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TITLE: Biomarker Development for Diagnosis, Surveillance, and Prognosis for Adrenocortical Carcinoma (ACC)

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14. ABSTRACT

We will assess the prospective utility of novel biomarkers in the clinical management of ACC. We hypothesize that serum steroids can specifically diagnose ACC, measure ACC burden, and detect recurrence; we also hypothesize that molecular biomarkers including tumor DNA methylation will predict ACC recurrence, progression, and selective response to adjuvant therapy. We will prospectively recruit ~200 participants with ACC or ACA from UM, A5, and ADIUVO-2. Diagnosis of ACC or ACA will be established by histology or imaging. Aim 1a: In patients with ACC or ACA, we will measure serum steroids by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify ACC-specific steroid markers. Aim 1b: In patients with ACC, we will measure serum steroids as in Aim 1a with parallel imaging surveillance to identify steroid markers that predict recurrence. Aim 2a: In patients with ACC, we will measure tumor DNA methylation and determine if it predicts recurrence, progression, response to systemic therapies, and death. Aim 2b: In patients from ADIUVO-2, we will measure tumor DNA methylation and determine if it predicts adjuvant therapy-specific recurrence/survival. We will perform exome sequencing of paired tumor/germline DNA to prospectively identify genetic factors that predict response to adjuvant therapies.

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1. Introduction

The adrenal glands are paired endocrine organs that produce steroid hormones and catecholamines critical for life. Adrenocortical carcinoma (ACC) is a rare cancer of these glands affecting ~1 individual/million/year worldwide. While ACC is rare, ~10% of the population bear benign adrenal lesions (largely adrenocortical adenomas [ACA]). Differentiating localized ACC from ACA is challenging, requiring extensive imaging workup. However, imaging often cannot rule out ACC, and exposes patients to collateral radiation. Half of all patients with ACC are diagnosed with surgically resectable localized disease. However, up to ~75% of all patients with ACC develop metastatic disease for which therapies are limited and prognosis remains dismal; <10% these patients survive 5 years after diagnosis. Early diagnosis of a recurrence is essential for appropriate management. Currently, mitotic activity in the primary tumor is the best predictor of recurrence, but aggressive disease course is frequently observed among "low-risk" patients. Additionally, patients are usually surveilled with extensive imaging exams post-operatively, which is expensive and exposes patients to high doses of radiation. These statistics highlight significant gaps in the knowledge of optimal strategies for Diagnosis, Surveillance, and Prognosis of ACC. The goal of this proposal is to assess the prospective utility of novel biomarkers, including serum steroids and tumor DNA methylation, in diagnosis, risk stratification, and disease surveillance of patients with ACC This proposal will utilize samples prospectively obtained for the UM Endocrine Oncology Repository; the "American-Australian-Asian Adrenal Alliance" (A5), a large international collaborative network for adrenal research; and A5-initiated clinical trial ADIUVO-2 (NCT03583710), aiming to evaluate adjuvant therapies in patients with high grade ACC, randomized to receive mitotane alone or plus chemotherapy.

2. Keywords

adrenocortical carcinoma, ACC, DNA methylation, CIMP-high, steroidomics, LC-MS/MS, steroids, adjuvant therapy, adrenal cancer, adenoma, tumor, prospective, predictive, biomarker, adrenal, hormones

3. Accomplishments

The major goals of this project, as stated in two Specific aims in the SOW, are:

- Specific Aim 1. Evaluating the use of steroid profiles in Diagnosis (Aim 1a) and Surveillance (Aim 1b) of adrenocortical carcinoma (ACC) patients. In patients with ACC or adrenocortical adenomas (ACA), we will measure serum steroids by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify ACC-specific steroid markers (Aim 1a). In patients with ACC, we will measure serum steroids with parallel imaging surveillance to identify steroid markers that predict recurrence (Aim 1b).
- Specific Aim 2: Evaluating the use of tumor DNA methylation in stratifying ACC patients into risk groups (Prognosis). We will measure the methylation levels of a single locus, G0S2, and prospectively assess the utility of G0S2 hypermethylation in ACC risk stratification (Aim 2b), and in predicting response to different types of adjuvant therapy (Aim 2b).

For this reporting period, pertinent tasks as proposed in the SOW and **accomplishments** are described below:

- Specific Aim 1:
    - Major Task 1 & 2: We have enrolled 58 patients with ACC and 45 patients with ACA (total of 103 patients). We also received 72 additional serum samples from our collaborators at MD Anderson. We have finalized the majority of steroid measurements from serum samples as proposed, including Delta-4-steroids and steroid sulfates; we have processed samples from. Initial basic limited clinical data have been completely abstracted for these patients and we are in the process of abstracting more detailed clinical data. Steroids were analyzed and quantified using 2D liquid chromatography-tandem mass spectrometry scanning for 4 sulfated steroids and 25 Δ4 steroids in MRM mode. A third method to analyze Δ5 steroids has been re-validated after supply chain related reagent shortages, and samples will be extracted and analyzed in the coming few months during no-cost extension. We have begun the biostatistical analysis for the 4 sulfated steroids and 25 Δ4 steroids for differences and diagnostic potential to differentiate benign and malignant adrenal masses. According to our preliminary results, a linear discriminant analysis-based classifier identified that some of the

sulfated steroids had the highest discriminative power to distinguish ACC from ACA, with 17-Pregnenelone-sulfate achieving a sensitivity of 0.8 and specificity on 0.92 for diagnosing ACC.

- Specific Aim 2:
  - Major Task 1: Enroll 100 participants with ACC; collect relevant clinical data and biospecimens.
    - Subtask 1 and 2: We have performed targeted bisulfite sequencing to assess *G0S2* methylation in a retrospective cohort (from A5) of 100 formalin-fixed paraffin-embedded (FFPE) samples. Twenty-five of these samples featured in our previous publication (*Mohan & Lerario; Clinical Cancer Research, 2018*) and served as controls for validation of our FFPE method (in the original publication we measured *G0S2* methylation in snap-frozen samples). We observed a 100% concordance between *G0S2* measurements performed in snap frozen and FFPE samples, validating our new approach. In the heatmap depicted below (Figure 1), we show the distribution of the methylation values of CpG sites spanning the *G0S2* locus. We are currently work to increase the number of samples by enrolling cases from our retrospective cohort from MEOR, and to perform the statistical correlations between the methylation data and clinical parameters.

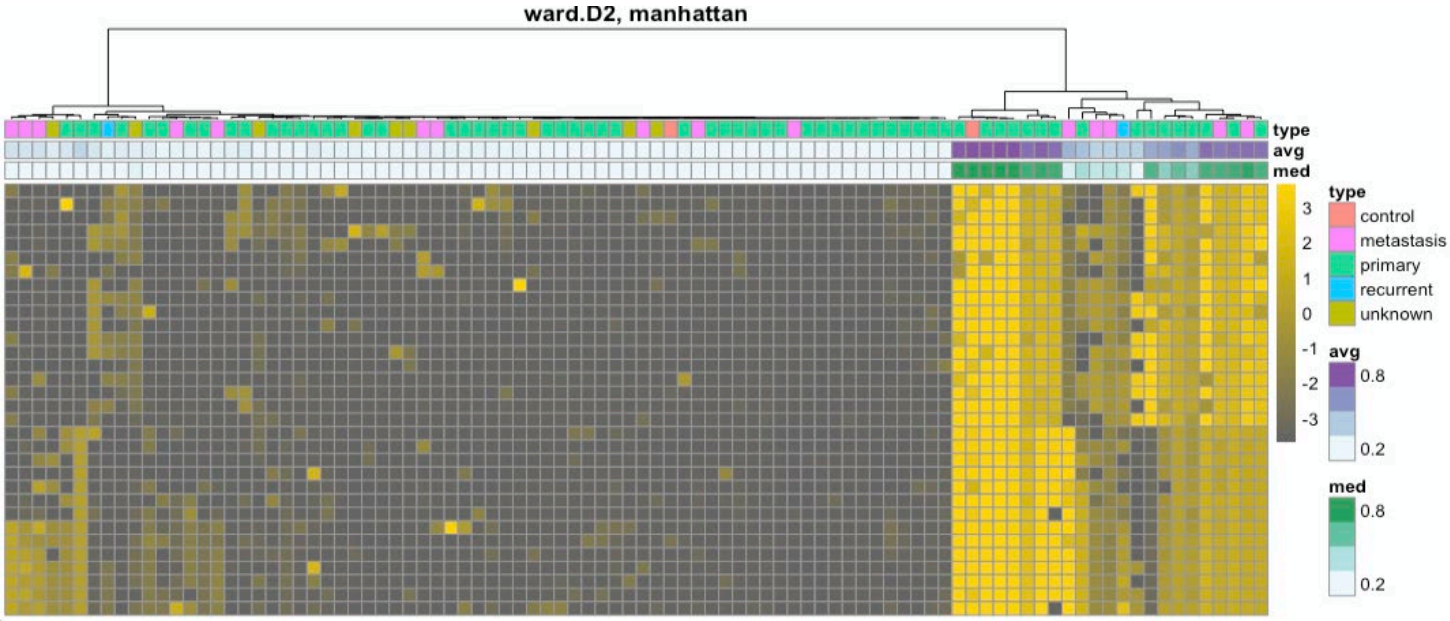

Figure 1: Heatmap depicting the distribution of CpG methylation values spanning the *G0S2* locus measured by targeted bisulfite sequencing. Similarly to our previous publication (*Mohan & Lerario, Clinical cancer research, 2018*), a binary pattern of DNA methylation can be observed, with ~1/3 of samples exhibiting the CIMP-high signature (clustered together in the right side of the chart). Each row represents a CpG position spanning the *G0S2* locus; each column is a sample. Sample types, average, and median methylation for each sample are shown in the top tracks. Methylation values were logit-transformed before clustering. Hierarchical clustering was performed using the ward.D2 algorithm and the Manhattan distance as the dissimilarity metric.

Training opportunities and professional development:

Rachel Scheske – undergraduate. Gaining experience in steroid analysis. Desmare van Rooyen, post-doc, teaching experience in the laboratory setting with undergraduates.

Dissemination of results to communities of interest:

Steroid data (Aim 1) was presented at ENDO 2023 (Chicago) as a poster entitled "*Serum Steroid Biomarkers dor the Differential Diagnosis of Adrenocortical Carcinoma (ACC) and Adrenocortical Adenoma (ACA)",* authored by Desmaré van Rooyen, Rachel Scheske, William E. Rainey, Antonio M. Lerario, Richard J. Auchus, Gary D. Hammer, Tobias Else.

4. **Impact:** Nothing to report at this time.

5. **Changes/Problems:** We experienced a significant delay in patient enrollment, sample collecting and processing due to lockdowns and access restrictions to facilities related to COVID-19. As a result, we fell short of our target of the 240 samples for this reporting period. We are working with our collaborators to fill this gap. For aim 2, we were able to substantially increase the number of cases by procuring FFPE samples from our retrospective cohort. With the inclusion of retrospective cases, we were able to compensate for the slow accrual rate of prospective cases that we unexpectedly experienced during this time.

6. **Products:** Nothing to report.

7. **Participants & Other Collaborating Organizations**

   Individuals who have worked in this project during this reporting period are listed below. There are no changes from the previous submission of this information and no change in active or other support of the PD/PI or senior/key personnel since the last reporting period.

Name: Gary D. Hammer
Role: Initiating Principal Investigator
Research Identifier: http://orcid.org/0000-0001-6843-3628
Nearest person month worked: 1
Contribution to Project: Dr. Hammer has supervised the entire study and lead meetings with all members of the research team.

Name: Richard J. Auchus
Role: Partnering Principal Investigator
Research Identifier: https://orcid.org/0000-0001-6815-6181
Nearest person month worked: 1
Contribution to Project: Dr. Auchus has supervised his arm of the study and shared his expertise regarding LC-MS/MS analysis as we plan for sample acquisition during team meetings.

Name: Tobias Else
Role: Partnering Principal Investigator
Research Identifier: https://orcid.org/0000-0002-2262-0011
Nearest person month worked: 1
Contribution to Project: Dr. Else has supervised his arm of the study, facilitated acquisition of IRB approval, shared his expertise regarding serum sample analysis.

Name: Antonio M. Lerario
Role: Co-Investigator
Research Identifier: https://orcid.org/0000-0002-8336-6432
Nearest person month worked: 1
Contribution to Project: Dr. Lerario has worked on optimizing sample acquisition for G0S2 methylation analysis and has shared bioinformatics expertise to evaluate study design and number and type of samples required for biological/clinical significance during team meetings.

Name: Dipika R. Mohan
Role: Graduate Student
Research Identifier: https://orcid.org/0000-0002-6334-9416
Nearest person month worked: 1
Contribution to Project: Ms. Mohan has worked with Dr. Lerario to optimize sample acquisition for G0S2 methylation analysis and evaluate study design, and has worked with Ms. Brand and Dr. Else to obtain IRB approval.

Name: Sarah Brand
Role: Coordinator of A5
Research Identifier: N/A
Nearest person month worked: 1
Contribution to Project: Ms. Brand has led submitting and obtaining IRB approval, and will also assist in enrolling and consenting patients at the University of Michigan and managing clinical data.

Name: Patrick O'Day
Role: Technician
Research Identifier: N/A
Nearest person month worked: 1
Contribution to Project: Mr. O'Day has prepared and optimized mass spectrometer for measurement of steroid profiles from plasma samples.

Name: Desmare Van Rooyen
Role: Post-doctoral Fellow
Research Identifier: N/A
Nearest person month worked: 3
Contribution to Project: Dr. Van Rooyen optimizes the mass spectrometry analysis and extraction. She will prepare samples for further analysis and interpret results.

Partner organizations which are collaborating with this project:

- Dr. Mouhammed Habra, MD (**MD Anderson Cancer Center**, Houston, TX).

- Dr. Anand Vaidya, MD **(Brigham and Women's Hospital** Boston, MA).
- Dr. Diane Reidy, MD (Memorial Sloan Kettering Cancer Center, New York, NY).
- Dr. Nitya Raj, MD (Memorial Sloan Kettering Cancer Center, New York, NY).

8. Special Reporting Requirements: Not needed.

9. Appendices:

Abstract of the work presented at ENDO 2023 meeting (Chicago):

Serum steroid metabolomics showed sex differences in adrenocortical carcinoma (ACC) vs adrenocortical adenomas (ACA)

Desmaré van Rooyen[1,2], Rachel Scheske[1,2], William E. Rainey[1,2], Antonio Marcondes Lerario[2], Richard J. Auchus[2,3], Gary D. Hammer[1], Tobias Else[2]

[1] Department of Molecular and Integrative Physiology, University of Michigan

[2] Division of Metabolism, Endocrine, and Diabetes, Department of Internal Medicine, University of Michigan

[3] Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA

Background:

Adrenal tumors are very common, with the vast majority being benign adrenocortical adenomas (ACA). Adrenocortical carcinomas (ACC) are only a very small proportion of adrenal tumors. Distinction from one another relies on invasive and costly protocols which are often inconclusive. Research into the use of steroid biomarkers as a non-invasive alternative diagnostic tool has been the focus of various steroid metabolomics studies. In this study, we aimed to identify serum steroid biomarkers to distinguish ACA from ACC.

Methods: This study population comprised of 103 patient samples, including 45 (31 female) and 58 (40 female) respective samples from ACA (age 32-90) and ACC (age 16-77) patients presenting with disease at the time of sampling but not on mitotane treatment. Serum steroid levels (4 sulfates and 25 Δ4 steroids) were measured using liquid-chromatography tandem mass spectrometry (LC-MS/MS). ACA and ACC sample data were compared within the sex groups. Data is expressed as the geometric means with 95% Cls with statistical significance determined using a two-tailed Mann-Whitney test.

Results: Significance in steroid levels differed between male and female samples. ACC patients were characterized by significantly elevated levels of pregnenolone-sulfate (Preg-S), 17-hydroxypregnenolone-sulfate (17OHPreg-S), 11-deoxycorticosterone (DOC), 17-hydroxyprogesterone (17OHProg), and androstenedione (A4) in ACC vs ACA. In addition, dehydroepiandrosterone-sulfate (DHEA-S; $p<0.01$), androstenediol-sulfate (A5-S; $p<0.05$), 11-deoxycortisol ($p<0.0001$), cortisol ($p<0.05$) and testosterone (T; $p<0.01$) were significantly increased in female ACC patients only.

Conclusion: Significant differences in plasma steroids are observed in ACC vs. ACA patients with more steroids altered in female patients. Serum steroid profiles, particularly considering steroid sulfates, might serve as an adjunct, easy to obtain diagnostic tool to differentiate ACA and ACC.

Keywords: Adrenocortical carcinoma; serum steroids; LC-MS/MS