Fresh Tissue Clinical Trial: Exploring Predictive Factors for Triple Negative Breast Cancer

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SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this study will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable Health Canada regulations and ICH guidelines.

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List of Abbreviations

BC	Breast Cancer
BCSC	Breast Cancer Stem Cell
CDK	Cyclin-dependent Kinase
CRF	Case Report Form
GCP	Good Clinical Practice
ICH	International Conference on Harmonisation
MaSC	Mammary Stem Cell
REB	Research Ethics Board
TNBC	Triple Negative Breast Cancer

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1 Introduction/Background

Successful diagnosis and treatment of breast cancer (BC) is complicated by the tremendous heterogeneity of the disease [1]. Breast cancer stem cells (BCSCs) may be the driving force, in part responsible for heterogeneity within a tumour. BCSCs represent a dangerous population of cells within the tumour and are more resistant to treatment [2-6]. Their ability to evade treatment often makes this population of cells the driving force behind patient relapse [2-6]. One subtype of BC known to harbor a high population of BCSCs, lack susceptible targets, and be more resistant to therapeutic options is triple negative breast cancer (TNBC) [7, 8]. Thus understanding the mechanisms which control expansion of this population may provide essential insight into better therapeutic options for treatment and eradication of this disease.

TNBC has elevated populations of cells with stem cell properties/ markers. This is further highlight by the fact that TNBC often exhibit loss of checkpoints and tumour suppressors (p16, p19, p53) and an increase in cell cycle proliferation genes (CDK2, Spy1) [9-11]. The ability to restore cell cycle checkpoints represents an attractive therapeutic approach to simultaneously halt cell proliferation and prevent unwanted expansion of the BCSC population, thereby preventing relapse [12-14]. Stratification of patient populations based on not only receptor status, but also a cell cycle profile may help to better predict which patients will respond favourably to emerging therapeutic options involving direct targeting of cell cycle mediators. Additionally, this information will held shed light on the mechanisms regulating expansion of the BCSC population and how to better target this highly resistant population of cells.

BCSCs retain many of the characteristics of normal mammary stem cells (MaSCs). These include similar marker expression, the ability to give rise to multiple progeny and the ability to self-renew [5]. MaSCs are normally held in a state of quiescence to prevent unwanted expansion of this population allowing for normal development of the gland and preserving the lifespan and genomic integrity of the cells [15]. MaSCs are critical to the normal function of the female mammary gland, especially during pregnancy and lactation when expansion of this population is required to generate the different subsets of cells needed for lactation to occur [16]. The ability of MaSCs to expand both the population of both SCs and differentiated progeny is dependent on the regulation of symmetric and asymmetric division [17]. Symmetric division allows for rapid expansion of the MaSC population, will asymmetric division allows for simultaneous maintenance of the SC population and expansion of differentiated progeny. The balance between symmetric and asymmetric division is a tightly controlled process regulated by a variety of cell cycle mediators. CDK inhibitors such as p21 and p27, and tumour suppressors such as p53, play a critical role in mediating differentiation of the gland and limiting expansion of the SC population [18-21]. Loss of p53 has been shown to promote a switch from asymmetric to symmetric division leading to an expansion of the SC population and reduced differentiation [22]. Given that loss of p53 is common in BC, this could represent an important loss of tumour suppression thereby driving expansion of a potentially dangerous population of cells.

The expansion of MaSCs is not only controlled by CDK inhibitors and tumour suppressors, but also by cell cycle mediators which promote cell cycle progression and expansion of this population, which is required at certain stages of development. Spy1 is an intra-cellular cell division regulator that promotes proliferation and expansion of cells [23-30]. Levels of Spy1 are tightly regulated during normal mammary gland development, highlighting an important role for this protein in mediating development of the gland [31]. A mouse model with increased levels of Spy1 in the mammary gland, the MMTV-Spy1 mouse, has been developed to study the role of Spy1 in mammary development and tumour initiation and progression. This mouse was shown to have increased rates of proliferation and an increase in the stem cell population. Additionally, when challenged with a known carcinogen, the MMTV-Spy1 mice are significantly more susceptible to mammary tumour formation when compared to their control littermates. Thus, further investigation into the potential role of the expanded SC population in increasing susceptibility is an important avenue research that could yield exciting new therapeutic targets. Furthermore, using human BC and brain cancer cell lines, Spy1 has been shown to play a role in the expansion of both brain and breast cancer stem cell populations, and can affect stem cell division properties in gliomas [32]. Mechanistically, Spy1 also drives cells past senescent and apoptotic barriers, resulting in resistance to a wide variety of drugs in cell systems and animal models [25, 33, 34]; an important finding given that CSC populations are known to be more resistant to therapy and can drive resistance. The ability of Spy1 to expand BCSC population points to a potential role for Spy1 in driving the initiation and/or progression of TNBC, given that Spy1 levels are upregulated in this disease.

2 Study Objectives

Study Objectives

1. Determine whether the expression of Spy1, Cyclins or MAPK/Myc signaling tested in a tissue microarray correlate with the TNBC subtype patient outcomes, using fresh tissue from the patients' diagnostic biopsies.

2. Assess the role of Spy1 in mammary stem cell renewal and differentiation using MMTV-Spy1 mice, and using the breast tissue material from the patients. Mammary cells will be transplanted into the mice with varying dilutions. These mammary cells will have over-expression or knock-down levels of Spy1 and cyclins. The Porter lab and Dr. MacNicol lab will study the role of Spy1 in asymmetric division through its effect on Notch and PKH26.

3. Examine the role for Spy1 and cyclins in treatment response in patients with TNBC. Expressions of Spy1 and cyclins will be manipulated in patient-derived cells, and then labeled and injected into zebra fish. The fish will be exposed to standard of care treatment for TNBC in combination with carboplatin and/or CDK inhibitors (ie Dinaciclib, NU2058). Tumour burden will be assessed by confocal imaging.

4. Collect ongoing data for patient 5 year recurrence free survival (RFS) and overall survival (OS). Data collected from the above objectives regarding expression levels of markers, the mammary stem cell population and response to treatment will be correlated with the data collected on RFS and OS.

3 Patients and Methods

3.1 Study Design

3.1.1 General Design

This clinical trial is aimed at assessing expression of various biomarkers, as well as response to treatment in novel treatment study systems in the lab which will enable researchers to better stratify patient populations and improve diagnostic tools. Data obtained from this study will also be correlated with patient RFS and OS. This project proposes to examine whether Spy1 up-regulation in triple negative breast cancer affects tumorigenesis by de-regulating stem cell renewal. Patient tissue samples will be used to establish a cell cycle profile to better stratify patient populations, with a focus on the levels of Spy1 within each sample. The BCSC population will also be studied to establish if Spy1 may be enhancing this population and be a driving force behind the expansion of this population. Importantly, samples will be used in an *in vivo* drug screening assay using a zebrafish screening platform we have established in the lab. This screening tool will be used to test the response to standard chemotherapy regimens and carboplatin and/or CDK inhibitors and correlate this with our findings about the cell cycle profile and BCSC population in these samples. Spy1 levels will be manipulated using lenti-viral approaches in the obtained tissue samples to determine if decreasing or increasing levels of Spy1 alters BCSC characteristics or response to treatment in the zebrafish drug screening platform.

Approximately 20% of patients will not be confirmed TNBC cases. This tissue will still be acquired at the time of surgery prior to an official diagnose of TNBC or otherwise. Those samples that ultimately are not TNBC will still be utilized in the studies and serve as controls for the TNBC patient samples. This will allow for direct comparison of TNBC to other subtypes of breast cancer and will be valuable controls in the studies.

3.1.2 Primary Outcome Variable

The proposed hypothesis is that Spy1 regulates the expansion and fate of mammary stem cells and that this plays a key role in the establishment of the breast cancer stem cells (BCSC). The expectation is Spy1 directly regulates drug resistance in this population of cells, and can be used as a prognostic indicator and novel therapeutic target for patients with triple negative breast cancer.

3.2 Subject Selection and Withdrawal

3.2.1 Inclusion Criteria

1. Age 18 years or older.

2. Patient has provided informed consent to participate in this study by signing and dating an REB-approved written informed consent form in accordance with regulatory guidelines.

3. Diagnosis of breast cancer made by core biopsy. Patients that have < 10% positivity in the estrogen receptor, < 10% positivity in the progesterone receptor and HER2 negative may be eligible. Patients that do not have this information available at the time of surgery may be included and assessed as controls if the final pathology report identifies > 10% positivity in the estrogen receptor, > 10% positivity in the progesterone receptor or does not find the patient to be HER2 negative (HER2 negativity defined by current institutional standards).

3.2.2 Exclusion Criteria

1. In the opinion of the surgeon, or pathologist, the patient would have insufficient tissue to donate for research purposes.

3.3 Study Procedures/Method

3.3.1 Tissue acquisition

1. Potential participants may be identified by the Breast Screening Program navigator, radiologist, or surgeon. If a breast biopsy is positive for malignancy, the radiologist or surgeon will inform the Breast Screening Program navigator.
2. When the potential participant presents to the surgeon's office or to the Breast Screening Program, the surgeon or the Breast Screening Program navigator will introduce the trial to the patient.
3. The breast screening clinic navigator will obtain consent.
4. Once consent is obtained, the Breast Screening Program navigator will inform the Clinical Trials staff at the WRH Cancer Program of the consented patient, and the date and time of the surgery.
5. The Clinical Trials staff will register the patient onto the trial, and provide the appropriate information to both the blinded study members in Dr. Porter's lab and the unblinded study member who will attend the surgery to obtain tissue.
6. The surgical suite manager will be informed by the Clinical Trials Staff that the patient is a participant of this clinical trial, and that a member of the Study team will be present in the surgical area to accompany the tissue to pathology.
7. The unblinded member of Dr. Porter's study staff will be arrive prior to surgery to bring a copy of the consent form as well as a form to remind the surgeon and pathologist that the patient is part of the clinical trial and the tissue is not to be placed in formalin.
8. Standard surgical procedure will proceed.

9. After resection of the tumour, the breast cancer fresh tissue will be transported back to the pathology department, using standard procedures, but with no formalin fixation.
10. The member of the Porter lab research team will accompany the tissue to pathology department. The pathologist will confirm the diagnosis of breast cancer. Approximately 10% of the fresh tumour, will be released by the pathologist to the unblinded study staff from Dr. Porter's lab. The fresh tissue will be placed in RPMI Medium (STEMCELL Tech) and will be transported chilled in a sealed container back to the University of Windsor.
11. Six months after registration into the clinical trial, the Clinical Trials Study staff will request one archived tissue block from the WRH pathology department. This will be shipped to Dr. Porter's lab at the University of Windsor.

3.3.2 Processing of the Cancer Specimen

A number of *in vitro* and *in vivo* assays will be conducted on the fresh tissue sample received. Assays will be prioritized, and the number of assays completed will be dependent on the amount of tissue received. Samples will be processed as follows, with the assays listed in order of priority:

1) Genomic analysis: Tissue will be flash frozen and cDNA will be isolated for a cDNA array for determination of the cell cycle signature.
2) *In vivo* analysis
    a. Mouse PDX Models: a fragment of tissue will be inserted into the inguinal gland of NSG mice to allow for propagation and expansion of the tumour. The resulting tumour tissue can be stored for further downstream analysis (ie genomic testing, protein expression, *in vitro* cell work etc)
    b. Zebrafish drug screening system: cells will be isolated from a portion of the tumour and will be injected into the zebrafish. Response to therapy will then be assessed by treating the tumour cells with differing chemotherapeutic agents/CKIs by adding the agents to the water. The difference in mean fluorescent intensity will be used as a measure of response to therapy
3) A portion of the tumour will be flash frozen to use for extraction of protein and/or RNA to assess protein/RNA levels of cell cycle mediators, tumour suppressors, etc.
4) Genomic analysis: Tissue will be flash frozen and genomic DNA will be isolated to assess the status of various genes of interest including p53 and BRCA1.
5) Tissue microarray (TMA) analysis: Using the archived tissue block, a TMA will be constructed to assess expression of various biomarkers. This will allow for direct comparison of expression levels between various patient samples at the same time as multiple samples will be placed on one TMA.

3.4 Statistical Plan

3.4.1 Sample Size Determination

The focus of this study is on TNBC, and we require 90 samples to make meaningful conclusions about the data acquired and perform proper statistical analysis. Since approximately 20% of patients will be of an unknown diagnosis at the time of acquiring the tissue, we anticipate another 30 samples that will not be TNBC. This brings the total number

of patients on the study to 120 to include both the required 90 TNBC samples and 30 of unknown diagnosis at the time of registration on the trial.

3.4.2 Statistical Methods

Statistical analysis of all results obtained will be conducted under the guidance of Dr. Hussein from the University of Windsor. For the zebrafish studies, a Fisher's exact test will be used to provide a proportional statistical analysis on the difference between control and chemotherapy exposed groups. The one-sided Fisher's exact test will be used to calculate the sample size of 30 embryos per treatment. The embryos are each individually exposed; therefore, this will represent an individual test subject. We can then compare control and treated groups statistically using one-way analysis of variance (ANOVA). For comparison within the treated groups, of those treated with a different chemotherapy regimen, we can use an alternative statistical test, Kruskal-Wallis analysis of variance on ranks. For mouse xenograft studies, tumour burden will be studied using a comprehensive longitudinal regression model and overall survival will be monitored. Expression analysis data will be correlated retrospectively to patient response. A two-sided Student's t-test will be used to compare the differences between patient samples and breast cancer subtypes.

Results will be given as mean + standard deviation and p values will be determined. In the event that the statistical tests yield a p-value less than or equal to 0.05, then the conclusions from the data will be interpreted as a statistically significant difference between treated and control or between individual patients/groups.

4 Data Handling and Record Keeping

The following data will be collected on all participants from their medical record at the Windsor Regional Hospital. Data will be collected onto Case Report Forms and sent to the University of Windsor Study staff without any patient identifiers other than the participant study number, the pathology sample reference number, and the participant date of birth. The only exception will be the unblinded surgical information that will be required for the University of Windsor Study staff responsible for obtaining the fresh tissue at the time of surgery.
Data will be collected from the participant's medical record as outlined in the table below:

Timepoint	Data to be collected and submitted
Registration of Participant	• Blinded Registration Form to UofW Study Staff • Unblinded Surgical Information Form to unblinded U of W Study Staff Only
6 months after study registration	• Chemotherapy Administration Form • Archived Tumour Tissue Submission Form
Annually from Study Registration	• Patient Status Form

4.1 Confidentiality

To protect the participants' identity and privacy of participants the samples will be labeled with a unique study number or 'code' before they are sent to the research lab at the University of Windsor, but not with any personal identifiers. Only the pathology identification number and the study number will be used. The code linking the personal identifiers to the sample will be kept by the Windsor Regional Hospital Cancer Program study staff in a secure location at the Cancer Centre. Decoding can only be done by the study investigator or an individual authorized by the study investigator.

5. Publication Plan

Data obtained from this research study will be shared at conferences and published in peer reviewed journals. The principal investigators as well as those that have contributed significantly to the project will be listed as contributing authors on publications.

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