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TITLE: Advancing Our Understanding of the Etiologies and Mutational Landscapes of Basal-Like, Luminal A, and Luminal B Breast Cancers

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Advancing Our Understanding of the Etiologies and Mutational Landscapes of Basal-Like, Luminal A, and Luminal B Breast Cancers

We are conducting a five-year population-based case-control breast cancer study to identify how various breast cancer risk factors differ in their relationships to different molecular subtypes of breast cancer and to further characterize molecular differences between these subtypes. To address the existing research gaps regarding the etiologies of different molecular subtypes of breast cancer we will employ state of the art multidisciplinary approaches to advance our understanding of the epidemiology and mutational landscapes of basal-like, luminal A, and luminal B tumors. Our original goal was to recruit about 2,700 women in Western Washington who have been diagnosed with breast cancer to compare to 900 women who have never been diagnosed with breast cancer, but control ascertainment has been unacceptably low, so on 8/25/15 we submitted a request to modify the SOW to drop the control group and replace it with an additional 80 to 100 ER+ cases. Participation in this research includes a detailed telephone interview, collection of breast tissue and oral samples and medical record abstraction. Breast tissue samples will be reviewed and tested at Fred Hutchinson Research Center and special tissue analyses will also be performed at the Michigan Center for Translational Pathology. This research may eventually be of help in developing clinically important insights and treatment protocols for future breast cancer patients. There are no major findings from this study yet as data collection is currently in progress.
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INTRODUCTION:

We are conducting a five-year population-based case-control breast cancer study to identify how various breast cancer risk factors differ in their relationships to different molecular subtypes of breast cancer and to further characterize molecular differences between these subtypes. To address the existing research gaps regarding the etiologies of different molecular subtypes of breast cancer we will employ state of the art multidisciplinary approaches to advance our understanding of the epidemiology and mutational landscapes of basal-like, luminal A, and luminal B tumors. Originally this study intended to include 900 newly diagnosed first primary triple negative (TN) invasive breast cancer cases, 1800 randomly selected age-matched estrogen receptor positive (ER+) cases, and for comparison, a population-based control group of 900 women without breast cancer. Control ascertainment did not go as planned due to significant changes in telephone equipment and practices, so the control response rate is unacceptably low. Consequently we made a request to modify the Statement of Work (SOW) on 8/25/15, which was approved. With the new SOW, we dropped the control group and replaced it with an additional 80 to 100 estrogen receptor positive (ER+) cases. In terms of the impact of this change on our specific aims, it impacts only Aims 1 and 4. Aim 1 is still achievable but will involve case-case comparisons instead of case-control comparisons. This is a viable and valuable approach given that the risk factors we will assess are all well-established and so we will still be able to determine which of these factors is differentially associated with the different subtypes of breast cancer we are assessing. Thus, the etiologic information this aim seeks will still be obtained. Similarly, risk prediction models for breast cancer overall are well-established and well-validated. So Aim 4 will also be revised to focus on case-case comparisons to identify risk prediction models that distinguish risk across breast cancer subtypes. The additional cases will provide us with more statistical power for our specific aims, and in particular increase the number of recurrences that we will identify which is critical to Aim 5. This modification does not impact the work or budget of Dr. Chinnaiyan’s component of this project as there were never any plans to provide samples from controls to his lab for testing.

Study participants are residents of Western Washington and their participation in this study includes a telephone interview that will elicit detailed information on a variety of established and suspected breast cancer risk factors. At the end of the interview participants will be asked to donate an oral tissue specimen for future genetic testing. Medical record reviews will also be conducted to ascertain treatment and outcome (recurrence) information for cases. Additionally, tumor tissue specimens from all TN and ER+ cases will be centrally reviewed at the Porter Lab and all tumors will be tested for markers enabling us to identify the TN cases that are basal-like, and the ER+ cases that are luminal A vs. luminal B. Tumor tissue from confirmed basal-like, luminal A, and luminal B cases will be sent to the Chinnaiyan Lab for mutational analysis. 133 cases from each group will be used for discovery work, and the remainder will be reserved for independent validation of promising candidates.

This research may eventually be of help in developing clinically important insights and treatment protocols for future breast cancer patients. There are no major findings from this study yet as data collection is currently in progress.
BODY:

Task 1. Develop Interview Instrument, Other Study Materials, and Tracking System, Months 1-3, completed under Dr. Li’s supervision at Fred Hutchinson Cancer Research Center (FHCRC):

a. Refinement of interview instrument, Months 1-2:
   The telephone interview that will be used in this study has already been developed, refined through field testing, and is currently in use. Dr. Li and Ms. Taylor will review it again to see if any additional data elements should be collected based on any newly reported data relevant to our study aims.

   Status: Complete, the questionnaire is currently in use in the field.

b. Development of other study materials, Months 1-2:
   Several other materials will be prepared including: approach letters to potentially eligible cases and controls; an interview consent form; a HIPAA compliant authorization to access personal health information; tumor tissue (cases only), medical records, and pharmacy records release forms; and oral tissue specimen donation consent forms. Again, these forms will be adapted from our current work for use in this study.

   Status: Complete, all of the documents listed above were approved and are currently in use in the field.

c. Development of tracking system, Months 1-3:
   We will modify the computerized tracking systems we currently use in our other studies to fit the specific needs of this project. This system will allow for up-to-date tracking of study progress and retrieval of information on any aspect of the study as needed.

   Status: Complete, the tracking system is working efficiently.

Task 2. Obtain Institutional Review Board (IRB) Approval for Human Subjects Research, Months 1-6, completed under the supervision of Dr. Li at FHCRC and Dr. Chinnaiyan at the University of Michigan:

a. Obtain approval from the Fred Hutchinson Cancer Research Center and the University of Michigan IRB, Months 1-2:
   Drs. Li and Chinnaiyan and their staff will lead the preparation and revision of documents necessary to obtain this approval.

   Status: Complete, local IRB approval has been obtained at Fred Hutchinson and at the University of Michigan.

b. Obtain approval from the United States Army IRB, Months 1-4:
   Drs. Li and Chinnaiyan and their staff will lead the preparation and revision of documents necessary to obtain this approval.

   Status: Complete, DOD IRB approval has been obtained at Fred Hutchinson and at the University of Michigan.
Task 3. Training of Study Staff, Months 1-6, completed under Dr. Li’s supervision at FHCRC:

a. Training of office based staff, Months 1-6:
   Dr. Li will lead the finalization of all study protocols and the training of the study coordinator and program assistant on the implementation of these protocols.

   Status: Complete, the study manager and program assistant have been trained. They worked closely with the Dr. Li to finalize and implement the study protocols and study staff has begun data collection.

b. Training of field staff, Months 4-5:
   Under Dr. Li’s supervision, the study coordinator, Ms. Taylor, will oversee the training of the study’s field and random digit dialing (RDD) interviewers. To support this and on-going training, Ms. Taylor will develop a question by question (QxQ) specification manual that details the approach to be used for each question in the interview form.

   Status: Complete, the QxQ and other training materials are complete. The telephone interviewers completed their training and are currently working in the field. The interviewers are experienced and well versed on our study specific procedures. They received extensive training on confidentiality, obtaining informed consent, administering the questionnaires, phone protocols, and collecting oral specimens. With the approved modified SOW, the RDD interviewer was trained to complete telephone interviews with the case group, since controls are no longer being recruited.

Task 4. Case Identification, Months 3-45, completed under Dr. Li’s supervision at FHCRC:

a. Identification of potentially eligible cases from the cancer registry Months 3-45:
   All potentially eligible cases will be ascertained through the Cancer Surveillance System (CSS), a population-based cancer registry covering 13 counties in western Washington State that participates in the NCI SEER Program.

   Status: Complete, cases have been ascertained through the Cancer Surveillance System.

b. Verification of potentially eligible cases Months 3-45:
   Ms. Taylor will review the cancer registry abstracts and appended pathology reports to determine the eligibility of breast cancer cases. Lists of newly diagnosed cases will be generated twice each month from CSS files.

   Status: Complete, CSS files for this study were downloaded and reviewed by the study manager each month.

c. Physician notification letters, Months 3-45:
   The physicians of eligible patients will be notified in writing of our intention to contact their patients, to request updated contact information for these patients, and to solicit any reasons why patients may not be suitable for this study.

   Status: Complete, per the Cancer Surveillance System (CSS), prior physician notification and physician permission is optional for research subjects diagnosed on or after January 11, 1992, so we only sent physician notification letters when we could not find selected tumor marker results or address and phone information for participants.
However, living cases identified through the CSS are first approached with a letter from the CSS. As of October 2010, before any study can contact potential participants and their physicians, the CSS sends each potential participant a letter explaining that they are in the cancer registry, the registry’s purpose, and that we are conducting a breast cancer study. The registry letter also gives potential participants a phone number to call within 10 days to opt out of the study. If the potential case does not call the registry in the specified time to opt out, a detailed approach letter and brochure explaining the study are sent. The CSS letter was used in the field for this study.

**Task 5. Control Identification, Months 5-45, completed under Dr. Li’s supervision at FHCRC:**

a. Identification of controls, Months 5-45:
   General population controls with no prior history of breast cancer will be identified through RDD using a system that automates the administration, execution, and tracking of the RDD process.

   **Status:** Complete. RDD control ascertainment was halted last year after a revised Statement of Work was reviewed and approved. The RDD interviewer was trained to complete telephone interviews with the case group.

**Task 6. Approach to Study Subjects, Months 5-48, completed under Dr. Li’s supervision at FHCRC:**

a. Initial approach letter, Months 5-48:
   Cases and controls will be approached about the study through a letter describing the study’s purpose and procedures, and advising them that an interviewer will call soon.

   **Status:** Ongoing, cases are currently being approached for this study. An enrollment table is attached as Appendix 1. Per the approved modified SOW, controls are no longer being approached.

b. Initial telephone contact by a study interviewer, Months 5-48:
   Within one week of the initial mailing, a trained interviewer will call the subject to answer any questions, verify eligibility, and schedule the interview. Then a letter confirming the appointment will be sent to subjects.

   **Status:** Ongoing, the interviewing processes are underway for cases. An enrollment table is attached as Appendix 1. Per the approved modified SOW, controls are no longer being approached.

**Task 7. Conduct of Interviews, Months 6-49, completed under Dr. Li’s supervision at FHCRC:**

a. Administration of study interview, Months 6-48:
   All interviews will be conducted over the telephone. At the time of the interview the consent form will first be reviewed, any questions participants have will be answered, and consent will be obtained. We will enroll and interview a total of 900 TN cases, 1800 ER+ cases, and 900 controls. 700 TN cases and 900 ER+ cases are already been enrolled through another of Dr.
Li’s funded studies, so through this project we will enroll an additional 200 TN cases, 900 ER+ cases, and all 900 controls.

_Status: Ongoing_, the interviewers are currently working in the field answering participant questions, obtaining consents and conducting telephone interviews. A total of 1787 cases and controls have been enrolled in the study. Case enrollment is on target and proceeding as expected. 1658 cases have been enrolled and 566 are in process for the interview. (700 cases were enrolled over the past year.) Our request to modify the SOW to stop control ascertainment was approved and we added about 90 more ER+ cases. 129 controls have been enrolled. (2 controls were enrolled over the past year.)

b. Request for additional authorizations and release forms, Months 6-48: HIPAA compliant authorizations to collect personal health information will be sought including:
   i. A tumor tissue release so that specimens can be ascertained and centrally reviewed by the Porter Lab;
   ii. A medical records release so we can review medical records including radiology reports from prior mammograms;
   iii. A pharmacy records release that gives us permission to contact their usual pharmacies to verify and supplement reported medication use in future ancillary studies.

_Status: Ongoing_, the interviewers review all the consents with the participant at the conclusion of the interview and this process is going well and as expected. The majority of the participants sign all the consents.

c. Request for oral tissue specimen, Months 6-48:
   All subjects will be asked to donate an oral tissue sample using a provided Oragene kit. Participants will be asked to return their oral tissue sample along with all of their signed consent forms in a pre-paid envelope via the U.S. postal service.

_Status: Ongoing_, the interviewers request the oral sample at the conclusion of the interview and this process is going well and as expected. The majority of the participants provide an oral sample.

d. Editing and coding of completed interviews, Months 6-48:
   Interviewers will edit each interview within three days of their completion. Next, one of our staff members who has extensive editing experience will edit and code the completed questionnaires. Lastly, Ms. Taylor will conduct a final edit of all questionnaires and determine which subjects need to be re-contacted so that missing or incomplete data can be collected.

_Status: Ongoing_, the interviewers, editor, and study manager are conducting edits and final review for all completed interviews.

e. Validation of interview data, Months 7-49:
   Ms. Taylor will review a random 5% sample of voice recordings from completed interviews as a quality controls check.

_Status: Ongoing_, quality control procedures are underway.
Task 8. Tumor Tissue Review and Testing, Months 5-48, completed under Dr. Li’s supervision at FHCRC:

a. Identification of tumor tissue specimens of interest, Months 5-48:
Ms. Taylor will review all cancer case abstracts assembled by CSS to identify the tumor tissue specimens of interest for this study including hospitals where they were ascertained, specimen characteristics, and specimen numbers. Tissue from surgeries post neo-adjuvant therapy will not be requested. This information will be entered into our study tracking system and used to generate hospital specific tissue request lists for this study that will be batched and requested from local hospitals quarterly by CERC staff under Ms. Taylor’s supervision.

Status: Ongoing, the study coordinator is identifying, prioritizing, and requesting tissue specimens. Tissues specimens for this study are being batched by provider and requested quarterly.

b. Receipt and processing of specimens, Months 9-48:
We will request that all tissues be sent directly to the Porter Lab where Porter Lab staff will be responsible for tracking their receipt and organizing them for further processing by the Lab.

Status: Ongoing, we ask tissue providers to send the tissue specimens directly to the Porter Lab. Upon receipt, each specimen is checked-in, tracked and prepared for analysis by the Porter Lab staff.

c. Review and testing of specimens, Months 9-48:
Staff in the Porter Lab will conduct complete histopathologic reviews of each specimen obtained. They will also determine if the correct specimens have been received and if they are sufficient for further testing using immunohistochemistry (IHC). If additional tissue is needed, the Porter Lab informs the CERC group of this so any additionally surgical specimens potentially available can be requested. All ER+ cases will be evaluated for Ki-67 using IHC so that luminal A and luminal B cases can be distinguished from each other. All triple-negative cases will be evaluated for EGFR and cytokeratin 5/6 using IHC so basal-like cases can be identified.

Status: Ongoing, the review and testing of tissue specimens is underway at the Porter Lab. The PIs and staff for the study and the Lab at FHCRC meet monthly to review the study’s progress and to address any issues or concerns.

Task 9. Review of Medical Records, Months 7-51, completed under Dr. Li’s supervision at FHCRC:

a. Ascertainment and review of radiology reports, Months 7-51:
Medical records of breast cancer cases enrolled will be reviewed from date of diagnosis forward to ascertain information on breast cancer treatments and disease recurrences through the present date. Our medical record abstraction team lead by Ms. Zuanich has over 10 years of experience collecting data of this type from breast cancer patients in our region and interacting with each of the local hospitals and health care providers providing medical care to them.

Status: Ongoing, the medical record abstract team has been trained and is working in the field and in the office, abstracting from electronic and paper medical records. Study staff are entering provider information into the tracking system so we are able to generate lists by institution which will allow us to assign field work to the abstractors most efficiently.
Task 10. Integrative sequencing of specimens collected for this proposal, Months 7-60, completed under Dr. Chinnaiyan’s supervision at the University of Michigan:

a. Process nucleic acids from FFPE specimens and ensure quality control, months 7-60:
Extraction using QIAGEN FFPE RNAeasy and DNA protocols will be followed by analysis with the Agilent BioAnalyzer 2100 using RNA Nano and DNA 1200 reagents. If necessary DNA will be further fragmented using Covaris S2 adaptive focused acoustics shearing.

Status: Ongoing, we have optimized conditions to process FFPE specimens. All quality control measures are in place including determination of RNA quality, tumor content and genetic finger-print analysis to ensure sample integrity. A total of nine shipments of samples from FHCRC to the Chinnaiyan Lab were made over the past year. In total, samples from 266 patients have been sent.

b. Construct exome and captured transcriptome libraries. Sequence tumor and germline biospecimens (n=400 patients), months 7-60:
We will carry out whole exome sequencing of the tumor and matched germline specimens, and gene fusion assessment of the tumor transcriptome. Molecularly barcoded libraries for exome and transcriptome sequencing will be constructed using Illumina TruSeq protocols. Capture of the exome libraries and transcriptome libraries will be done using Roche EZ Exome v2 reagents and protocols. The three libraries for each patient will be multiplexed into a single lane and sequenced using Illumina TruSeq SBS v3 flowcells and reagents.

Status: Ongoing, we have extracted tumor DNA and RNA from all 266 FFPE samples that have been sent to Michigan. Normal DNA has been extracted for 266 of these cases thus far. Library preparation using tumor RNA is complete for all 266 cases, and library preparation using normal and tumor DNA is complete for 256 cases. All of this work is proceeding according to schedule.

c. Optimize our integrative sequencing approaches, incorporating improved methods and reagents for both increased speed and sequencing yield, months 7-60:
We will optimize and incorporate improved methods for library construction, such as transposon based addition of adapters, as they become available. We anticipate a continuing improvement in Illumina SBS reagents and procedures both increasing the speed and reducing the cost for each sample, as has been the case since the introduction of the technology.

Status: Ongoing, we have protocols in place that are optimized for carrying out the integrative sequence analysis. These include physically separate sample preparation module, reagent preparation module and post amplification module. Dedicated equipment is used throughout the sample and library preparation and downstream sequencing and analysis. We will bring online, new reagents and methodologies as they are developed and become available. We have incorporated the Illumina v4 chemistry and seen the anticipated improvement in both quality of sequenced bases and turnaround time on the sequencers.

d. Validation of mutations identified by integrative sequencing by targeted resequencing and QPCR methods, months 50-60:
Somatic point mutations and small indels will be validated by PCR amplification of the identified exons in tumor and matched normal DNA followed by PCR cleanup using Agencourt Ampure XP reagents and then sequencing on the ABI 3500 Genetic Analyzer using BigDye v3 protocols and reagents. Candidate gene fusions nominated from paired-end transcriptome sequencing and analysis will be validated by SYBR Green based QPCR using
primers designed using Primer 3 to encompass the fusion junction on a panel of index case and control sample cDNAs.

Status: Not started, not scheduled to begin until month 50.

Task 11. Bioinformatics analysis of integrative sequencing results, Months 7-60, completed under Dr. Chinnaiyan’s supervision at the University of Michigan:

a. Analyze sequencing data to identify significant variants, months 7-60:
   Using our in house developed tools, ChimeraScan, SNP detection and exome copy number, as well as available tools such as GATK and Snowshoes, we will analyze the patient sequence data.

   Status: Ongoing, all informatics analysis pipelines are in place. A web-based portal is in place that integrates data from a number of parallel analyses that are run by bioinformatics staff as well as coupled with a sample tracking LIMS system. Analysis pipeline consists of tumor content analysis, a SNP “fingerprinting” QC analysis, somatic and germline mutation calls, insertions, deletions, copy number alterations, gene fusions, gene expression, and zygosity analysis from DNA and RNA sequence libraries. Results are integrated with lab and clinical data and public datasets such as COSMIC and Ensembl. We have generated data from 127 samples for which analyses are complete.

b. Interpret and translate sequence variants, months 7-60:
   Identified mutations will be further analyzed for effects on reading frame and protein structure and function using analysis and prediction tools such as PolyPhen and PhastCons.

   Status: Ongoing, Analysis tools are in place and are being used as samples undergo completed sequencing analysis. We have recently incorporated additional mutation evaluation tools, such as CADD (Combined Annotation Dependent Depletion) into the pipeline. We have fully implemented a bioinformatics pipeline for the presence of small indels in the sequence data.

c. Optimize bioinformatics approaches, months 7-60:
   We will continue to refine and test our bioinformatics pipelines and evaluate and incorporate new analysis tools and approaches as they are developed.

   Status: Ongoing, we have a robust informatics pipeline in place supported by a web-based portal (see above). We have added LOH analysis for uncovering deleterious / tumor suppressor genes in the tumor samples. We have completed investigations on the feasibility of extracting reliable expression information from the FFPE derived transcriptome libraries. We have published an evaluation of the capture transcriptome method we developed for use with FFPE and shown excellent performance in both expression data as well as gene fusion detection. (Cieslik, “The use of exome capture RNA-seq for highly degraded RNA with application to clinical cancer sequencing” Genome Research 2015.) The portal will be undergoing upgrades to accommodate new features and analysis pipeline as they are developed, for example integrating alternate splicing and epigenetic analyses.

d. Incorporate primary sequencing results of the 400 patients in the discovery set with results from patients in the validation set, months 12-60:
   Using a combination of Sanger based resequencing and pools of multiplexed PCR products resequenced on Illumina next-generation sequencing equipment, we will screen any recurrently mutated gene appearing in our 400 fully sequenced cases, across the entire set of
available samples. Likewise, we will screen any functionally recurrent gene fusion across the complete sample set, using QPCR methods and further validation by Sanger sequencing of PCR products.

Status: Ongoing, complete sequence analysis for all accrued patients is ongoing as data are generated. Complete analysis of exome and transcriptome is now complete for 228 cases.

Task 12. Data Analysis and Manuscript Preparation, Months 52-60, completed under the supervision of Dr. Li at FHCRC and Dr. Chinnaiyan at the University of Michigan:

a. Data analysis, Months 52-60:
Dr. Tang, under the direction of Dr. Li, will lead the analyses of all epidemiologic data and the histopathologic data from the Porter Lab. Dr. Chinnaiyan and his team will lead the analyses of the mutational data generated in his lab. Data analysts from both groups will work together to realize the aims involving data collected from Dr. Li’s field work and Dr. Chinnaiyan’s lab.

Status: Not started, not scheduled to begin until month 52.

b. Manuscript preparation, Months 56-60:
Drs. Li and Chinnaiyan will lead the preparation of the multiple anticipated manuscripts that will describe the results of this study with the assistance of Dr. Porter and study staff.

Status: Not started, not scheduled to begin until month 52.

KEY RESEARCH ACCOMPLISHMENTS:
Data collection is underway.

REPORTABLE OUTCOMES:
None.

CONCLUSION:
None.

REFERENCES:
None.

APPENDIX:
Table 1 Study Enrollment

SUPPORTING DATA:
None.
Appendix 1

Advancing our Understanding of the Etiologies & Mutational Landscapes of Basal-Like, Luminal A, & Luminal B Breast Cancers

Table 1: Enrollment 9/9/16

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