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TITLE: Identification of a Genomic Signature Predicting for Recurrence in Early Stage Ovarian Cancer

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14. ABSTRACT The second year of the grant required the following tasks: 1. RNAseq of 400 training specimens (Months 12-18) 2. Import raw data into public databases (Months 12-18) 3. Generate preliminary gene signature through bioinformatic and statistical analysis (Months 18-24). In year 1) we had identified 592 early-stage high-grade ovarian cancers with 5-year follow-up, clinical annotation and accurate pathological review (228 recurrent and 364 non-recurrent), 2) established a specimen repository and clinical data inventory at MGH, 3) micro-dissected and isolated RNA from 110 tumors, and 3) optimized the preparation of cDNA libraries using NuGene WT-Ovation FFPE System V2. Given the fact that RNA sequencing is in its early stage of application, and application of this technology to FFPE tissue is still being fully developed, we have been working a work-process with different Nextgen facilities to successfully apply this technology to our FFPE samples. This included sequencing a sample test of 10 tumors and comparing the sequencing results of these early stage samples with publicly available RNAseq data for early and advanced ovarian cancers. Once the SOP were set we have been able to sequence the first 100 samples of our biorepository.					
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Introduction

All patients with high-risk early stage ovarian cancer are treated with comprehensive surgery followed by chemotherapy over a four to six month period. Yet it is clear that many of these women are cured by surgery alone. The overtreatment results from our inability to accurately identify patients who will not likely recur with surgery alone. This ultimately exposes these patients to both short and long term toxicities from chemotherapy. An objectively measurable characteristic (i.e. biomarker) that could accurately predict for ovarian cancer recurrence would be of great clinical value much like *Oncotype DX* has done for triaging early stage breast cancer patients. This ovarian biomarker would enable health care providers to provide a more tailored approach to ovarian cancer patients. We have identified a preliminary but promising genomic signature (i.e. characteristic expression of a set of genes) that can be applied to surgically attained ovarian specimens and predicts for cancer recurrence. While we do not expect this precise signature to validate, it is proof of principle that this type of genomic tool can be identified. This project proposes to generate and validate a recurrence signature for early stage ovarian cancer. A key bottleneck precluding the validation of cancer-related signatures, in general, lies in the large number of specimens needed to ensure that the signature is clinically valuable. This proposal will utilize a larger number of early stage ovarian cancer specimens obtained from an international consortium of clinical research groups to identify a genomic signature which can accurately identify patients who will suffer tumor recurrence. The stratification of patients according to risk of recurrence will allow those patients at high risk to receive more intense therapy and those at low risk to avoid chemotherapy toxicities. This will provide patients with early stage ovarian cancer a more personalized approach in addition to reducing overall costs of treatment. The identification of a recurrence signature will occur over the three years of the grant and due to our industrial collaborations, we expect the genomic signature to rapidly transition into a commercially available tool. In addition, all specimens will undergo extensive genomic analyses to generate a publically available database of genetic changes within early stage ovarian cancer to help researchers worldwide identify biomarkers that can aid early detection and inform novel targets for therapy. This will provide a unique database which will complement existing publically available genomic data. This project will leverage unique individual banks of stored specimens and associated clinical data present in the collaborating but disparate organizations. This will allow this clinically important question to be addressed and fulfill an important unmet need.

KEYWORDS: Early Stage Ovarian Cancer, genomic predictive signature, recurrence, RNAseq

Research Accomplishments

Past accomplishments and goals for this period: The first year of the grant required: 1) IRB approval from all the Consortium collaborative Institutions, 2) specimen collection and inventory, 3) initial specimen processing. All the collaborating Institutions have obtained IRB approval to identify early stage/high grade tumors from their respective pathology service and to ship these tumors together with their clinical annotations to Massachusetts General Hospital (MGH). MGH has obtained IRB approval for using these cancer FFPE samples to identify molecular features that distinguish recurrent and non-recurrent tumors through RNA sequencing. We have then: 1)

identified 592 early-stage high-grade ovarian cancers with 5-year follow-up, clinical annotation and accurate pathological review (228 recurrent and 364 non-recurrent), 2) established a specimen repository and clinical data inventory at MGH, 3) micro-dissected and isolated RNA from 110 tumors, and 4) optimized the preparation of cDNA libraries using NuGene WT-Ovation FFPE System V2.

During this second year of the project we have set up the optimal sequencing protocol, by testing 10 samples at different next generation sequencing core facilities, and generated a standard operating procedure. We have achieved following key accomplishments:

Development of a standardized protocol for FFPE ribonucleic acid extraction: We have finished extracting 100 FFPE early-stage ovarian cancer specimens (50 recurrent and 50 non-recurrent) to prepare RNA samples for subsequent next-generation sequencing. A standardized protocol was developed for RNA extraction. To minimize the interference from tumor stroma derived expression profile, cresyl violet guided macro-dissection was introduced to ensure at least 80% tumor cell content within the samples subjected to nucleic acid extraction. Cresyl violet forms non-covalent, easily-reversible binding to nucleic acids and allows distinguishing tumor tissue from stroma. The staining provided by Cresyl violet, is comparable to traditional dyes such as hematoxylin but, unlike hematoxylin, it does not chemically modify DNA or RNA and interfere with downstream profiling study (Figure 1).

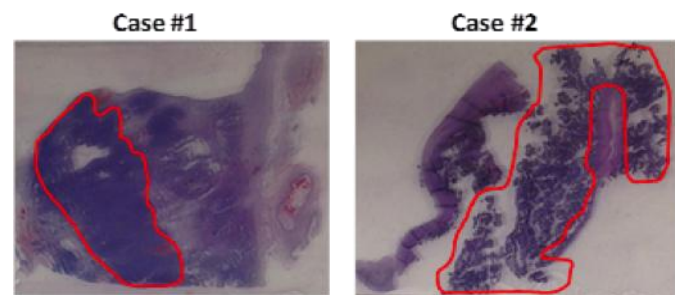


Figure 1. Cresyl violet guided macrodissection to enrich tumor component (circled by red line). In brief, deparaffinized and rehydrated FFPE tumor sections were briefly dipped into 0.5% (v/v, dissolved in 50% EtOH) cresyl violet for 30 seconds. Excessive dye was washed sequentially by 70% and 90% EtOH. Sections were then dehydrated in 100% EtOH and air-dried before macro-dissection using a sterile, RNase-free scalpel.

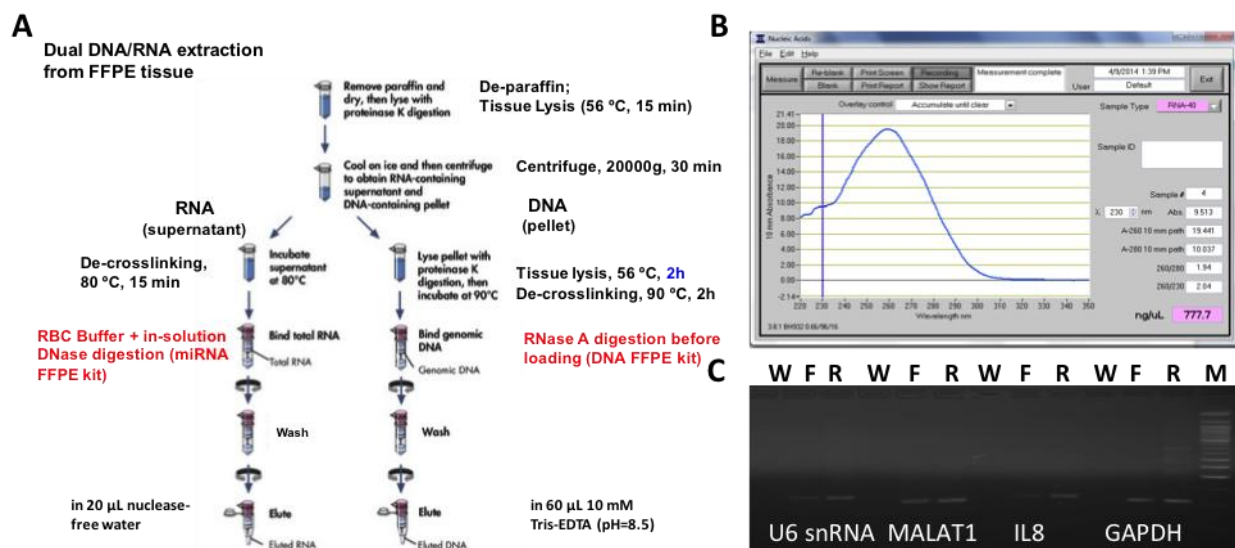


Figure 2. Flow-chart of standardized nucleic acid extraction (A) and related quality control (B and C)

Dual DNA/RNA extraction from FFPE sections was then carried out by sequential use of QIAGEN miRNeasy FFPE kit (217504) and QIAGEN QIAamp® DNA FFPE Tissue Kit (56404). De-paraffinized, macrodissected FFPE tissues were briefly digested with Proteinase K in Buffer PKD (QIAGEN) to release the RNA into solution while the after-digestion pellet contained primarily DNA that was processed by the QIAGEN DNA FFPE kit for a parallel project recently funded by DOD (W81XWH-14-1-0194) aimed at interrogating the genomic profiles of early-stage ovarian cancers.

After de-crosslinking to reverse the formaldehyde modification, the RNA in the solution was precipitated with increased strength of salt (guanidine HCl) and isopropanol to recover all RNA species including the mRNA and long and small non-coding RNAs (e.g. miRNA). Standard QIAGEN low-volume QIAGEN columns with preferential binding to RNA were used for RNA recovery and clean-up. The quality of the extracted RNA was checked by Nanodrop as well as qRT-PCR for various transcripts representing small RNA (U6), long non-coding RNA (MALAT1) and mRNA (IL8 and GAPDH) (Figure 2).

Selection of Next generation sequencing core facility for RNAseq: 10 tumor samples (5 recurrent and 5 non recurrent) were sequenced at 3 different core facilities. The sequencing results were analyzed and compared to publicly available sequences in TCGA database for early- and late-stage ovarian cancers. Based on these results we have selected the facility at the Center for Molecular Oncologic Pathology (CMOP), Dana Farber Cancer Institute (Harvard Medical School, Boston MA). The whole library construction and RNAseq procedures will be carried out following a Standard Operating Procedure (SOP) that will be replicated throughout all the samples. Briefly:

- RNA concentration is measured by Picogreen assay (Life Tech). RNA quality control is performed on Agilent 2100 Bioanalyzer. Agilent RNA 6000 nano kit is used for QC RNA with a minimum concentration of 5ng/uL.
- TruSeq Stranded total RNA kit is used for library preparation. The library construction protocol was optimized for degraded RNA according to guidelines from Illumina. Heating fragmentations are eliminated while only chemical fragmentations are used. Total RNA is put into RNA purification where ribosomal RNA and human mitochondrial RNA are removed by binding to magnetic micro particles with specific probes. Remaining messenger RNA and other non-coding RNA are used for generating library.
- Purified RNA is reverse transcribed to cDNA and then complementary DNA strand are synthesized to form stable double strand DNA. After 3' end adenylation, a 6 nucleotide adaptor is ligated into the dsDNA. Libraries are enriched by 15 cycles of PCR amplification, as indicated by manufacture.
- The samples preparation is automated on BioMek FXP automation workstation (Beckman Coulter). Batches of 48 samples are processed in parallel.

This SOP has been established by the CMOP core facility and has been demonstrated to generate robust and reliable data from FFPE RNA samples. At the CMOP a similar procedure was successfully applied to clinical prostate and lung cancer samples. For both studies paired fresh frozen (FF) and FFPE were used. The samples from both cancer types showed excellent depletion of ribosomal RNA (a major concern for non polyA selected library preparation methods), we observed less than 1% of the reads mapping to the ribosomal genes. Over 80% of the sequenced reads aligned uniquely to the human genome, a percentage comparable to the sequencing results from the frozen specimens. We observed correlations over 0.9 between the technical replicates for FFPE samples, and correlations ranging from 0.8 to 0.98 between FFPE and FF pairs. The prostate study was designed to perform biological validation of the RNA-Seq from FFPE – using paired tumor and normal specimens we were able to distinguish malignant and normal tissue using a panel of genes known to be differential expressed between these two tissue types.

Sequencing of 100 tumor samples: we are presently sequencing the initial 100 samples (50 recurrent and 50 non recurrent). The sequences are being analyzed (real-time) to help reinforce the power calculation of our study and determination of the optimal ratio of recurrent versus non recurrent tumors to be used for the training stage of the study. This is important to avoid using an excessive number of samples that can be other ways used for other studies.

Procedures for “Batch effect” control: Considering the relative large size of the proposed study, we noticed that the batch effect might have significant impact on data analysis for our signature development. In consultation with Dr. Victoria Wang, biostatistician from the Dana Farber Cancer Institute, we have established a two-tier of strategy to reduce the batch effect. 1) From bioinformatic prospective, standard surrogate variable analysis / principle component analysis (sva/pca) will be used to estimate artifacts introduced by factors irrelevant to biology such as sample source, sample age and technical variations. 2) We also set up SOP to minimize the technical variations during the wet-lab procedures. The latter includes: 1) performance of all extractions by only one dedicated post-doctoral fellow, and 2) tight quality control (e.g. repetitive assaying of the same sample). To perform the bioinformatics analysis of potential batch effects we have generated a fully annotated sample datasheet that records the following parameters for each sample: tumor block age, cutting-to-extraction time, tumor volume used for extraction (estimation based on number of 10µm slides), tumor purity (70 to >90% purity), DNA and RNA yield, type of stromal pattern, stromal versus tumor TILs infiltration pattern.

Plans for the next reporting period to accomplish the goals: The objectives for this current year were to: 1) RNAseq of 400 training specimens, 2) import raw data into public databases, 3) generate preliminary gene signature through bioinformatic and statistical analysis.

We have established a detailed protocol to sequence RNA extracted from FFPE early-stage/high grade tumors that were collected in our biorepository. Because the RNAseq technique was not yet fully established for FFPE samples, this procedure revealed to be more complex than expected. However, the lengthy and detailed preliminary studies we have performed will allow a rapid analysis of the remaining number of tumors required to generate a preliminary gene signature that will be further validated by QRT-PCR in the next project period.

Results disseminated to communities of interest: We have created a news letter that is being distributed every 2 months to communities of interest. This news letter updates the communities on the status of the project and keeps them engaged. It may be used to ask for more material. Please find attached the first version of the letter that was submitted when this project started.

Actual or anticipated problems or delays and actions or plans to resolve them: we continue to have accrual problems as many cases identified do not have the proper follow-up, pass pathologic confirmation, and sufficient tissue. As such we have continued to expand the consortium by including new institutions.

IMPACT

Impact on the development of the principal discipline(s) of the project: Creation of a well annotated biorepository of early-stage tumors allows performing correlative clinical and genomic studies on these tumors that are so poorly characterized and yet significantly affect the life of so many women. Establishment of a detailed protocol for RNAseq on RNA extracted from formalin-fixed paraffin-embedded (FFPE) samples brings advancement in this novel genomic technology (RNAseq) and its broader application to cases where fresh frozen material is not available. Because most patient tumor specimens are kept as FFPE samples by the hospital, application of RNAseq to these samples allows biologic characterization of rare tumors.

Impact on other disciplines: Nothing to report

Impact on technology transfer: We anticipate that genomic discoveries in this project will have commercial application.

Impact on society beyond science and technology: Nothing to report

INTEGRATED GENOMIC ANALYSIS OF EARLY STAGE HIGH GRADE OVARIAN CANCER

Newsletter

Early Stages Ovarian Cancer consortium

Coordinating Center:

Massachusetts General Hospital

Principal Investigator:

Michael J. Birrer

Background

The standard of care for high-grade early stage ovarian cancers remains identical to their advanced stage counterparts. Platinum based therapy only benefits 50% of women with early-stage ovarian cancer resulting in **over treatment** and unnecessarily exposure to **toxic side effects for the remaining 50%** of women with this disease.

Objective

We have received a Federal Fund to create a biorepository of FFPE early-stage high-grade ovarian cancers to perform a multi-component genomic analysis including: **DNA copy number variation, RNA sequencing.**

We hypothesize that through integration of these multi-demonstrational genomic data we will be able to identify and validate very specific markers that distinguish recurrent from non-recurrent high-grade early-stage OCs.



Winter is coming, samples are being collected!

We are now obtaining samples from yours and from other institutions. Our last update just before this issue is **up to 175 samples.**

Our goal is to collect 200 recurrent and 200 non recurrent FFPE tumor tissues with 5 years medical follow up (if not relapsed cases).

What we need

- FFPE samples;
- early stage/high grade epithelial ovarian cancer (grade2 and 3 for old WHO classification);
- serous or endometrial histology (mixed cells tumors with at least one of these histology features represented are also eligible);
- from patients that had recurrences or not, with 5 years follow up (if not relapsed cases) and clinical data.

If you still don't have our eProtocol form, you can find it attached. You can send us block or RNase free cut slides, please see the protocol.

What we are now doing

The Pilot study had just started. We were able to extract Nucleic Acids from each of sample obtaining a reasonable amount of DNA and RNA.

...continues....

INTEGRATED GENOMIC ANALYSIS OF EARLY STAGE HIGH GRADE OVARIAN CANCER - *Newsletter***What we need you to do:**

Thank you for your collaboration, we are briefly moving forward on that project and any help will be really valued!

When you find samples in your Institution /group the following steps will be:

Submit and obtain an approval from your IRB the form you can find here attached:

- [IRB form for submission](#)
- [IRB 2014 Approval Update](#)

Fill the eProtocol form from page 3 for each sample you are going to send and, if it is possible, send also the original Pathologic report;

Contact us to arrange the Shipment!

If you request help for samples inclusion criteria, section protocol, IRB documentation, samples shipment, please send an email to lceppi@mgh.harvard.edu

Early Stage Ovarian Cancer Consortium, Massachusetts General Hospital.

Do not reply to this email, please send all the correspondence to: Lorenzo Ceppi at lceppi@mgh.harvard.edu



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