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Estrogen-DNA Adducts as Novel Biomarkers for Ovarian Cancer Risk
and for Use in Prevention

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14. ABSTRACT The purpose of this research is to determine the association between ovarian cancer and (1) imbalances in estrogen metabolism that lead to higher levels of estrogen-DNA adducts and/or (2) genetic polymorphisms in selected enzymes that metabolize estrogens. The first year of the grant has been spent collecting urine and saliva samples from cases and controls, establishing the protocol to purify the maximum amount of DNA from saliva samples, synthesizing estrogen metabolite, conjugate and DNA adduct standards for MS/MS analysis, optimizing the UPLC-MS/MS method, and setting up a database in ACCESS to analyze data collected from the subject questionnaires. All of these analyses will be carried out in the second year of the grant.					
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Introduction

Ovarian cancer is the fifth leading type of cancer in women in the U.S., but first in gynecological cancer mortality. Our inability to diagnose ovarian cancer at an early stage is a major problem. If ovarian cancer is initiated by imbalanced estrogen metabolism leading to estrogen-DNA adducts that generate mutations in critical genes in ovarian epithelial cells, analysis of estrogen-DNA adducts could provide a tool for early diagnosis. In this project we are analyzing 40 estrogen metabolites, conjugates and DNA adducts in urine samples and genetic polymorphisms in four selected estrogen-metabolizing enzymes in DNA saliva samples from 50 women diagnosed with ovarian cancer and 50 matched controls. The goal of this project is to determine whether increased levels of estrogen-DNA adducts and/or specific genetic polymorphisms are associated with ovarian cancer.

In the first year of this project, we enrolled subjects, began collecting samples, and established experimental protocols. Our progress on the specific tasks planned for the first year was as follows.

Task 1. Obtain approval of the protocol from the OCRP Human Research Protection Office (Sp. Aims 1 & 2, Months 1-6).

Approval of our protocol by the OCRP Human Research Protection Office was received on July 7, 2010.

Task 2. Prepare and recruit subjects (100 total) into the study (Sp. Aims 1 & 2, Months 1-6).

Specimen cups and Oragene kits for collecting saliva and isolating DNA were purchased for Dr. Hall's clinic to use in this study. The Carolinas Medical Center (CMC) began to identify potential subjects.

Task 3. Begin recruiting subjects (at least 50) into the study and collecting urine and saliva samples (Sp. Aims 1 & 2, Months 7-12).

Because of the time needed to obtain approval of our protocol (4 months) and obtaining supplies for collecting saliva in Oragene kits (2 months due to an error by the supplier), recruiting subjects began in September, 2010. Recruitment of subjects at CMC has been slower than anticipated because (1) a data manager at CMC resigned, leaving the site unable to carry out screening to find potential subjects for an extended period of time; (2) a nurse was gone on maternity leave, also hindering recruitment, (3) it was discovered that the CMC financial department had not executed the subcontract with UNMC and the study was temporarily suspended until the subcontract was finalized; and (4) an additional 11 potential subjects were disqualified for various reasons, including age and history of other types of cancer.

Staff at CMC are doing the following to accelerate recruitment so that we can meet the goals of the project: (1) reviewing the clinic appointment schedule to identify the first post-op visit for patients diagnosed with ovarian cancer; (2) flagging charts of potential subjects with reminders to the physician that the patient may be eligible for the study; (3) highlighting the importance of the study at monthly Gyn Oncology research meetings; (4) involving clinic nurses, chemotherapy coordinators and the nurse navigator in helping to identify potential subjects; and (5) developing a list of potential controls for the project.

An ACCESS database has been created by Dr. Beseler for the subject questionnaire data. The database will facilitate extraction for analysis and linking of matched cases and

controls. Data from the first nine questionnaires has been entered into the database. A coding plan has been developed in order to expedite analysis once the genotyping is completed and the urine samples are processed.

Task 4. Begin processing urine samples and analyzing them by UPLC-MS/MS (Sp. Aim 1, Months 8-12).

Although we have not had urine samples to analyze, we have accomplished several tasks in preparation for the analyses. First, we purchased or synthesized the required estrogen metabolite, conjugate and DNA adduct standards. These are summarized in Table 1. Synthesis of the remaining standards will be complete by the end of April. We have also established the conditions for solid phase extraction of urine samples and UPLC-MS/MS analyses.

Table 1. Standards

	Standard	Name	Amount in dry stock (mg or g)
Parent	1	E ₂	purchased
	2	E ₁	purchased
Metabolites	3	2-OHE ₂	900 mg
	4	2-OHE ₁	850 mg
	5	4-OHE ₂	600 mg
	6	4-OHE ₁	400 mg
	7	16 α -OHE ₂	purchased
	8	16 α -OHE ₁	purchased
COMT	9	2-OCH ₃ E ₂	purchased
	10	2-OCH ₃ E ₁	purchased
	11	4-OCH ₃ E ₂	6 mg
	12	4-OCH ₃ E ₁	purchased
	13	2-OH-3-OCH ₃ E ₂	purchased
Internal standard	14	2-OH-3-OCH ₃ E ₁	purchased
Quinone conjugates	15	2-OHE ₂ -1-SG	8 mg
	16	2-OHE ₂ -4-SG	7 mg
	17	2-OHE ₁ -1+4-SG	10 mg
	18	2-OHE ₂ -1-Cys	4 mg
	19	2-OHE ₂ -4-Cys	6 mg
	20-1	2-OHE ₁ -1-Cys	need to synthesize
	20-4	2-OHE ₁ -4-Cys	need to synthesize
	21	2-OHE ₂ -1-NACys	need to synthesize
	22	2-OHE ₂ -4-NACys	need to synthesize
	23	2-OHE ₁ -1+4-NACys	need to synthesize
	24	4-OHE ₂ -2-SG	15 mg
	25	4-OHE ₁ -2-SG	10 mg
	26	4-OHE ₂ -2-Cys	need to synthesize
	27	4-OHE ₁ -2-Cys	need to synthesize
	28	4-OHE ₂ -2-NACys	4.5 mg
	29	4-OHE ₁ -2-NACys	5 mg
Depurinating adducts	30	4-OHE ₂ -1-N7Gua	purifying synthesized product
	31	4-OHE ₁ -1-N7Gua	in process
	32	4-OHE ₂ -1-N3Ade	need to synthesize
	33	4-OHE ₁ -1-N3Ade	need to synthesize
	34	2-OHE ₂ -6-N3Ade	5 mg
	35	2-OHE ₁ -6-N3Ade	4 mg

Task 5. Begin purifying DNA from saliva samples (Sp. Aim 2, Months 8-12).

We have optimized conditions for purifying DNA from saliva samples collected by use of an Oragene kit by collecting and extracting DNA from three volunteers. We obtained 100 μ l of DNA per sample with concentrations of 285 ng/ μ l, 261 ng/ μ l and 475 ng/ μ l. The 260/280 ratios ranged from 1.72 to 1.81, indicating very little contamination with proteins. The purity and amounts are more than adequate for PCR amplification and genotyping of the four single nucleotide polymorphisms, CYP1A1 (I462V), CYP1B1 (V432L), COMT (V158M) and NQO1 (P609S).

Task 6. Order and test primers for analysis of the SNPs (Sp. Aim 2, Months 8-12).

We have not yet ordered and tested primers for analysis of SNPs. The first batch of samples was shipped from CMC to UNMC during the last week of March, 2011. In early April we will order the primers and test them.

Key Research Accomplishments

1. Obtained approval of the protocol.
2. Synthesized or purchased estrogen metabolite, conjugate and DNA adduct standards for UPLC-MS/MS.
3. Optimized purification of DNA from saliva.
4. Established an ACCESS database to analyze data obtained from subject questionnaires.
5. Recruited 9 subjects and obtained urine and saliva samples, as well as completed subject questionnaires.
6. The first samples were shipped to UNMC.

Reportable Outcomes

None, as expected, because the results need to be evaluated as a whole, when collection and analysis of samples and subject data are complete.

Conclusion

This study continues to be important because of the potential to establish a tool to diagnose ovarian cancer at an early stage. The results of this study are expected to lead to studies of ovarian cancer prevention by use of specific antioxidants, already being studied for breast cancer prevention. The study got off to a slow start, but is now poised to be completed as planned.