The Spindle Assembly Checkpoint- A Predictor of Anthracycline Sensitivity in Breast Cancer Patients.

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Laboratory Medicine and Pathobiology
University of Toronto

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2016

Abstract

Identifying drug response remains a major challenge in breast cancer patients treated with adjuvant chemotherapy, as some patients relapse following treatment. Breast cancer consists of differences in tumour biology, which potentially correlates to differential treatment response patterns in patients. Resistance to commonly used chemotherapeutics such as anthracyclines poses an obstacle within the clinical setting, and no clinically validated biomarker exists to identify patients who will respond to treatment. Through analysis of the BR9601 trial we have been able to identify the spindle assembly checkpoint (SAC) as a potential predictor of anthracycline sensitivity, allowing us to further analyze this mechanism at the molecular level using anthracycline resistant breast cancer cells. To further elucidate the mechanism, we interrogated the SAC signal within our resistant cell lines and found that the SAC signal was significantly downregulated in the resistant cells. Investigation into SAC dysregulation could represent a mechanism for identifying anthracycline response.
Acknowledgments

This work was completed under the supervision of Dr. John Bartlett and Dr. Melanie Spears of the Transformative Pathology lab at the Ontario Institute for Cancer Research (OICR).

I would like to thank my supervisor Dr. John Bartlett for his guidance and support throughout this project. I would like to sincerely thank Dr. Melanie Spears for her ongoing input, guidance and leadership in designing this project. Thank you to my committee members, Dr. Paul Hamel (Department of Laboratory Medicine and Pathobiology), Dr. Irene Andrulis (Department of Laboratory Medicine and Pathobiology) and Dr. Rima Al-Awar (Department of Pharmacology and Toxicology) for their support throughout this project. I would like to sincerely thank all the members of the Transformative Pathology lab for their tutelage, patience in my training and support for the duration of my project. I would like to specifically thank Linda Liao and Nicola Lyttle for their assistance with developing and maintaining the resistant cell lines, Dr. Marsela Braunstein for her assistance with the flow cytometry analysis and Nazleen Lobo for her expertise and assistance with the siRNA knockdown design.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AI</td>
<td>Aromatase inhibitors</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase-promoting complex/cyclosome</td>
</tr>
<tr>
<td>ASCO</td>
<td>American Society of Clinical Oncology</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BCSG1</td>
<td>Breast cancer specific gene 1</td>
</tr>
<tr>
<td>Bub1R1</td>
<td>Budding-uninhibited-by-benzimidazoles-related-1</td>
</tr>
<tr>
<td>Bub1B</td>
<td>Budding-uninhibited-by-benzimidazoles-1-homolog-beta</td>
</tr>
<tr>
<td>CCK-8</td>
<td>Cell-Counting-Kit-8</td>
</tr>
<tr>
<td>Cdc20</td>
<td>Cell division cycle 20</td>
</tr>
<tr>
<td>CH17CEP</td>
<td>Chromosome 17 centromere enumeration probe</td>
</tr>
<tr>
<td>CMF</td>
<td>Cyclophosphamide-methotrexate-fluorouracil</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle value</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in-situ</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EBCTCG</td>
<td>Early Breast Cancer Trialists’ Collaborative Group</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EpiR</td>
<td>Epirubicin resistant</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERbB</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>Epi-CMF</td>
<td>Epirubicin-cyclophosphamide- methotrexate-fluorouracil</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in-situ hybridization</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory concentration at 50%</td>
</tr>
<tr>
<td>IBC</td>
<td>Invasive breast cancer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>Mad2</td>
<td>Mitotic arrest deficient 2</td>
</tr>
<tr>
<td>MCC</td>
<td>Mitotic checkpoint complex</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistant genes</td>
</tr>
<tr>
<td>MINDACT</td>
<td>Microarray in node negative disease may avoid chemotherapy</td>
</tr>
<tr>
<td>NEAT</td>
<td>National Epirubicin Adjuvant Trial</td>
</tr>
<tr>
<td>NPI</td>
<td>Nottingham prognostic index</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-targeting control</td>
</tr>
<tr>
<td>OSM</td>
<td>cytokine Oncostatin M</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PTTG1</td>
<td>Pituitary tumor-transforming 1</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinyl difluoride</td>
</tr>
<tr>
<td>RFS</td>
<td>Relapse-free survival</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAC</td>
<td>Spindle Assembly Checkpoint</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SNCG</td>
<td>Synuclein gamma</td>
</tr>
<tr>
<td>TAILORx</td>
<td>Trial Assigning IndividuaLized Options for Treatment(Rx)</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
</tr>
<tr>
<td>TNM</td>
<td>tumour, nodes, metastasis</td>
</tr>
<tr>
<td>TOPIIA</td>
<td>Topoisomerase type II-alpha</td>
</tr>
</tbody>
</table>
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Chapter 1

1 Introduction and Background

1.1 Breast Cancer Epidemiology and Etiology.

According to the Canadian Cancer Society, breast cancer is the most commonly diagnosed cancer in women with an estimated 25,000 new cases in 2015, it is also the 2nd leading cause of death from cancer among women in Canada\(^1\). With the increased use of mammography screening\(^2\) and effective therapies administered following primary treatment\(^3\) the age standardized mortality rate of breast cancer has decreased by 44% since 1986\(^1\). Relative to lung cancer (Figure 1), the breast cancer mortality rate in Canada has decreased considerably with similar statistics observed in the United Kingdom, United States and Australia\(^1\). The age-standardized incidence rate (Figure 2) for breast cancer has largely remained consistent throughout the years 1986-2015.

Figure 1\(^4\): Age-standardized mortality rates (ASMR) for breast, lung and colorectal cancer in Canadian women. The ASMR rate has decreased by 44%, with 32 deaths out of a population of 100,000 in 1986, to 17 deaths in 2015. (Canadian Cancer Society Stats, 2015).

Figure 2\(^4\): Age-standardized incidence rate (ASIR) for breast, lung and colorectal cancer in Canadian women. The ASIR has stabilized in recent years but remains quite high in comparison to lung and colorectal. (Canadian Cancer Society Stats, 2015).
According to the World Health Organization 2012, globally, breast cancer represented the second (1.67 million cases) most common form of cancer incidence, representing 25% of all cancers\(^5\). Breast cancer incidence increases with age, according to the Surveillance, Epidemiology and End Results (SEER) program increase in breast cancer incidence was reported every 10 years from 30-70 years of age\(^6\), further supporting the observation that the majority of breast cancers are found in post-menopausal women. Although incidence is higher in more developed countries (i.e. North America), survival following detection and subsequent treatment is higher\(^7\), attributed to the increase of screening in comparison to less developed regions. Additional risk factors implicated in breast cancer incidence include: a prior family history of breast cancer, genetic factors, obesity following menopause, increased breast density, increased exposure to hormones (i.e. hormone replacement therapy), contraceptives and alcohol\(^8\). Research from the American College of Physicians has indicated that both increased breast density and prior familial history of breast cancer were associated with a significant 2-fold increase of risk of acquiring the disease in women aged 40-49 years\(^9\).

Figure 3\(^5\): WHO Global Breast Cancer statistics among male and females: incidence (blue) and mortality (red). (GLOBOCAN,2012). Incidence of breast cancer is highest in more developed regions with mortality fairly stable and lower in both developed and less developed regions.
Evidence has suggested that earlier pregnancies coupled with breastfeeding can decrease the risk of acquiring breast cancer\textsuperscript{10,11}. Both pregnancy and breastfeeding decrease the overall number of menstrual lifecycles, decreasing exposure to endogenous hormones which are risk factors of breast cancer\textsuperscript{12}. Furthermore, it has been hypothesized that a correlation between lobule differentiation during pregnancy and a decreased risk of acquiring breast cancer exists\textsuperscript{13}.

### 1.2 Breast cancer pathology

The majority of breast cancers are classified as carcinomas\textsuperscript{14,15} a cancer of the epithelial cells, specifically as either in-situ (within confined regions of the breast) or invasive carcinomas. \textit{In-situ} carcinomas are divided into cancers that are localized within the breast ducts or lobules and do not invade surrounding tissues (Figure 4).

![Figure 4: Anatomy of a normal breast. Representation of ducts and lobules (For the National Cancer Institute © 2011 Terese Winslow, U.S. govt. has certain rights).](image-url)
Ductal carcinoma in situ (DCIS) refers to cancer that arises within the breast ducts and remains within the same location. Identified as a non-lethal cancer, DCIS may transition or become a precursor for invasive breast cancer (IBC)\textsuperscript{17}, which becomes an important consideration to take into account when administering treatment. IBC refers to cancer that has invaded surrounding tissues within the breast, escaping the duct and/or the lobule regions. IBC is believed to progress through different stages prior to becoming invasive; one of these stages includes in situ carcinoma. However, screening mammography has greatly improved the frequency and early detection of DCIS, currently accounting for 20\%-30\% of all diagnosed breast cancers in patients\textsuperscript{18}. Early detection of DCIS has allowed for the development of effective therapies (i.e. breast-conserving surgeries)\textsuperscript{19,20} that allow patients to avoid harsher therapies such as mastectomies.

1.3 Predictive and Prognostic markers

Predictive and prognostic markers are studied with the intent of determining the appropriate form of therapy for breast cancer patients taking into account clinical outcomes such as recurrence or death. Prognostic markers identify the probable course of the cancer in untreated patients amidst a population, evidence from these markers allows for the identification of appropriate therapy. Predictive markers identify groups of patients that would be receptive to the administered therapy from those that would not\textsuperscript{21}.

1.3.1 Breast Cancer Staging- Nottingham Prognostic Index (NPI)

Staging, a prognostic factor used within the clinical setting, classifies the extent of spread of the cancer within a patient (i.e. early vs metastatic stage), a common staging system that has been used by The International Union Against Cancer (among many other organizations) is the tumour-node-metastasis (TNM) system\textsuperscript{22}. The classification system relies on analysis of the size of the tumour (T), the number and location of lymph nodes with cancer cells (N) and if the cancer has metastasized to other regions (M). Grading classifies breast cancer cells based on appearance compared to normal cells, observed under a microscope. According to the Canadian Cancer Society, the grading system observes three features of the tumour: number of cells actively dividing, change of the size and shape the cells nuclei and percentage of tubular structure\textsuperscript{22}. A high grade indicates of rapidly dividing cells, vastly different from normal cells.
These factors, such as stage and grade, assist in determining how effective and successful a specific treatment for a patient will be. Currently there are tools and algorithms that take into consideration the stage and grade of a tumour used within the clinical setting to determine treatment decisions for patients. One such tool is the Nottingham Prognostic Index (NPI), which assists in predicting patient clinical outcome (i.e. survival) and in turn stratifies breast cancer patients based on their prognosis. The NPI system relies on the examination of three significant prognostic indicators: a patient’s tumour size, lymph-node status and tumour grading. The advantages of the NPI system include utilizing the mentioned clinical factors in determining treatment decisions, however its disadvantages lie in its inability to account for breast cancer heterogeneity and as a result falls short in explaining treatment failure. Classification of breast cancer based on tumour grade, nodes, hormonal receptors and type assist in patient therapy design. The study and observation for the presence/absence of hormonal receptors estrogen(ER), progesterone(PR) and human epidermal receptor-2(HER2) within breast cancer patients further represents indicators of response to administered therapy. These receptors are traditional biomarkers, observed/identified within patients as predictors of treatment response.

1.3.2 Estrogen and Progesterone receptors

Estrogen receptors (ER) presence or absence is a predictive marker used within the clinical setting for determining patient sensitivity to endocrine therapy. ER isoforms include: ER-α and ER-β with ER-α overexpressed in over half of all breast cancers. Both ER isoforms are transcription factors, activated by estrogen, which in turn controls specific genes.

Patients with ER positive (ER+ve) tumours are treated with endocrine based therapy such as tamoxifen. The mechanistic action of tamoxifen relies on its ability to function as an ER antagonist and in turn prevent the transcription and replication of ER activated genes. Various studies have researched the impact of potential predictive biomarkers and treatment impact in breast cancer within clinical trials, for example The Early Breast Cancer Trialists Collaborative Group (EBCTCG) combine multiple studies to perform a randomised meta-analysis on early breast cancer treatment outcomes. The EBCTCG conducted a meta-analysis of approximately 21,000 patients with early breast cancer, to identify ER’s role as a predictive biomarker, they demonstrated ER+ve patients treated with tamoxifen had reduced recurrence and breast cancer mortality, while this benefit was not observed in ER-ve tumours. Aromatase inhibitors (AI) have also been studied as a potential therapeutic for ER+ve breast cancer patients, in contrast to
tamoxifen, AI inhibits or suppresses aromatase in cancer cells, which is responsible for estrogen synthesis\textsuperscript{31}. The meta-analysis conducted by the EBCTCG using patients with ER\textsuperscript{+}ve tumours and early breast cancer were administered AI as an initial endocrine therapy as opposed to tamoxifen, observed over a 5-year period. The patients demonstrated a significant decrease of 30\% in recurrence\textsuperscript{32} within the first year. Tamoxifen, however, remains a prominent therapeutic option for ER\textsuperscript{+}ve early breast cancer patients as indicated by the American Society of Clinical Oncology (ASCO). ASCO has indicated that there is no significance or increase in benefit for any particular subset of patients of an AI versus tamoxifen treatment option\textsuperscript{33}.

Similar in function to ER, progesterone receptors (PR) also function as transcriptional factors that once bound to its receptor allows for transcription and the production of specific proteins to take place. Expression of ER-\(\alpha\) has been observed to regulate the expression of PR, as such the expression of PR is indicative of a functional ER-\(\alpha\) expression within the tumour\textsuperscript{34,35} serving as a predictive marker of endocrine based therapy.

1.3.3 Human epidermal receptor-2 (HER2)

The ErbB/ human epidermal receptor (HER) family of proteins consist of four members of membrane-bound receptor tyrosine kinases: HER1(ErbB1), HER2 (ErbB2), HER3(ErbB3) and HER4(ErbB4)\textsuperscript{36}. The ErbB/HER family of receptors are involved in signaling pathways that include; cellular proliferation, apoptosis, cellular motility and differentiation. However, due to the receptors prominent role in cell growth, if not tightly regulated will result in uncontrolled cell growth, leading to tumourigenesis\textsuperscript{37}. Recombinant proteins have been utilized to specifically target the HER family receptors and in turn compete with ligands for binding (extracellular) or preventing receptor dimerization (intracellular). HER2 overexpressed tumours respond positively to antibody-based therapies most commonly, Trastuzumab (herceptin), a humanized monoclonal antibody used within the adjuvant treatment setting for invasive breast cancers over expressing HER2. Its use within the clinical setting has improved both recurrence-free survival (RFS) and overall survival (OS) in HER2\textsuperscript{+}ve patients\textsuperscript{38,39,40}. A study conducted by Romond \textit{et al.} 2005 analyzed the results of two clinical trials in which patients with surgically removed HER2\textsuperscript{+}ve tumours were administered Trastuzumab. Patients administered Trastuzumab along with standard of care chemotherapeutics (doxorubicin and cyclophosphamide) demonstrated an improved outcome; in addition, patients experienced a decrease of 67\%(p=0.015) in risk of death relative to those who had not been administered Trastuzumab\textsuperscript{40}. 
As such, determination of HER2 status in breast cancer patients is important for determining the effectiveness and usability of Trastuzumab as a form of therapy. HER2 expression, by immunohistochemistry (IHC) or fluorescent in-situ hybridization (FISH), within the cells can lead to the administration of targeted therapy (i.e. Trastuzumab).

Although ER, PR and HER2 expressions have excluded some non-responsive patients from targeted therapies, they remain incomplete as predictive markers of response lacking in clinical validity and ineffective in determining alternative treatment for resistant patients.

1.4 Breast Cancer molecular subtypes

Breast cancer is a molecularly heterogeneous disease with biologically distinct subtypes, as a result molecular classification of the disease has the potential to determine clinical outcome and therapy design. Evidence of breast cancer heterogeneity is observed within tumour pathology, tumour histological factors (i.e. staging, grading) and clinical factors (i.e. hormonal receptors). Therefore, studying molecular differences in breast cancer supports this trend. Histologically similar tumours detected through the NPI system may differ in prognosis and respond to treatment differently, molecular heterogeneity within breast cancer may account for the differences observed in treatment responses.

Breast cancer is classified into molecular subtypes: luminal (A and B), HER2-type and basal-like. Through gene expression profiling, unique molecular tumour classes (i.e. luminal, HER2-type and basal-like) were identified and related to the unique molecular biology and features of the tumour. Each of the subtypes are defined by distinct treatment responses and prognosis. However, obstacles such as resistance to therapy may arise in some patients regardless of subtype similarity, in which case clinical outcomes are never guaranteed. Patients with luminal breast cancers, containing positive ER and PR hormones and low grade tumours, respond positively to hormonal therapies, with the luminal A subtype exhibiting good prognosis. HER2-type, a less common subtype containing an overexpression of the HER2 gene, has been associated with poor outcomes, higher histological grade and aggressive tumours, with patients responding positively to Trastuzumab. Basal-like tumours lack hormonal receptors (ER-ve, PR-ve, HER2-ve) and are identified with a high histological grade, the majority of which are classified as triple negative breast cancer (TNBC) subtypes. Patients with this subtype cannot be treated with hormonal therapies, as a result alternative treatments such as
Chemotherapies are administered. Table 1 demonstrates the current classification of breast tumours according to molecular expressions, prognosis and treatment options.

<table>
<thead>
<tr>
<th>Molecular Subtypes</th>
<th>Triple negative</th>
<th>HER2-type</th>
<th>Luminal B</th>
<th>Luminal A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor expression</td>
<td></td>
<td>HER2⁺ve</td>
<td>ER⁺ve, PR⁺ve</td>
<td></td>
</tr>
<tr>
<td>Prognosis</td>
<td>Poor</td>
<td>Good</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Therapy</td>
<td>Chemotherapy</td>
<td>Trastuzumab</td>
<td>Endocrine</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Breast cancer molecular subtypes (Modified from Wong E, Rebelo J. 2012)
Coloured arrows indicate of the strength of the prognosis within each subtype. Red- poor prognosis and blue- good prognosis. TNBC subtypes contain poor prognosis (on the higher end of the spectrum) whilst HER2-type is on the lower end.

1.5 Treatment

1.5.1 Surgery

Surgery represents primary treatment for breast cancer patients, administered to: completely remove the tumour, observe if the tumour has invaded lymph nodes and treat recurrent tumours. Surgery may involve either breast conserving or mastectomy depending on the extent and spread of the disease. According to the American Cancer Society, breast conserving surgery targets only the cancerous tissue leaving the breast, mastectomy involves removal of the entire breast in addition to lymph nodes if needed. According to the National Institutes of Health, breast-conserving surgery followed by radiation is the preferred primary treatment for early breast cancer patients as opposed to mastectomy, early and routine mammography screening may allow patients to avoid mastectomy in the event of breast diagnosis and reduce mortality.

1.5.2 Adjuvant treatment- Chemotherapy

According to the American Cancer Society and Cancer Research UK, chemotherapy maybe administered to a patient: prior to surgery (neoadjuvant) where the drug is administered to shrink the tumour prior to excision or following surgery (adjuvant) to ensure of recurrence free survival as well as to account for micro-tumours that may have evaded the primary treatment.
Patients within the advanced stages of the disease or those with the risk of their cancer metastasizing may also be administered chemotherapy to delay the spread of the cancer. In most cases, chemotherapy is administered as a cocktail or in combinations of more than one drug known as chemotherapy regimens. The American Cancer Society indicates of at least five common chemotherapeutic drugs administered for early breast cancer patients which include the standard of care chemotherapy regimen cyclophosphamide-methotrexate-fluorouracil (CMF), taxanes and anthracyclines. Chemotherapeutics such as vinorelbine, with increased side effects and direct impact on quality of life, would be administered to late stage breast cancer patients with the objective of delaying metastasis. The adjuvant treatment process that patients undergo following primary treatment consists of being administered an anthracycline or taxane-based regimen as adjuvant therapy, relapse into disease is measured within 6-10 months. Response rates are initially positive (~30-70%) however some of the responses are transient with patients relapsing or displaying resistance, patients are then administered second-line therapy drugs (i.e vinblastine).

1.5.2.1 Anthracyclines

Anthracyclines are an effective and widely used chemotherapeutic used to treat breast cancer patients following primary treatment. Anthracyclines, originally derived from Streptomyces peucetius, include Daunorubicin, which was the first of the anthracyclines developed displaying anti-tumour activity in mice. Over the years, numerous anthracycline derivatives have been developed; epirubicin and doxorubicin are used commonly in the clinic. Anthracyclines target tumour cells by intercalating with DNA strands causing damage, formation of free radicals and binding to the Topoisomerase II-DNA complex, this in turn promotes double-stranded breaks and degradation of the DNA. The benefits of anthracyclines have been demonstrated in a meta-analysis conducted by the EBCTCG over a period of 10 years, in which breast cancer patients treated with an anthracycline based regimen observed a lower percentage of tumour recurrence and breast cancer mortality in comparison to patients treated with CMF. This represented a 2.6% and 4.1% absolute gain respectively. However, anthracyclines have been implicated in an increased probability of patients acquiring cardiotoxicities due to repeated use of the drug over time, in addition to the risk of acquiring leukemia.

Effective chemotherapeutics, such as anthracyclines, are beneficial if administered to the right patient, the challenge arises in maximizing anthracyclines effectiveness whilst minimizing its
toxicity. Predictive biomarkers of anthracycline benefit have been researched, however no clinically validated marker has been identified, and this may due to an incomplete understanding of the molecular mechanisms responsible for anthracycline sensitivity within breast cancer patients.

1.6 Predictive markers of anthracycline benefit

Studies have indicated of HER2 overexpression in approximately 15%-20% of early breast cancer patients, overexpression of HER2 is associated with a negative prognosis and generally increased recurrence of the disease. Research has focused on both HER2 and Topoisomerase IIα (TOPIIA) as potential predictive biomarkers of anthracycline benefit.

Topoisomerases, are responsible for ensuring that the DNA supercoiling is not excessive but maintained to allow for protein-interactions. Topoisomerases can be split into two functionally distinct types: type I which are responsible for single stranded breaks of the DNA and type II which allows for double stranded breaks of DNA through which another double stranded DNA may pass. Isoforms of type II topoisomerases include topoisomerase IIα and topoisomerase IIβ. Both isoforms differ in biochemical properties, function and localization, TOPIIA isoform is linked to cellular proliferation and as such relevant to DNA replication. Type II topoisomerase enzymes cleave both DNA strands which in turn serves to manage DNA supercoils and tangles. Anthracyclines target the TOPII- DNA complex causing breaks and deregulation, this has led to the initial assumption that TOPIIA overexpression is a potential target of anthracycline sensitivity. Based on previous work completed to date, in vitro studies have indicated that over expression of the TOPIIA enzyme could result in increased sensitivity to anthracyclines. Anthracyclines directly target TOPIIA within the cells, as such the predictive impact of TOPIIA overexpression/aberration has been extensively studied with regards to anthracycline benefit. Studies have indicated TOPIIA aberrations as predictive for anthracycline benefit in breast cancer patients, however not all results have been conclusive. Romero et al. 2011 demonstrated the lack of correlation between TOPIIA copy number aberrations and TOPIIA gene expression in breast cancer, further research and evidence is required for determining associations between TOPIIA and anthracycline sensitivity.

Previous studies identified a potential relationship between HER2 overexpression and anthracycline sensitivity, however evidence supporting the role of HER2 as a predictive
marker of anthracycline sensitivity has been inconsistent\textsuperscript{75,71,79}. A meta-analysis conducted by Di Leo et al. 2011 consisted of five randomized adjuvant trials that observed HER2 and TOPIIA as potential markers indicative of anthracycline benefit in breast cancer patients. The results did not support the significance of either of the markers as exclusive markers of anthracycline sensitivity in patients that contained either an amplified HER2 signature or amplified/deleted TOPIIA\textsuperscript{76}. Both the TOPIIA and HER2 genes are located on the long arm of chromosome 17, which further adds to the interest of studying these genes in the context of breast cancer therapy. Genes located on chromosome 17 have been observed to being either downregulated/overexpressed/abnormal, as in the case of both HER2 and TOPIIA\textsuperscript{77}.

### 1.7 Ch17CEP duplication

Human chromosome 17 is implicated in a number of genetic diseases as observed through the genes that are located on this chromosome. Genes located on the arms of chromosome 17 which include BRCA1 (early-onset breast cancer marker), p53 (DNA damage), HER2 and TOPIIA (potential breast cancer predictive markers). Chromosome 17 abnormalities are observed in breast cancer, these abnormalities include whole chromosome variations, chromosomal structural changes and gene copy number variations\textsuperscript{78}, which can be detected through fluorescence in situ hybridization techniques (FISH). The region of interest is the alpha-satellite pericentromeric region of chromosome 17 where duplication, within this region is detected by utilizing a centromere enumeration probe (CEP)\textsuperscript{79}. CEP17 includes binding to the region of interest, enumerating the chromosome copy number and identifying the duplicated region within the chromosome\textsuperscript{80}. Studies have correlated the duplication of the alpha-pericentromeric region of chromosome 17 with anthracycline sensitivity in breast cancer patients, resulting in improved OS and RFS\textsuperscript{81,82}.

In a recent meta-analysis of five trials completed by Bartlett et al. 2015, FISH was performed for CEP17, HER2 and TOPIIA to determine their roles as predictive biomarkers. In this study adjuvant chemotherapy was assessed by comparing patients treated with cyclophosphamide-methotrexate-fluorouracil against those treated with an anthracycline regimen. The results demonstrated patients with abnormal gene expression of CEP17 (duplication) and TOPIIA (aberrations) significantly benefiting from the anthracycline treatment in both OS and RFS, relative to patients with normal CEP17/TOPIIA gene expression observing no significance in treatment. HER2 over expression did not display any significance and was not predictive of
treatment benefit. Patients exhibiting the CEP17/TOPIIA abnormalities demonstrated a 38% decrease in risk of relapse when treated with anthracycline based regimens compared to those patients treated with standard chemotherapeutic CMF\textsuperscript{83}. However, the alpha-pericentromeric region of chromosome 17 (Ch17CEP) contains heterochromatin which generally consists of repetitive and inactive DNA sequences\textsuperscript{84}. Therefore, no identified or known biological function exists that would further explain the association of Ch17CEP duplication and anthracycline benefit in breast cancer patients.

1.7.1 Chromosomal Instability

Ch17CEP describes duplication of a specific region within chromosome 17, chromosomal instability (CIN) a hallmark of solid tumours, is characterized by either a gain or loss of whole chromosomes or specific regions of the chromosome. CIN has been correlated to both sensitivity and resistance to chemotherapeutics\textsuperscript{85}. Extensive research has been conducted with regards to CIN’s role in cancer progression, specifically observing the mechanism of CIN development and subsequent effects\textsuperscript{86,87}. Causative mechanisms of CIN include two general types: type I and type II. Type I mechanisms include cell cycle processes responsible for chromosomal maintenance that include; spindle assembly during mitosis, repair machinery and G2M/G1S phase checkpoints\textsuperscript{88}. It is within these processes that CIN may arise if the mechanism is compromised or dysregulated. For example, within the cell cycle if chromosomes are misaligned during the metaphase stage of mitosis or if there are misaligned spindle fibers, chromosomal aneuploidy may occur leading to CIN. Type II mechanisms appear to be involved in physiological processes rather than genetic processes.

Studies of patients whose tumours contain CIN have been predictive of poor clinical outcomes in cancer subtypes which included breast cancer\textsuperscript{89,90}. A study completed by Munro et al. 2012, demonstrated that tumours with high CIN presence correlated with Ch17CEP duplication in the BR9601 clinical trial\textsuperscript{91}. Furthermore, the study observed patients containing a high CIN experienced a decreased risk of mortality when treated with an anthracycline regimen relative to the standard of care chemotherapeutic administered (i.e. cyclophosphamide-methotrexate-fluorouracil). To explain the correlation observed, Ch17CEP is a potential surrogate marker for CIN. Surrogate markers are used within the clinical setting as tests to assess improved outcomes to a specific treatment, in addition to indirectly correlating to the clinical outcome\textsuperscript{92}. A correlation was found to exist between CIN and the spindle assembly checkpoint (SAC) in
13 breast cancer cells, a study carried out by Yoon et al. 2002 observed breast cancer cell lines with high levels of CIN also contained defective mitotic spindle checkpoints as demonstrated through flow cytometry. Type I mechanisms of CIN directly impair chromosomal processes that includes the SAC signal, dysregulation of the SAC signal may provide a potential mechanistic understanding of Ch17CEP duplication and in turn anthracycline sensitivity.

1.7.2 Spindle Assembly Checkpoint

A study conducted by Hoyt et al. 1991 observed a mutant strain of Saccharomyces cerevisiae was unable to undergo cell cycle arrest during the mitotic phase due to a loss of microtubule function. As a result, they were able to identify three genes required for normal cell cycle arrest: Mad1, Mad2 and Mad3 (BubR1 in humans). Further research led to the understanding that these genes are conserved in all eukaryotes and function as a mechanism within the prometaphase/metaphase stage of the cell cycle. As mentioned in the previous section, dysregulation within the SAC signal and its components (kinetochores, mitotic checkpoint complex proteins, sister chromatid cohesion, centrosome aberrations) will lead to CIN resulting in aneuploidy within the cells.

The SAC mechanism ensures that dividing cells receive the correct number of chromosomes by halting mitosis if chromosomes are misaligned at the metaphase stage of the cell cycle, correct attachment involves spindle fibers attaching to the centromere of each chromosome at either poles of the cell. In a normal cell, activation of the APC/C by Cdc20 allows the cell to transition from the metaphase stage into the anaphase stage, this is preceded by the degradation of cyclin B, securin and the separation of the sister chromatids by separase. In the event of checkpoint activation, due to chromosomal misalignment, the cell is blocked from entering the anaphase stage of the cell cycle. As seen in Figure 5, to rectify the misalignment a cohort of member proteins within the SAC signal, the mitotic checkpoint proteins; BubR1, Bub3, Mad2 and Mad1 are activated. BubR1 and Mad2 directly bind to Cdc20 negatively regulating the APC/C and halt progression into anaphase. If components within the SAC mechanism are defective, cells may enter anaphase prematurely which in turn may give rise to aneuploidy and CIN. In addition to this, cells with a defective SAC are resistant to a range of anticancer drugs which includes anthracyclines and taxanes, this further supports research and interest into studying the SAC signal as a mechanism indicative of chemotherapeutic benefit within breast cancer patients.
Figure 5: Schematic of the SAC signal within the G2M phase of the cell cycle.

In normal cells the checkpoint is turned off, allowing for the APC/C to activate a cascade of signals which in turn allows for the cell to begin anaphase. Improper chromosomal alignment results in checkpoint activation, as a result the cell will arrest during metaphase.

Based on the chromosomal alignment within the cell, the SAC signal may remain turned off (cell progresses into anaphase) or turned on. In which case if the SAC signal is activated upon detection of improper chromosomal alignment mitotic arrest will occur. If the SAC signal remains activated this may result in a mitotic buildup and cell death. However, in the event of a dysregulated SAC, cells with improper chromosomal alignment may bypass the checkpoint and proceed with mitosis resulting in potential aneuploidy within the daughter cells.

Direct silencing of the SAC signal has been shown to result in dire consequences, for example complete absence of SAC member components was observed to result in developmental defects in mice\textsuperscript{105,106}. Interestingly research largely supports the possibility of a weakened SAC signal rather than a compete loss of the signal as a potential precursor for tumour formation within cells\textsuperscript{107}. This observation would support the rational that the SAC signal is dysregulated (not inhibited) in which case cells would progress within the cell cycle resulting in aneuploidy and chromosomal aberrations such as duplication of the centromeric region of chromosome 17 (Ch17CEP). It remains to be seen whether SAC dysregulation is a precursor of tumour...
development or if it is a result of it, in either case studying the SAC signal as the mechanistic explanation for Ch17CEP will assist in further determining the appropriate methodology for chemotherapy benefit within breast cancer patients.

1.7.3 BubR1

BubR1, a serine/threonine kinase, is a major component of the SAC signal when turned on. As part of the MCC (mitotic checkpoint complex), it binds to Cdc20 preventing it from binding to the APC/C\(^{108,109}\) causing the cell to arrest in the presence of chromosomal misalignment. In addition, BubR1 interacts with CENP-E\(^{110}\), a kinetochore motor, indicating of BubR1’s involvement in kinetochore tension and of its versatile roles within the SAC signal. Studies have shown of the importance of BubR1 within the SAC signal and cell viability. Kops et al. 2004 observed, through the use of small-interfering RNA’s, apoptosis within BubR1 kinase inhibited cells or cells with reduced BubR1 protein levels\(^{111}\). The study demonstrated that low BubR1 expression within cells resulted in a weakened SAC and as a result cells with misaligned chromosomes proceeded into anaphase. The study showed the importance of BubR1 activity to a sustainable SAC signal. Furthermore, it was reported through the study that a 50% reduction in BubR1 kinase activity was enough to compromise the SAC signal, considered a lethal consequence for cells including tumour cells. As a result, BubR1 has emerged as a highly desirable entity to study concerning inhibition of cancer cell growth. In the clinical landscape, studies have demonstrated that BubR1 overexpression in breast cancer is associated with poor survival and tumourigenesis\(^{112,113}\). The gene that codes for BubR1, \textit{BUB1B}, is overexpressed in breast cancer at both the transcriptional and translational levels\(^{114}\) with a strong association observed between \textit{BUB1B}, other mitotic checkpoint genes and an increased risk for breast cancer\(^{115}\).

1.7.4 Mad2

Mad2 functions alongside BubR1 within the MCC when the SAC signal is turned on. Its role, along with BubR1, focuses on the detection of misaligned chromosomes during the metaphase stage of the cell cycle. Previous studies have observed that overexpression of Mad2 in breast cancers (as well as other cancers) results in poor prognosis\(^{113,116}\). The importance of Mad2 to the checkpoints function was assessed in a study conducted by Michel et al. 2001, in which Mad2 was silenced in human cancer cell lines as well as mice primary embryonic fibroblasts. The cells displayed a defective checkpoint in which premature aneuploidy and CIN were
observed; the cells continued to cycle even after exposure to spindle inhibitors were administered. In addition, the mice with decreased levels of Mad2 developed lung tumours. The study further noted of the possibility that other members of the SAC mechanism might be implicated in tumour progression through the loss of the checkpoints function, which in turn could result in both aggressive tumour development and resistance to specific chemotherapeutics.

1.7.5 Securin

Securin, PTTG1, along with cyclin B are degraded prior to the anaphase stage of the cell cycle. Prior to its degradation, securin is bound to separase, the protein responsible for cleaving the cohesion rings of the sister chromatids, which is released as the cell progresses into anaphase. Securin overexpression has been observed in breast cancer patients, Solbach et al. 2004, observed a direct correlation between securin mRNA overexpression and tumour recurrence. High Cdc20 and high securin immunoexpression correlate to patients with a higher risk of aggressive breast cancer, aneuploidy and poor survival.

1.7.6 Breast cancer specific gene-1: Synuclein gamma

SNCG is a member of the synuclein family of neuronal proteins involved in the pathogenesis of neurodegenerative diseases and largely expressed in brain tissue. High SNCG expression has been observed in aggressive breast cancer tissue relative to normal tissue. Identified as breast cancer specific gene-1(BCSG1), studies carried out using both in vivo and in vitro models have found that SNCG expression both increases and promotes breast cancer cell proliferation and metastasis. Positive SNCG expression within breast cancer has been significantly correlated to late stage breast cancer tumours. In a study conducted by Wu et al. 2003, 38.8% of clinical breast cancer samples from a co-hort of 79 patients contained SNCG mRNA expression. In addition, 79% of late stage breast cancers within the same co-hort were SNCG positive.

Relevant to this project, a yeast-two hybrid screen conducted by Gupta et al.2003 indicated of an interaction between BubR1 and SNCG. Through this study, SNCG expression was observed to cause chromosomal instability within breast cancer cells during the cell cycle and affect the mitotic checkpoint. Through the work of Miao et al. 2014, it was observed that the SNCG-BubR1 interaction (Appendix I) resulted in a structural change directly effecting BubR1
kinase activity and binding patterns with Mad2 and Cdc20\textsuperscript{130}. The paper notes that BubR1 is not degraded or completely silenced by SNCG, rather it results in a structural change, which in turn impairs the activity of BubR1 or decreases its overall efficiency. Furthermore, the group confirmed through a neoadjuvant clinical trial that patients with SNCG positive tumours were in fact resistant to the effect of chemotherapy (induced-apoptosis). To counter the inhibitory effect of SNCG on the mitotic checkpoint complex, Inaba \textit{et al.} 2005 induced overexpression of BubR1 within breast cancer cell lines that expressed SNCG, as a result the inhibitory effect decreased\textsuperscript{131}. SNCG has also been studied as a potential biomarker of microtubule toxin sensitivity within breast cancer cells. Zhou \textit{et al.} 2006, showed that inhibition of SNCG expression increased sensitivity to paclitaxel treatment within breast cancer cell lines\textsuperscript{126}. Due to its interaction with BubR1 and inhibitory effect on the mitotic complex, upregulation of SNCG expression within breast cancer cells may be a target for reversing resistance within anthracycline resistant cell lines.

1.7.7 \textit{In-vitro} chemotherapy resistant cell lines

The use and development of \textit{in-vitro} chemotherapy-resistant cancer cell lines have been highly valuable in the study of molecular mechanisms of resistance and drug sensitivity\textsuperscript{132,133,134}. Chemotherapeutic resistant cell lines, have been used over the years to study mechanisms of drug resistance, dating back to the earliest \textit{in vitro} model developed in 1970\textsuperscript{135}. The model consisted of developing actinomycin D resistant cells from a parental Chinese hamster cell line, through an incremental dose treatment method. This in turn resulted in a 2500-fold increase in resistance seen in the resistant cell lines relative to the parental. Our laboratory has successfully generated epirubicin resistant cell lines representative of the four major breast cancer subtypes (luminal A and B, TNBC and HER2-type). This work demonstrated that increased resistance observed in the epirubicin resistant (EpiR) breast cancer cell lines resulted in cells that are able to proliferate and function with the addition of epirubicin relative to their parental cell lines\textsuperscript{136}. The development of an \textit{in vitro} cell line model allows for a more focused analysis of the molecular mechanisms responsible for the decreased sensitivity in addition to studying the SAC signal, specific proteins (i.e. BubR1, Mad2, SNCG) and their direct impact on the cells.
1.8 The BR9601 clinical trial

The current issue of administering chemotherapy to patients is the lack of benefit that some patients exhibit following administration. This results in unnecessary side effects and increased costs. The BR9601 clinical trial assessed the effectiveness of anthracyclines as adjuvant chemotherapy to treat early breast cancer patients. Patients recruited into this trial were pre- and post-menopausal women with completely excised, histologically confirmed breast cancer, and confirmed to receive chemotherapy as an adjuvant treatment. Patients were randomized into two treatment arms (Figure 6), those in the control treatment arm (red) received 8 cycles of CMF (cyclophosphamide 750mg/m², methotrexate 50mg/m², fluorouracil 600mg/m²) administered every 21 days. Those in the test treatment arm (blue) received 4 cycles of Epi-CMF (epirubicin 100mg/m²) every 21 days and 4-cycles of CMF. Patients were monitored over a 10-12 year period.

The hypothesis developed at the time of the BR9601 trial initiation was that Epi-CMF (epirubicin in combination with CMF) would result in significant benefit for patients in recurrence free survival (%) and overall survival (%) relative to the CMF treatment arm. The results of both the BR9601 and NEAT (a trial designed in parallel with similar objectives) clinical trials was reported by Poole et al. 2006, in which patients treated with an Epi-CMF regimen experienced a 7% increase in both OS (HR: 0.67, 95% confidence interval 0.55-0.82, p<0.001) and RFS (HR:0.69, 95% confidence interval 0.58-0.82, p<0.001) respectively, over a 5-year period relative to patients treated with only CMF. The hazard ratios (HR) indicated that patients treated with an anthracycline regimen demonstrated a decrease in risk of relapse and death compared to patients treated with only CMF. As such the hypothesis was proven correct and both trials were instrumental in highlighting the effectiveness and significance of Epi-CMF as an adjuvant chemotherapeutic for early breast cancer patients.

The results of the BR9601 trial highlighted the effectiveness of anthracyclines as adjuvant chemotherapies for breast cancer patients, the trial also provided an opportunity to further examine predictive markers of anthracycline benefit. Since treatment benefit was established, determining a marker that would identify a population of patients that would benefit from anthracycline regimens was the next logical step. As such, the SAC signal and member proteins (BubR1, Mad2 and securin) were tested for correlation to anthracycline sensitivity. The results
from this work provided the foundation for studying the molecular mechanism of the SAC signal and member proteins using an *in vitro* cell line model.

Figure 6 79,81,83,91,136,138,139. Schematic representation of the BR9601 clinical trial patient randomization into respective treatment arms (4 cycles), CMF (8 cycles).
1.8.1 Preliminary Results

Based on the work completed within the BR9601 trial, we set out to further identify patients within the trial that would benefit from the Epi-CMF treatment. As such, the goal for this particular aim was to identify a potential correlation between SAC member protein expression levels in breast cancer patients and anthracycline sensitivity.

The SAC signal was analyzed as a predictive marker in the BR9601 trial by IHC. The work completed in this section serves as the foundation for the development of this project and subsequent results. Through IHC, specific proteins of the SAC signal, BubR1, Mad2 and securin, were analyzed for expression levels and treatment benefit. High expressions of all three proteins were detected in the tumours analyzed independent of treatment administered. High BubR1 expression was significantly associated with a reduced OS (HR=1.87, 95% confidence interval 1.20-2.91, p=0.005) and RFS(HR=1.52, 95% confidence interval 1.00-2.32, p=0.047), further supporting what has been identified in the literature. High securin expression was significantly associated with increased OS(HR=0.64, 95% confidence interval 0.43-0.95, p=0.024) and RFS(HR= 0.56, 95% confidence interval 0.38-0.82, p=0.003) and Mad2 demonstrated no significant associations with OS and RFS.

Further analysis included the formulation of a dysregulated SAC signature that would identify patients that benefitted from the anthracycline treatment versus CMF. The signature was formulated based on the initial analysis of the individual markers within the samples: high BubR1, low securin and low Mad2 expressions defined the dysregulated SAC signature. Based on this signature 296/321 patients contained at least 1 or >1 dysregulated SAC protein with 15.2% of the patients identified as high SAC dysregulation (all 3 proteins dysregulated). Furthermore, a significant correlation was observed between increased SAC dysregulation and decreased OS (HR=3.09, 95% confidence interval 1.79-5.33, p<0.0001) and RFS(HR=3.06, 95%confidence interval 1.79-5.22, p<0.0001).

Finally, the effects of the identified markers were assessed on RFS and OS between patients receiving the Epi-CMF versus the CMF treatment regimen. As demonstrated in Figure 7, patients containing high BubR1 expression benefited from the Epi-CMF (blue) treatment relative to the CMF (red), these patients also experienced an increase in RFS and OS. There was no significant association observed in patients containing low BubR1 expression. Following a multivariate regression analysis, which accounted for more than one outcome variable at a time.
as well as adjusting for: age, nodal status, grade, tumour size, HER2 and ER status, the hazard ratio for the treatment by marker effect in BubR1 was 0.38 for OS (95% confidence interval 0.13-1.05, p=0.064) and 0.39 for RFS (95% confidence interval 0.14-1.04, p=0.061). Hazard ratios indicated of an approximate 50% decrease in risk of recurrence or death in patients containing high BubR1 expression and treated with Epi-CMF (Figures 11B and 11D). Patients with low BubR1 expression demonstrated a hazard ratio of 1 which indicated of no benefit or difference in treatment administered (Figures 11A and 11C). In addition, high SAC dysregulation, through observations of BubR1, Mad2 and securin, was associated with increased OS and RFS in patients treated with Epi-CMF relative to CMF treatment.

Figure 7: Kaplan Meir survival curves of patients displaying low and high BubR1 expression. %Relapse free survival A. with low BubR1 expression, B. with high BubR1 expression, %Overall Survival C. low BubR1 expression, D. high BubR1 expression. Blue curve- CMF and red curve- Epi-CMF. The OS treatment by marker effect of BubR1 was determined with a hazard ratio of 0.38 (~40% decrease in risk), 95% confidence interval 0.13-1.05 and p=0.064. For the RFS treatment by marker effect of BubR1, hazard ratio 0.39, 95% confidence interval 0.14-1.04 and p=0.061.
The BR9601 study highlighted the significance of high BubR1 expression as an independent predictor of anthracycline benefit in breast cancer patients, as well as identifying a signature for SAC dysregulation to be studied at the molecular level. We believe that the SAC signal represents a potential predictive marker of anthracycline sensitivity within breast cancer patients and through this work we would like to highlight the molecular evidence supporting SAC signal dysregulation and its correlation to anthracycline sensitivity.
1.9 Hypothesis and Aims

The aims of this project are to study the SAC as a potential molecular marker of anthracycline sensitivity through the use of isogenic epirubicin resistant breast cancer cell lines. Using an *in vitro* cell line model, we aim to identify SAC dysregulation and potential correlation to epirubicin resistance within our resistant cell lines. We hypothesize that the SAC is a potential biomarker for anthracycline sensitivity in breast cancer patients.

*Aim 1:* To validate the SAC signal and its surrounding pathways as a biomarker using anthracycline resistant cell lines.

Our goal for this aim was to validate the SAC member protein expression levels at the molecular level through western blot and qPCR. Based on our previous clinical work with the BR9601 trial, we hypothesize that there will be significant downregulation of the SAC member proteins, indicative of a dysregulated SAC signal, within the resistant cell lines. We set out to determine the SAC signal expression and in turn validate the potential correlation between SAC signal dysregulation and sensitivity to epirubicin.

*Aim 2:* To identify a key druggable target, which would reverse the resistance and re-sensitize the resistant cell lines to epirubicin.

Our goal for this aim was to identify a specific target that would reverse the resistance in the EpiR cell lines. The ultimate goal would be to provide an alternative treatment for patients unable to receive an anthracycline-based regimen. We hypothesize that the druggable target would interact with the SAC signal inducing dysregulation and decreased sensitivity to epirubicin.
Chapter 2

2 Materials and Methods

2.1 Breast cancer cell line culture

The breast cancer cell lines MDA-MB-231 and ZR-75-1 were purchased from ATCC and cultured in DMEM mixed with 10% FBS and 1% L-glutamine (Gibco, Burlington, Canada). Epirubicin resistant (EpiR) cell lines were generated in-house by exposing native cells to incremental doses of epirubicin concentrations and culturing the resistant populations, resistance was defined when the inhibitory concentration at 50% (IC50) of the resistant cell lines exceeded that of the native cell line counterpart. IC50 assays were carried out using the Cell Counting Kit-8 (Dojindo,Cedarlane Burlington Canada) and calculated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). As Table 2 demonstrates, a consistent concentration of epirubicin was added to the resistant cell lines to maintain resistance within the cells (25nM-MDA-MB-231, 10nM-ZR-75-1).

<table>
<thead>
<tr>
<th>Breast Cancer Cell Line</th>
<th>Hormonal Receptor Expression</th>
<th>Subtype</th>
<th>EpiR1000nM Highest Tolerated Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>ER^-ve/PR^-ve/HER2^-ve</td>
<td>TNBC</td>
<td>25nM</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>ER^+ve/PR^-ve/+ve/HER2^-ve</td>
<td>Luminal B</td>
<td>10nM</td>
</tr>
</tbody>
</table>

Table 2: ATCC breast cancer cell lines and hormone receptor expressions.

2.2 CCK8 assays

CCK-8 assays assessed the cell lines proliferation/cell viability by exposing cells to increasing doses of epirubicin diluted in dimethyl sulfoxide (DMSO): 0nM/DMSO (control), 0.3nM, 3nM, 10nM, 30nM, 100nM, 300nM, 1000nM, 3000nM, 10,000nM. Cells were plated on a 96-well plate in triplicates and left for 24 hours to adhere. The cells were then exposed to increasing concentrations of epirubicin, incubated for 72hrs at 37°C, 5%CO2, following which 10µl of the CCK-8 assay (Dojindo,Cedarlane Burlington Canada) was administered to each well. Plates were analyzed using the iMark Microplate Absorbance Reader (Bio-rad Laboratories Inc) at 450nM wavelength. The assay functions on a colorimetric reaction, where dehydrogenase
within live cells reduce the tetrazolium salt (WST-8), producing a yellow-orange colour change. Dose response curves, generated through GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA), were then used to calculate and illustrate the IC50 values of the cell lines. Fold change values calculated as a ratio of IC50 EpiR / IC50 native.

2.3 Flow Cytometry

For cell cycle analysis, cell lines were synchronized using a double thymidine block protocol\(^{140}\), following which the cells were collected at the following time points: 0hrs, 4hrs, 8hrs, 10hrs, 12hrs, 16hrs, 24hrs, 32hrs, 48hrs. Cells were trypsinized, washed with PBS, centrifuged and the pellet fixed with 80% ethanol overnight. Following which, cells were incubated for 1hr with 2mg/mL RNase and 0.1mg/mL of propidium iodide (Sigma-Aldrich) prior to analysis. Flow cytometry analysis, completed with BD FACSCanto II (BD Biosciences, Mississauga Canada), was utilized for determining cell cycle time points within the respective cell lines, specifically identifying the G2M phase time point. Analysis was completed using a univariate Watson pragmatic algorithm\(^{141}\) (FlowJo software, version 10), G1 represents cells within the G0/G1 phase and G2 represents cells within the G2/M phase\(^{142}\).

2.3.1 Double Thymidine block

A double thymidine block includes a primary block (17-18hrs), releasing cells into fresh media (7-9hrs) and a secondary block (17-18hrs) allowing for a synchronized S phase entry. A stock solution of 100nM of thymidine (Sigma-Aldrich) was made up and filter sterilized prior to use, this was further diluted to 2nM per well (6-well plate) and added to the media. The thymidine was stored at 4°C and refreshed every month.

2.4 Western Blots

Prior to collection of the lysate, cells were synchronized and then collected according to the G2M phase time points determined through flow cytometry analysis (10hrs MDA-MB-231 and 12hrs ZR-75-1). Lysate was collected from both the native and resistant cells lines using 1X RIPA buffer and quantified using the Pierce ThermoFisher Scientific BCA Protein Assay Kit. Final protein concentrations were calculated using iMark Microplate Absorbance Reader (Bio-rad Laboratories, Inc) at 550nM wavelength. OD readings of the samples and 9 bovine serum albumin standards (BSA), were used to calculate lysate concentrations. In addition to the volume of lysate, 5X loading buffer and 1X RIPA buffer were added to bring the total volume to
40µl. Samples were heat-shocked at 97°C for 6mins, centrifuged and loaded into the respective wells. Following which, gel electrophoresis was completed for 2hrs at 75-82V. The proteins were transferred overnight (onto a PVDF membrane) within a gel tank at 4°C with a voltage of 20V. Following which the PVDF membrane was blocked for 1hr in 5% skim milk(diluted in 1X TBS-Tween20) and incubated with a primary antibody overnight at 4°C. The membrane was washed for three-15min intervals with 1X TBS-Tween20, incubated for 1hr at room temperature with an HRP-linked secondary antibody and 0.2µl of Precision Protein Streptactin HRP conjugate (Bio-rad Laboratories, Inc). The blot was washed again for three-15mins intervals, treated with BM chemiluminescence western blotting substrate (Sigma-Aldrich) for 1min and visualized using the gel imaging ChemiDoc MP System (Bio-rad Laboratories, Inc).

2.4.1 Antibodies

The antibodies used primarily for this project included BubR1 and Mad2. Table 3 depicts the proteins used throughout this project and the antibody dilutions administered based on optimization work completed.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>BubR1 (BD Transduction Lab)</td>
<td>1°- 1:1000, 2°- 1:2000</td>
</tr>
<tr>
<td>Mad2 (BD Transduction Lab)</td>
<td>1°- 1:1000, 2°- 1:2000</td>
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<tr>
<td>Cyclin B1 (Cell Signaling)</td>
<td>1°- 1:1000, 2°- 1:2000</td>
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<tr>
<td>Cyclin E1 (Cell Signaling)</td>
<td>1°- 1:1000, 2°- 1:2000</td>
</tr>
<tr>
<td>GAPDH (Cell Signaling)</td>
<td>1°- 1:1000, 2°- 1:2000</td>
</tr>
<tr>
<td>Actin (Protein tech)</td>
<td>1°- 1:10,000, 2°- 1: 10,000</td>
</tr>
</tbody>
</table>

Table 3: Antibodies and respective dilutions routinely used for western blot analysis. 1°-primary and 2° secondary
2.4.2 Densitometry

Densitometry calculations were conducted to further provide a quantitative analysis of the western blots produced. Utilizing Biorad Image Lab (Image Lab software, version 4.0.1) the adjusted volume intensity of each band was displayed and compared against the adjusted volume intensity of its actin counterpart (normalization) yielding a ratio of which was used to determine the level of up-or down regulation (Table 4).

<table>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Native 0hrs</td>
<td></td>
<td>2,995,482.81</td>
<td></td>
<td>2,339,689.41</td>
<td>1.280290791</td>
</tr>
<tr>
<td>2</td>
<td>EpiR 0hrs</td>
<td></td>
<td>4,179,795.43</td>
<td></td>
<td>2,212,224.62</td>
<td>1.889408245</td>
</tr>
<tr>
<td>3</td>
<td>Native 10hrs</td>
<td></td>
<td>3,761,316.49</td>
<td></td>
<td>4,068,982.55</td>
<td>0.924387472</td>
</tr>
<tr>
<td>4</td>
<td>EpiR 10hrs</td>
<td>BubR1</td>
<td>3,666,893.33</td>
<td>Actin</td>
<td>3,987,547.48</td>
<td>0.919586124</td>
</tr>
</tbody>
</table>

Table 4: Sample densitometry calculation of BubR1 normalized to its actin control. The adjusted volume intensity for BubR1 is normalized against its native counterpart which is then used to compare native and EpiR up or downregulation of the protein.

2.5 qPCR

RNA was extracted from cell lines using the RNeasy Mini Kit (Qiagen, Toronto Canada). A total of 20µg of RNA was analyzed for each sample using Taqman Gene Expression Assays. The samples used for the qPCR runs included cells synchronized at 0hrs, 10hrs, 12hrs, the purpose of including cells at 0hrs would allow for comparison between cells collected at the G2M phase against cells collected at the G1 phase. Reactions were run in triplicates using the Applied Biosystems Viia 7 real-time PCR instrument and Viia 7 software (ThermoFisher Scientific, Burlington Canada). Transcript levels were determined using the $\Delta\Delta$Ct method. Primers used included BubR1, Mad2 and SNCG, the endogenous control was RPL37A and the reference sample (normalization) was the native cell line collected at 0hrs.
The ΔΔCt method represents a set of calculations that produces a final value representative of the test gene normalized expression level (requires a reference sample to be used). In this case the value relays the fold change of the test gene (upregulated or downregulated) relative to the control used (0hrs Native) represented by a 1. The ΔΔCt values were calculated from the Ct values that are the qPCR experimental run output, the Ct value indicates the number of cycles the machine completed in order to detect a real signal from the sample. A low Ct value indicates of abundant target nucleic acids (i.e. < 38). For the experimental work completed, Ct values as well as delta delta Ct values were obtained from each qPCR run through an excel spreadsheet, the final fold value was calculated. Table 5 represents a sample calculation completed in which Ct values are obtained for control and test genes, the Ct test values are subtracted from the Ct control values (i.e. Test 1 0hrs – Control 1 0hrs). An average of these values is obtained and subtracted (Ct test average – Ct control average). This value is used for fold change calculations.

<table>
<thead>
<tr>
<th></th>
<th>Control 1</th>
<th>Control 2</th>
<th>Control 3</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct test – Ct reference</td>
<td>2.819</td>
<td>3.069</td>
<td>3.620</td>
<td>2.254</td>
<td>2.381</td>
<td>2.498</td>
</tr>
<tr>
<td>Average</td>
<td>3.169</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.377</td>
</tr>
<tr>
<td>Delta Ct test – Delta Ct reference</td>
<td></td>
<td></td>
<td></td>
<td>-0.792</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fold Change (2^-ΔΔCt)</td>
<td></td>
<td></td>
<td></td>
<td>1.73</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Therefore the BubR1 in the ZR Native 12hr sample is upregulated by 1.73 fold relative to the reference sample ZR Native 0hrs.

Table 5: ΔΔCt sample calculations following qPCR run. Control: RPL37A. Test: BubR1. Reference Sample: ZR Native 0hrs
2.6 siRNA knockdowns

Knockdowns were completed using a Dharmacon pooled ON-TARGETplus Human BUB1B siRNA. The sample was diluted to 20,000nM (using a 5X siRNA diluent) and aliquotted into 25µl volumes. The final concentration was determined through a Nanodrop2000 (ThermoFisher Scientific, Burlington Canada). Knockdown validation was completed through western blot by collecting cell lysate at four time points (24-96hrs) to ensure of complete protein knockdown, controls included siGAPDH (+ve control), siNTC(-ve control), lipofectamine-only and media-only controls. The controls ensured of selective/specific knockdown of BubR1 prior to running the CCK-8 assays as well as ensuring that the knockdown would occur during the required time points. The impact of the knockdowns was assessed using CCK-8 assays and completed in both native cell lines (MDA-MB-231 and ZR-75-1). IC50 survival curves were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).
Chapter 3

3 Results

3.1 Isogenic EpiR cell lines, MDA-MB-231 and ZR-75-1, display 8-11X more resistance to epirubicin relative to native cells.

EpiR cell lines generated from native cells MDA-MB-231 and ZR-75-1 demonstrated an average 8-fold and 11-fold increase in resistance to epirubicin respectively (Figure 8A). EpiR cell lines demonstrated a significant increase in resistance to epirubicin relative to the native cells, MDA-MB-231 EpiR cells versus native (p=0.00026, unpaired t-test) and ZR-75-1 EpiR cells versus native (p=0.0015, unpaired t-test). Results from this work confirmed the utility of the in vitro cell line model, validating epirubicin resistance in EpiR cells relative to the native cells.

![A. MDA-MB-231 Survival Curve Analysis](image)

![B. MDA-MB-231 and ZR-75-1 Survival Curve Analysis](image)

<table>
<thead>
<tr>
<th></th>
<th>MDA-MB-231</th>
<th></th>
<th>ZR-75-1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicates</td>
<td>Native</td>
<td>EpiR</td>
<td>Fold</td>
<td>Native</td>
</tr>
<tr>
<td>1</td>
<td>48.2</td>
<td>1103</td>
<td>22.9</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>113.6</td>
<td>836.3</td>
<td>6.2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>94</td>
<td>1091</td>
<td>11.6</td>
<td>3</td>
</tr>
<tr>
<td>Average</td>
<td>91.9</td>
<td>1010.4</td>
<td>11</td>
<td>Average</td>
</tr>
<tr>
<td>SD</td>
<td>42.7</td>
<td>156.9</td>
<td>8.5</td>
<td>SD</td>
</tr>
<tr>
<td>P(T&lt;=t)</td>
<td>0.00026</td>
<td></td>
<td></td>
<td>P(T&lt;=t)</td>
</tr>
</tbody>
</table>

Figure 8: A. Dose response curve of MDA-MB-231 and ZR-75-1 Native vs EpiR cell lines. Red curve- native cell lines, blue curve- EpiR cell lines. B. Half-maximal inhibitory survival curve analysis (IC50) for three separate experiments. IC50 values are in nanomolar concentrations with the fold change representing IC50 resistant/IC50 native.
3.2 Cell Cycle Analysis: G2M phase time points 10hrs & 12hrs.

Figure 9 represents plots produced using FlowJo (FlowJo software, version 10), which depict the percentage of cells in different phases of the cell cycle at specific time points following a double thymidine block synchronization\(^{140}\). At 10hrs, the percentage of native MDA-MB-231 cells within the G2 phase was 31.3% relative to 24.4% of cells within the G1 phase and 36.7% (G2) of cells relative to 29.6% (G1) for the EpiR cell line. At 12 hrs, the percentage of native ZR-75-1 cells within the G2 phase was 48.3% relative to 31.7%(G1) and 40.7%(G2) relative to 31.6%(G1) for the EpiR cell line. These results indicate a 10-12hr window for transition to the G2 phase, with the cells completing a full cell cycle by the ~16-24hr time points.

![Figure 9: FlowJo analysis of synchronized cells collected at 0hrs, 8hrs, 10hrs, 12hrs and 24hrs (N=1). G1- purple peak and G2- green peak. Cell lines include MDA-MB-231 Native, MDA-MB-231 EpiR, ZR-75-1 Native and ZR-75-1 EpiR.](image-url)
3.2.1 Double thymidine block synchronization confirmed through cyclin B and E.

Cyclins B and E expression observed through western blot and densitometry (Figure 10) was analyzed for each of the cell lines following flow cytometry analysis. Figure 10A demonstrates, MDA-MB-231 native, cyclin E band intensity was highest at the 0-4hrs time points and again at 24hrs (representing the cells re-entry into the cell cycle). Cyclin B band intensity was highest at the 8-12hr time points representing the cells entry into the G2M phase of the cell cycle. Figure 10B, MDA-MB-231 EpiR, cyclin E band intensity was highest at the 24hr time point and cyclin B band intensity was highest from the 4-12hr time points. Figure 10C, ZR-75-1 native, cyclin E band intensity was highest at the 0-8hr time points and again at 32hrs. Cyclin B band intensity was undetectable at the 0hr time point and highest at the 12hr time point. Figure 10D, ZR-75-1 EpiR, cyclin E band intensity was highest at the 0-4hrs time points and again at the 24hr time point. Cyclin B band intensity was highest at the 12hr time point.
Figure 10: Western blots and densitometry analysis of cyclins B, E normalized against actin. MDA-MB-231. A. Native, B. EpiR and ZR-75-1, C. Native, D. EpiR. Purple- cyclin E, orange- cyclin B
3.3 Analysis of BubR1 and Mad2 at the G2M phase time points.

Western blotting was performed on the EpiR and native cell line lysates using the conditions optimized for determination of expression during the G2M phase; 0hrs (cells released following first block), 10hrs for MDA-MB-231 cell line and 12hrs for ZR-75-1 cell lines. Figure 11 represents western blot analysis of BubR1 and Mad2 for each of the cell lines, the blots show the down regulation of BubR1 expression at both the 0hr and 12hr time points within the ZR-75-1 EpiR cells relative to the native cells. For the MDA-MB-231 cells, the bands display similar intensity within the 10hr time point. Mad2 protein expression (Figure 11C and 11D) was observed to not change within the native and EpiR cells at the 10hr and 12hr time points for either cell lines.

Figures 11A and 11B demonstrates both the western blots and densitometry for BubR1(n=3) and Mad2 (n=2) normalized against actin. Although no significant difference was observed between the native and EpiR protein expressions (t-test unpaired), for both BubR1 and Mad2 a decrease in band intensity was observed at 10hrs for the MDA-MB-231 EpiR cells. Figures 11C and 11D demonstrated no significant difference in band intensity between the cell lines, however a decrease in BubR1 and Mad2 band intensity was observed at both 0hrs and 12hrs for the ZR-75-1 EpiR cells relative to the native cells.
Figure 11: Western blots and densitometry analysis of BubR1 and Mad2 normalized against actin. MDA-MB-231
A. BubR1, B. Mad2 and ZR-75-1. C. BubR1, D. Mad2. Red- native, blue- EpiR.
In addition to western blots, qPCR analysis was performed. The analysis demonstrated BubR1 and Mad2 gene expression downregulation within the EpiR cell lines relative to the native cells. Figure 12 depicts the average of three separate experiments carried out confirming the downregulation of both BubR1 and Mad2 within the cell lines using the native 0hrs cell line as normalization. Figure 12A demonstrates significantly lower BubR1 gene expression in the MDA-MB-231 EpiR cell lines relative to the native cells with a 37% decrease in BubR1 gene expression at 0hrs (p=0.017, unpaired t-test) and 41% decrease at 10hrs (p=0.0044, unpaired t-test). For Mad2 (Figure 12B), the EpiR cell lines demonstrate a 22% decrease in gene expression at 0hrs (p=0.030, unpaired t-test) and 41% decrease at 10hrs (p=0.011, unpaired t-test). Increase in gene expression observed within the native MDA-MB-231 cells from 0hrs to 10hrs is significant only for BubR1 (p=0.013, unpaired t-test) and Mad2 (p=0.12, unpaired t-test).

Figure 12C demonstrates significantly lower gene expression in the ZR-75-1 EpiR cell lines relative to the native cells with a 30% decrease in BubR1 gene expression at 0hrs (p=0.012, unpaired t-test) and 38% decrease at 12hrs (p=0.0021, unpaired t-test). For Mad2 (Figure 12D), the EpiR cell lines demonstrate a 24% decrease in gene expression at 0hrs (p=0.019, unpaired t-test) and a 25% decrease at 12hrs (p=0.031, unpaired t-test). Increase in gene expression observed within the native ZR-75-1 cells from 0hrs to 12hrs is significant only for BubR1 (p=0.007, unpaired t-test) and Mad2 (p=0.08, unpaired t-test).
Figure 12: Gene expression analysis of BubR1 and Mad2 at G2M phase time points MDA-MB-231 A. BubR1, B. Mad2 and ZR-75-1 C. BubR1, D. Mad2. Red- native, blue- EpiR.
3.4 Increased SNCG gene expression observed within EpiR cell lines.

Figure 13A shows significant BubR1 downregulation within the MDA-MB-231 EpiR cells relative to the native cells by 48% at time points 0hrs (p=0.0018, unpaired t-test) and 47% at 10hrs (p=0.00036, unpaired t-test). Mad2 (Figure 13B) shows significant downregulation within the EpiR cells relative to the native cells by 10% at time points 0hrs (p=0.044, unpaired t-test) and 35% 10hrs(p=0.0083, unpaired t-test). In contrast, Figure 13C shows significant upregulation of SNCG within the MDA-MB-231 EpiR cells relative to the native cells at 0hrs(p=0.014, unpaired t-test) and 10hrs(0.0012, unpaired t-test). SNCG within the MDA-MB-231 EpiR cells increased by 1029% at 0hrs(p=0.014, unpaired t-test) and 819% at 10hrs(p=0.0012, unpaired t-test). The downregulation of BubR1 and the upregulation of SNCG potential correlation within the MDA-MB-231 EpiR cells was significant at 0hrs(p=0.013, unpaired t-test) and 10hrs(p=0.00099, unpaired t-test).

Figure 13D shows significant downregulation of BubR1 within the ZR-75-1 EpiR cells relative to the native cells by 29% at 0hrs(p=0.020, unpaired t-test) and 35% at 12hrs(p=0.0067, unpaired t-test). Significant downregulation of Mad2 (Figure 13E) within the EpiR cells was observed by 27% at 0hrs(p=0.047, unpaired t-test) and 25% at 12hrs(p=0.040, unpaired t-test). In contrast, SNCG(Figure 13F) was significantly upregulated in the EpiR cell lines relative to the native cells at 0hrs(p=0.024, unpaired t-test) and 12hrs(p=0.0034, unpaired t-test). SNCG within the ZR-75-1 EpiR cells increased by 76% at 0hrs(p=0.024, unpaired t-test) and 50% at 12hrs(p=0.0034, unpaired t-test). The downregulation of BubR1 and the upregulation of SNCG potential correlation within the ZR-75-1 EpiR cells was observed to being significant at 0hrs(p=0.014, unpaired t-test) and 10hrs(p=0.030, unpaired t-test).
Figure 13: Gene expression analysis of SNCG, BubR1 and Mad2. MDA-MB-231 A. BubR1, B. Mad2 C. SNCG. ZR-75-1 D. BubR1, E. Mad2, F. SNCG. Red- native, blue- EpiR.
3.5 Time course (24-96hrs) knockdown of BubR1 in native breast cancer cell lines.

A time course study of BubR1 knockdown was performed to estimate the duration of knockdown of the BubR1 protein. Western blot and CCK-8 assays were performed following downregulation of the *Bub1B* gene using siRNA. Figures 14 and 15 represent examples of western blot confirming knockdown for both native cell lines. Controls included *siGAPDH* *siNTC*, lipofectamine-only and media-only. As demonstrated in Figures 14 and 15, the *siGAPDH* control was successful in silencing GAPDH and *siBub1B* was also successful in silencing BubR1 (target protein) over a 96hr time period.
Figure 14: Western blot knockdown of BuBR1 and GAPDH in MDA-MB-231 native cell line.

Figure 15: Western blot knockdown of BuBR1 and GAPDH in ZR-75-1 native cell line.
3.6 Significant increase in epirubicin resistance observed following knockdown of BubR1 in native cell lines.

BubR1 knockdown in both MDA-MB-231 and ZR-75-1 resulted in a significant 3-fold (p=0.04, ANNOVA single factor) and 2-fold (p=0.05, ANNOVA single factor) increase in resistance to epirubicin respectively (Figure 16) relative to the controls. Figure 16A demonstrates one example of triplicate dose-response curves for MDA-MB-231, the siBub1B knockdown cell line demonstrated the highest IC50 of 404.1nM, siNTC 119.9nM, lipofectamine-only 138.6nM and media-only 173nM. The dose response curve of ZR-75-1 (one example from an n=3), siBub1B knockdown cell line demonstrated an IC50 value of 319.2nM, siNTC 158nM, lipofectamine-only 117.2nM and media-only 125.3nM. The average IC50 values for each control and test were used to determine the final IC50 value across all three individual runs. As seen in Figure 16B, the media-only control demonstrated the lowest standard deviation for both cell lines relative to the other controls used.
Figure 16: A. Dose response curve of native siBub1B knockdown MDA-MB-231 and ZR-75-1. Green curve- siBub1B (target), purple curve- siNTC, orange curve- lipofectamine only and blue curve- media only curve. B. Half-maximal inhibitory survival curve analysis (IC50) for three separate experiments. IC50 values are in nanomolar concentrations.
Chapter 4

4 Discussion

The purpose of this study was to analyze the SAC signal and member proteins within our EpiR cells as the mechanistic explanation for Ch17CEP duplication and anthracycline sensitivity. Based on the findings of this study, we believe that a dysregulated SAC signal, demonstrated by a downregulation of BubR1 and Mad2, is a driver of anthracycline resistance. Preliminary results from the BR9601 trial indicated that patients containing low BubR1 expression did not benefit from the Epi-CMF treatment, this was further identified as a causal relationship as demonstrated by a knockdown of BubR1 within the native cell lines which was followed by a significant increase in resistance to epirubicin.

MDA-MB-231 and ZR-75-1 were chosen for this project due to the similar molecular subtype of these cell lines to the patients analyzed within the BR9601 trial. Patients within this trial were representative of those who would receive chemotherapy as adjuvant treatment. Our proliferation study demonstrated both the MDA-MB-231 and ZR-75-1 EpiR cell lines had a significant increase in resistance compared to native cells. This experiment falls in line with the work completed by Braunstein et al. 2016, in which resistant MDA-MB-231 cell lines expressed an average of 67-fold increase in resistance and resistant ZR-75-1 cell lines expressed a 7-fold increase\textsuperscript{136}. Importantly, concerning these generated EpiR cell lines was the observation that multi-drug resistant genes (MDR), cells with the reduced ability of accumulating drug\textsuperscript{143}, were not upregulated in the cell lines studied for this project. The fact that MDR genes are not upregulated in the respective cell lines indicates that an alternative independent mechanism is at work and responsible for the increased resistance within the EpiR cells.

Western blots and qPCR analysis showed significant downregulation of both BubR1 and Mad2 in both cell lines at all time points (0hrs, 10hrs, 12hrs). The downregulation could be due to a number of reasons some of which being: the drug directly impacts the SAC protein expression levels, the structure and/or the function of the proteins has been altered by another target (upstream or downstream of the SAC signal). Our lab has previously observed, through western blot analysis that the hormonal receptor expression levels (ER, PR, HER2) of the EpiR breast cancer subtypes had not changed from their native counterparts\textsuperscript{136}. This observation supports the fact that epirubicin is not directly influencing the cell lines protein expression levels and we can
safely assume that the expression of BubR1 and Mad2 are independent of epirubicin addition to the cells. SNCG was significantly upregulated in the EpiR cell lines relative to the native cells, whilst both BubR1 and Mad2 were downregulated. The upregulation of SNCG and downregulation of BubR1 within the EpiR cells was also significant, further supporting the interaction of BubR1 and SNCG indicated within the literature. We observed a dramatic upregulation of SNCG within our MDA-MB-231 cell lines (TNBC); supporting previous results that demonstrated high SNCG expression in aggressive breast cancer subtypes.

By mimicking BubR1’s downregulated expression in the EpiR cell line, a knockdown of BubR1 within the native cells correlated to an increase in epirubicin resistance. This finding supports the work completed in the BR9601 trial where patients with low BubR1 expression did not benefit from Epi-CMF, demonstrating that decreased BubR1 expression, due to a dysregulated SAC signal, is responsible for increased epirubicin resistance within our cell lines. Although we did not study cellular viability in our knockdowns, we did observe the native knockdown cells (siBub1B) growing at a slower rate relative to the controls during the 96hr collection period. Interestingly, the control cells (siNTC, siGAPDH, lipofectamine-only, media-only) grew quickly (cells stacking), observed through the microscope. This observation indicates towards the importance of BubR1 in cellular proliferation, if so, further work is required to determine how the EpiR cells are able to continue proliferating with the decreased BubR1 expression and if this decrease is directly related to the increase in resistance observed.

The results of the BR9601 clinical trial further supports the molecular analysis work that we have completed thus far concerning the dysregulation of the SAC signal and its correlation to increased epirubicin resistance. The results of the trial indicated that patients containing low BubR1 expression (Figures 7A and 7C) did not observe any significant benefit from the Epi-CMF treatment relative to the patients that contained a high BubR1 expression (Figures 7B and 7D) and significantly benefited from the treatment. The results of the trial indicated of BubR1’s significance as an independent predictor of anthracycline benefit with increased OS(HR:0.38, 95% confidence interval 0.13-1.05, p=0.064) and RFS (HR:0.39, 95% confidence interval 0.14-1.04, p=0.061), our molecular work further supports this conclusion. We believe that decreased BubR1 expression, indicative of a dysregulated SAC signal, is linked to increased resistance to epirubicin as observed at both the molecular and clinical levels.
Analysis of key cell cycle markers through flow cytometry and western blots, cyclin B and cyclin E, further confirmed the association between the SAC signals expression and the G2M phase time point. In order to assess the SAC signal within the generated in vitro model it was important to determine the specific cell cycle time point in which the SAC signal would normally function within (mitotic phase). The time points observed for the G2M phase in this experiment falls in line with the work completed by Whitfield et al. 2002, where HeLa cells were synchronized using a double thymidine block, cells progressed into the G1S phase at 0-4hrs, entered a synchronous G2M phase within 7-8hrs and completed a full cycle within the 14-16hr timeframe. Furthermore, supporting the G2M phase time points we have identified through our work, a study published by Harshman et al. 2014 observed a G2M phase 8-12hr window for their MDA-MB-231 cells following a double thymidine synchronization.

Figures 10A and 10B, show cyclin B band intensity is highest during the 8-16hrs time points with cyclin E band intensity highest at the 24hr time point within the MDA-MB-231 cell lines. Similar trends are seen within the ZR-75-1 cell lines (Figures 10C and 10D). The western blots and densitometry indicate of lower cyclin B band intensity towards the end of the G2M phase; however, it is difficult to determine if this is due to a degradation and if in fact the SAC signal is functional within these cells. It is possible that the SAC signal is turned off or bypassed due to inhibition or dysregulation within the signal itself, thus enabling the cell to function normally regardless of potential CIN or aneuploidy. This would explain the presence and absence of cyclin B at specific time points within the cells.

For cyclin E, there appeared to be two distinct bands as seen in Figure 10. Multiple bands of cyclin E have been observed in breast cancer cell lines, with some of these bands expressing lower-molecular isoforms of the protein (34-49kDa). A study conducted by Keyomarsi et al. 1994, supported the presence of an altered cyclin E protein through analysis of breast cancer tumour tissue against normal breast tissue, the western blots presented a cyclin E of multiple isoforms (35-50kDa) within the aggressive breast cancer tissue relative to the normal tissue. Cyclin E has been actively researched as a potential prognostic marker of breast cancer, with some positive preliminary results as presented by Keyomarsi et al. 2002. Although the cyclins represent an excellent model for studying synchronization, it would appear that these proteins, specifically cyclin E, are impacted and altered in cancer cells and as a result expression cannot be guaranteed. For the future, a multivariate or multi-dimensional algorithm could be used for
flow cytometry in which case cells would be sorted according to each population (i.e. G0, G1, G2 and M phase) as opposed to the univariate method.

Our results thus far support the possibility of a dysregulated SAC signal within the EpiR cell lines and that this dysregulation may be a driver of anthracycline resistance, further representing a causal relationship. Two hypotheses arise from this analysis; 1) either BubR1 and Mad2 function is directly inhibited or altered rather than degraded, by a target, resulting in the subsequent dysregulation of the SAC signal or 2) the SAC signal has been inhibited or dysregulated by mutations of an upstream component that in turn impacts BubR1 and Mad2 function and as a result resistance is observed.

In support of the first hypothesis, SNCG upregulation within the EpiR cells appears to impact the SAC signal through its interaction with BubR1. However, the reasoning behind this upregulation or the specific interaction this protein has with BubR1 remains to be seen. The dramatic increase of SNCG expression within the EpiR cell lines leads, particularly as seen in the MDA-MB-231 cells, to the assumption that hormonal receptors presence/absence may play an indirect role in SNCG expression. Interestingly, SNCG has been observed to stimulate the ERα signaling pathway149 and protect Hsp90150, a chaperone protein of HER2, further promoting cancer cell proliferation. However, Cirak et al. 2015 did not observe any significant associations between high SNCG expression and hormonal receptors presence or absence within taxane resistant breast cancer cell lines151.

Cirak et al. 2015 studied the interaction of BubR1 and SNCG as potential predictive and prognostic markers within a clinical trial. Their results indicated of a significant correlation with low BubR1 expression and increased taxane sensitivity, whilst high SNCG expression (62% of patients) was significantly associated with decreased taxane sensitivity151. From our results and those noted within literature, low BubR1 expression coupled with a high SNCG expression seems to correlate with decreased chemotherapeutic sensitivity (our work has been with anthracyclines, literature has largely worked with taxanes). As a result, research has observed the druggability of SNCG with small molecules and potential re-sensitization of resistant cells. One methodology reported, has been the use of oncostatin-M (OSM)152, a member of the interleukin-6 family of cytokines, as a transcriptional suppressor of SNCG within breast cancer cell lines153. The study observed a decrease in SNCG mRNA expression as early as 30mins following administration, by 2 days the level of SNCG mRNA was decreased to 90%.
Alternatively, an SNCG knockdown could be completed within the EpiR cell lines. Miao et al. 2014 completed a knockdown of SNCG within SKBR3 (HER2-type breast cancer) and MDA-MB-231 cell lines, by inhibiting SNCG expression the docetaxel-mediated apoptosis response significantly increased within the cells relative to the controls, where SKBR3 witnessed a 35% increase in apoptotic efficiency and MDA-MB-231 cells witnessed a 24% increase\(^\text{130}\).

To further explain the decreased BubR1 expression observed in the patients of the BR9601 trial and our EpiR cell lines we turn to CIN. CIN has also been studied for its potential as a predictive marker of anthracycline sensitivity, it has been linked to SAC dysregulation and benefit from chemotherapy regimens containing anthracyclines\(^9\). A recent study conducted by Spears et al. 2015, identified a 4 gene signature related to CIN (CIN4) as a potential independent predictor of anthracycline sensitivity\(^15\), the study also indicated that the CIN4 marker contained genes involved in DNA repair (i.e. SAC) concluding that dysregulation within these mechanisms may also lead to anthracycline sensitivity. Our work supports the presence of a dysregulated SAC signal within our resistant cell lines, in addition this dysregulation has been correlated to an increase in resistance. This finding supports the assumption that a dysregulated SAC signal as observed through BubR1 expression or a CIN4 gene signature may infer anthracycline benefit in early breast cancer patients.

Further work is required to confirm the impact of BubR1 silencing on the proliferative ability of cells. The work of Wang et al. 2004, found that a deficiency of BubR1, through a gene trapping method, resulted in death for early mice embryos\(^15\). Baker et al. 2004, observed mutant mice with low levels of BubR1, these mice developed aneuploidy, increased senescence and a variety of features that mimic physiological aging\(^15\). Interestingly, Schnerch et al. 2013 observed significant downregulation of BubR1 in acute myeloid leukemia cell lines\(^15\), re-introducing BubR1 to these cell lines through an inducible retrovirus mechanism re-sensitized the cells to spindle toxins (i.e. taxanes). Since the knockdown experiments conducted were transient, future experiments should include the development of a stable knockout BubR1 cell line to further validate the increase in epirubicin resistance (the IC50 values might increase as a result) and importantly the impact on cellular viability and proliferation. We believe that BubR1 effects the cells proliferative ability where growth is slower in comparison to the control cell lines, however it is still unclear if the impact is detrimental to the cells where the knockout of BubR1 could cause cell death. Implementing a permanent knockout (i.e. CRISPR/Cas9) of BubR1
within the cell lines would require additional proliferation assay type experiments due to BubR1’s prominent role in the SAC signal; this in turn might have some adverse effects on the cells.

In support of the second hypothesis, the SAC signal and member proteins may be dysregulated by mutations of an upstream component. Maciejczyk et al. 2013 observed a correlation between BubR1 overexpression and poor survival in early breast cancer patients\textsuperscript{112}. In addition to breast cancer, high BubR1 expression has been associated with poor prognosis in bladder, stomach, kidney, ovarian and large intestine cancers\textsuperscript{158,159,160,161}. Interestingly Lee et al. 2009\textsuperscript{161} attributed the increase in BubR1 expression within ovarian cancer to an increased mitotic index and increased proliferation of tumour cells. Overexpression of BubR1 within these cells seems to be correlated to uncontrolled proliferation or cells that are aneuploid, which could indicate of a dysregulated SAC signal. A dysregulated SAC signal would result in aneuploid cells, bypassing mitotic arrest, proliferating and in the case of cancer cells, surviving anthracycline induced apoptosis\textsuperscript{162}. An increased mitotic index opens the cell to increased mutations which in turn results in either cell death or cells surviving with genetic instability (i.e. increased BubR1 expression in breast cancer). It is quite possible that the decreased BubR1 and Mad2 expressions within the EpiR cell lines are caused by accumulated mutations from upstream genes such as p53, BRCA1 and BRCA2. It has been reported that mutations in BRCA2 impacts BubR1 acetylation processes, producing a weakened SAC signal\textsuperscript{163} and mice deficient in BRCA1 were found to contain decreased Mad2 expression, again leading to a weakened SAC signal\textsuperscript{164}. The buildup of these mutations may result in a dysregulated SAC, promoting CIN, increasing heterogeneity of the tumour and as a result an increase in resistance to chemotherapy\textsuperscript{161}. This assumption would support the hypothesis that upstream mutations may be responsible for the dysregulated SAC signal. Tumour suppressor genes such as p53, BRCA1 and BRCA2, located upstream of the SAC signal, are notable candidates that have been studied extensively in the context of breast cancer prognosis and tumour proliferation.

The findings generated from clinical trials, such as the BR9601, to study biomarkers cannot succeed without a thorough understanding of the molecular mechanisms at work. This project has focused on studying the molecular mechanism of the SAC signal and its member proteins within an \textit{in vitro} cell line model, our main objective has been to utilize the molecular information to further inform and support the clinical findings we have demonstrated thus far.
The presence of a dysregulated SAC signal and the upregulation of SNCG within our EpiR cell lines are potential drivers of anthracycline resistance, with SNCG demonstrating promise as a druggable target for reversing the resistance. Based on our molecular and clinical evidence, BubR1 expression is predictive of anthracycline benefit.
Chapter 5

5 Conclusion

Through our research, we have observed a downregulation of BubR1, Mad2 and an upregulation of SNCG within epirubicin resistant breast cancer cells, which in turn may be indicative of SAC dysregulation and an increase in epirubicin resistance. Furthermore, evidence from our clinical and molecular work supports BubR1 as a promising independent predictive marker of anthracycline benefit. Determining the appropriate treatment for breast cancer patients remains important for the improvement of patient’s overall survival and relapse-free survival. The use of adjuvant chemotherapies have been clinically demonstrated to improve patient survival\textsuperscript{165}, which translates to the continued use of chemotherapy in the adjuvant treatment setting for breast cancer patients. According to a study conducted in 2011 by the American Cancer Society, 34% of US female patients with late-stage breast cancer received chemotherapy and radiation as the adjuvant form of treatment following mastectomy\textsuperscript{166}. Chemotherapy is widely used within the breast cancer adjuvant treatment space, issues such as recurrence following treatment influences both the patient and the clinical practice. A study conducted on the cost of initial cancer treatment in Ontario by de Oliveira \textit{et al.} 2013 found that chemotherapy use increased by 8\% in those aged 19-44 years and 17.2\% in patients 45 years and older. The study found that chemotherapy costs increased by 5-fold in all breast cancer patients, concluding that the cost of chemotherapy directly influenced Ontario’s health care budget\textsuperscript{167}. Until an alternative to chemotherapeutics is developed with fewer side effects, increased benefits and low-costs, it is here to stay and we are required to further optimize chemotherapy for patients to ensure that the right treatment is given to the right patient.

The issue of drug resistance within patients and the inability to stratify patients into treatment arms, which in turn results in unwarranted side effects and a delay in the treatment process, remains a serious and detrimental downfall of the current chemotherapeutic landscape. As a result, determining a predictive marker within patients would aim to bypass the side effects and ensure patients receive effective treatment. The ultimate goal of our work is to support the introduction of personalized medicine into breast cancer patient’s treatment plan. The benefits of introducing personalized medicine into the treatment space has been highlighted within literature and current clinical studies being carried out such as the TAILORx and MINDACT
projects. Interestingly, a 21-gene panel\textsuperscript{168}, utilized as an assay test for ER\textsuperscript{+ve} and lymph node –ve breast cancer was reported as determining the likelihood of patients experiencing tumour recurrence, in addition to determining the benefit, if any, of being administered chemotherapy. Utilizing personalized medicine and gene expression-profiling tests, such as the example mentioned, have been predicted to save costs in comparison to the current health care costs associated with administering chemotherapy to patients\textsuperscript{137,167,169}. Through our work we have shown evidence of patients, with high BubR1 expression, benefiting from anthracycline regimens, furthermore our work has laid the foundation for analysis into the potential of BubR1 as a predictive marker of anthracycline benefit. From this, we have established epirubicin resistant breast cancer cell lines in order to study the mechanism behind epirubicin resistance; this work was prompted by our group’s earlier identification of Ch17CEP as both a consistent and significant predictor of anthracycline benefit in a number of clinical trials. By utilizing an \textit{in vitro} cell line model, we have been able to identify SAC dysregulation within the EpiR cell lines and in turn identify SNCG as a potential target for reversing the resistance within our cell lines.

Future directions include identifying SNCG expression within the BR9601 clinical trial through IHC; we believe that similar to the qPCR work carried out, SNCG should be upregulated in patients with low BubR1 expression and those that did not benefit from the Epi-CMF treatment. Following this step, we are interested in targeting SNCG through a knockdown experiment within the EpiR cell lines and observing the resistance levels, in this case we believe that IC50 values will decrease. Furthermore, developing knockout cell lines of BubR1 utilizing techniques such as CRISPR/Cas9 would allow us to study the impact of BubR1 silencing on cellular viability, impact on other SAC member proteins and further confirming the increase in resistance through a complete and stable BubR1 knockout. It is difficult to continue optimizing chemotherapeutic regimens that would maximize benefit when we have not identified an effective method of stratifying patients who would benefit from those that would not. The identification and validation of predictive markers of treatment benefit represent the future of personalized medicine, which aims to improve therapy design, reduce current side effects and costs of chemotherapy administration (i.e. resistance, toxicities). Through this work we aim to identify the mechanisms behind anthracycline resistance at the molecular level which will further lay the foundation and groundwork for studies aimed at developing biomarkers that would detect adjuvant treatment resistance and benefit in breast cancer patients.


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Cancer Research UK. “When Chemotherapy is used?” *Cancer Research UK* 2015


Cummings J, Smyth JF. “DNA Topoisomerase I and II as targets for rational design of new anticancer drugs.” *Ann Oncol* 7- 4-1993: 533-545.

Cardoso F, Durbecq V, Larsimont D, Paesmans M, Leroy JY, Rouas G, Sotiriou C, Renard N, Richard V, Piccart MJ, Di Leo A. “Correlation between complete response to anthracycline-


Logarinho E and Bousbaa H. “Kinetochore-microtubule interactions “in check” by Bub1, Bub3 and BubR1: The dual task of attaching and signalling.” Cell Cycle 7-12-2008: 1763-1768.


Dano K. “Active outward transport of daunomycin in resistant Ehrlich ascites tumor cells.” *Biochim Biophys Acta* 323-3-1973:466-83.


