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TITLE: Overcoming Deficiencies in the Early Detection of Endometrial Cancer: Evaluating Exosomal Proteins as Novel Biomarkers of Disease

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CONTRACTING ORGANIZATION: The Ohio State University

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14. ABSTRACT

High-grade serous ovarian carcinoma (HGSOC), the most common epithelial ovarian cancer, is associated with a particularly poor prognosis as most patients are diagnosed at an advanced stage owing to a lack of early detection as well as due to the eventual development of platinum-resistant. Exosomes are an attractive source of biomarkers as they carry cargo (proteins, microRNAs, and lipids) from their cells of origin, are highly stable, and can be obtained from any biological fluid using non-invasive methods. We have identification differentially expressed and unique exosomal proteins (TMEM205, CD1B, ENPL, SA-A2, EP-CR and FAS) in platinum-resistant HGSOC, however it has not yet been proven or validated as a clinical tool. Thus, there is a critical need to establish novel methods for exosomal isolation and to validate their use in detecting potential biomarkers in HGSOC. This will pave the way for exploring the clinical implications that serum exosomal proteins could have as platinum-resistance markers, prognostic indicators and therapeutic targets.

15. SUBJECT TERMS

Obesity, Endometrial Cancer, exosome, Microfluidic chip, and Biomarkers

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1. INTRODUCTION

Endometrial cancer (EC) is the fourth-most common cancer in women in the US. Obesity and the associated unopposed estrogen is more strongly associated with the development of Type-1 EC. Type 1 EC represents 70-80% of all endometrial cancers. Due to the obesity epidemic, the clinical importance of identifying these malignancies has come to the forefront. Recent clinical evidence has demonstrated an *increase in the incidence of EC* in pre-menopausal women likely secondary to the obesity epidemic with a projected rise of 42.13 cases per 100,000 women by 2030. Type 1 EC is often diagnosed in early stages due to symptom presentation but even earlier detection is optimal for reduction of needing aggressive treatment such as radiation adjuvant therapy. Additionally, this biomarker would be able to be utilized for detection of recurrence when patients are in surveillance. This opportunity for earlier diagnosis is clinically important as it has been demonstrated that obesity impacts oncological outcomes with up to a 7.5-fold increase in mortality in obese patients. Currently, there are *no clinically-utilized biomarkers* for obese-mediated EC. Despite the fact that many different biomarkers have been tested, those discovered lack sensitivity or specificity to detect early-stages of EC (8-10). Therefore, there remains a *critical need* to identify and validate biomarkers that are both sensitive and specific to early-stage obese-mediated Type-1 EC allowing for earlier intervention.

HYPOTHESIS & OBJECTIVE: Based on our preliminary results, our **central hypothesis** is that unique exosomal proteins can serve as sensitive and specific biomarkers that will detect early stage of obese EC. The **overall objective** of this proposal is to validate the identified exosomal protein candidates and to determine their sensitivity and specificity using larger cohorts of EC patients and non-cancer controls to identify potential diagnostic early biomarkers.

The proposed research will address the FY21 Military Health Focus Area of "Gaps in early detection/diagnosis", because outcomes will provide innovative and clinical information on EC, particularly in the women's Veteran population and their close family members associated with obesity EC. This higher prevalence of these obese mediated EC in military Veterans and their family will determine a higher risk for EC. Currently no clinically-utilized biomarkers for EC. The impact of this proposed study is identification and validation of exosomal protein as novel biomarkers for early detection of EC. The findings of this study will benefit all women affected by EC, including female military service members, family members, as well as other military beneficiaries

SPECIFIC AIMS:

Specific Aim 1: To validate the microfluidics chip for exosome isolation and to optimize the exosomal proteome in EC by ELISA, Luminex and Proximity extension assay (PEA).

Specific Aim 2: To determine the ability of identified candidate exosomal proteins to detect early-stage of EC independently and in combination with other markers using a multinomial regression model.

2. KEY WORDS

Endometrial Cancer

Obesity

Exosome

Microfluidic chip

Biomarkers

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goal of this study is to validate the identified exosomal protein candidates and to determine their sensitivity and specificity using larger cohorts of EC patients and non-cancer controls to identify potential diagnostic early biomarkers.

What was accomplished under these goals?

We have identified the significance of key findings in SA1

(i) Developed a MFD chip for exosome isolation in serum samples;
(ii) Validated our MFD chip with known EVs isolation techniques
(ii) Exosomes are highly elevated in obesity associated serum samples;

Aim 1: To validate the microfluidics chip for exosome isolation and to optimize the exosomal proteome in EC by ELISA, PEA and Luminex

Microfluidic devices have provided the ability to efficiently capture exosomes based on specific membrane biomarkers, but releasing the captured exosomes intact and label-free remains a challenge. We present a herringbone-grooved microfluidic device, which is covalently functionalized with antibodies against cancer exosome membrane proteins (CD9, CD63 and TSG101) (**Fig.1** and **Fig. 2**) to isolate exosomes from small volumes ovarian cancer and EC serum samples. The *objective* of this aim is to validate the utility of our novel microfluidic device (MFD) for exosome isolation for proteomic biomarker studies in order to translate the technique into clinical use. Based on our preliminary results, our *working hypothesis* is that our novel MFD isolates exosomes with greater purity and quality in a shorter time in clinical samples. The *rationale* for this aim is that successful completion of this study will lead to the MFD forward in clinical translation for exosome isolation. To test this objective and hypothesis, we will use 125 patient serum samples to validate the performance of our MFD to isolate and purify exosomes and to compare to known exosome isolation techniques (ultracentrifugation and commercial kits). Specifically, we will evaluate in SA1:

1.1. To validate the utility of our novel MFD device for exosome isolation for proteomic biomarker studies in order to translate the technique into clinical use.

1.2. To optimize the identified exosomal candidate proteins (Table 1) by ELISA, PEA, and Luminex

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 8 months). **Completed 100%**

Approach 2. MFD validation for exosome isolation and quantification using four different techniques:

Milestone # 2. Developed Microfluidic chip validation for exosome isolation using ultracentrifugation, NTA, dot blot and Western blot assay (Year 1: 8-12 months). **Completed 80%**

RESULTS

1. Development of microfluidics device (MFD) for the isolation of exosomes. In collaboration with Dr. Derek Hanford from the Department of Biomedical Engineering at The Ohio State University (refer to the support letter), we have innovated a distinctive microfluidic device (MFD) tailored for exosome isolation (depicted in Fig. 1). Our primary objective revolves around advancing the clinical application of this device for efficient exosome isolation. Conventional techniques for exosome isolation within research settings pose technical complexities, involving labor-intensive ultracentrifugation, substantial sample volumes, and protracted timeframes. Additionally, existing commercially available kits are burdened by high costs and lack specificity. Our study surmounts these limitations by introducing an original microfluidics-based approach, enabling exosomal isolation from minute sample volumes and yielding a more substantial quantity of high-quality exosomes compared to established methodologies. Our innovative MFD captures and releases intact exosomes devoid of labeling (termed the "Catch and Release" system; see Fig. 1B), facilitating subsequent processing untainted by contamination. Precisely, our device integrates surface antibody capture within a modified PDMS channel, along with a bespoke elution protocol that effectively liberates exosomes from antibodies without residual components. Consequently, we achieve the purification and isolation of intact exosomes, guided by their surface markers and without the interference of extraneous antibodies. This unique attribute sets our device apart from commercially available chips, endowing it with the potential to serve as a rapid screening tool in clinical applications. Our ongoing efforts encompass exosome isolation from both cell lines and clinical samples, resulting in time and cost savings while ensuring straightforward clinical translation.

2. Microfluidics device (MFD) validation for the isolation of exosomes. In partnership with Dr. Derek Hanford (Co-I) from the Department of Biomedical Engineering at The Ohio State University, we have successfully engineered a distinct microfluidic device (MFD) tailored for exosome isolation (as illustrated in Fig. 1). Our overarching aim revolves around advancing the application of this innovative device for exosome isolation, specifically within clinical samples. Validation of exosome isolation and purification was executed using our novel MFD across both the Principal Investigator's (PI) and Co-Investigator's (Co-I) laboratories (Drs. Pollock and Hansford), as depicted in Fig. 2A-C. A comparative analysis was performed against established methodologies including ultracentrifugation and a commercial kit. Remarkably, the MFD demonstrated superior outcomes in terms of yield, cost-effectiveness, and operational efficiency when contrasted with the ultracentrifugation and Exo-Quick techniques (as demonstrated in Fig. 2D). This achievement marks a significant stride towards the clinical-translational application of our approach, particularly in processing patient samples. By conserving time and cost while concurrently enhancing yield, specificity, and quality, our MFD presents a pivotal advancement in facilitating the transition towards clinical implementation.

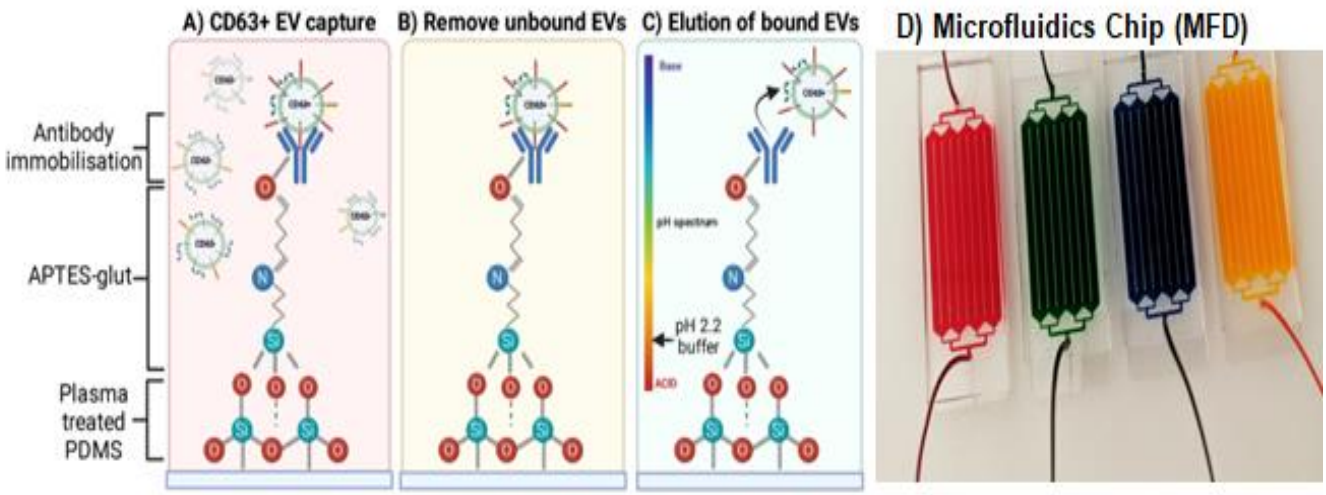

Fig. 1. EV capture and release system using antibody-functionalized microfluidic devices showing (A) covalent functionalization of PDMS surface chemistry with antibody to target EV antigens of interest and EV capture from biofluids, (B) removal of unbound EVs using PBS, (C) elution of selectively captured EVs using acidic buffer prior to immediate downstream neutralization. (D) Image of microfluidic device (MFD). (E) EVs isolation by MFD and confirmation of EVs size was limited by NTA and quantification EVs population by image stream flowcytometry (ISF) in the early HGSOC patient' and control (without cancer) serum samples.

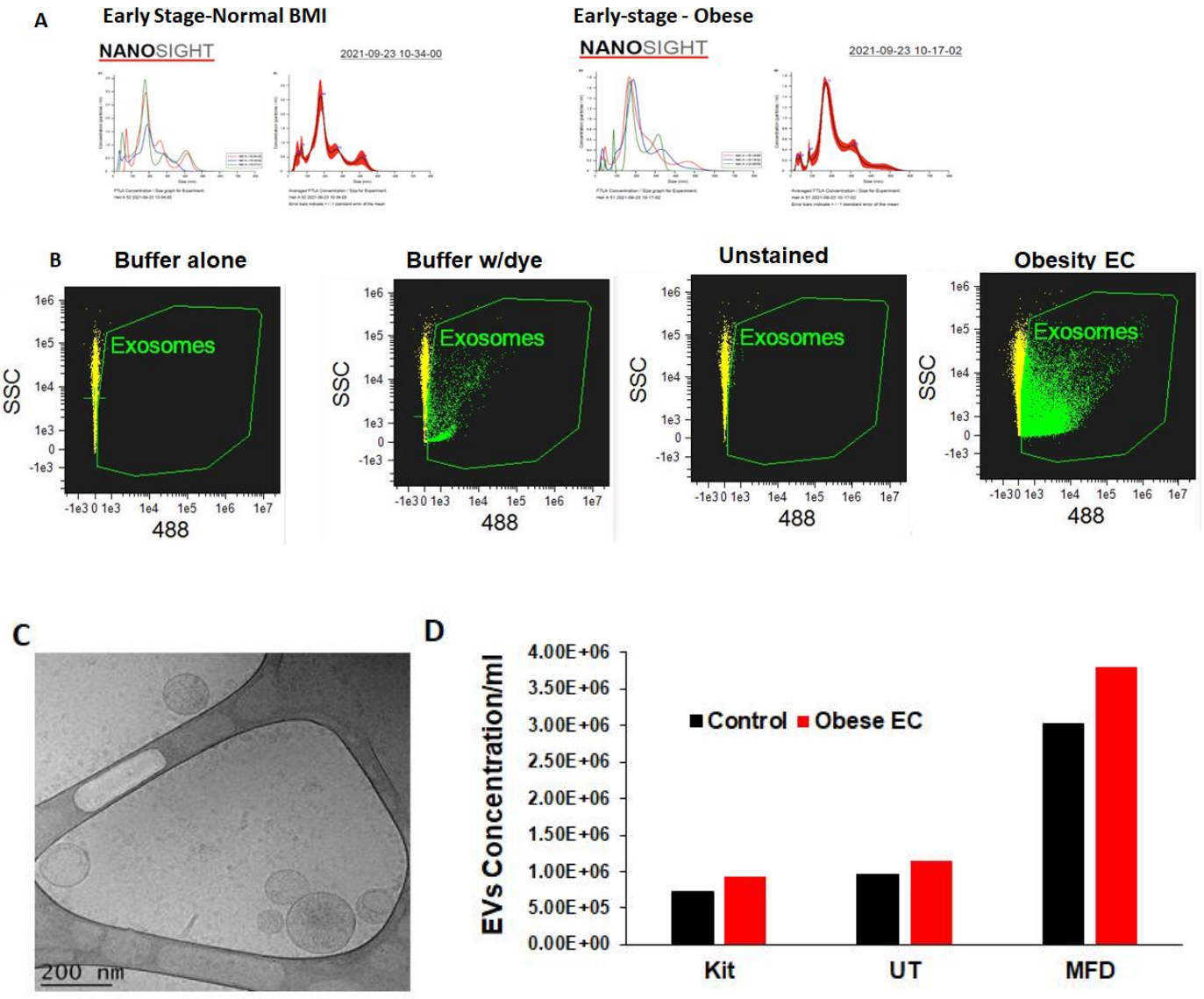

Figure 2. Exosome isolation by MFD and characterization. **A)** NTA for exosome isolated by microfluidic chip in PI and Co:I labs showing reproducible results (n=5)**. B)** Quantification of Exosomes by image stream flow cytometry in obese EC serum samples using microfluidic chip in PI labs (n=5). C**)** Morphological characterization and size measurement of exosomes (indicated by red arrows) by Cryo transmission electron microscope (TEM). D**)** Higher exosome yield is obtained in the Microfluidics "catch & release" chip (MFD) compared with other conventional methods UT and commercial Kit (n=7 **p< 0.005, ***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 manuscript describing the data presented in the report and plan to submit it in July end.

For year 2, we plan to complete our proposed experiments from Aim 1, To determine if ENPL regulates exosome secretion through MRP2 in OC cells and Does ENPL associate with exosomes to mediate OC chemo-resistance?. Currently we have preliminary data that is under analysis state.

Initiate experiments from Aim 2 July middle.

4. IMPACT

1. Impact on the development of the principal discipline (ovarian cancer) of the project: Identifying the role of endoplasmin in the development of platinum-resistance of OC: Our preliminary results showing that ENPL could play a role in in promoting OC progression and chemoresistance through the exosome secretion payways. Based on these findings, we will evaluate novel mechanisms linking ENPL with exosomal secretion, including, how ENPL expression plays a role in exosome release and contributes to OC chemoresistance through the MRP2 activation in OC in SA1 and SA2. This finding will potentially lead to the identification of novel biomarkers and therapeutic targets for chemoresistant OC.

2. Impact on the development of other disciplines: Our study can have impact on all other solid tumors. By identifying the role of ENPL and combining the approaches of blocking exosome secretion with cisplatin treatment approaches, the outcome of combination thearpy can me enhanced and made for successful for the patient.

3. Impact of the technology transfer: Translational Technology - Microfluidics device: 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.

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

5. CHANGES & PROBLEMS

Changes: Nothing to report

Problems: We encountered an issue during the development of the MFD chip for extracellular vesicle (EV) isolation, characterized by chip leakage. This concern was effectively resolved by applying a PDMS material as a sealing solution.

- **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
Project Role: PI
No Change

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

Name: Lianbo Yu
Project Role: Biostatistician
No Change

Name: Derek Hansford
Project Role: Co:I
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

Jing Zhao (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:

1. Additional OC platinum resistant and sensitive serum samples was provided by **Dr. Larry Maxwell, MD** at **Inova Schar Cancer Center** for evaluate the clinical significance of ENPL expression as a marker of platinum resistance and survival in OC patient samples.

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

8. Special Reporting Requirements

Nothing to report

9. APPENDICES