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Identifying Novel Biomarkers for Early Detection of Ovarian Cancer Using Exosomes

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CONTRACTING ORGANIZATION: The Ohio State University

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: High-grade serous ovarian cancer (HGSOC) accounts for over 75% of all epithelial OC and the majority of patients with HGSOC are diagnosed with advanced-stage disease due to a lack of early detection methods. Exosomes serve as a potential and unique source of biomarkers as they can be obtained via a patient blood draw, are highly stable, and reflect the information of their cell of origin. Due to these features, there is growing interest in the use of exosomal cargo (proteins, microRNAs and lipids) as a source of serum biomarkers. However, exosomal isolation and protein detection has not yet been proven or validated as a clinically effective method for early disease detection. Thus, there is a critical need to establish methods for exosomal isolation and to validate their use as biomarkers that can detect the early stages of HGSOC in order to improve the survival rates of patients with this disease.

15. SUBJECT TERMS

Exosome, exosomal proteins, early detection, Biomarkers and MFD chip

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1. INTRODUCTION

Ovarian cancer (OC) is the deadliest of all gynecologic cancers. High-grade serous ovarian cancer (HGSOC) accounts for over 75% of all epithelial OC and the majority of patients with HGSOC are diagnosed with advanced-stage disease due to *a lack of early detection methods*. Notably, there is a drastic difference in survival depending on the stage of disease at the time of diagnosis: the 5-year survival rate for patients diagnosed with epithelial ovarian cancer at stage I/II is 66.9-81.3%, whereas this is reduced to 33.3-41.3% for patients diagnosed at more advanced stages. Biomarkers, such as proteins that originate from HGSOC cells, are an attractive option for early disease detection. Exosomes serve as a potential and unique source of biomarkers as they can be obtained via a patient blood draw, are highly stable, and reflect the information of their cell of origin. Due to these features, there is growing interest in the use of exosomal cargo (proteins, microRNAs and lipids) as a source of serum biomarkers. However, exosomal isolation and protein detection has not yet been proven or validated as a clinically effective method for early disease detection. Thus, there is a *critical need* to establish methods for exosomal isolation and to validate their use as biomarkers that can detect the early stages of HGSOC in order to improve the survival rates of patients with this disease.

Hypothesis: Based on our preliminary results, our *central hypothesis* is that exosomal proteins can serve as sensitive and specific biomarkers that will detect early stage of HGSOC.

SPECIFIC AIMS & APPROACH:

Specific Aim 1: To validate the microfluidics chip for exosome isolation and to standardize the exosomal proteome by ELISA, Proximity Extension Assays (PEA) and Luminex. We will use 100 serum samples (25 of each control, benign, early and late stage HGSOC) to further validate the performance of our novel microfluidic chip to isolate exosomes and compare to known exosome isolation techniques, a key step toward clinical translation of the technology. Using the exosomes isolated from the validated MFD chip, the exosomal candidate protein profile expression will be evaluated and standardized by ELISA, PEA, and Luminex.

Specific Aim 2. To determine the ability of identified candidate exosomal proteins to detect early-stage disease independently and in combination with other markers using a multinomial regression model. In this aim, we will be using an independent set of 800 serum samples from patients with early stage HGSOC (180) and advanced-stage HGSOC (210), benign (200), and age-matched healthy controls (210). First, exosomes will be isolated from a training cohort of 300 samples and the candidate biomarker proteins will be evaluated by ELISA, PEA and Luminex. The protein data for each disease state will be used to develop a multinomial logistic regression model. The best performing biomarkers determined by the training cohort will then be validated in an additional 500 patient samples. The sensitivity & specificity of the exosomal biomarkers will be compared to that of clinically utilized biomarkers, such as CA125 & HE4 as well as evaluated in combination with these widely used biomarkers.

2. KEY WORDS

Ovarian Cancer

Exosome

Biomarkers

Early detection

Exosomal proteins

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goal of this study is to identify the novel exosomal proteins, as potential biomarkers for high-grade serous ovarian cancer.

What was accomplished under these goals?

We have identified the significance of key findings in SA1

(i) Validation of MFD chip for exosome isolation in serum samples;

(ii) Identified the exosomal candidate proteins are highly elevated in early stage of HGSOC;

Aim 1: To identify the serum exosomes proteins that are differentially expressed in platinum-resistant HGSOC samples. Thus, the *objectives* of this aim are to identify the exosomal proteins that are unique to platinum resistance in HGSOC, and to validate the utility of our microfluidic device for exosome isolation. The microfluidic device will be used to identify serum exosomal proteins that are unique to platinum-resistant HGSOC compared to platinum-sensitive HGSOC and controls by LC-MS/MS.

1) Procurement, processing, and handling of human samples Prepare forms for approval of Animals use and protocols involved.
Prepare IRB forms for approval of human sample use and protocols involved.

Milestone # 1 human sample use approval (Year 1: month1-3): **Completed 100%.**

Approach 1. Developed a microfluidics device (MFD) for exosome isolation.

Milestone # 1. Developed Microfluidic chip standardization of the method and validation for exosome isolation (Year 1: 1 to 6 months). **Completed 100%**

1.3. MFD validation for exosome isolation: (i) Nano particle tracking analysis (NTA) and Image stream flow-cytometry (ISF):

Milestone # 2. Isolated exosome confirmed by different methods (Year 1: 7 to 10 months). **Completed 70%**

1.4. LC-MS/MS and Shotgun method identified exosomal proteome (Table 1) standardization by ELISA, Luminex and PEA
Currently working on exosome samples analysis using LC-MS/MS study

Publication: None.

RESULTS

1. Exosome Isolation, confirmation and quantification: The FITC-Labelled EVs (green) captured from our MFD channels are shown in (**Figure 2Ai-Aii), V**esicle size and morphology was confirmed by cryo-TEM and SEM (**Figures 2Aiii, 2Aiv**). Exosome surface markers CD9, CD63 and TSG101 with epithelial specific markers EpCAM confirmed vesicle lineage from the epithelial cells in controls and HGSOC samples (**Figures 2B-C**). When evaluated by ISF and NTA, a sufficient amount of exosome secretion levels and EVs proteins (500ng to 5µg/µl) was detected in controls, early- and advanced-stage HGSOC samples using our novel MFD coated with three different exosome surface markers, including CD9, CD63 and TSG101 (**Figures 2D-F**). Overall and EpCAM-positive EVs concentrations were higher in advanced-stage HGSOC samples compared to early-stage HGSOC and control samples (**Figures 2E, 2F**). There was no difference in exosome size among groups.

2, Evaluation of exosome proteome profiles in high grade serous ovarian cancer compared to controls:
LCMS/MS and Shotgun Proteomics. The subset of proteins elevated in early-stage HGSOC samples relative to control samples based on their difference in expression and fold change are summarized in **Table 1**. This is termed our discovery dataset of candidate proteins.
IPA. When the discovery set of candidate proteins were analyzed by IPA, many of those that were differentially expressed were found to have a significant role in cancer regulatory networks, such as cancer cell proliferation, survival, tumor progression and metastasis. These results are shown in **Figure 2**.
PEA. Using PEA, we again identified a subset of proteins that were significantly elevated in early-stage HGSOC serum EVs compared with controls (**Table 2**). These include ANG-2, CD40, CXCL1, FAS, GAL1, IL-6, IL-8, MMP-1, PDL1, and STAT3.

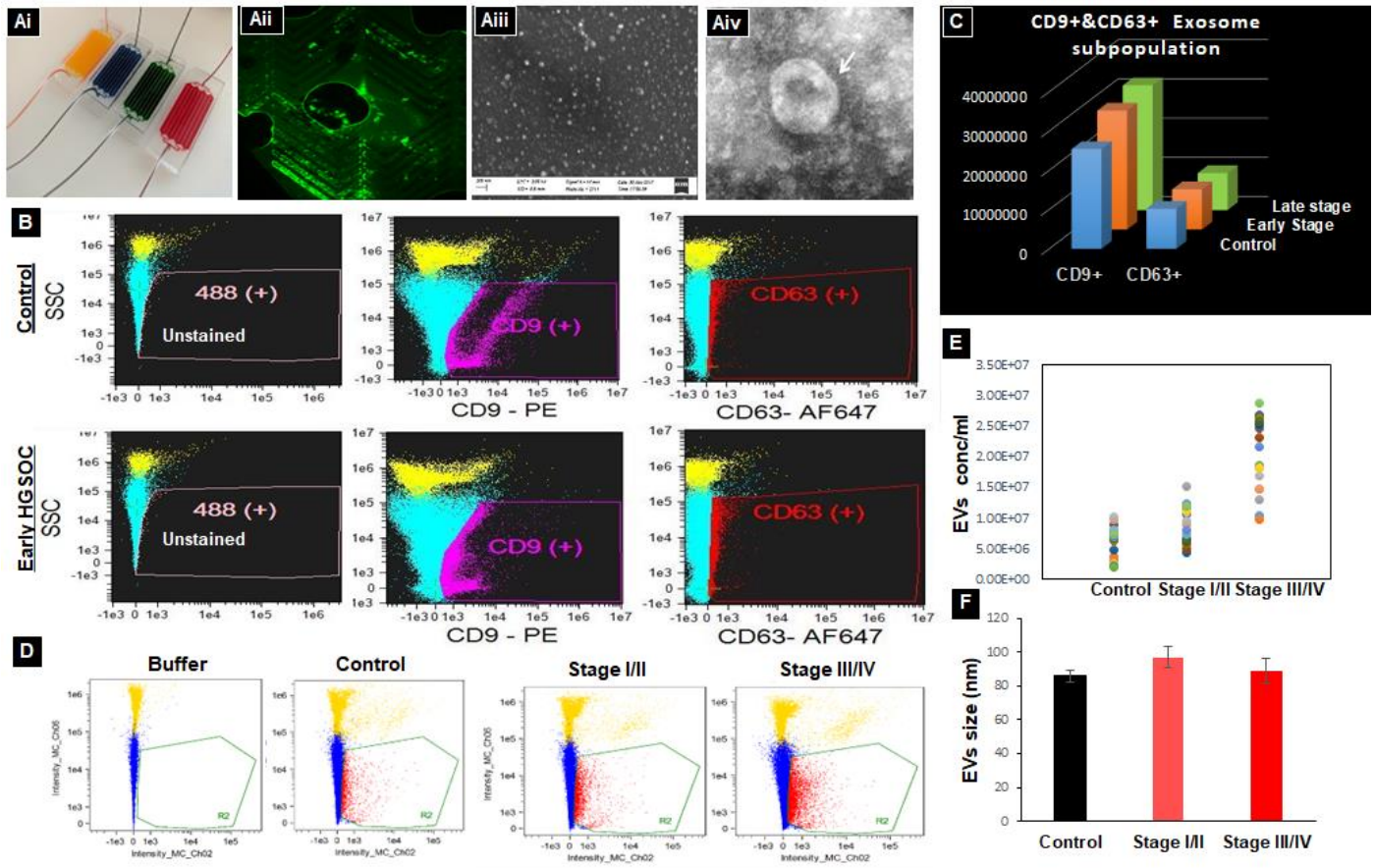

Figure 1. Exosome isolation and confirmation. Ai) Image of Microfluidic device (MFD), MFD channels functionalized with CD9, CD63 and TSG101 antibodies, the flow is shown in different colors. **Aii**) Exo-FITC labelled Exosome (green) capture in MFD channels. **Aiii and iv)** Morphological characterization and size measurement of EVs (indicated by red arrows) by (**iii**) Cryo and (**iv**) classical transmission electron microscope (TEM). **B and C)** Confirmation of exosome specific marker CD9 and CD63 population by flowcytometry in the different patient' serum samples. **D)** Exosome particles confirmed by image stream flow cytometry (the bar surrounding color indicated exosome count) in control, benign and HGSOC serum stage I/II and stage IV samples. **E and F**) Nanoparticle tracking analysis (NTA) for exosome quantification and size confirmation, isolated by microfluidic device (n=5).

Table 1. Candidate proteins upregulated in EVs from HGSOC early stage compared to control patient samples by LC-MS/MS

Proteins	p<value
AGRIN	0.02
CPNI	0.005
CFH	0.01
CMET	0.003
Cyto P450 2E1	0.01
CXCL3	0.002
FAS	0.002
G6-MP	0.01
HGF	0.02
IL-6	0.009
IL-8	0.01
MAPK	0.001
MMP3	0.003
NID1	0.001
PDL1	0.001
Pim-2 oncogene	0.03
PZP	0.02
SPP24	0.05
STAT3	0.001
TETN	0.05
TETR	0.01
VEGFA	0.03

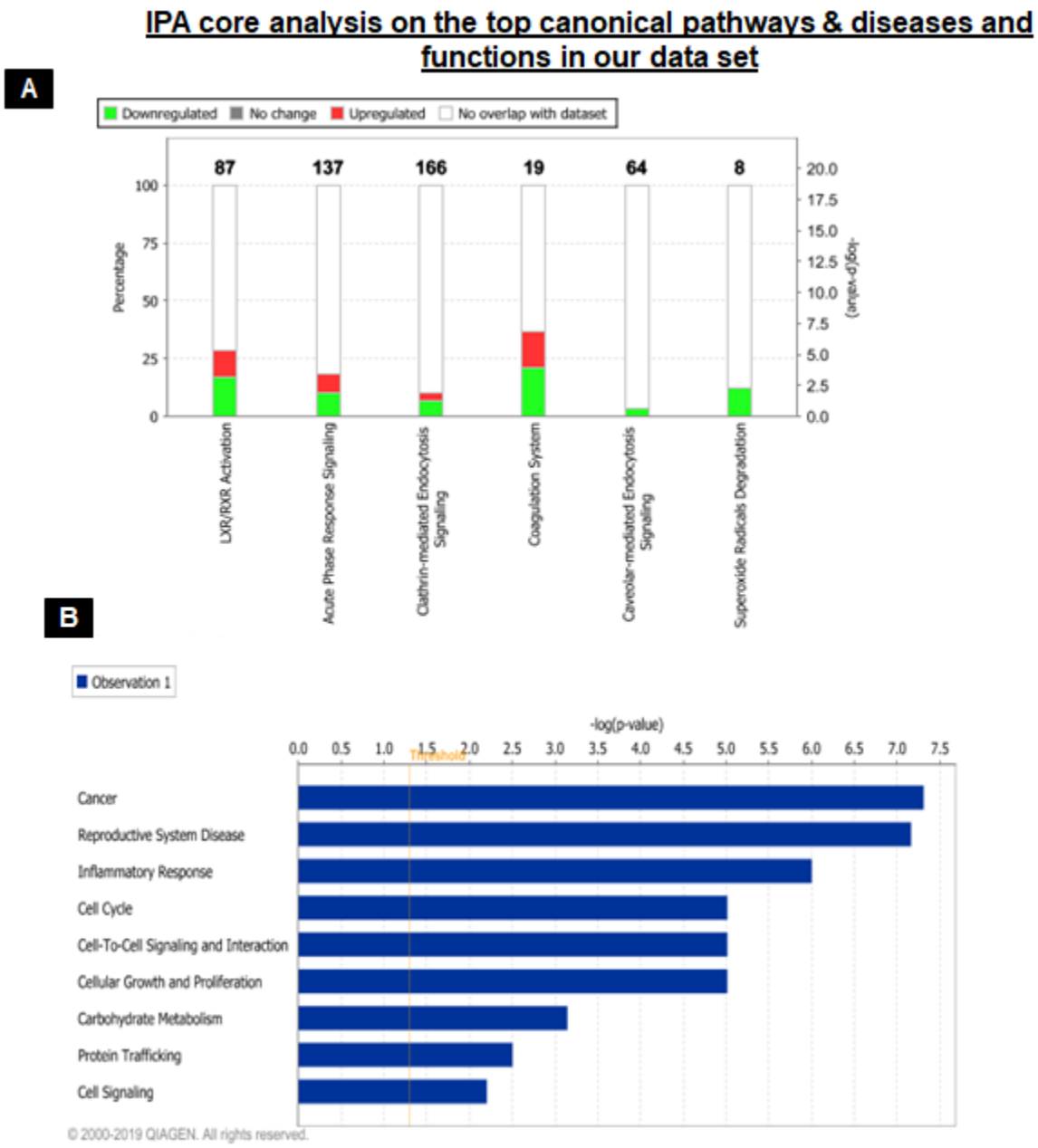

Fig 2: IPA comparative core analysis reveals **(A)** the top canonical pathways and the percentage of upregulated and downregulated proteins identified in our dataset expressed as log (P value) in Early HGSOC stage(I&II) Vs Control healthy patient samples. **(B)** The top disease and functions associated with the proteins identified in our dataset are expressed as log (P value) above a significance threshold based on Fisher exact test ($P < 0.05$).

Table 2. Serum exosomal proteins that were differentially expressed in early stage HGSOC samples compared to benign samples by PEA (n=12).

Protein	p-value
ANG-2	0.001
CD40	0.016
CXCL1	0.041
EGF	0.046
FAS	0.037
GAL1	0.015
IL-6	0.006
IL-8	0.026
MMP-1	0.013
PDL1	0.001
STAT3	0.001

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Currently we are finalizing the exosomes isolation and confirmation, LC-MS/MS data describing the data presented in the report and finish the remaining study by this year end 2023..

For year 2, we plan to complete our proposed experiments from Aim 2, To validate the clinical significance of patient serum exosomal protein expression as a biomarker for HGSOC. We will be using an independent set of 800 serum samples from patients with early stage HGSOC and advanced-stage HGSOC, benign, and age-matched healthy controls. The protein data for each disease state will be used to develop a multinomial logistic regression model. The best performing biomarkers determined by the training cohort will then be validated in an additional 500 patient samples. The sensitivity & specificity of the exosomal biomarkers will be compared to that of clinically utilized biomarkers, such as CA125 & HE4 as well as evaluated in combination with these widely used biomarkers.

Initiate experiments from Aim 2 January 2024..

4. IMPACT

1. Impact on the development of the principal discipline (ovarian cancer) of the project: To identify potential biomarker, we performed preliminary studies on serum from patients with early-stage HGSOC, benign ovarian disease and controls (without cancer) to assess their exosomal protein profile using Mass spectrometry (LC-MS/MS). We have identified more than 15 exosome-derived proteins that are differentially expressed in serum from patients with early-stage HGSOC relative to serum from age-matched controls and patients with benign ovarian tumors, making these high-priority candidate biomarkers for HGSOC screening (**Table 1**). However, these preliminary studies need to be confirmed in a larger number of samples, and additional research is needed to identify the combination of serum proteins that will optimize sensitivity and specificity for detection of early-stage disease. Our study proposes to explore the biology and clinical value of exosomes as a source of protein biomarkers that can be used for the early detection of HGSOC.

2. Impact on the development of other disciplines: Our study can have impact on all other solid tumors. The expected outcomes of the proposed project is identification and validation of exosomal protein biomarkers within a novel multinomial regression model that can predict early-stage HGSOC

3. Impact of the technology transfer: Nothing to report

4. Impact on society beyond science and technology: nothing to report.

5. CHANGES & PROBLEMS

Changes: Nothing to report

Problems: We faced a problem with our developing MFD chip for exosome isolation using different HGSOC serum samples. It appeared that our chip yield exosome isolation is slow and low yeild. This significantly delayed our initial screening of exosome isolation and validation of the chip. We solved the problems within two months, modified the chip surface with our collaborator, and confirm the greater yield and quantification in different set of HGSOC patient samples.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  Nothing to report

- **Significant changes in use or care of human subjects**
  Nothing to report

- **Significant changes in use or care of vertebrate animals**
  Nothing to report

- **Significant changes in use of biohazards and/or select agents**
  Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations**
  Nothing to report

- **Journal publications.**
  Nothing to report

- **Books or other non-periodical, one-time publications**
  Nothing to report

- **Other publications, conference papers and presentations**
  Nothing to report

- **Website(s) or other Internet site(s)**
  Nothing to report

- **Technologies or techniques**
  We have developed a novel microfluidics based device to isolate intact exosomes with greater purity and quality in a shorter time that will allow for downstream processing. These factors are critical for moving forward in clinical translation and be directly applicable for exosome-based biomarker screening in patient serum samples.

- **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:

Name: Selvendiran Karuppaiyah, PhD
Project Role: PI
No Change

Name: Casey Cosgrove, MD
Project Role: Co-I
No Change

Name: Lianbo Yu, PhD,
Project Role: Biostatistician
No Change

Name: Kalpana Deepa Priya Dorayappan
Project Role: Post Doc Fellow
No Change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Active Support Changes:

Selvendiran Karuppaiyah (PI)

Now Active / Awarded:
DOD FY20 Ovarian Cancer Research Program - Clinical Translational Research
Award W81XWH2110427 Total Costs: 06/15/2021 – 06/14/2023 3 calendar months

Lianbo Yu (Biostatistician)

Active / Awarded:
DOD FY20 Ovarian Cancer Research Program - Clinical Translational Research
Award W81XWH2110427 Total Costs: 06/15/2021 – 06/14/2023 0.6 calendar months

Active / Awarded:
Role: **Biostatistician**
Nat In. Arthritis & Muscoskeletal & Skin
Title: Skeletal muscle in rheumatoid
arthritis K23AR068450 Total Costs: 09/012020 – 08/31/2021 2.4 calendar months

Active / Awarded:
Role: Biostatistician
National Institute of Neurological Disorders and Stroke
Title: Reducing infection susceptibility by immune function restoration in spinal cord injury
R01NS118200 Total Costs: 07/01/2020 – 06/30/2022 0.6 calendar months

Active / Awarded:
Role: Biostatistician
National Institute of Neurological Disorders and Stroke
Title: Implementation of machine learning workflows in primary brain tumor diagnostics R03NS116334 Total Costs: 06/01/2020 – 11/30/2021 0.6 calendar months

Active / Awarded:
Role: Biostatistician
NCI
Title: The translational regulation of pro-apoptotic genes
R01CA251753 Total Costs: 07/14/2020 – 06/30/2025 1.2 calendar months

Active / Awarded:
Role: Biostatistician
National Heart, Lung and Blood Institute
Title: ISGylation regulates lung endothelial inflammation
R01HL157164 Total Costs: 04/20/2021 – 03/31/2025 1.2 calendar months

Active / Awarded:
Role: Biostatistician
NCI
Title: Validating urine derived cancer cells (UDCC) – non-invasive and living liquid biopsies – in bladder cancer clinics
R33CA258016 Total Costs: 05/01/2021 – 04/30/2024 0.60 calendar months

What other organizations were involved as partners:

Nothing to report on any other personnel's and relationships.

8. Special Reporting Requirements

Nothing to report

9. APPENDICES N/A