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TITLE: Somatic mutation rate as determinant of breast cancer penetrance in BRCA1/2 familial cases

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Somatic Mutation Rate as Determinant of Breast Cancer Penetrance in BRCA½ Familial Cases

Analysis of how mutations accumulate in pretumor tissue, although widely presumed to occur, has been extremely difficult to study. This is principally because, with most such mutations being unique to individual cells within a tissue, their detection is technically challenging. In this study we propose to apply "Single Cell Multiple Displacement Amplification (SCMDA)" that we recently developed for high accuracy detection of a spectrum of mutations from single nucleotide substitutions to indels and aneuploidy in individual cells within pre-tumor tissues of women who inherited mutations in the BRCA1 or BRCA2 genes. We hypothesize that mutations from single nucleotide substitutions to indels, large genomic rearrangements, and aneuploidy accumulating as consequence of defects in homology dependent DNA repair in mammary epithelial cells are the underlying cause of increased cancer risk in these women. We further hypothesize that estrogen, which is known to generate metabolites that directly damage DNA, mechanistically acts as a modifier of BRCA1/2 cancer penetrance by working in concert with the BRCA1/2 repair defects to increase the somatic mutation rate in the cells of BRCA1/2 carriers. In Aim 1, we will utilize SCMDA to test if mutation frequencies are elevated in individual BRCA1/2 heterozygous mammary epithelial cells. In Aim 2, we will directly test the hypothesis that estrogen increases mutation frequencies in BRCA1/2 mutant cells.
1. Introduction
Cancer is a genetic disease caused by mutations accumulating in somatic cells during aging, environmental exposure or other endogenous factors (i.e. hormone exposure). How somatic mutations in non-tumor tissue result in tumor initiation remains largely unknown, in part due to the technical difficulties of studying non-clonal casual mutations in tissues. By leveraging in house generated approaches including “Single Cell Multiple Displacement Amplification (SCMDA)” in this project we aim to map all forms of mutations from single nucleotide substitutions to indels, large genomic rearrangements, and aneuploidy to test the hypothesis that accumulation of these mutations underlie increased cancer risk. As a model we selected women who inherited germline mutations in BRCA1 or BRCA2 because of their intrinsic defect in the homologous repair (HR) pathway. We further hypothesize that estrogen (from endogenous production, contraceptives, pregnancy, hormone replacement therapy), which is known to generate metabolites that directly damage DNA, mechanistically acts as a modifier of BRCA1/2 cancer penetrance by working in concert with the HR repair defects to increase the somatic mutation rate in the cells of BRCA1/2 carriers. Our hypothesis is being tested along two specific aims: in Aim 1, we utilize SCMDA to test if mutation frequency in normal mammary epithelial cells is increased in BRCA1/2 carriers relative to aged matched control women undergoing reduction mammoplasty purely for cosmetic reasons. In Aim 2 using primary organoids obtained from BRCA1/2 carriers or controls exposed to mammary gland estrogen levels we are defining how the hormonal microenvironment of the mammary epithelium influence genomic instability and promoted transformation by acquisition of a cascade of genetic event converging to tumorigenesis.

2. Keywords
Breast cancer, BRCA1, BRCA2, germline mutations, mutations, genomic instability, transformation, estrogen, 17β estradiol, inherited cancer, DNA damage

3. Accomplishments

**Major goals of the project and accomplishments:**

**Goal 1: to collect and isolate mammary epithelial cells.** Tissue for this study is being obtained from the Cooperative Human Tissue Network (CHTN). During this funding period we obtained six tissue samples for the control group from women of 28-42 y.o. undergoing plastic surgery reduction mammoplasty and indicating no previous family history of breast cancer. Six tissue samples considered as early onset cancer risk group were also collected from cancer-free female patients carrying a pathogenic mutation in BRCA1 gene and, hence, undergoing preventive care mastectomy. Normal tissue samples were also collected upon oncology surgery from two patients with early onset breast cancer (<45 y.o.), carrying pathogenic mutation in BRCA 1 or BRCA 2 gene (2 samples). All tissues were collected fresh at the time of surgery from the eastern division of the Cooperating Human Tissue Network (CTHN) and shipped over night at room temperature in transportation media. Mammary gland tissues, 0.5-2 g, were dissected into smaller pieces of ~3-4 mm and digested with Collagenase III and hyaluronidase to obtain mammary organoids for subsequent digestion into single cells. To enrich for luminal or basal mammary epithelial cells, single cells were stained with and antibody against CD49f and CD326 (EpCAM) as well as lineage specific markers CD31, CD45, CD235a, CD140b before sorting to separate basal epithelial cells (Lin- CD49f+/high EpCAM-/low) and luminal epithelial cells (Lin- CD49f-/low EpCAM+/high) as shown in Figure 1. Single cells obtained from both populations were deposited into Eppendorf tubes and stored at -80°C for further analyses.

**Goal 2: to perform whole genome single cell sequencing with the goal to establish if mammary epithelial cells of BRCA1/2 carriers have increased genomic instability.** After enrichment single primary mammary epithelial cells subjected to whole genome amplification (WGA) using our advanced single cell multiple displacement amplification method (SCMDA) as described in the application. To control the quality of amplified single cell MDA products we routinely use the 8-target locus-dropout tests. Qualified samples (4 single cell MDA products per each individual) are next subjected to library preparation and whole-genome sequencing using protocols described in the application and as reported by us [PMID: 28319112]
**Goal 3:** to run analytical pipelines and biostatistical analyses to evaluate genomic instability index across experimental groups. As sequencing data become available single cell WGS and its matching reference genome are analyzed using our custom in house generated pipelines described in [PMID: 28319112; PMID: 32064334; PMID: 30992375]. Currently, analysis has been completed for 14 samples. As new samples are collected they will flow their sequencing results into the analytical pipeline.

**Goal 4:** to establish *in vitro* model of 3D mammary organoids exposed to 17β estradiol. For studying the consequences of 17β estradiol (E2) on genomic instability and transformation of mammary epithelial cells of BRCA1/2 mutation carriers and controls we explored two models: mammary organoids in which partially digested mammary epithelial tree tissue is embedded in extracellular matrix hydrogel (Matrigel) and spheroids organized from patients derived dissociated single cells grown in Matrigel (Figure 2). Both 3D models have been cultured in basal control media or in the presence of serial dilutions of E2 ranging from 0.02 ng/ml to 20 ng/ml, with the highest concentration (20μM) reflecting the local E2 levels estimated in the mammary tissue; all E2 concentrations remain within the physiological range detected in the blood of women in the third trimester of pregnancy. While both models preserve similar cell viability in culture up to 7 days, partially digested organoids had lower yield and showed limited response to 20μM E2 exposure as measured by growth and branching (Figure 2A-D). On the contrary spheroids obtained from digested cells could be cultured for up to 24 days (Figure 2B-E) and were highly responsive to E2 exposure at 20μM concentration as measured by the number of organoids per field of view and their size (size distribution is preliminary observation). Phosphorylated gamma-H2AX staining to measure DNA damage indicates increased frequency of cells with foci of DNA repair in 20μM E2 exposed cultures relative to untreated controls. Based on these results, at the end of year one, we have established a robust model of culture of primary human mammary epithelial cells that retain response to E2 treatment. Thus, we will base our future studies on the mammary spheroid model. In addition, we also demonstrated the ability to dissociate the organoids at the end of the E2 treatment and isolate viable single cells for future SCMDA analysis.
Major goals for the next reporting period:

**Goal 1:** to collect and isolate mammary epithelial cells. We will continue the collection of additional samples for analysis of mutations to reach the sample size proposed (n=20).

**Goal 2:** to perform whole genome single cell sequencing with the goal to establish if mammary epithelial cells of BRCA1/2 carriers have increased genomic instability. Our preliminary analyses support the hypothesis that mammary epithelial cells obtained from BRAC1/2 mutation carriers have increased level of somatic mutations compared to age matched non-carrier controls; we will continue these analyses and apply appropriate statistical tests.

**Goal 3:** to run analytical pipelines and biostatistical analyses to evaluate genomic instability index across experimental groups. During the next period of funding we will finalize our preliminary observations by completing the analysis of human primary mammary epithelial cells isolated from BRCA1/2 carriers and controls to meet the proposed sample size. We will perform appropriate statistical comparisons to determine if significant differences between BRCA1/2 mutation carriers and control exist. We will also begin the SCMDA assay and analysis of

**Goal 4:** to establish *in vitro* model of 3D mammary organoids exposed to 17 β estradiol. We will continue perfecting the 3D mammary organoids model and ensure its robustness to evaluate E2 treatment response. Primary basal and progenitor cells will be tested for their effective reorganization into 3D structure when grown in Matrigel and compared to pooled cells to ultimately study their response to E2 treatment. In parallel we will begin isolating single cells from 3D organoids established from BRCA1/2 carriers and controls exposed to E2 or not in order to carry on genomic studies. A portion of the organoids culture will formalin fixed and paraffin embedded to study the genomic changes occurring in mammary epithelial cells in response to E2 exposure.
4. Impact
Nothing to report

5. Changes/Problems
We had difficulties in obtaining samples with sufficient material for FACS enrichment of epithelial subtypes, especially from BRCA1/2 mutation carriers. In order to obtain a sufficient number of mammary epithelial cells for efficient enrichment of subtypes by FACS we require a minimum of 20mg of tissue. To overcome limitations in obtaining sufficient amount of tissue for processing by the Cooperative Human Tissue Network (CHTN) we requested to broaden the tissue search from the eastern division to other collection sites part of the Mid-Atlantic, Southern and Western division.

Work on this project was substantially affected by the pandemic caused by SARS-CoV-2. Following NY State regulations from the second week of March until mid-June, the Albert Einstein College of Medicine was allowed to grant laboratory access only to essential personnel or individuals working in SARS-CoV-2 laboratories. Now that these state requirements have resulted in highly successful reductions in SARS-CoV-2 infections and COVID-19 hospitalizations and deaths, NY State is in the process of gradually reducing restrictions, with New York City, in which we are located, following the slowest course. Our institution is still operating with limited and staggered shift personnel to maintain safe physical spacing at the time of submission of this progress report (July 25). As a result, we were unable to perform sequencing as planned; samples have been collected and we have libraries prepared ready for sequencing; currently we are in the process of submitting these samples. Likewise, organoids experiments proposed in Aim 2 were delayed. Culture of organoids is now underway. To overcome potential further delays in case of future shut down caused by the pandemic we are exploring cryopreservation of organoids that can be cultured 3D at later time.

6. Products
Nothing to report

7. Participants & Other Collaborating Organizations

Name: Cristina Montagna
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-2343-5851
Nearest person month worked: 2.4
Contribution to Project: Dr. Montagna supervised the project and directed the daily operations; she was responsible for preparing the Institutional Review Board (IRB) application and coordinating the primary tissue collection.

Name: Tao Wang
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID): 0000-0003-0581-1251
Nearest person month worked: 0.6
Contribution to Project: Dr. Wang provided statistical support with the study design and preliminary data analysis including QC and evaluation of sample size based on the preliminary data obtained.

Name: Susan Klugman
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID): 0000-00001-8775-008X
Nearest person month worked: 0.24
Contribution to Project: Dr. Klugman was instrumental for the selection of patients to be included in our study; her expertise in genetic counseling and functional consequences of BRCA1 or BRCA2 mutation on genome instability provides the project insight into effect of germline mutations on tumor risk.
Name: Yi Zhang  
Project Role: Post Doc  
Researcher Identifier (e.g. ORCID ID): K-4058-2014  
Nearest person month worked: 9  
Contribution to Project: Dr. Zhang processed the samples for organoids and established the 3D in culture model to study the effect of estrogen on mammary epithelial cells as described in specific aim 2.

Name: Elaine Maggi  
Project Role: Post Doc  
Researcher Identifier (e.g. ORCID ID): 0000-0002-3387-106X  
Nearest person month worked: 1.5  
Contribution to Project: Dr. Maggi provided support with single cell DNA amplification and library preparation for the experiment described in specific aim 1.

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

- What other organizations were involved as partner? 
  “Nothing to Report”

8. Special Reporting Requirements

9. Appendices