker profile of inherent platinum resistance) does not currently exist. Additionally, no other population-based genomic study has been able to properly contextualize somatic sequencing with as much accurate treatment information as the ORIEN database provides. Capitalizing on these innovative features will allow for the identification and validation of novel biomarkers that can be used to predict response to cytotoxic chemotherapy agents in the frontline setting. This may lay the ground work to change the upfront treatment paradigm in ovarian cancer.

5.4 Experimental Approach

We plan to utilize the results of the epithelial ovarian cancer tumor organoid mutation and differentially expressed gene (DEGs) analysis as the discovery set [50]. The

identified platinum resistance signature will be validated with the ORIEN Avatar database and a tissue microarray (TMA).

Aim 1: We will evaluate the performance of our prediction models based on the external ORIEN cohort. Included subjects will be divided into “Type I" and “Type II" subtypes based on the reported histology and grade. Low grade serous and other types of cancers that fit the “Type I” subtype are usually inherently platinum resistant. Often times, carboplatin is not even offered to these patients since the efficacy us low [5]. Thus, we will mostly focus on the “Type II” subtype since almost all of these patients are treated with carboplatin.

Statistical measures such as sensitivity, specificity and the area under the receiver operator characteristic (ROC) curve and area under the ROC curve (AUC) will be calculated. It has been reported that a prediction model based on clinical factors yielded an AUC of 0.63 [51]. We will assess whether our prediction model outperforms the model reported in the literature (i.e. whether the AUC from our model is greater than 0.63). We expect our model will achieve an AUC of 0.8.

Finally, we will compare gene mutation and expression profiles between “Type I” and “Type II” subjects in order to identify similarities that may explain carboplatin resistance in the “Type I” group. Specifically, the Fisher’s exact test will be used to compare gene mutation frequency and the edgeR method [19] will be used to compare gene expression level between the two subtypes. Multiple comparisons adjustment will be performed based on the Benjamini-Hochberg procedure. Differentially mutated/expressed genes will be identified based on a false discovery rate threshold of

0.05. In addition, gene set enrichment analysis (GSEA) will be performed to identify enriched pathways.

Aim 2: All ovarian cancer subjects enrolled in TCC/ORIEN at the UKMCC will be screened for available banked tumor tissue. Formalin fixed paraffin embedded (FFPE) blocks will be screened for quality with hematoxylin & eosin (H&E) sections and reviewed by a pathologist. Subjects will be divided in to "Type I" and "Type II" ovarian cancer groups. The included specimens will be further stratified based on clinical sensitivity to carboplatin and divided into two additional groups: carboplatin sensitive and carboplatin resistant. Next, two cores of each block will be incorporated into a TMA block. Immunohistochemical (IHC) staining of the TMA block for proteins of interest will be performed by the University of Kentucky Biospecimen Procurement and Translational Pathology Shared Resource Facility (UK BPTP SRF).

5.5 Preliminary Data and Progress

We have already taken the following steps toward completion of this study:

- Applied for and been awarded a $25,000 grant from the Oncology Research Information Exchange Network (ORIEN) led by co-principal investigators Justin W. Gorski, MD and Frederick R. Ueland, MD.
- Drafted and obtained approval for the study "Utilization of Integrated Genetic Analysis to Predict Clinical and Therapeutic Outcomes in

Gynecologic Oncology" from the University of Kentucky Intuitional Review Board (IRB), Protocol #50767, Re-Approved 1/11/21.

- Drafted and obtained approval for a statement of work (SOW) to lead an ORIEN inter-member project to build a clinically stratified database of ovarian cancer somatic WES and RNASeq data compiled from nine institutions throughout the United States. We have successfully recruited the following institutions to join our efforts:

    1. Moffitt Cancer Center
    2. University of Colorado
    3. Rutgers Cancer Institute of New Jersey
    4. University of Southern California Norris Comprehensive Cancer Center
    5. University of Utah Huntsman Cancer Center
    6. University of Oklahoma Stephenson Cancer Center
    7. Roswell Park Cancer Institute
    8. Indiana University Simon Cancer Center

Aim 1: We have obtained genomic and clinical data for a total of 269 ORIEN Avatar subjects (Table 5). We are able to explicitly classify 28 patients as "Type 1" and 167 patients as "Type 2". Unfortunately, we have 74 patients who are not able to be explicitly classified since the grade is listed as "unknown, not applicable or cannot be assessed" in the clinical data from other ORIEN institutions.

Table 5. Number of epithelial ovarian cancer ORIEN Avatar database subjects stratified based on histology and grade.

Type of Ovarian Cancer	Number of Subjects
Type I	28
Type II	167
Unknown	74
Total	267

A sample of 167 "Type II" ORIEN Avatar patients in the validation cohort (assuming 25% platinum resistant and 75% platinum sensitive [52]) will provide 98% power to detect a significantly higher AUC of our prediction model compared to the model in the literature based on a one-sided z-test at 5% significance level.

Aim 2: We have recruited the University of Kentucky Biospecimen Procurement and Translational Pathology Shared Resource Facility (UK BPTP SRF) to build the tissue microarray. 99 total UKMCC subjects have been identified as having available tumor tissue. FFPE blocks are currently being screened for quality and then will be divided into "Type I", "Type II", "carboplatin sensitive" and "carboplatin resistant" cohorts as previously described.

5.6 Study Limitations

Possible Interdependence of Aims: It may be viewed that Aim 2 is dependent on Aim 1. However, no matter the outcome of Aim 1, creation of a large clinically-stratified genomic database of ovarian cancer patients (Aim 1) and an ovarian cancer tissue

microarray (Aim 2) are valuable resources that will provide insight into the mutational, gene expression and proteomic landscape of platinum-resistant ovarian cancer.

5.7 Next Steps

Aim 1: Currently, the clinical data from ORIEN member institutions is being cleaned and analyzed. This laborious process is necessary to be able to properly stratify subjects into carboplatin sensitive and carboplatin resistant cohorts. Once the subjects are properly stratified, we will be able to run the mutation and gene expression analysis as proposed above.

Aim 2: The University of Kentucky Biospecimen Procurement and Translational Pathology Shared Resource Facility (UK BPTP SRF) is currently screening a total of 99 tumor specimens from UKMCC ovarian cancer subjects and then they will stratify the tumor samples and build the tissue microarray. Once, the mutation and gene expression validation analysis is complete, we will compile a correlative list of candidate proteins and begin validating antibodies for immunohistochemistry staining.

APPENDIX

APPENDIX 1. COMPLETE LIST OF DIFFERENTIALLY EXPRESSED GENES

A. Complete List of Upregulated Differentially Expressed Genes

Gene Symbol	logFC	PValue	FDR
AQP1	8.72296804	1.46E-15	2.26E-11
TMEM178B	6.48927461	1.30E-14	1.01E-10
RELN	7.08324445	1.29E-13	6.68E-10
ZNF723	8.99862304	1.08E-12	4.20E-09
HAVCR1	9.87035567	3.29E-11	1.02E-07
FXYD2	8.37493724	8.24E-10	2.13E-06
TGM3	6.38140039	1.01E-09	2.24E-06
OGFRL1	3.64876223	1.25E-09	2.41E-06
LIPC	5.51188931	2.91E-08	4.31E-05
ADGRF2	9.0513764	3.06E-08	4.31E-05
SLC20A2	3.04718904	3.58E-07	0.00046273
CCBE1	6.5099096	4.69E-07	0.00051891
SIM2	6.32271726	1.18E-06	0.00120412
HTR6	7.94986715	1.24E-06	0.00120412
TP63	4.97541291	1.40E-06	0.00127749
ARHGAP29	3.03290502	1.56E-06	0.00134327
ZNF702P	3.39126355	1.80E-06	0.00146738
MT1A	6.24789528	2.21E-06	0.00171313
GLS	3.79936543	3.06E-06	0.00226043
SIRPG	7.54913104	4.01E-06	0.00282587
ABHD12B	6.73069519	4.50E-06	0.00303535
CRLF1	6.73263901	4.82E-06	0.00311334
ERVH48-1	8.03551356	5.38E-06	0.00333667
DPP4	3.68879445	7.31E-06	0.00416751
DSCAML1	3.47224113	8.06E-06	0.00416751
SNX25	3.18652714	8.16E-06	0.00416751
BTBD16	6.53206726	8.34E-06	0.00416751
ARHGAP4	3.84509158	1.56E-05	0.00734072
JAG1	2.59779767	1.82E-05	0.00805056
GABRB3	4.91616659	1.86E-05	0.00805056
ANKRD2	4.86381221	1.87E-05	0.00805056
KCNQ3	5.42149835	1.93E-05	0.0080697
SLC66A1L	4.78522635	2.16E-05	0.0088237
UGT2B17	5.41070458	2.97E-05	0.01180982
TNFSF18	5.73794232	3.10E-05	0.01194273
KCNJ15	4.26008825	3.16E-05	0.01194273

ABCA4	3.69699855	3.51E-05	0.01284988
KHDRBS2	5.6197865	3.68E-05	0.01284988
PGBD5	3.79280018	3.74E-05	0.01284988
SMOC2	4.76300444	3.90E-05	0.01284988
ZNF385D	6.24138246	3.90E-05	0.01284988
COL14A1	4.65727301	4.80E-05	0.01516819
PRKCB	4.12248354	4.91E-05	0.0152132
COBLL1	2.92011069	5.13E-05	0.01545876
VAV3	3.40533782	5.19E-05	0.01545876
PLAU	3.88691296	6.20E-05	0.01812394
TRIM59	2.76657057	6.48E-05	0.01859316
C8orf34	4.43174171	8.34E-05	0.02349993
EGFR	2.23102572	8.91E-05	0.02421952
EN2	4.79824185	0.00012275	0.0317034
CHST9	4.50491972	0.00012562	0.03191452
SIM1	5.50671866	0.00013074	0.03231461
TM4SF18	5.06827989	0.00015439	0.03648978
FAM157B	5.06391749	0.00015541	0.03648978
SLC24A4	6.00919652	0.00018787	0.04261436
PRDM6	5.24389641	0.00018974	0.04261436
COL4A1	2.68641041	0.00022885	0.0499514

B. Complete List of Downregulated Differentially Expressed Genes

Gene Symbol	logFC	PValue	FDR
MAPK1	-10.072382	2.43E-09	4.19E-06
ARNT2	-9.9226013	3.89E-07	0.00046344
STRA6	-5.394013	6.80E-06	0.00405016
RBP1	-5.0549638	8.05E-06	0.00416751
ANTXR1	-5.0680235	1.13E-05	0.00545274
LTBP1	-4.1972806	3.59E-05	0.01284988
AXIN2	-5.6951996	4.47E-05	0.0144202
SLFN11	-3.931175	8.66E-05	0.02396011
PHACTR1	-5.3994256	9.58E-05	0.02559225
LYPD1	-4.7769573	0.00011584	0.0304256
NES	-6.2220916	0.00013137	0.03231461
PPM1F	-8.2123502	0.00015368	0.03648978
IQGAP2	-6.557849	0.00018348	0.04243936
CDKN2B	-8.6152355	0.00021212	0.04695916

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VITA

Justin Wayne Gorski, MD

EDUCATION

Doctor of Philosophy Candidate at University of Kentucky College of Clinical and Translational Science (8/2018 – Present), Thesis Title: Utilizing Patient-Derived Epithelial Ovarian Cancer Tumor Organoids to Predict Carboplatin Resistance.

Gynecologic Oncology Fellowship (7/2018 – Present) at University of Kentucky Markey Cancer Center, Lexington, Kentucky.

Obstetrics, Gynecology and Reproductive Science Residency (7/2014 – 6/2018) at Icahn School of Medicine at Mount Sinai, New York, New York.

Medical Doctorate (5/2014) at Drexel University College of Medicine, Philadelphia, Pennsylvania.

Bachelor of Science (5/2008) in Molecular Genetics at University of Rochester, Rochester, New York.

PROFESSIONAL EXPERIENCES

Research Assistant in the Department of Neurological Surgery (9/2008 - 7/2010) at Columbia University Medical Center, New York, New York.

National Space Biomedical Research Institute Fellow in the Cardiovascular Laboratory (5/2007 – 8/2008) at National Aeronautics and Space Administration: Johnson Space Center, Houston, Texas.

National Science Foundation Research Experiences for Undergraduates Fellow in the Department of Neurological Oncology (5/2006 – 8/2006) at Roswell Park Cancer Institute, Buffalo, New York.

Research Intern in the Department of Pathology (5/2005 – 8/2005) at the Mount Sinai Medical Center in New York, New York.

HONORS & AWARDS

T32: Oncology Research Training for Surgeon Scientists Post-Doctoral Fellowship (2018 – 2020) at University of Kentucky, Lexington, Kentucky, Principal Investigator: B. Mark Evers, T32 CA160003.

University of Kentucky STAR Program: Bronze Award for Compassion (2021)

Best Overall Poster Presentation at OncLive® National Fellows Forum: GynOnc (2019), Miami, Florida.

House Staff Medical Student Teaching Award (2015 and 2017) at Icahn School of Medicine at Mount Sinai, New York, New York.

Alma D. Morani, M.D. Award in Gross Anatomy (2014) at Drexel University College of Medicine, Philadelphia, Pennsylvania.

Take Five Undergraduate Fellowship (2007 – 2008) at University of Rochester, Rochester, New York.

Benjamin A. Gilman International Scholarship (2007 – 2008), U.S. Department of State Bureau of Educational and Cultural Affairs, Washington, District of Columbia.

Summer Research Fellowship at National Space Biomedical Research Institute (2007), Houston, Texas.

National Science Foundation Research Experience for Undergraduates Fellowship (2006) at Roswell Park Cancer Institute, Buffalo, New York.

PEER REVIEWED PUBLICATIONS

Gorski JW, Zhang Z, McCorkle JR, DeJohn JM, Wang C, Miller RW, Gallion HH, Dietrich CS, Ueland FR, Kolesar JM. Utilizing Patient-Derived Epithelial Ovarian Cancer Tumor Organoids to Predict Carboplatin Resistance. Biomedicines. 2021 Aug 16;9(8):1021. doi: 10.3390/biomedicines9081021. PMID: 34440225.

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Ducruet AF, Gigante PR, Hickman ZL, Zacharia BE, Arias EJ, Grobelny BT, Gorski JW, Mayer SA, Connolly ES Jr. Genetic determinants of cerebral vasospasm, delayed cerebral ischemia, and outcome after aneurysmal subarachnoid hemorrhage. J Cereb Blood Flow Metab. 2010 Apr;30(4):676-88. doi: 10.1038/jcbfm.2009.278. Epub 2010 Jan 13. PMID: 20068580

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