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PRINCIPAL INVESTIGATOR: Adrian V. Lee, Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA

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Adrian V. Lee, Ph.D.

E-Mail: leeav@upmc.edu

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4200 Fifth Ave,
Pittsburgh,
PA 15260

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14. ABSTRACT
High-grade serous ovarian cancer (HGSOC) is known for its lack of early detection, limited therapies, and high rate of recurrence. Recent advances in transcriptomic sequencing have identified drug-targetable, pathogenic fusion genes in solid cancers. We hypothesize that fusion genes are commonly acquired or enriched in relapsed HGSOC and contribute to the enhanced malignancy observed in recurrent disease. We assembled a cohort of 18 patient matched pairs of chemotherapy naïve and resistant HGSOC and performed RNA sequencing. We noted transcriptional similarity between the patient-matched pairs of samples, but several recurrent transcriptional remodeling events were noted. Some fusions acquired in the chemotherapy-resistant HGSOC are found in HGSOC cell lines. One of these (CCDC6-ANK3) is expressed in an HGSOC cell line and when knocked-down preliminary data shows decreased growth. We are currently examining the expression of these fusions in patients treated with neo-adjuvant chemotherapy (pre and post therapy) and examine the biologic function of prioritized RNA fusion events.

15. SUBJECT TERMS

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1) INTRODUCTION:

High-grade serous ovarian cancer (HGSOC) is known for its lack of early detection, limited therapies, and high rate of recurrence. Greater than 80% of patients with late-stage HGSOC recur after an initial response to chemotherapy, with the majority of relapsed tumors developing deadly resistance to subsequent chemotherapies. The generation of fusion mRNA transcripts is an oncogenic event in many cancer types. Recent advances in transcriptomic sequencing have identified drug-targetable, pathogenic fusion genes in solid cancers. We hypothesize that fusion genes are commonly acquired or enriched in relapsed HGSOC and contribute to the enhanced malignancy observed in recurrent disease. The goal of this proposal is to test this hypothesis with the following specific aims; 1) To define the presence and relative expression of fusion mRNA transcripts in primary and recurrent high grade serous ovarian cancer (HGSOC). 2) To establish the prevalence and clinical importance of identified pathogenic gene fusions 3) To determine the biological effect, and mechanistic action, of fusion candidates acquired in relapsed disease. This study will provide novel targets and biomarkers for a cancer with limited options. This pilot project will develop key preliminary data critical for further analysis of RNA fusions in recurrent HGSOC and may identify new prognostic markers and ultimately therapeutic targets for reversing HGSOC chemoresistance, reducing recurrence, and extending patient survival.

2) KEYWORDS:

High grade serous ovarian cancer, chemotherapy resistance, RNA fusions, prognosis, recurrence, sequencing

3) ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1) Identify fusion transcripts in recurrent HGSOC	**Timeline**	**Progress**
Major Task 1 RNA-sequencing of recurrent HGSOC	Months	
Local IRB/IACUC Approval	0	Completed
Submission of institution's IRB approval and related material for DoD's HRPO approval	0-1	Completed
Receive HRPO approval or exempt finding before initiating relevant tasks	1-3	Completed
Subtask 1 Pathology analysis of 20 pairs of primary and recurrent HGSOC	3-4	Completed
Subtask 2 Isolation of RNA from 20 pairs	3-5	Completed

Subtask 3 Sequencing using NextSeq500 in sequencing core at Pitt	5-6	Completed
Milestone(s) Achieved – RNA-seq data from primary and recurrent Pitt tumors (n=23 total – 3 pairs performed for preliminary data)		Completed
Major Task 2 Bioinformatic analysis and validation of fusions		
Subtask 1 Analysis of RNA-seq by mapping with STAR and calling fusions using Fusion MetaCaller. Analysis of gene expression using STAR and Deseq	6-7	Completed
Subtask 2 Validation of candidate fusions using RT-PCR and Q-RT-PCR	7-8	Partially completed
Subtask 3 Validation of a select number of fusions by FISH	8-10	Not started
Milestone(s) Achieved: Validated fusion mRNAs present in recurrent HGSOC		Partially completed
Specific Aim 2) Establish the prevalence and clinical significance of identified fusion genes		
Major Task 3 Isolate RNA and measure fusion using Nanostring		
Subtask 1 Procure 60 FFPE recurrent samples and 200 primary HGSOC	4-8	Completed
Subtask 2 Isolate and measure RNA from 260 samples	8-10	Completed
Subtask 3 Develop NanoString codeset based upon fusions from Aim 1	10-14	Not started
Major Task 4 Bioinformatic analysis of NanoString data		
Subtask 1 Analyze specific fusion levels (using probes across the fusion) and predict fusion using the 5'/3' ratio of mRNA levels for all genes	16-18	Partially completed
Subtask 2 Correlate fusion levels with outcome (progression free survival and overall survival)	17-19	Partially completed

Milestone(s) Achieved: Correlation of fusion mRNA presence and levels with clinical outcomes in HGSOC		Partially completed
Specific Aim 3) Determine the biological effect, and mechanistic action, of fusion candidates acquired in recurrent disease		
Major Task 5 Development of HGSOC cell lines with knockdown or overexpression of fusion genes		
Subtask 1 Panel of ovarian cancer cell lines (n=45) from CCLE with RNA-seq data already available and downloaded from cgHub screened for fusions using Fusion MetaCaller.	14-18	Completed
Subtask 2 Knockdown of biologically relevant fusions from Aims 1 and 2 in HGSOC cell lines endogenously expressing the fusion	18-20	Partially completed
Subtask 3 Overexpression of fusions in cell lines which don't express any of the identified fusions.	18-20	Not started
Milestone(s) Achieved – HGSOC cell lines expressing or not expressing fusion genes		Partially completed
Major Task 6 Examine the phenotype of HGSOC cell lines with and without gene fusions		
Subtask 1 Analyze phenotypic differences (proliferation, colony formation, anoikis, migration, invasion)	20-24	Partially completed
Subtask 2 Analyze response to chemotherapy (cisplatin, doxorubicin)	20-24	Not started
Milestone(s) Achieved: Data on how HGSOC fusions affect chemo-resistance and progression		Not completed

What was accomplished under these goals?

For this reporting period describe:

1) major activities

In this project we have performed a comprehensive analysis of transcriptomic changes in patient-matched frozen pairs of chemotherapy naïve and treated recurrent HGSOC and compared results to publicly available data. Numerous identified fusions were validated by RT-

PCR and preliminary studies on the biological importance performed. A study of RNA fusions in HGSOC before and after neo-adjuvant chemotherapy is underway and will be reported within the next year.

2) specific objectives

The major tasks of the proposal were to 1) Define the presence and relative expression of fusion mRNA transcripts in primary and recurrent high grade serous ovarian cancer (HGSOC). 2) To establish the prevalence and clinical importance of identified pathogenic gene fusions 3) To determine the biological effect, and mechanistic action, of fusion candidates acquired in relapsed disease.

3) significant results or key outcomes, including major findings, developments, or conclusions

Specific Aim 1) Identify fusion transcripts in recurrent HGSOC

Major Task 1 RNA-sequencing of recurrent HGSOC

Local IRB/IACUC Approval

Written and approved

Submission of institution's IRB approval and related material for DoD's HRPO approval

Written and approved

Receive HRPO approval or exempt finding before initiating relevant tasks

Written and approved

Subtask 1 Pathology analysis of 20 pairs of primary and recurrent HGSOC

Completed. The details of the unique cohort of patient-matched chemotherapy naïve and treated HGSOC are described in the Yr 1 report.

Subtask 2 Isolation of RNA from 20 pairs

Completed. All samples had a RIN score above 7.5 and this made them suitable for downstream sequencing.

Subtask 3 Sequencing using NextSeq500 in sequencing core at Pitt

Completed. All QC metrics were excellent.

Major Task 2 Bioinformatic analysis and validation of fusions

Subtask 1 Analysis of RNA-seq by mapping with STAR and calling fusions using Fusion MetaCaller

Completed. We have previously detailed analysis of the data based upon FusionCatcher which has very high specificity. Fusion RNAs were called with FusionCatcher v0.99.7b. We identified fusions in the chemotherapy naïve (early, E) and recurrent (late, L) HGSOC. Nearly all recurrences harbored "preserved" fusions—fusion transcripts detected in both the early and late lesion. Although no fusions acquired in the recurrent sample were present in more than one patient, fusions of particular interest were identified. Because preserved fusions (present in both early and late samples) were found to be common in ovarian cancer recurrences, we searched for preserved fusion genes that were shared in multiple samples. CCDC6-ANK3 was found to harbor distinct breakpoints in two different HGSCOC samples and in the OVCA3 cell line. These breakpoints were confirmed with RT-PCR.

Since the main goal of the proposal was to study recurrent fusions, we applied a more comprehensive approach to fusion detection, using three different algorithms StarFusion(v2.6),

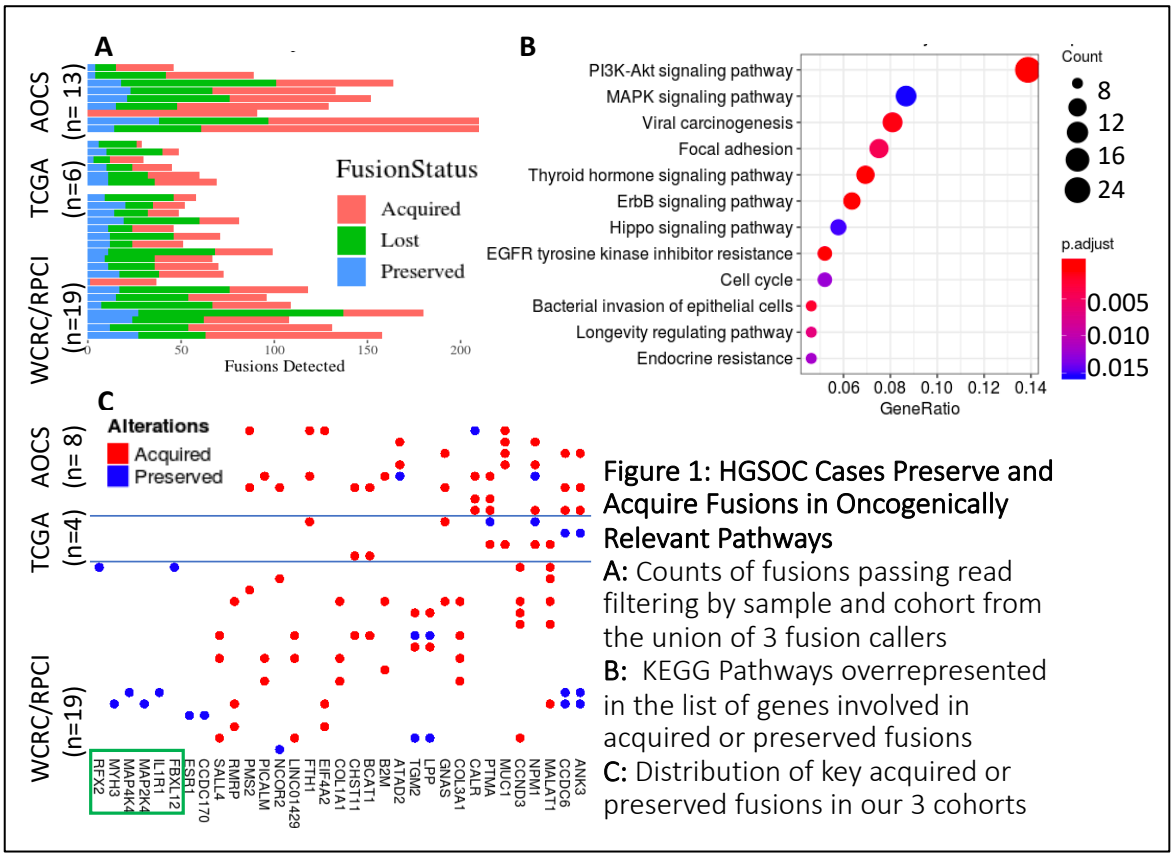

Figure 1: HGSOC Cases Preserve and Acquire Fusions in Oncogenically Relevant Pathways

A: Counts of fusions passing read filtering by sample and cohort from the union of 3 fusion callers

B: KEGG Pathways overrepresented in the list of genes involved in acquired or preserved fusions

C: Distribution of key acquired or preserved fusions in our 3 cohorts

FusionCatcher(v1.1), and FusionZoom, and incorporating the most current software releases into our pipeline .

Figure 1 shows the results from fusions identified in the three cohorts of patient-matched pairs of HGSOC using the three different algorithms. The WCRC/RPCI is our new cohort. TCGA is The Cancer Genome Atlas and AOCS is the Australian Ovarian Cancer Study. Note that nearly every HGSOC acquires additional RNA fusions when they recur (Figure 1A).

We further examined the genes and pathways recurrently affected by fusion genes after rigorous filtering, shown in Figure 1B. Notably, kinase signaling, and focal adhesion pathways were among those most frequently bearing fusions in our cohort. Similarly, certain genes were recurrently fused in our cohorts, the top 25 of which are shown in Figure 1C.

We next developed an approach to identify the most appropriate fusions for further study. All of the acquired fusions i.e. those found only in the late recurrent HGSOC, were filtered for a number of parameters including number of sequencing reads which span the fusion breakpoint. These were also examined for their potential to be a driver oncogenic fusion event using OncoFuse (score >0.75) and examined for their presence in HGSOC cell lines.

Analysis of gene expression using STAR and Deseq

Completed. As detailed in the Year 1 report we performed unsupervised hierarchical clustering and found that nine patient-matched pairs of HGSOC clustered in the same doublet clade of their patient-matched primary, suggesting a profound transcriptional conservation between the recurrence and the early lesion. Differential expression analyses revealed heterogeneous expression between the patient-matched samples, only uncovering 39 differentially expressed genes. The most significantly upregulated gene in late ovarian cancer was NTRK2, showing upregulation in the majority of recurrences. Since resistance mechanisms in advanced cancers may be mutually exclusive, and thus would be missed by conventional differential expression analyses, we performed analysis focusing on outlier expression gains and losses—particularly in genes that are clinically actionable. Four clinically actionable genes showed outlier increases in at least one-third of late disease samples versus their matched early disease lesion—INHBA, IGF1 NTRK2 and EPHA3.

Subtask 2: Validation of candidate fusions using RT-PCR and Q-RT-PCR

Completed. In the year 1 report we showed initial RT-PCR validation of several RNA fusions – we noted that we were able to validate nearly all of the bioinformatically identified fusions. This is an important feat given the challenges of specificity in these algorithms. Figure 2 shows an example of 4 different RNA fusions detected by RT-PCR. Rt-PCR products were sequenced, and this validated that we had specifically amplified the fusion mRNA product. Interestingly, while the 4 fusions we show here were identified as increased in the recurrent disease, and this validated by RT-PCR, we did identify fusions which were identified as increased in recurrence but upon RT-PCR were found in the primary cancer. Thus, while specificity is high, sensitivity for the RNAseq may be low.

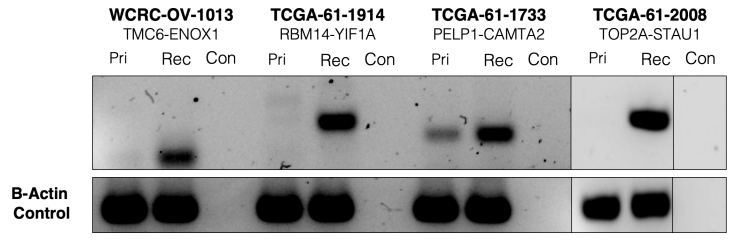

Figure 2: Relapse acquired / enriched fusions and fusion expression. RT-PCR validation with expression of fusion partners. RT-PCR with breakpoint flanking primers—Pri (Primary), Rec (recurrence) and Con (control – no template). All bands shown are of expected size and Sanger sequencing confirmed breakpoints.

Many of the fusions we validated at the expression level had low OncoFuse scores, indicating that they are likely not drivers of the disease, and may simply be aberrant genomic events. Consistent with this, none of them were found in HGSOC cell lines. We thus next focused on recurrently detected, high OncoFuse score, in-frame fusions, one example of which, FBXL12—RFX2, is depicted below. We found many supporting reads for this fusion, depicted in Figure 3A, and confirmed the expression of the fusion junction with RT-PCR and Sanger Sequencing the produce, as shown in 3B-C.

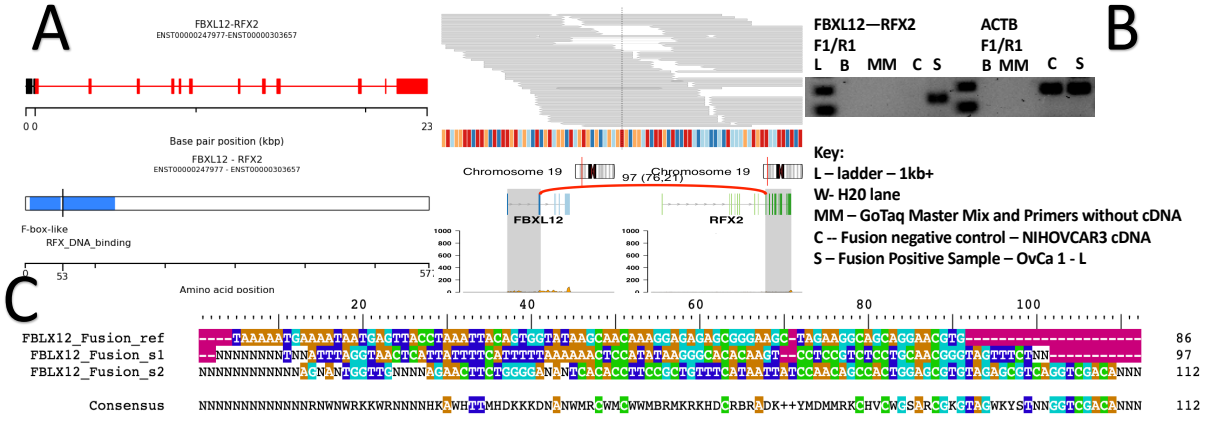

Figure 3: Detection and Validation of Candidate Fusion FBXL12—RFX2: A) Computational support for the detection of FBXL12—RFX2, demonstrating the reads supporting the fusion junction, and the resulting exon and protein structure. B) RT-PCR detection of FBXL12—RFX2 fusion junction. C) Multiple sequence alignment for RT-PCR amplified FBXL12—RFX2 junction sequencing results.

Further validation studies revealed more fusions that are recurrent in the HGSOC samples, and one particular fusion CCDC6-ANK3 was present in two HGSOC samples and also a HGSOC cell line NIH OVCAR-3 (Figure 4).

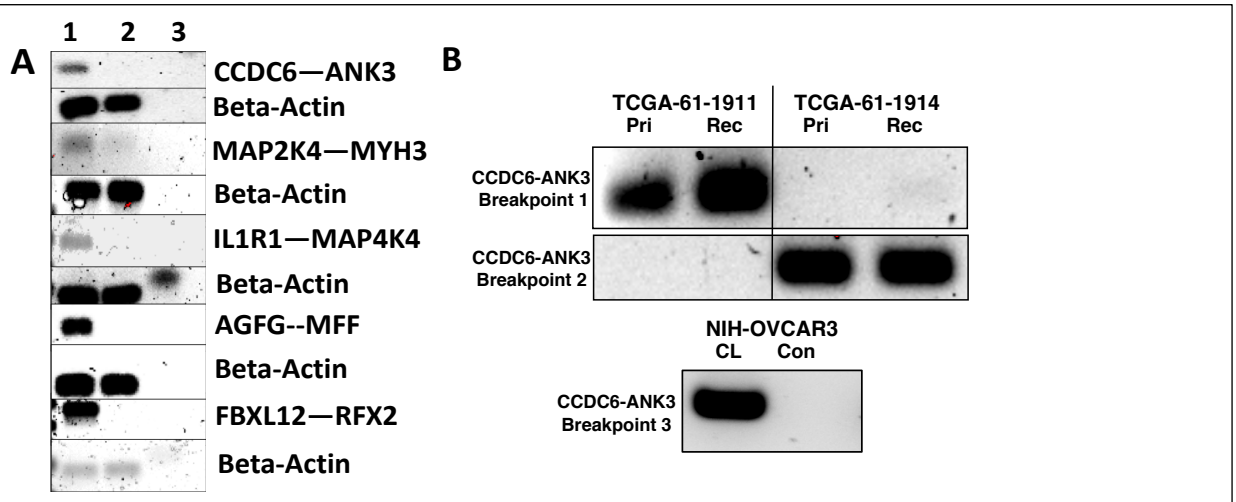

Figure 4: Expression of RNA fusions in HGSOC tumor and cell lines. A) RT-PCR of 4 different RNA fusions and beta actin as a control. 1- fusion positive sample. 2 – fusion negative sample. 3- water control. All fusions were sequenced to confirm specificity. B) CCDC6-ANK3 fusion detected in 3 HGSOC tumors and the NIH-OVCAR03 cell line. For two of the HGSOC tumor the breakpoint is at a different intro (Breakpoint 1 and 2). Pri – primary, Rec- recurrence, CL – cell line, Con- control.

Subtask 3 Validation of a select number of fusions by FISH

Not performed. This wasn't completed due to the technical challenges of FISH which we attempted with our core facility but wasn't successful.

Specific Aim 2) Establish the prevalence and clinical significance of identified fusion genes

Major Task 3 Isolate RNA and measure fusion using NanoString

Subtask 1 Procure 60 FFPE recurrent samples and 200 primary HGSOC

Completed. The identification of the HGSOC for use in this project has proven challenging and taken more time than expected. We identified 1,006 cases of HGSOC treated at our hospital and attempted to extract clinical information on them. However, while primary treatment and therapy is well documented in our cancer registry, this wasn't the case for recurrence, and we were unable to obtain clinical information critical to selection of cases for analysis.

As an alternative source of chemotherapy naïve and treated HGSOC we examined patients treated with neo-adjuvant chemotherapy. Importantly, we identified 174 cases which received neoadjuvant chemotherapy and thus would allow us to examine changes in fusion expression before and after neo-adjuvant chemotherapy. These samples were examined by a pathologist and scored with regards to the quality and size of the specimen. We have obtained FFPE slides for 40 pairs of slides, from which tumor epithelium were micro dissected for RNA extraction and sequencing.

Subtask 2 Isolate and measure RNA from 260 samples

Completed. Samples from Major Task 3 Subaim 1 above (pre and post neo-adjuvant chemotherapy) were examined by a pathologist and scored with regards to the quality and size of the specimen. We obtained FFPE slides for 40 pairs of slides, from which tumor epithelium were macro-dissected and RNA isolated.

Subtask 3 Develop NanoString codeset based upon fusions from Aim 1

Not started. This was not started as RNA for analysis was not available due to delays in obtaining clinical samples. However, we would like to note that since writing the original proposal, the cost of sequencing has dropped significantly. In light of the recent successes of targeted RNA-sequencing for fusion detection in clinical cohorts, we expect to use RNA sequencing for this approach when we examine the samples above. We expect this will be completed soon.

Major Task 4 Bioinformatic analysis of NanoString data

Subtask 1 Analyze specific fusion levels (using probes across the fusion) and predict fusion using the 5'/3' ratio of mRNA levels for all genes

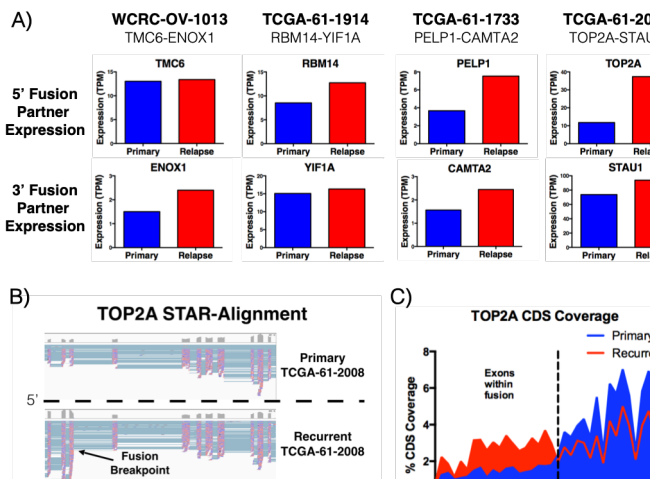

Figure 5: 5'/3' exon imbalance in expression fusion genes. (A) Normalized RNA-seq expression values (transcripts per million) of primary (blue) and relapsed tumor (red) from respective fusion-containing cases. **(B)** Anatomy of *TOP2A-STAU1* overexpression. Aligned RNA-seq reads to TOP2A fusion region in primary and recurrent tumor. **(C)** Coding sequence (CDS) coverage of TOP2A with proportion of reads mapping to each exon normalized to total number of reads covering gene in each sample.

We examined the expression of the fusion genes. We didn't have NanoString data to achieve this and thus performed the analysis on the original RNAseq data we obtained from the frozen primary and recurrent HGSOC samples. In four different fusions, we found differences in exon usage in the recurrent disease, with expression of the 5' partner and 3' partner increasing in all cases (Figure 5). We next examined where there were differences in expression of the exons within a single RNA fusion TOP2A-STAU1. Briefly, we quantified RNAseq counts per exon (an output of Salmon) and examined exon usage across the TOP2A gene in both the primary and recurrent tumor in which it was discovered (TCGA-61-2008). Note that for the first half of TOP2A gene, the exons are more highly expressed in the recurrent disease, yet the 3' exons are under-expressed in the recurrence, likely due to translocation to STAU1.

Subtask 2 Correlate fusion levels with outcome (progression free survival and overall survival)

As most fusions were found in very few samples, a result consistent with several other studies, we didn't have the power to assess the prognostic significance of individual fusion genes. However, we did assess the prognostic significance of expression of the individual gene components within the fusions to determine if this tells us about the biology of HGSOC. We have previously reported that expression of STAU-1 is associated with poor outcome in HGSOC (see Figure 6). This is interesting, as STAU-1 is translocated to TOP2A a target of several chemotherapies.

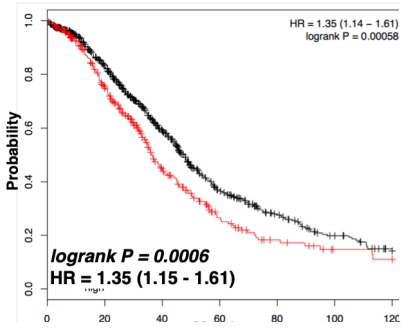

Figure 6: STAU1 overexpression associated with poor prognosis in OvCa. Kaplan-Meier overall survival analysis of 1137 patients with high (red, highest 333) vs low (black, lowest 804) *STAU1* OvCa tumor expression. JetSet probe ID: 213037_x_at, 10 year follow-up threshold (patients censored at threshold).

Given the recurrence of the CCDC6-ANK3 fusion in recurrent disease, and presence in the NIH OVCAR3 cell line, we examined the prognostic significance of both of these genes in HGSOC using the MetaGXOvarian package in Bioconductor. We note that high levels of CCDC6 are strongly associated with poor outcome (HR 1.48, p>3.8x10-E5), and high expression of ANK3 shows a trend to poor outcome (Figure 7).

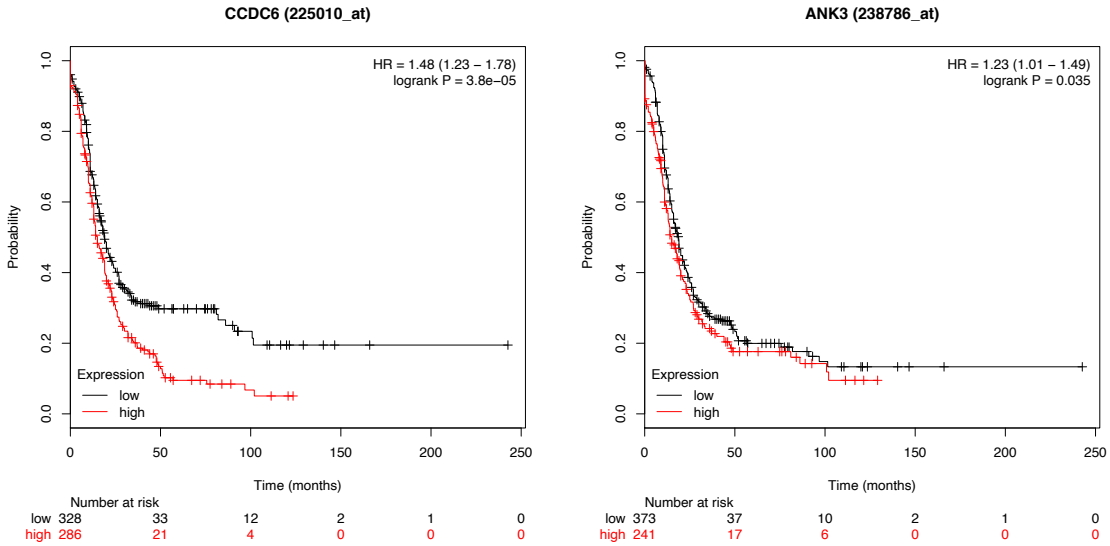

Figure 7: CCDC6 overexpression associated with poor prognosis in HGSOC. Kaplan-Meier overall survival analysis of patients with high (red) vs low (black) *CCDC6 (left panel) and ANK3 (right panel)*. Probe IDs for expression are shown.

Specific Aim 3) Determine the biological effect, and mechanistic action, of fusion candidates acquired in recurrent disease

Major Task 5 Development of HGSOC cell lines with knockdown or overexpression of fusion genes

Subtask 1 Panel of ovarian cancer cell lines (n=45) from CCLE with RNA-seq data already available and downloaded from cgHub screened for fusions using Fusion MetaCaller.

Completed. We downloaded RNAseq data from 45 ovarian cancer cell lines from the public Cancer Cell Line Encyclopedia (CCLE) and identified RNA fusions using three different RNA fusion calling algorithms. This generated a list of fusions which we cross-referenced with those found in HGSOC tumors and resulted in the identification of CCDC6-ANK3 being expressed in 2 cases of HGSOC and in the NIH OVCAR-3 cell line. The fusion list from the cell lines will be made publicly available as a resource for others to examine.

Subtask 2 Knockdown of biologically relevant fusions from Aims 1 and 2 in HGSOC cell lines endogenously expressing the fusion

Partially completed. We chose to knockdown CCDC6-ANK3 due to its expression in 2 HGSOC cases and the NIH OVCAR-3 cell line. We designed siRNA across the junction of the fusion – reasoning that this sequence should be highly specific for the fusion and this sequence isn't present in normal cells or other cell lines. We designed two different siRNA in case one didn't work. NIH OVCAR-3 cells were transfected, and expression of the fusion measured using primers specific for the fusion product. Figure 8 shows successful downregulation of CCDC6-ANK3.

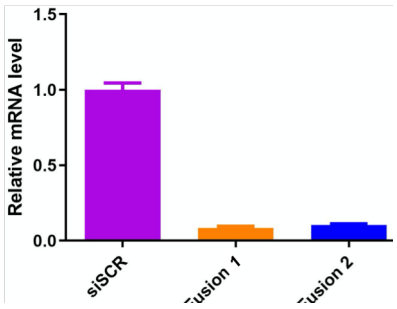

Figure 8: siRNA knockdown of CCDC6-ANK3 in NIH OVCAR3 cells. Cells were transfected with control scrambled siRNA (siSCR) or two different siRNA (siFusion 1 and 2). CCDC6-ANK3 was measured by Q-RT-PCR correcting for GAPDH and presented as relative mRNA level compared to siSCR.

Subtask 3 Overexpression of fusions in cell lines which don't express any of the identified fusions.

Not started as we focused on the downregulation of fusion genes.

Major Task 6 Examine the phenotype of HGSOC cell lines with and without gene fusions

Subtask 1 Analyze phenotypic differences (proliferation, colony formation, anoikis, migration, invasion)

Partially completed. We examined the effect of CCDC6-ANK3 downregulation on growth of NIH OVCAR-3 cells. Cells were transfected with siRNA as above, and then measured growth. Figure 9 shows that knockdown of the fusion with siFusion 2 strongly inhibited growth of cells compared to the control siSCR. Despite good knockdown of CCDC6-ANK3, siFusion 1 inhibited growth but to a lesser extent. This result was repeatable, and we are investigating the discrepancy in this growth phenotype between the two different siRNA.

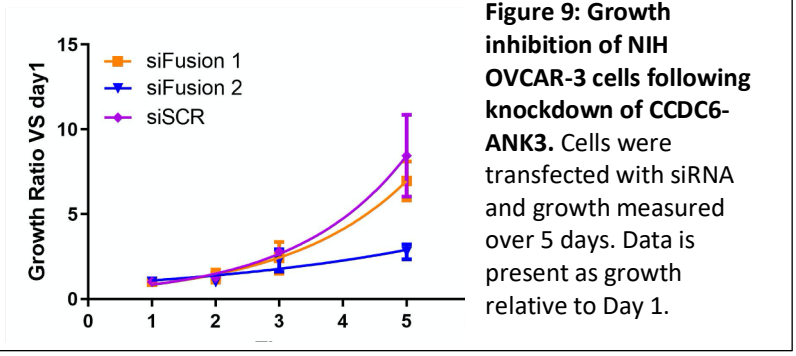

Figure 9: Growth inhibition of NIH OVCAR-3 cells following knockdown of CCDC6-ANK3. Cells were transfected with siRNA and growth measured over 5 days. Data is present as growth relative to Day 1.

Subtask 2 Analyze response to chemotherapy (cisplatin, doxorubicin)

Not completed.

What opportunities for training and professional development has the project provided?

Several trainees received training in cell and molecular biology and learned about ovarian cancer. Nolan Priedigkeit participated in the year 1 study as part of his PhD thesis in the Lee laboratory. He graduated and received his PhD and subsequently his MD. He is currently in Internal Medicine PGY-1 at Brigham Women's Hospital and plans to have a career in medical oncology and the study of women's cancers. The project was then taken over by a Human Genetics student Zeynep Erdogan who received her Masters degree and has now left the laboratory. The project was then carried out by John Willis, who is just about to graduate with a Masters degree and return to medical school. His recent work will be written up in a final publication in the coming year.

How were the results disseminated to communities of interest?

We presented our work as a poster at the AACR Addressing Critical Questions in Ovarian Cancer Research and Treatment, October 1 - 4, 2017, Wyndham Grand Pittsburgh. We received strong positive feedback. Subsequent Work has been presented at the University of Pittsburgh's Integrated Systems Biology Symposium in Feb 2019.

Describe how the results were disseminated to communities of interest.

We presented our work as a poster at the AACR Addressing Critical Questions in Ovarian Cancer Research and Treatment, October 1 - 4, 2017, Wyndham Grand Pittsburgh. The work was also presented as both a poster and talk at the 2019 ISB symposium.

We will complete the sequencing of our FFPE cohort and run that data through our existing molecular characterization pipeline, integrating our findings with our existing cohorts of HGSOC pairs to identify driver genes and pathways of rapid recurrence and chemoresistance. This work will be reported in a manuscript and all of the data made publicly available.

Other achievements.

None

Stated goals not met.

While we were able to complete the majority of the study, there was work where we didn't reach our stated goals, and the reasons are presented above. The change of personnel was a challenge with both Zeynep Erdogan and John Willis originally enrolling as PhD students but leaving with masters degrees. This turnover of personnel slowed progress. We were not able to retrieve primary and recurrent HGSOC for validation and found an alternative dataset of neoadjuvant chemotherapy treated HGSOC, however, obtaining these samples took a long time. The biological functions of fusion genes was started but not completed, but preliminary data suggests a proliferative function for CCDC6-ANK3 and warrants further study on survival and metastasis.

4) IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We describe RNA fusions in chemotherapy naïve and resistant HGSOC, and the finding of acquired fusions in all of the cases suggests there maybe biological drivers of recurrence. Reports from other groups during this funding period indicate a potential role for these RNA fusions in HGSOC.

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project.

What was the impact on other disciplines?

We believe our approach of identifying and validating fusion RNAs may have relevance to others in different fields. We will make all computer code to replicate our approach publicly available.

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

We are using new methods for RNA fusion discovery, validation, and then driver prediction which will add to others work and help guide this field forward. For example, the use of RNA fusion driver prediction algorithms is nascent, and our prediction and then functional validation will deliver data and help improve these algorithms and approaches.

What was the impact on technology transfer?

Nothing to Report

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

Nothing to Report

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

Nothing to report

5) CHANGES/PROBLEMS:

Describe any changes in approach during the reporting period and reasons for these changes

We faced a challenge obtaining a validation set of naïve and chemotherapy resistant HGSOC samples. To overcome this, we focused on neoadjuvant treatment, where each patient is guaranteed to have a pre-biopsy and subsequent resection. Utilizing this approach, we identified a suitable cohort which underwent pathology review and we successfully extracted RNA. We should note however that obtaining these samples took an inordinate amount of time, but they are now ready for sequencing.

Since writing the proposal, the costs of sequencing have dropped considerably. Furthermore, the advantage of NanoString for FFPE samples is decreased as we have recently reported outstanding performance of capture exome RNA sequencing on breast cancer FFPE specimens (PMID: 28878133), and targeted capture sequencing has successfully been used for the detection of fusions in clinical samples (PMID: 30918253). We will therefore now use RNA sequencing instead of NanoString for our validation study.

Actual or anticipated problems or delays and actions or plans to resolve them

We had a delay in the procurement of the second set of human HGSOC samples. The identification and procurement of human samples is always a challenge. Samples must be identified, and then clinical characteristics identified to make sure that the correct samples are procured. However, the neoadjuvant patient cohort was identified, and RNA has been isolated.

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Delays in identifying and procuring tissue as noted above.

Changes that had a significant impact on expenditures

Nothing to report

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period.

Nothing to report

6) PRODUCTS:

Publications, conference papers, and presentations

We presented our work as a poster at the AACR Addressing Critical Questions in Ovarian Cancer Research and Treatment, October 1 - 4, 2017, Wyndham Grand Pittsburgh.

B55 Recurrent transcriptional remodeling events and acquired fusion RNAs in relapsed ovarian cancers. Nolan Priedigkeit, University of Pittsburgh, Pittsburgh, PA, United States.

https://www.aacr.org/Documents/OVA17_Poster_B_for_Web.pdf

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7) PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one-person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name: Adrian V. Lee, Ph.D. – no change

Name: Peter Lucas – no change

Name: Robert Edwards – no change

Name: George Tseng – no change

Name: Zeynep Erdogan graduated with an MS and left the lab. She was performing computational analysis on fusion RNAs, which is now being performed by John Willis.

Name: Li Zhu - no change

Name: Nolan Priedigkeit graduated with his MD/PhD and left the project. He was replaced by John Willis who is a similar trained PhD student who is working on the project. John's salary is currently supported by the graduate program of the University of Pittsburgh and no funds have been required from this grant.

What other organizations were involved as partners?

Organization Name: Roswell Park Cancer Institute (RPCI)

Location of Organization: Buffalo, NY

Partner's contribution to the project (identify one or more)

Financial support; N/A

In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff); N/A

Facilities (e.g., project staff use the partner's facilities for project activities); N/A

Collaboration (e.g., partner's staff work with project staff on the project); RPCI will analyze the RNAseq data in collaboration following transfer under MTA/DUA. This work is in-kind, and no financial support is provided.

Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); N/A

8) SPECIAL REPORTING REQUIREMENTS

Nothing to report

9) APPENDICES

CLINICAL CANCER RESEARCH

Genetics and Molecular Drivers

Abstract B55: Recurrent transcriptional remodeling events and acquired fusion RNAs in relapsed ovarian cancers

Nolan Priedigkeit, Sarah Taylor, Shannon Grabosch, Jahnik Kurukulasuriya, Peter C. Lucas, Silvia Liu, Ester Elishaev, Amit Lugade, Kevin Eng, Anda Vlad, George C. Tseng, Kunle Odunsi, Robert P. Edwards, and Adrian V. Lee

DOI: 10.1158/1557-3265.OVCA17-B55 Published August 2018

Abstracts: AACR Special Conference: Addressing Critical Questions in Ovarian Cancer Research and Treatment; October 1-4, 2017; Pittsburgh, PA

Abstract

Background: The majority of ovarian cancers (OvCa) are exquisitely sensitive to primary cytoreductive surgery and platinum-based agents, yet up to 80% of late-stage disease will relapse and develop deadly resistance to subsequent therapies. Identifying molecular mediators driving this acquired resistance is essential to improve the tragic 12- to 18-month prognosis for patients with recurrent disease. We hypothesize that relapsed ovarian cancers —which are largely uncharacterized—are molecularly distinct from primary disease and acquire druggable vulnerabilities throughout their life histories. To test this hypothesis, we undertook a transcriptome-wide analysis of 19 longitudinally collected patient-matched pairs of chemotherapy-naïve and recurrent cancers.

Materials and Methods: Illumina TruSeq Total RNA-sequencing was performed on 19 flash-frozen patient-matched pairs of primary and recurrent OvCa. Time to recurrence was up to 64 months with a median of 25 months—a shared variant analysis confirmed all paired samples were patient-matched. Adapter-trimmed RNA-sequencing reads were quantified with k-mer based lightweight-alignment (*Salmon v0.8.2*) and transcript-abundance estimates were collapsed to gene-level with *tximport*. Differentially expressed genes were determined with *DESeq2* using a paired model to account for patient-matched samples. To identify gains and losses in clinically actionable genes (*DGIdb 2.0*), pair-specific, outlier fold-change thresholds were defined as Q1/Q3 -/+ [1.5 X IQR], using each pairs'

expression fold-change values (recurrence vs. primary) as the distribution. These discrete, longitudinal transcriptional remodeling events (LTREs) in relapsed OvCa were then assessed for recurrence across all cases. Given that OvCa is thought to be driven largely by genomic structural variation, fusion RNAs were then called with *FusionCatcher v0.99.7b*. Identified fusions were filtered for cancer specificity by discarding fusions detected in normal tissue (Human Protein Atlas and BodyMap). The same fusion analysis was performed on CCLE OvCa cell line RNA-seq and selected fusions were validated with RT-PCR and Sanger sequencing.

Results: A suite of genes were consistently upregulated in OvCa recurrences, the most significant being *NTRK2* (adjusted p-value < 0.001)—a targetable tyrosine kinase. LTREs were also common with the most shared LTRE gains in recurrences being *INHBA* (44%) and *IGF1* (39%). 18 of 19 (95%) recurrent cancers acquired cancer-specific fusion RNAs that were undetectable in the primary lesion. An in-frame, recurrence-acquired fusion between *TOP2A*, a target of doxorubicin and known chemoresistance mediator, and *STAU1* was confirmed with RT-PCR. Lastly, we discovered recurrent (2 of 19 cases), in-frame *CCDC6-ANK3* fusions that persisted throughout therapy in both the primary and relapsed lesions, each with distinct breakpoints. A *CCDC6-ANK3* fusion was also validated in the chemoresistant OVCAR3 cell line. 15 of 19 cases (79%) harbored additional preserved fusions, albeit none were shared between cases.

Conclusions: Collectively, these results define multimodal transcriptomic mechanisms of ovarian cancer evolution in late disease. Considering that some acquisitions are highly recurrent and readily druggable (*NTRK2*, *IHBA*, *IGF1*), further preclinical studies are demanded and currently ongoing. Lastly, we establish acquired fusions involving known chemoresistance modulators and preserved fusion transcripts—which are maintained throughout therapy—as common somatic events in OvCa. Because fusion breakpoints are cancer specific, they may serve as promising patient-specific nucleotide targets and biomarkers.

Citation Format: Nolan Priedigkeit, Sarah Taylor, Shannon Grabosch, Jahnik Kurukulasuriya, Peter C. Lucas, Silvia Liu, Ester Elishaev, Amit Lugade, Kevin Eng, Anda Vlad, George C. Tseng, Kunle Odunsi, Robert P. Edwards, Adrian V. Lee. Recurrent transcriptional remodeling events and acquired fusion RNAs in relapsed ovarian cancers. [abstract]. In: Proceedings of the AACR Conference: Addressing Critical Questions in Ovarian Cancer Research and Treatment; Oct 1-4, 2017; Pittsburgh, PA. Philadelphia (PA): AACR; Clin Cancer Res 2018;24(15_Suppl):Abstract nr B55.

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Recurrent HGSOC tumors are enriched in fusions with ECM-receptor signaling genes

Introduction: High Grade Serous Ovarian Cancer, the deadliest gynecologic cancer, develops chemoresistance in recurrence following chemotherapy(cite). The genome of HGSOC is notably rich in structural variation(cite), and the acquisition of gene fusions in recurrent HGSOC has been suggested as a potential driver of the phenotypic evolution of HGSOC.

Materials and Methods: 150bp-Paired-End RNA Seq was performed on 19 pairs of matched chemonaive and chemo-treated recurrent HGSOC samples. Additional sequencing data from the TCGA and AOCS was obtained from DBGAP. Using an ensemble of fusion callers(FusionCatcher v1.0, STARFusion v1.4, MapSplice2, pizzly), and the fusion prioritization algorithms Oncofuse and FusionZoom, we generated a list of acquired fusions that might drive HGSOC evolution. Concurrent alignment/mapping with STAR and SALMON was performed prior to differential gene expression analysis with DESeq2 and limma-voom. Fusions were prioritized based on their driver score, influence on gene expression, detection by multiple algorithms, frame, and supporting reads. High priority fusions were compared to databases of known fusions using FusionHub. High Priority fusions were screened in tumor samples and cell lines via PCR with primers designed against novel exon-exon junctions.

Results: Fusion calling with our ensemble approach revealed considerable variability between the outputs of different fusion callers. FusionCatcher called the most fusions per sample, though it categorized the majority of called fusions as false positives. Notably, we did not see a greater number of fusions called for chemonaive vs chemo-treated samples. Of the fusions called by our ensemble, >25 recurrent fusions were reported. Many of these fusions were false positives involving pseudogenes or homologues. Fusions caller were enriched in genes from ECM-receptor signaling, Receptor Tyrosine kinase($q=0.002$), and cyclin D($q=0.07$) associated events in G1 pathways($q=0.01$). Chemotherapy treated samples overexpressed genes associated with ECM-Receptor Signaling($q=0.0005$) and Focal Adhesion($q=0.004$) pathways. Of the recurrent acquired fusions detected in our pipeline, expression the fusion ACTB—MTPAP was associated with an enrichment in immune-related genes.

Discussion: Fusion calling on our paired HGSOC dataset failed to uncover any highly recurrent acquired fusion genes, or a notable increase in fusions detected between chemonaive and chemo-treated samples. However, the enrichment of fusions in gene pathways that are also differentially expressed in our paired dataset pointed to a set of potentially related fusions in common pathways. By introducing these fusions into HGSOC model cell lines from the Cancer cell line encyclopedia, we will attempt to characterize the influence of acquired gene fusions in ECM-receptor signaling pathways on malignancy associated phenotypes including proliferation, chemosensitivity, and motility.