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Preclinical Models Of Metastatic Disease

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PRECLINICAL MODELS OF METASTATIC DISEASE

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MECHANISMS OF DRUG RESISTANCE IN METASTATIC MODELS

by

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ABSTRACT

Breast cancer is a disease that continues to take the life of approximately 40,000 men and women in the United States each year [1]. Tumor drug resistance represents a major problem for the treatment of many breast cancer patients. The mechanisms underlying such resistance remain to be elucidated, and one approach to study this issue involves the development of tumor drug-resistant breast cancer models in mice. HER-2 positive breast cancer is considered to be one of the most aggressive subtypes of breast cancer and has become a major target for treatment. HER-2 status is a routinely checked marker for breast cancer due to as many as 1/5 of patients overexpressing this gene, thus making it an ideal model to approach drug resistance in tumors. In this project, two human breast cancer models, derived from the HER-2 positive BT747 and MDA-MB-361 cell lines, will be characterized. Parental and drug sensitive populations were studied, which included the evaluation of the levels of protein expression of components of the HER-2 signaling pathway. A second approach involved intracardiac injection of tumor cells in mice to generate widespread metastases, and the subsequent evaluation of relative drug resistance to chemotherapy in metastases to different organs. Lastly, intracranial injection of cancer cells employed to address the increasing incidence of brain metastasis in breast cancer patients.

My dissertation aims to uncover mechanisms by which cancer cells develop drug resistance, developing models of late-stage breast cancer and the combination of these models with metronomic chemotherapies.

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1: INTRODUCTION

Cancer will claim the life of approximately 12 million lives worldwide in the year 2020[1]. Cancer is uncontrolled and malignant growth of cells, which results from a collection in mutations in specific genes. These changes include the upregulation of oncogenes and downregulation (or inactivation) of tumor suppressor genes [2]. There are a number of characteristics that can differentiate a benign tumor from a malignant one, and chief among these is the ability of the latter to metastasize (i.e., spread to other parts of the body). As cancers grow, they can accumulate additional mutations that can help them grow even in hypoxic environments, in those lacking specific nutrients [3], or even in the presence of administered anti-cancer drugs. Some cancers, when diagnosed, appear to contain subpopulations of cells intrinsically resistant to anti-cancer drugs, while in other cases tumors can develop such resistance *de novo* during treatments (so-called acquired resistance). In addition, cancer cells can gain the ability to bypass many apoptotic signals through a diverse repertoire of pathways, so they continue to grow and proliferate. Current therapies for cancer include surgery, radiation, chemotherapy, with the most problematic cancers being those resistant to chemotherapy and radiation after surgery is no longer feasible. Additional therapies include targeted therapies that stop cancer cell progression through targeting receptors responsible for growth and survival, or by activating the immune system (via targeting molecules such as CTLA-4 or PD-1). Although these approaches have been useful in increasing the survival of breast cancer patients (e.g., metastatic HER-2 positive breast cancers show significant responses to anti-HER-2 drugs in combination with chemotherapy), the death rate for this disease remains high.

To improve our understanding of these issues, I have studied models that reflect different aspects of tumor progression and drug resistance and I have coupled this analysis with the administration of metronomic chemotherapy.

1.1 Drug Resistance in Cancer

Treating disseminated cancers has met with limited success, in part because tumors can develop resistance to conventional chemotherapy. For example, ovarian cancers and small cell lung carcinomas respond well to chemotherapy and regress, but may subsequently develop acquired drug resistance [4]. Other types of cancers such as melanomas and prostate cancers are difficult to treat as they are reportedly intrinsically resistant to chemotherapy [4]. Current hypothesis suggest that cancers can evade therapy through different mechanisms, some of which are tumor cells specific and other cause changes in the microenvironment [5], such as the via the overexpression of Vascular Endothelial Growth Factor (VEGF).

Drug resistance can also arise through dysregulated apoptosis providing an imbalanced environment leading to irreversible changes in a cell that can render chemotherapeutics less effective [4]. The expression of some cytokines has also been implicated in aiding tumor cells to evade chemotherapy [6]. However, there is uncertainty over the different mechanisms by which cancer become drug resistant to current therapies, and that is in part what this dissertation seeks to address.

1.2 Metastasis

Metastasis occurs when a tumor seeds and begins to grow at a distant site. Most cancer fatalities are a consequence of metastasis and lack of response to chemotherapy. Patients are not infrequently diagnosed with cancer at an advanced stage, with tumors that have already metastasized, making it extremely difficult to eradicate the disease. One complicating factor can be the heterogeneity

of tumors, which means they may contain subpopulations intrinsically resistant to currently available therapies (even before any treatment is administered). During progression to late stage cancer, tumor cells can begin to develop characteristics that allow them to lose cell polarity, cell to cell adhesion, which can result in the tumor becoming invasive, thus increasing the likelihood for metastasis to occur [7]. Tumor progression can also be promoted by (non-malignant) cell populations residing in the tumor microenvironment, one example of this is the tumor associated macrophages (TAMs) that can secrete proteins that facilitate the local invasion of malignant cells [8]. This includes TAMS destruction of the basement membrane and invading the stroma and that can help through the secretion of proteases [9]. Next, tumor cells must infiltrate the blood circulation, intravasate, and survive long enough to exit the blood stream as to invade secondary sites. Assuming these circulating tumor cells survive (presently, available data suggest most cells do not survive in the circulation), they can invade a distant organ, arrest, and give rise to a secondary tumor (i.e., a metastasis).

1.3 Specific Aims

Specific Aim 1: Characterization of cell line models of HER-2 positive breast cancer.

Two HER-2 positive breast cancer cell lines were used to develop new *in vivo* tumor models to study the disease. BT474 and MDA-MD-361 cell lines have been selected for tumorigenicity, and/or acquired drug resistance, after implantation in mice. The 'parental' cell lines were compared against the selected 'variant' subpopulations to determine if there are significant differences in protein and gene expression levels. These models will be used to further evaluate responses to anti-HER-2 therapies (which are routinely used clinically for the treatment of these types of cancer), and the development of resistance to such therapies.

Specific Aim 2: Widespread Metastasis

Intracardiac injection of breast cancer cells was used to create a widespread metastasis model *in vivo*. Once models were established, these were studied to determine if the spread of cancer to different organs produces tumor deposits with different levels of drug resistance to given treatments. A second approach to study metastasis in late-stage cancers was to analyze the effect of metronomic chemotherapy in clinical patients. Together, these aim to test the hypothesis that drug resistance is organ dependent.

Specific Aim 3: Modeling Brain Metastasis

In order to create a breast metastasis models in the brain, intracranial injections were used to directly implant breast cancer cells *in vivo*. Tumor progression was monitored using the In Vivo Imaging System (IVIS). As tumors increased in size, they were randomized into experimental and control groups, which were then be used to test currently available chemotherapies using a metronomic approach.

2: BREAST CANCER

Breast cancer is a disease that affects over 3 million women worldwide and it is expected to take the life of over 40,000 women and men in the United States [1]. There are different subtypes of breast cancer and identification of specific subtypes for a given patient can help oncologists chose the most effective chemotherapy-based regimen to treat metastatic disease. For example, one routinely checked marker in breast cancers is HER-2. If a patient is diagnosed with HER-2 positive breast cancer (i.e., because of abnormally high expression of HER-2 in the tumor, the presence of HER-2) that defines the patient has having one of the most aggressive subtypes of breast cancer (i.e., HER-2 positive)– that is associated with a poor prognosis [9].

2.1 HER-2 positive Breast Cancer

HER-2 positive breast cancer is characterized by the overexpression of the Human Epidermal Growth Factor Receptor-2 (HER-2). Figure 1 shows cancer cells taken from a FVB/N-T (MMTVneu) 202Mul mouse (HER-2 positive) and 4T1 cells (HER-2 negative murine breast cancer cell line). HER-2 is endogenously expressed in different tissues such as the breast and ovaries. Is overexpressed in about 25% of breast cancer patients [10]. HER-2 as a surface tyrosine kinase receptor, which plays an important role in signaling for growth, proliferation, and survival in normal cells (Figure 2). Overexpression of HER-2 (or ErBb2), leads to enhanced cell proliferation and survival. Overexpression of HER-2 has been shown to promote tumor progression in mice, and in humans HER-2 overexpression in breast tumors is associated (as noted above) with a poor prognosis [10]. If a patient is diagnosed with HER-2 positive breast cancer, they are eligible for treatment with anti-HER-2 drugs

such as trastuzumab, trastuzumab emtansine, or lapatinib, which typically are given in combination with chemotherapy for those patients with metastatic disease. For example, Herceptin is used in combination with paclitaxel for the first line treatment of metastatic HER-2 positive breast cancer [11]. Although a number of patients respond to such treatment, drug resistance eventually develops in most cases.

Figure 1. HER-2 receptor comparison in murine cell lines. Protein analysis of FVB/N-T (MMTVneu) 202Mul/ and 4T1 murine cell lysates. FVB/N-T (MMTVneu) 202Mul/ shows expression of HER-2 protein and 4T1 is used as a negative control. Both lanes show tubulin as a loading control.

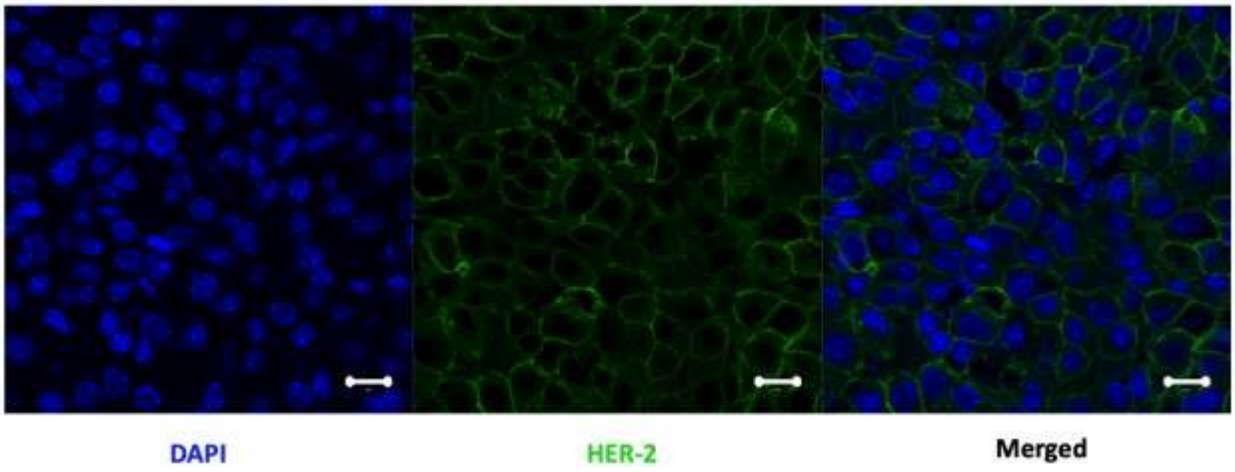


Figure 2. HER-2 transmembrane receptor visualized using florescence. Tumor tissue taken from FVB/N-T (MMTVneu) 202Mul/mice. These mice express HER-2 throughout the cells in their body.

2.2 HER-2 Receptor

The HER-2 protein is encoded by the ErBb2 gene. It is a tyrosine kinase receptor composed of an extracellular domain that can form dimers in the presence of any of its family of receptors. The intracellular domain of the protein can become phosphorylated following dimerization, and this initiates a signal transduction pathway (that stimulates cell division). The extracellular domain is made up of four different subdomains (Figure 3). Subdomain I and III can form binding sites for potential ligands and subdomains II and IV are cysteine-rich domains involved in dimerization [12]. The protein also has a dimerization arm, which is a short hairpin loop in subdomain II that contacts the partnering receptor.

2.3 HER-2 Targeted Therapy

Patients with HER-2 positive breast cancer are eligible for HER-2 targeted therapies, such as the anti-HER-2 antibody, trastuzumab, or the small molecule tyrosine kinase inhibitor lapatinib, among others. For example, trastuzumab is a monoclonal antibody that binds to the extracellular subdomain IV of HER-2 and keeps the receptor from dimerizing. This leads to the inhibition of the signaling cascade of both the Mitogen-Activated Protein Kinase (MAPK) and Phosphoinositide 3-kinase (PI3K/Akt) pathways which drive cell growth, proliferation, and cell cycle arrest [12]. Pertuzumab is another HER-2 targeted antibody that binds to its specific domain II, which prevents ligand-activated heterodimerization, inhibiting HER-2 driven proliferation (Capelan, 2012). Dimerization of HER-2 and HER-3 is the most mitogenic, potent, and most commonly found in cancer cells [10]. When combined with chemotherapy (e.g. paclitaxel), trastuzumab can improve the overall survival of patients with metastatic HER-2 positive breast cancer.

2.4 Resistance to HER-2 Targeted Therapy

Resistance to HER-2 targeted therapy is common among women with overexpression of this receptor and as many as 40-60% of HER-2 positive breast cancer patients do not respond [13].Some of the most commonly used HER-2 targeted therapies available are Trastuzumab (Trade name Herceptin), Lapatinib (Trade name Tykerb), Pertuzumab (Trade name Perjeta), and Trastuzumab emtansine dm-1 (Trade name Kadcyla). Herceptin is a monoclonal antibody that binds to domain IV of the HER-2 receptor, thus inhibiting dimerization and signaling cascade. Herceptin has been approved since 2008, for the treatment of HER-2 positive breast cancer. However, drug resistance remains a problem for many patients treated with Herceptin. Lapatinib is a small molecule tyrosine kinase inhibitor that targets the ATP-binding pocket in both EGFR and HER-2 and has been approved in the clinic for advanced breast cancer in combination with capecitabine after trastuzumab and chemotherapy regimens have failed [14]. p95HER-2 is a truncated form of HER-2 and the resistance to Herceptin is often linked to the loss of function of phosphatase and tensin homolog (PTEN), responsible for regulating the PI3K pathway. Mutations in PTEN or inactivation of PI3K pathway result in active HER-2 signaling, thus preventing inhibition of cell growth by trastuzumab [15]. The Nagata group suggested that tumors with loss of PTEN, have a lower response to trastuzumab than patients with PTEN-expressing tumors [16].Another group treated HER-2 positive breast cancer patients with trastuzumab and came to the conclusion that PTEN, PI3K, and HER3 play critical roles in its resistance mechanism [15]. Changes to HER-2 have also been implicated in drug resistance to HER-2 targeted therapy. While some cancers lose HER-2 amplification, others begin to accumulate the truncated version of HER- 2, p95HER2. p95HER2 is constitutively active, will respond to lapatinib, but not trastuzumab [16]. Trastuzumab is not effective in the interruption of the potent HER-2 and HER-3 heterodimers,

which contribute to the production of its ligands and together with the upregulation of HER-3, making trastuzumab futile [17], [18]. Resistance to lapatinib involves the mutation of the HER-2 tyrosine kinase domain [14].Trowe and his team found 17 mutations in the HER-2 tyrosinekinase domain that contribute to drug resistance to Lapatinib. The two mutations found typically associated with lapatinib resistance in HER-2 were L755S and T798I and T790M in EGFR [14].

2.5 Characterization of new HER-2 positive breast cancer cell lines

BT474 is a human breast cancer cell line, which was derived from a breast cancer brain metastasis that occurred in Caucasian female with a ductal carcinoma of the breast. The MDA-MD-361 cell line was derived from metastatic adenocarcinoma in a 40-year-old female. These breast cancer cell lines have been used for many years to study the aspects of HER-2 positive breast cancer [17]–[20]. Expression of various proteins, either members of the HER family of proteins or of downstream signal transduction mediator proteins, is being carried out as part of a characterization study of BT474 and MDA-MB-361, and of variants of these lines that were derived by the PI (Francia) – as detailed below. Such studies may provide additional data on potential biomarkers for drug resistance. For example, literature suggests that when HER-2 targeted therapy is administered, there is an increased expression of HER-3, another receptor belonging to HER family [21]. We have tested to determine if such an observations can be confirmed in our breast cancer models (Fig. 3).

MDA-MD-361 and BT474 cell lines were grown in RPMI at 37°C supplemented with 10%FBS and 2mM L-glutamine and in a humidified atmosphere with 5% CO_2.

For in vivo growth, cells were collected using 1% trypsin-EDTA, washed in PBS (Phosphate Buffered Saline), and $2x10^6$ cells were implanted orthotopically into the mammary fat pad of SCID mice. Tumors grew very slowly, and variants were eventually isolated and

passaged several times in vivo to generate variants with enhanced tumorigenicity up to three years (Fig. 3). In parallel, some tumors were allowed to grow to around 250mm^3, at which point the mice were treated with the antiHER-2 antibody trastuzumab (20mg/kg twice weekly) [20]. When primary tumors became resistant to the monoclonal antibody therapy and reached 500mm^3 in size, these were resected surgically (Figure 3), and drug resistant variants were grown in tissue culture.

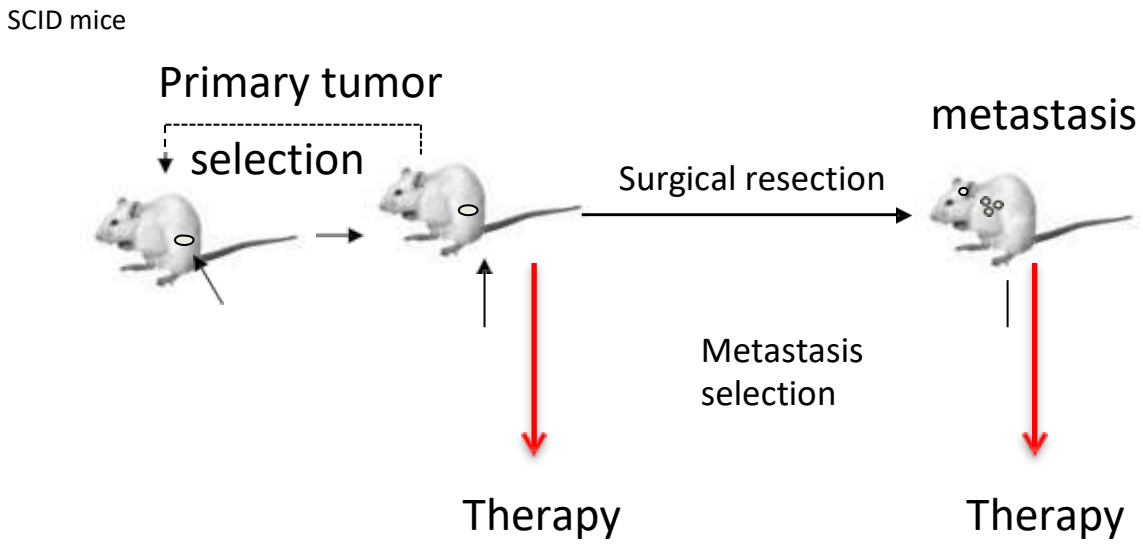


Figure 3. Tumor Selection in vivo. SCID mice implanted serially with HER-2 positive human tumors over a period of 3 years to select for drug resistant variants.

2.6 RNA Sequencing

RNA sequencing permits whole transcriptome analysis and can reveal differences in variant cell populations versus their parental counterparts. Through this process, we were able to begin to draw a map (in transcription) that reflect in part the process of adaptation through passaging of these cell lines. This analysis does not incorporate modifications, mutations in single-nucleotide polymorphism, and does not always reflect overall changes in gene expression at the protein level.

2.7 Preparation of RNA

RNA extraction was carried out using Trizol on fresh cell pellets grown in tissue culture. Once cells were homogenized with Trizol, equal amounts of chloroform and phenol were added, homogenized again until clumps were no longer visible. Mixture was then vortexed and left incubating on ice for 5 minutes. The mixture was then centrifuged for 15 min at 4 degrees to retrieve the supernatant (RNA). RNA was transferred to a new microtube with 500ml of isopropanol and stored at -20 degree. In order to assess RNA integrity, samples were run on a 1% agarose gel. The RNA-seq analysis was carried out by Otogenetics in Atlanta, GA.

2.8 Results

Examples of protein expression levels of MDA-MB-361 and BT474 (parental) cell lines, and variants are shown in Figure 4. To determine the the frequency of these changes in protein with the development of drug resistance. Extensive western blot analyses were also performed in proteins important in MAPK and PI3K/AKT pathways, such as AKT, using these cell lines. Figure 4B shows a decrease in protein expression in the variant cell line 361p3 in both Rag C and E- cadherin. E-cadherin is an important protein involved in cell to cell adhesion and loss of E-cadherin can suggest are more aggressive phenotype by encouraging detachment from the primary tumor.

The first step in the transition from epithelial to mesenchymal transition (EMT) is considered the loss of E-cadherin [22]. RagC is an important protein responsible for regulating the mTOR pathway responsible for growth, proliferation, and survival in a cell. Preliminary data shows that in the BT474 and BTLN2, EGFR a member of the HER family, is downregulated in the variant cell line. Cytochrome C expression is downregulated in the variant BTLN2 as well, which has been found to be downregulated in hypoxic tumors and known as a characteristic of tumor progression [23].

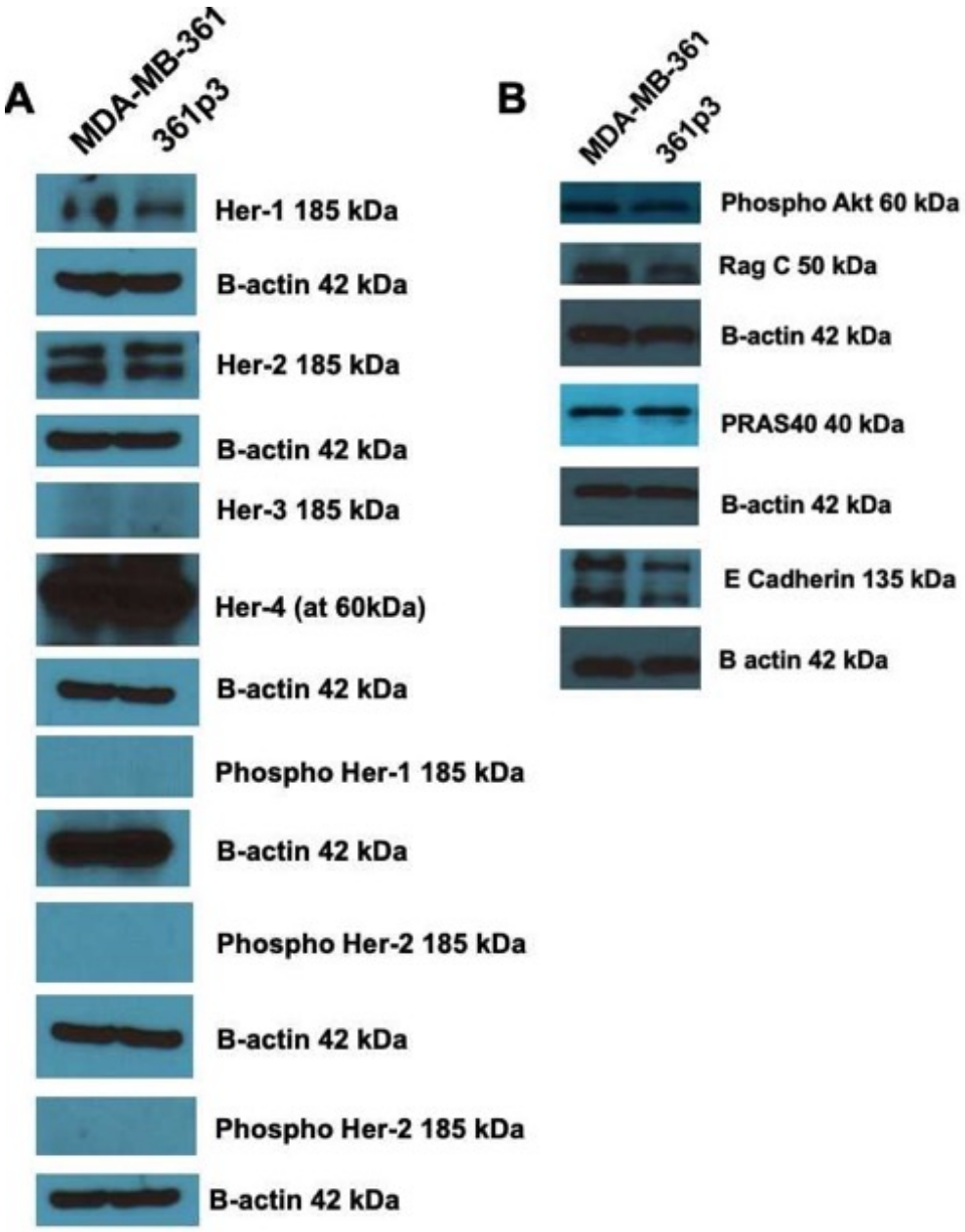


Figure 4. Protein expression in MDA-MB-361 models. A) Shows HER-2 protein analysis in MDA-MB-361 parental, and the tumorigenic variant 361p3. There seem to be no apparent change in the HER-2 receptors. B) Shows analysis of mTOR pathway, B-actin is used as a control. In the variant there is a downregulation of Phospho Akt and E-cadherin.

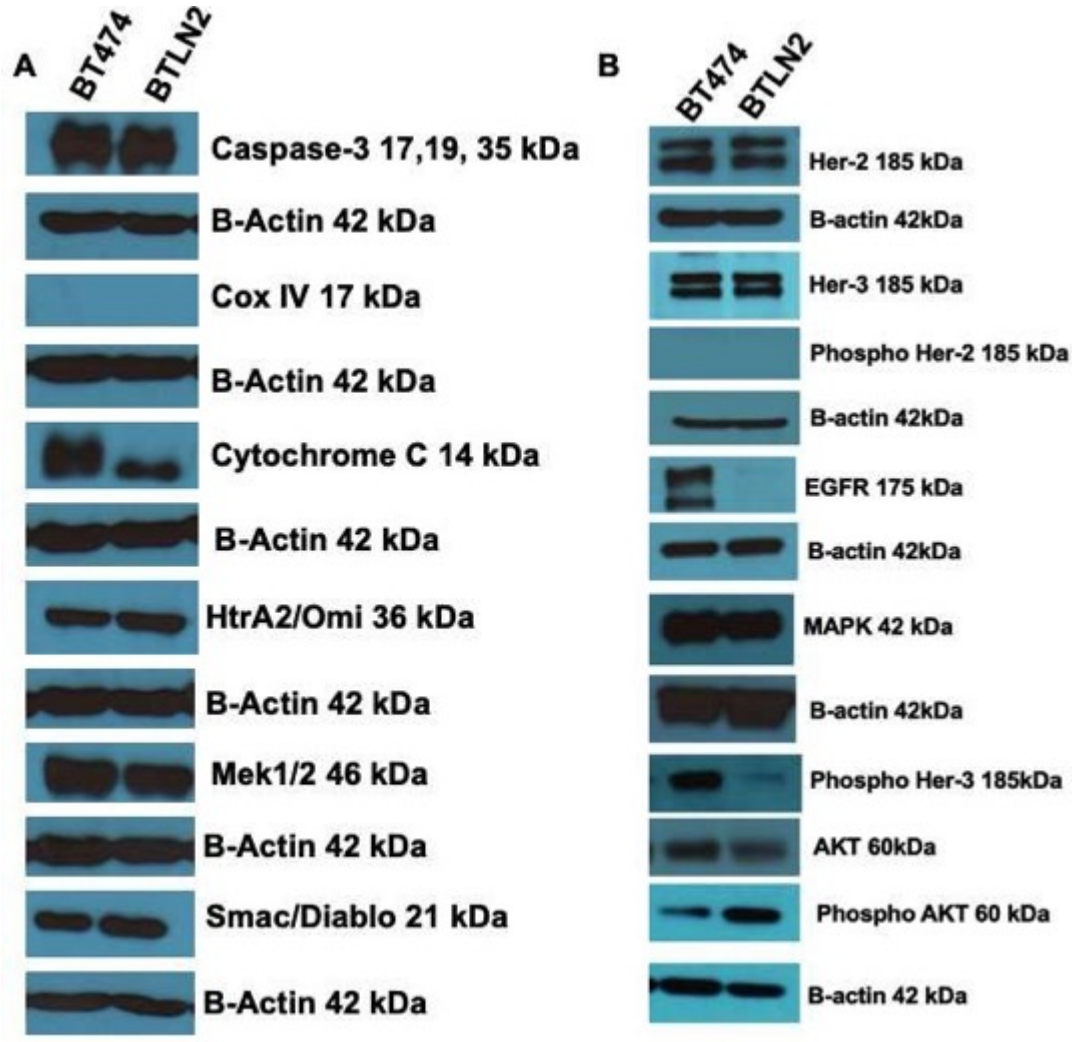


Figure 5. Protein expression of BT474 parental and BTLN2 variant cell lines. A. Expression of different proteins important for apoptosis. Changes are evident in Cytochrome C expression. B. Changes is protein expression of HER-2 family receptors are seen in BTLN2 variant such as EGFR and phospho HER-3. Changes in MAPK signaling proteins such as AKT and its phosphorylated form were also found.

2.9 Discussion

Breast cancer is the most common type of cancer diagnosed in women worldwide. It continues to be a major challenge although many breakthroughs in cancer research have been made. There is still a need to overcome and understand why drug resistance emerges. Identifying protein and gene expression differences in tumors before and after treatment is a way for us to unveil which of these is responsible for driving drug resistance in breast cancers. Our protein analysis supports our hypothesis that HER-2 positive breast cancer cells have different expression levels that allow these to become resistant to chemotherapy and give them the ability to invade other organs. One common example is the use of other survival compensatory programs such as Phosphatydil-inositol-3-kinase (PI3K) that enable the abnormal cell growth to continue [26]. Here, we present that there are changes in the PI3K pathway in the MDA-MB-361 cell line and its variant counterparts. Akt, an important component of the PI3K pathway, is responsible different processes in the cell such as regulating cell growth, motility, survival, and metabolism (Figure 5) [27]. Elevated levels of Akt is a similarity of many cancers. At the same time, the expression of Rag C, a mediator of the MAPK and mTOR pathways, is also downregulated. Once cells have acquired drug resistance, they can often express or downregulate proteins that helps them continue to grow, survive, and proliferate. Data suggests that there are changes in both parental and variant cell lines that may be responsible for the aggressive phonotype acquired by the administration of chemotherapy. Such rationale can be attributed to the change in expression of proteins such as Cytochrome C, RagC, Akt and EGFR all important for cell growth proliferation, division, and survival (Figures 4 & 5).

3: MODELS OF METASTASIS AND BIOMARKER STUDIES

Dissemination of tumor cells into other organs in the body is mediated by diverse cells surrounding the tumors. Cancer cells will begin to acquire changes that will enable them to leave the primary tumor and begin colonization in a distant organ. One of the most important processes regarding metastasis is a process termed, Epithelial-Mesenchymal Transition (EMT) [26]. EMT is a process where epithelial cells gain properties that are important for invasion, motility, and dissemination. This program is first seen during embryogenesis and in wound healing, but cancer cells learn to use this process to switch to a more motile and stem-like phenotype [28]. Transcription factors associated with EMT are often Slug, SNAIL, Twist, zinc-finger-E-box-binding (ZEB-1), and pair-related homeobox transcription factor 1 (Prrx-1) [28]–[30]. These transcription factors bind to E-boxes that repress the expression of E-cadherin, a protein that is commonly found in junctions of epithelial cells, thus assisting this transition from epithelial to mesenchymal phenotypes [28]. E-cadherin provide epithelial cells polarity and integrityin different tissues; therefore, its expression is crucial for the maintenance of cellular networks [31]. In cancer, E-cadherin expression is often replaced by N-Cadherin, which is typically seen in the process of embryogenesis and wound healing where cells must migrate to other tissues. Many cancers switch cadherin expression making cells lose polarity, gain invasiveness, and encourage cell survival after loss of normal cell and substrate interactions [31]. Many signaling pathways are involved in the progression of metastasis and induction of EMT such as TNF-b, which is commonly known as the ‘master inhibitor of cell cycle progression [32]. This cytokine is also necessary for the many different aspects of ECM degradation, remodeling, and its production, which are necessary for tumor cells to successfully detach from the primary tumor [32]. TNF-b has the ability to induce the expression Matrix Metalloproteinases (MMPs), which degrade both ECM (extracellular matrix) and basement

membrane (BM), essential for invasion to other organs. TNF-b regulates plasminogens that control degradation of proteins found in blood vessels that allow for intravasation into the bloodstream. Once BCCs find their way into the bloodstream, they continue to interact with other cells that will make their invasion feasible. BCCs will enter the bloodstream through a similar process; this is by remodeling the endothelial cells that line blood vessels, then circulating the bloodstream before exiting to begin colonization elsewhere. During the course of this process, cancer cells may cluster together to avoid elimination by the immune system such as natural killer cells that supervise the blood system for antigens. Cancer cells may become wedged in small capillaries and forcefully begin to colonize. Cancer cells can be attracted by the different cytokine and chemokine factors to homing tissues [33]. Different chemokines and cytokines will trigger circulating cancer cells and endothelial cells to express surface proteins and receptors important for rolling and docking of tumor cells into endothelial cells for intravasation [33]. E-selectin is a surface protein only found in cytokine-activated endothelial cells and is responsible for interacting with leukocytes allowing them entry into different tissues where inflammation and injury is taking place. For example, P-selectin glycoprotein ligand-1 expressed on BCCs will bind to E-selectin and permit rolling through vascularity to gain entrance to invade other tissues. Once the cancer cells dock, these will with loose Once in the bloodstream, these single cells or clusters of cells will encounter several cells of the immune system and at the same time BCCs will express different receptors on their surface that allow them to ‘home’ to different organs in the body.

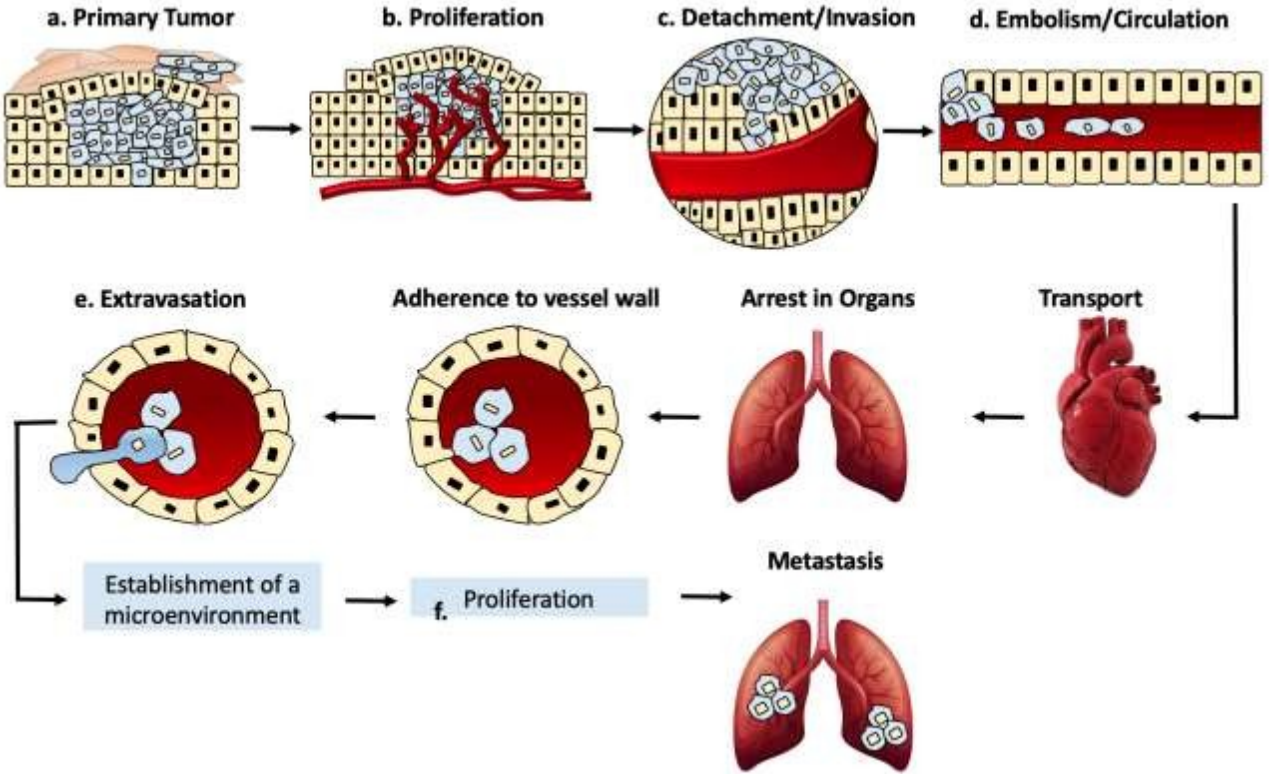


Figure 6. Formation of Metastasis. Diagram shows the many steps it takes for metastasis to occur. A. Primary tumor continues to grow. B. In order for tumor to grow past 2mm in size, a bed of capillaries must be established. C. Cancer cells begin to acquire invasive phenotypes that allow them to detach from primary tumor. Surrounding stroma can be reorganized and degraded to allow for intravasation to the blood vessels. D. Cells enter the blood vessels and use circulation to reach other organs. If these cancer cells survive circulation they may get trapped in capillaries and adhere to the blood vessel walls to begin extravasation. E. Cells will now exit the blood vessel and enter new tissues and organs. F. Cells will establish in new organs, proliferate, and give rise to a new secondary tumor or metastasis.

3.1 Widespread metastasis

According to the 'seed and soil' hypothesis, circulating tumor cells do not randomly metastasize to distant sites but rather preferentially grow in specific organs (i.e. the correct "soil") [34]. For example, patients suffering from breast cancer will most likely develop metastasis to the lung, bone, liver, and brain[33], but not to other organs such as the skin [35]. Developing a model that can reproduce this pattern *in vivo* helped unveil the underlying mechanisms, and allowed us to test if a metastasis in a specific organ (e.g., the lung) can be more resistant to anticancer therapies than metastasis to other sites. One way to establish widespread metastasis *in vivo* is to implant cancer cells directly into heart (i.e., into the mouse left ventricle) and follow the progression of the disease. Intracardiac injections have the advantage of allowing cancer cells to enter the blood stream, without directing them to a specific organ for metastatic growth. This is in contrast to, for example, to intravenous (tail vein) injection which invariably only produces lung metastases. In order to further understand cancer spread and drug resistance in different organs, plasma samples from gastrointestinal cancer patients enrolled in a clinical phase II trial were analyzed to determine how their cytokine profile varied in the course of treatment. Our original aim was to obtain samples of breast cancer patients (in line with our study on the above aims), but a perchance offer of samples from a gastrointestinal clinical trial, in line with our IRB, made it possible for us to address this question earlier than we had anticipated – albeit in a different cancer type.

Overall, the above approaches will allow us to address drug resistance in new preclinical models and in clinical samples.

3.2 Cytokines in Cancer

Cytokines are important signaling molecules that can stimulate and suppress the immune system. They are involved in a wide range of effects such as recruitment of a various cell types of the immune system and the downstream regulation of diverse cell signaling pathways. Macrophages, B lymphocytes, T lymphocytes, endothelial cells, and fibroblasts are some examples of cells known to release cytokines that can trigger an immune response [36]. They are important in a number of processes, signaling during infection, wound, cancer, and reproduction[36]. We evaluated the cytokine profile of cancer patients in a phase II clinical trial to see if we could correlate cytokine levels with progression free survival (PFS). These types of studies may allow for the future tailoring of specific therapies to those subgroups of patients most likely to show a response.

3.3 Methods Preclinical metastatic model

Balb/c, wild type (WT), and B6-JA18KO (KO) mice were used to generate preliminary data. The KO model lacks invariant natural killer T (iNKT) cells, allowing us to test whether such cells can alter the metastatic process. iNKT cells are important in the regulation of immune response via cytokine production [36]. First, we optimized the procedure in term of the number of cells injected per mouse. We empirically determined that the optimal number is $2x10^5$ in a 100ul volume for B16F1 melanoma cells, and $7x10^3$ EMT-6 breast cancer cells (in the same volume). In preliminary experiments the B16-F1 melanoma was chosen to better visualize the metastatic sites as the pigmented cells are black compared to the rest of the organs, Figure 5 shows an example of melanoma to different organ sites.

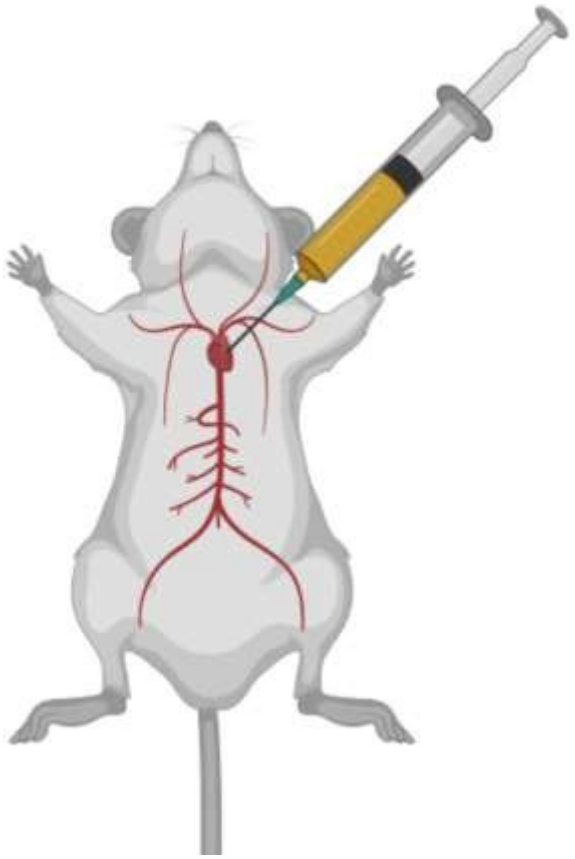


Figure 12. Modeling Metastasis though intracardiac implantation. Cancer cells such as EMT-6 and B.16 melanoma were injected in the left ventricle of Balb/c and immunodeficient mice to develop models of widespread disease.

3.4 EMT-6 breast cancer model

HER-2 positive, murine breast cancer cell line, EMT-6, was then injected (7 x 10^3 following the same protocol) into the heart of mice to test the efficacy of metronomic chemotherapy on late stage breast cancer. Balb/c mice (40) were implanted intracardiacally with 7 x10^3 EMT-6 cells, and 4 treatment groups were then set up to receive control, anti-CTLA-4 immunotherapy, gemcitabine chemotherapy or CTLA-4 plus chemotherapy. Metronomic Gemcitabine was administered 160mg/kg i.p. every three days and anti-CTLA-4 was administered 100ug i.p. on day one followed by 35ug i.p. on day 6, either alone or in combination.

3.5 Relevance of cytokines in cancer patients of a phase II clinical trial.

Allegrini, et al conducted a phase II clinical trial for patients with refractory gastrointestinal tumors [37]. 38 patients enrolled to receive UFT (100mg/day twice a day), Cyclophosphamide (50mg/day), and Celecoxib (200mg/kg twice daily). Celecoxib was used as an angiogenesis inhibitor. UFT is a prodrug that interferes with thymine synthesis, and Cyclophosphamide (CTX) is a DNA alkylating agent. Metronomic CTX has been reported to inhibit angiogenesis by targeting endothelial cells and upregulating thrombospondin-1 (TSP-1), a protein inhibits blood vessel formation[38]. Published studies suggest that metronomic cyclophosphamide can activate the immune system [39]. The purpose of the clinical trial was to evaluate the efficacy of a metronomic therapy in patients with late stage gastrointestinal cancers no longer responding to standard chemotherapy.

As noted above, metronomic chemotherapy is thought to act, at least for CTX, by activating the immune system. This led us to ask whether a complex therapy cocktail with CTX (plus the other drugs; UFT and celecoxib) would also lead to immune system activation. To test this, a 14-bead cytokine luminex panel was used, using the luminex technique, and applied to plasma samples

from the Allegrini trial. To evaluate how cytokine levels could correlate with response to chemotherapy in this phase II trial, analysis was carried out on plasma samples taken on Day 0 (i.e., before the start of treatment), Day 28, and Day 56 (Figure 14).

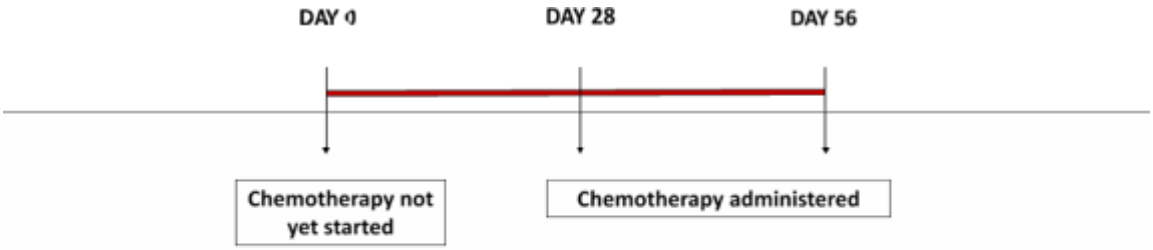


Figure 13. Plasma collection dates. Patients enrolled in clinical phase II trial had blood drawn on days 0, 28, and 56 for cytokine level analysis.

Plasma samples of patients in clinical phase II trial were received from our colleague (Dr. Bocci) at The University of Pisa and stored at -80°C. Samples of 38 patients were analyzed using Human Cytokine/Chemokine Magnetic Bead Panel Cat No. HCYTOMAG- 60K (Millipore) with 14 different analytes. The samples and beads were incubated as per manufacturer's protocol, then plate was read using the Millipore Luminex Analyzer.

3.6 Gemcitabine

Gemcitabine (2',2'-difluoro 2'-deoxycytidine, dFdC) is a widely used cytotoxic antimetabolite that is FDA approved for the use in many solid tumors such as bladder cancer, ovarian cancer, head and neck cancers, and breast cancer [40]. Gemcitabine is a cytidine analog that enters the cells through SLC28 and SLC29 nucleoside transporters, the latter being the most efficient in uptake [41].

Nucleoside kinases are responsible for converting cellular gemcitabine into its two active forms. One of the active forms of gemcitabine, dFd-CTP, incorporates into growing DNA and DNA synthesis is blocked immediately after another nucleoside is inserted. Masked chain termination occurs as DNA proofreading enzymes fail to remove dFd-CTP from the DNA strand because of its resistance to polymerase epsilon [42]. This leads to cell death in rapidly diving cells.

3.7 Anti-CTLA

Anti- CTLA-4 and Gemcitabine were used to combine the efficacy of immunotherapy and metronomic chemotherapy against HER-2 positive breast cancer in a widespread metastasis and brain metastasis models. Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) is a receptor found in the surface of T-cells that plays an important role in the inhibition of the immune system. There are two signals that must occur for a T-cell to become activated; the presentation of an antigen by antigen presenting cells (APCs) and binding of CD-28 on a T-cell and the B7 receptor of an APC. On the other hand, CTLA-4 will bind to B7 and have a negative effect on T-cell activation. By blocking this negative feedback of T-cell activation, anti-CTLA-4 antibody will allow for the continual activation of T-cells, encouraging the immune system to attack cancer cells [43].

3.8 Results

The use of this intracardiac model produced preliminary data suggesting some preferred sites for metastasis of B16-F1 melanoma cells. Thus, Figure 14 shows the distribution of metastases in different organs. The data suggest that there are preferred sites for metastases such as liver, and lung, and that the frequency can differ depending on the genetic background of the host. The probability of liver metastasis is found to be higher in WT models (95% CI) (Figure 15). These results show the feasibility of this approach, which were then used to set up therapeutic experiments of breast cancer metastases in mice. Next, widespread metastases was set up in Balb/c mice through

the implantation of EMT-6 breast cancer cells. Mice were then divided into groups, to be treated with control or with chemotherapy regimens. Analysis at autopsy was then used to determine if metastases at certain organs will promote drug resistance (as seen by an increase in metastases to those organs in mice treated with chemotherapy). In the EMT-6 breast cancer model, we saw that the combination of anti-CTLA-4 and Gemcitabine had the best percent survival when compared to the treatments alone and controls (Figure 16). Gemcitabine alone had the second-best percent survival at 24 days. Tumors cells extracted from each of the groups are now considered drug resistant and can be used to further understand drug resistance.

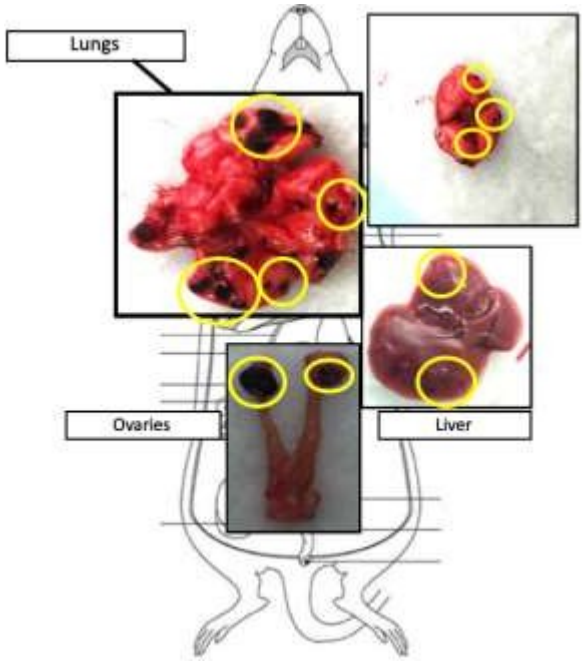
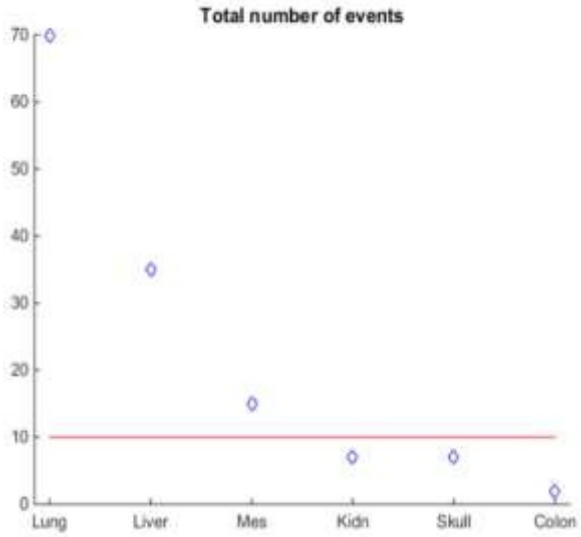


Figure 14. Metastasis of B16-F0 melanoma to different organ. B16-F1 melanoma cells were intracardiacally implanted into mice to establish a protocol and a model of widespread metastasis. Melanoma cells have deposited to the lungs, liver and ovaries of a mouse. The melanin produced by these cells allows the visualization of the metastasis to the naked eye (circled in yellow).



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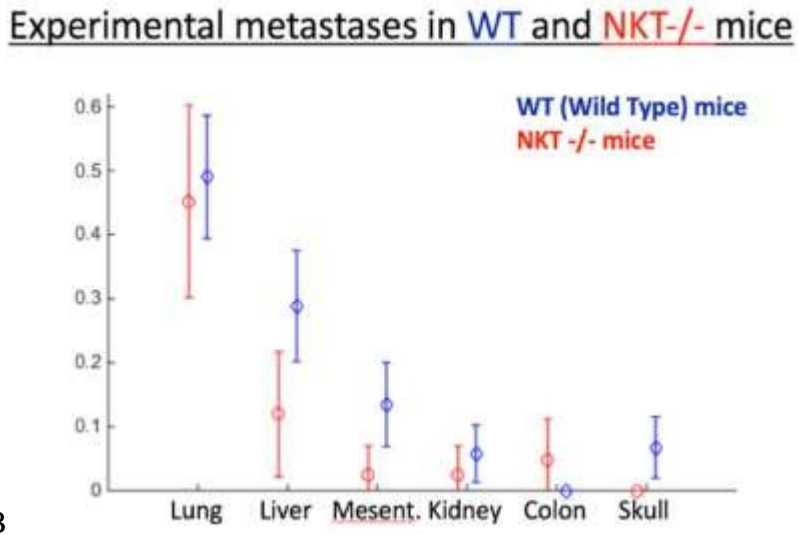

B

Figure 15. Total metastatic sites and correlation found in WT and B6-JA18KO models. A) This graph reflects the number oftimes an organ had metastasis in a mouse totaling both KO and WT models. B) WT mice are represented in blue and B6-JA18KO are represented in red. This is data suggests that there are statistically significant differences between the two models.

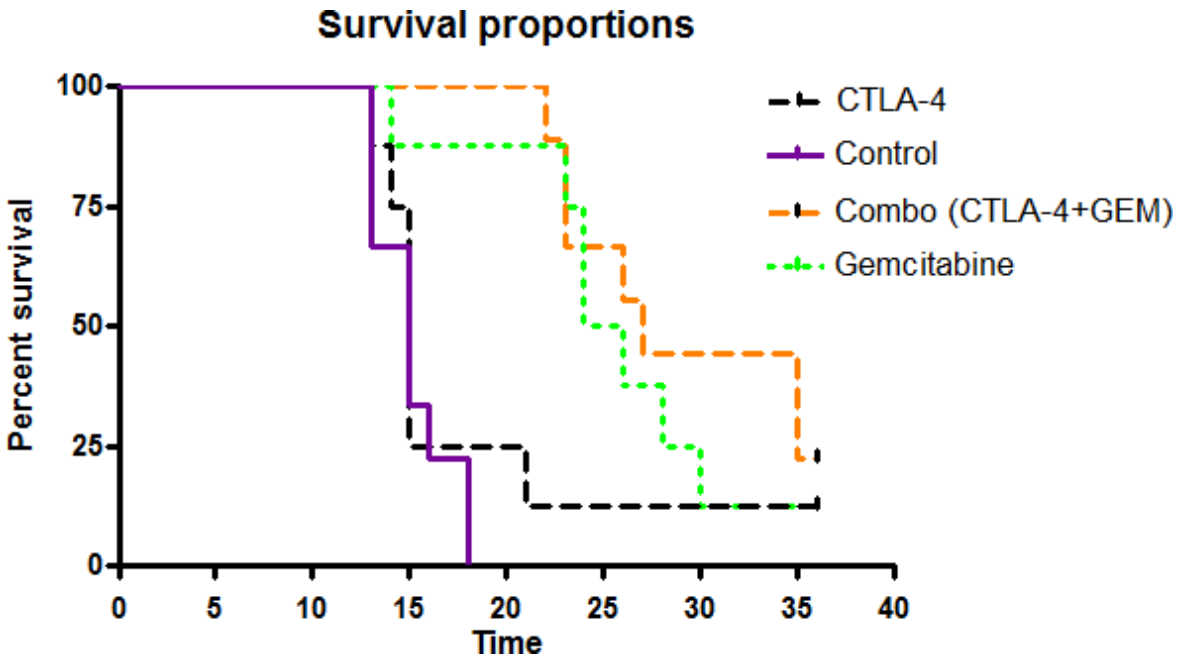

Figure 16. EMT-6 Breast cancer cells implanted in Balb/c mice. 40 mice were implanted breast cancer cell line, EMT-6 intracardiacally and split into 4 different groups, Gemcitabine 120mg/kg, anti-CTLA-4, combination of Gemcitabine and anti-CTLA-4, and control (PBS). Combination of immunotherapy and chemotherapy had a better percent survival than other experimental groups.

In the clinical samples analyzed, IL-6 and IL-8 plasma levels increased from baseline (at Day 0) to Day 28 after treatment began (Figure 17). IL-6 levels in the tumor microenvironment have been implicated in helping cancer cells by promoting survival, invasiveness, and metabolism [44]. On Day 56, there was a further increase IL-6 and IL-8 levels, but not in (for example) IL-12a levels (Figure 18). Patients who have better PFS, that is a longer period under treatment before disease progression was observed, had distinct cytokine profiles compared to patients who did not show much benefit from the metronomic therapy. Thus, at Day 56, patients with higher levels of IFNy and lower levels of sCD40L eventually showed better PFS (Figure 18 A&B) than the rest of the patients in this trial. Therefore, these cytokine levels, before metronomic chemotherapy started, appeared to be predictive markers for good responders to this treatment.

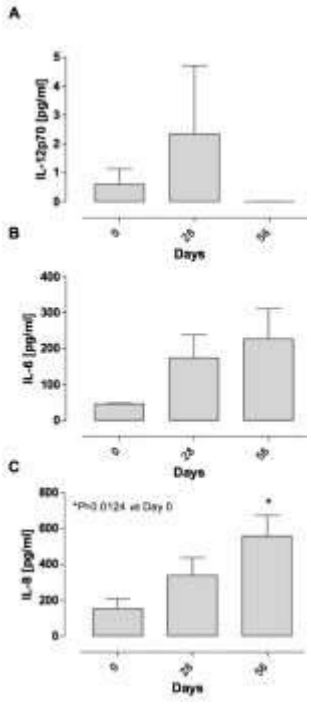

Figure 17. Selective Cytokine Levels. Plasma levels measured in patients with late-stage gastrointestinal cancer at Day 0, 28, and 56. There is an increase in both IL-6 and IL-8 as chemotherapy continues in gastrointestinal cancers.

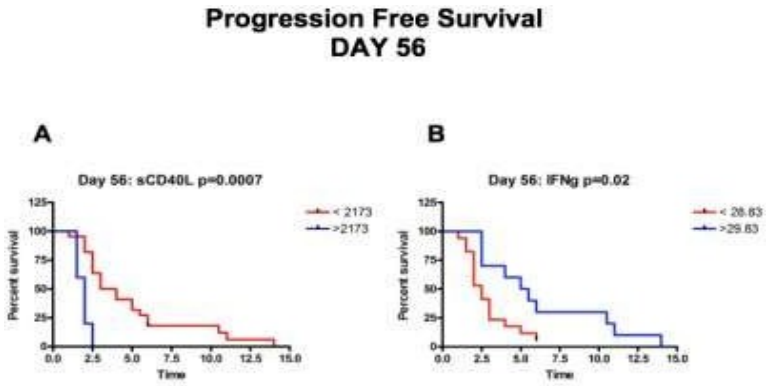

Figure 18. Progression Free Survival and plasma cytokine levels at Day 56. A) sCD40L plasma levels in patients below 2173pg/ml (p= 0.0007) show better PFS than those above this value. These values are consistent as well on days 0 and 28. B) IFNy levels above 29.83pg/ml (p=0.02) show better PFS.

3.9 Discussion

Metastatic disease remains the main challenge in the treatment of most cancers. Yet, the majority of cancer therapeutic studies involve the study of primary tumors rather than frank metastatic disease. As part of an effort to improve the *in vivo* models used to test new cancer therapies, we have developed a model of intracardiac implantation of EMT-6 breast cancer cells injected to the left ventricle of Balb/c mice. Our results show that the combination produced anti-tumor activity in the metastatic setting. In addition, similar experiments using the B16 melanoma conformed the usefulness of this intracardiac implantation technique to generate drug-resistant models of widespread metastatic disease. Coupled with this experiment, our analysis of cytokine levels at Day 56 confirmed that sCD40L and IFNy levels could be predictive markers for improved PFS. Thus, longer PFS is found in patients with lower levels of sCD40L and higher levels of IFNy (Figure 18). This data suggests that there are possible cytokines such as sCD40L and IFNy that can serve as biomarkers for patients who can benefit from this combination and regimen of chemotherapy. Additional studies using clinical samples and preclinical samples from *in vivo* mouse experiments will be necessary to confirm these results. Such studies will also be important to determine whether the potential biomarkers we identified are unique to gastrointestinal cancers, or if they can be applied to other tumor types such as breast cancers. These approaches improve our ability to study late stages of cancer progression, and to evaluate new therapeutic concepts in more relevant preclinical models.

4: MODELING BRAIN METASTASIS

Brain metastasis has become more and more common in women with breast cancer. Patient outcomes have improved leading to better survival thus increasing the incidence of brain metastasis. 15 to 30% of patients who achieve long-term survival are known to have breast cancer metastasize to the liver, bone, brain, and lungs. Breast cancer brain metastasis has poor prognosis, limited life expectancy and quality of life. To reach better prognosis and quality of life, an improvement in therapy must be achieved.

There is a higher chance of brain metastasis from breast or lung cancers than a brain primary tumor. HER-2 positive breast cancer patients are most likely to develop brain metastasis than breast cancers of other subtypes, such as luminal a and b subtypes. This can be attributed to the administration of HER-2 targeted therapy such as trastuzumab, which cannot cross the blood brain barrier (BBB) [45]. It is important to use HER-2 positive cell lines to represent the number of patients who succumb to cancer once the disease spreads to the brain since overall survival for such patients is about 16.5 months. Gene and protein expression can account for organotropism of breast cancer metastasis. Many BCCs can express certain chemokine receptors, such as CXCR4, that make them suitable for metastasizing to the brain where its ligand, CXCL12, is found in abundance [46], [47]. Sihto, et al., found an increased protein expression in primary breast cancer tumors and the site of metastasis. Breast cancer cells (BCCs) migrate to the brain in the same manner they would to other organs except that now they must penetrate the blood brain barrier (BBB). It is difficult for BCCs to survive in the circulation after detachment from the primary tumor and invasion to the brain proves to be a challenging task. The BBB is a composed of a layer of endothelial cells surrounded by a basement membrane, pericytes, and astrocyte foot processes, which make it difficult to invade [48]. Astrocytes are capable of synthesizing a wide range of cytokines that cancer

cells use to increase survival and invasiveness. These include IGF-1, TNFa, and PDGF-1 [47]. BCCs can rearrange the BBB by secretion of proteases [49]. Macrophages aid the invasion through the BBB by releasing Vascular Endothelial Growth Factor (VEGF), thus inducing proliferation and migration of endothelial cells (EC) and at the same time disrupting the tight junctions that hold ECs together [48]. There are different ways in which a BCC migrates to the brain. Once the BCCs attach to the endothelial cells, these are able to migrate through paracellular or transcellular migration. In order for either process to occur, interactions between vascular endothelial cells and BCCs must take place. For instance, vascular endothelial cell express Notch ligands that will interact with the Notch receptors expressed on the BCC, then intracellular junctions are disrupted and reorganized during paracellular migration [50].

4.1 Current Models of Metastasis

Current experimental models of metastasis include tail vein injection, carotid vein, intra-portal, and intracardiac. Tail-vein injection often results in lung metastasis, as this is the first set of capillaries that cells encounter when they enter the bloodstream [51]. Intracecal injection of cells tend to metastasize to the liver through the portal vein. The use of intracardiac injection permits the widespread metastasis throughout the body without forcing a tumor to a specific organ. Intracardiac implantation of cancer cells give metastasis to liver, lung, bone, mesentery, ovary, kidney, etc.

4.2 Stereotactic Surgery Methods

Mice were first prepared for injection following IACUC guidelines. Stereotactic equipment was inspected to make sure it is leveled to ensure proper site of injection. Mice were anesthetized with isoflurane 5% in ~1L/min oxygen from a precision vaporizer then placed in the stereotactic apparatus with its mouth and teeth properly fixed on mouthpiece for proper flow of anesthetic and oxygen (1-2% isoflurane for maintenance). Ophthalmic ointment (Lacri-lube) was put on the

mouse's eyes once the animal was placed on the stereotactic apparatus. Incision site was shaved and swabbed three times alternating 70% alcohol and chlorohexidine with one last application of chlorohexidine scrub. Buprenorphine (0.05mg/kg) was administered s.c. before the incision of the skull was made. Bregma and Lambda are clearly seen by the addition of 30% hydrogen peroxide using a swab directly on the skull. In order to make sure that the incision is made correctly, Bregma and Labmba were used as to establish a leveled cranium. From Bregma, the following coordinates were used: 2mm posterior to Bregma, 1mm to the left of Bregma, and 3mm deep. A Hamilton syringe needle was used to mark the site of injection, then a hand-held drill was used to drill through the cranium to expose the brain. $2.5x10^7$ BTGF1 ells were injected at a rate of 1ul/min over the span of three minutes. After cells had been injected, the needle was left in the brain for an additional minute to prevent any cells from spilling into other parts of the brain. Once needle is taken out of the brain, a piece of brain wax is smeared on top of the skull to conceal the incision made. Skin is sutured promptly, and topical antibiotic is applied over the incision to avoid infection of SCID mice. Post-surgical monitoring took place daily and an additional dose of buprenorphine (0.05mg/kg, i.p.) was administered 14 hours after surgery.

Tumor progression was monitored weekly after i.p. injection of Luciferase. Mice were then imaged using In Vivo Imaging System (IVIS) shortly after injection of luciferase. Luminescence was plotted using Prizm version 6.0.

4.3 Lapatinib

Lapatinib is an FDA approved tyrosine kinase inhibitor of HER-1 (EGFR) and HER-2 for use in combination with capecitabine, trastuzumab, or hormone therapy for advanced HER-2 positive breast cancer and gastrointestinal cancer patients. Lapatinib binds to the tyrosine kinase domain of the HER family receptors leading to the downstream signaling cascade, blocking tumor cell growth

[7]. Lapatinib has been widely used in the clinic to treat HER-2 positive breast cancer, but the use of other HER-2 targeted therapies are preferred due to efficacy. The use of Lapatinib is still preferred for cancers with truncated HER-2 and brain metastasis since it has the ability to easily cross the BBB [7]. To test the efficacy of Lapatinib in a metastatic breast cancer to the brain, it was administered orally via gavage (50mg/kg) to HER-2 positive cell line BTGF1.

4.4 Results

In the preliminary studies using Lapatinib as the drug of choice in brain metastasis models, our experimental group was not helpful in inhibiting tumor growth, shown as photons p/s in Figure 19. Mice in the control group had lower levels of luminescence throughout the span of the experiment. Due to these results, the drug of choice was changed to Gemcitabine to develop a second metastatic model of acquired drug resistance. Mice administered gemcitabine had larger tumors on average at the start of the experiment and had smaller tumors after the second week of treatment (Fig. 20). Luminescence was visualized weekly to determine tumor progression in these metastatic models (Fig. 21). Mice in the Gemcitabine group responded to treatment by week 2 and by week 5 these tumors began to show drug resistance.

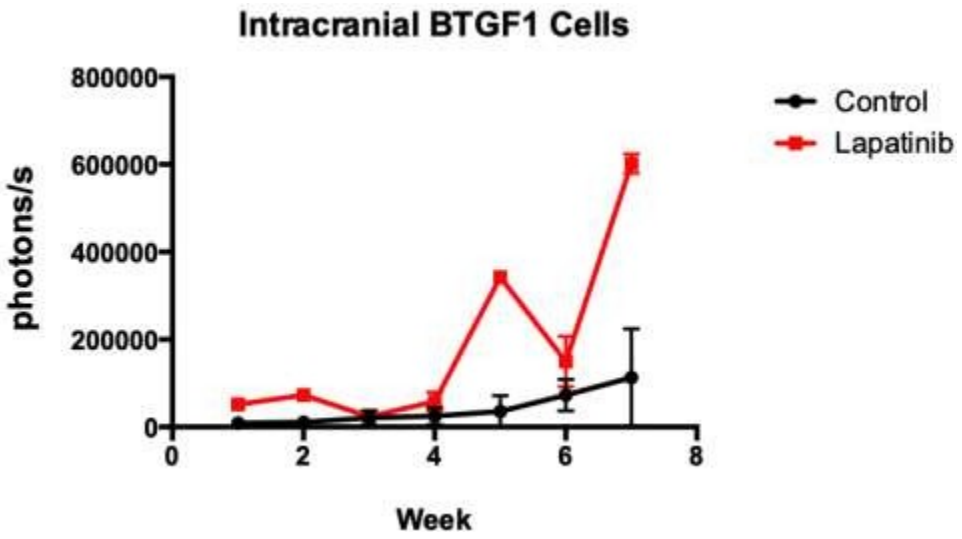

Figure 19. Lapatinib tested on BTGF1 brain metastasis model. Shows brain metastasis progression in both Control (PBS) and Lapatinib (50mg/kg) treated mice over the period of 7 weeks.

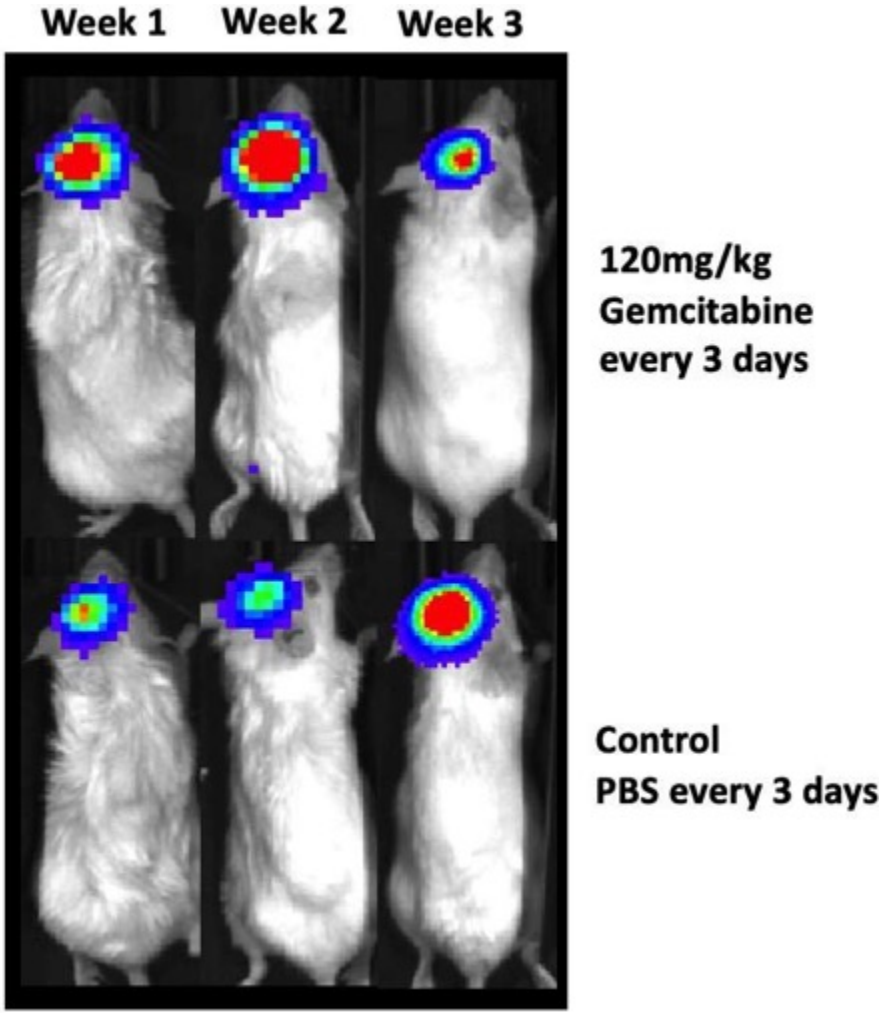

Figure 20. Visualization of luciferase positive, BTGF1 brain metastasis model. Top panel shows the efficacy of Gemcitabine (120mg/kg) given every 3 days i.p. On week 2, tumor luminescence increases, but after the 6th dose of metronomic Gemcitabine, the tumor begins to shrink at week 3. Bottom panel shows the increase in luminescence from week 1 to week 3, cancer continues to progress in control group.

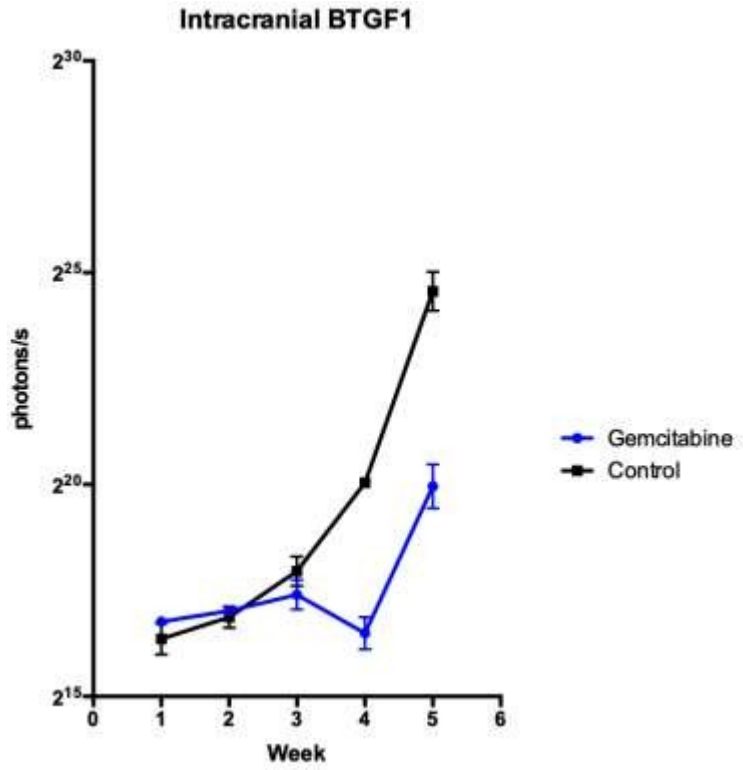

Figure 21. Tumor Growth curve of BTGF1 cells in SCID mice. SCID mice were injected $2.5x10^5$ BTGF1 cells intracranially. SCID mice (n=9) treated with Lapatinib (50mg/kg) via daily gavage. Control group (n=9) was administered PBS via daily gavage.

4.5 Discussion

Thanks to the many scientific advances and breakthroughs, the 10-year survival rate for breast cancer patients is now 85%. Unfortunately, this is not the case for metastatic breast cancer. Due to this increased survival rates for breast cancer patients, metastasis incidences to the brain are also on the rise. In this sense, breast cancer patients are living longer and now have higher chances of developing metastasis in their lifespan. It is important to note that brain metastasis from primary breast tumors are more common than a primary brain tumor. 24% of patients suffering from breast metastasis to the brain have a 2-year overall survival. Thus, it is imperative to model brain metastasis in vivo to study the mechanisms of disease taking place within these tumors to find potential therapeutic targets. The preferred site of invasion for patients treated with HER-2 targeted therapy is the brain [52]. The HER-2 positive brain metastasis models developed in this project have also been used to metronomically administer HER-2 targeted chemotherapy to improve treatment options. As the results suggested, the models have successfully been developed and therapy was tested against them. The next step in this project is to continue testing other therapies and combinations. One such treatment that can be used is Osimertinib, which is a tyrosine kinase inhibitor approved for small lung carcinomas that targets EGFR. Osimertinib in combination with other chemotherapies can target those mechanisms of drug resistance that HER-2 positive breast cancers usually present. Literature suggests that EGFR is often upregulated as a means for cancer cells to become drug resistant in HER-2 positive cancers [53].

5: CONCLUDING REMARKS

About 90% of cancer patients will die because of metastatic disease [28]. It is therefore important to develop models that recapitulate the steps by which cancers metastasize, and those include the detachment of cancer cells from the primary tumor, the survival the circulation, and the eventual arrest and growth at a distant organ. It is also important to study such preclinical models together with the administration of therapies that reflect current treatment options in the clinic. The use of advanced cancer models for experimental therapeutics should improve our understanding of the process of metastasis, and increase our knowledge of how cancer cells can stop responding to certain therapies. Such studies should eventually also allow us to develop new therapies. This project addressed the unmet need for HER-2 positive breast cancer models that can accurately represent late-stage disease in patients. There have been many improvements to patient care and treatment, but the mortality rates for breast cancer patients with metastasis continues to rise. The current models of disease are often not reproducible, too artificial, unreliable, or respond too well to the therapy administered. In order to overcome some of the current issues with modeling disease for such patients, several models of HER-2 positive breast cancer models were characterized, developed, and tested against metronomic chemotherapies. BT474 and MDA-MB-361 are two HER-2 positive breast cancer cell lines readily available and were used to select for more aggressive and drug resistant populations. After several cycles of implantation, these cells lines grew faster in vivo and gave us the opportunity for using them in a widespread model and brain metastasis model. These in vivo models were then tested against current FDA-approved therapies in a metronomic or low-dose fashion. These newly developed breast cancer models are important to study the differences in protein expression between primary breast cancer populations, and tumor variants selected for aggressive growth in vivo and/or resistance to anti-

cancer therapies. This work identified new markers of either cancer progression or of acquired drug resistance. Furthermore, using an in vivo model of widespread metastasis, by the intracardiac implantation of tumor cells in mice, relative drug resistance of a tumor cell population is dependent on the organ in which it is growing. These studies were conducted alongside analysis of plasma samples from cancer patients in a clinical trial. Specifically, I have looked at cytokines profiles of patients responding to, and not responding to, a metronomic chemotherapy treatment strategy. Certain cytokine profiles indicate a relative level of drug resistance in the tumor of a given patient, as indicated by reduced progression free survival compared to other patients. Altogether, these efforts have contributed to our understanding of how cancers respond to currently available therapies, and how drug resistance in some cases emerges.

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GLOSSARY

APCs Antigen Presenting Cells

BBB Blood Brain Barrier

BM Basement Membrane

CEACAMs Carcinoembryonic antigen-related cell adhesion molecules

CTLA-4 Cytotoxic T-Lymphocyte Antigen 4

CTX Cyclophosphamide

EC Endothelial Cell

EGFR Epidermal Growth Factor Receptor

EMT Epithelial to Mesenchymal Transition

HER-2 Human Epidermal Receptor 2

IGF-1 Insulin-like Growth Factor-1

IVIS In Vivo Imaging System

KO Knockout

MMPs Matrix Metalloproteinases

PBS Phosphate Buffered Saline

PDGF-1 Platelet Derived Growth Factor-1

PFS Progression Free Survival

PI3K Phosphoinositide 3-kinases

Prrx-1 Paired related homeobox 1

PTEN Ten-sin Homolog

TAM Tumor Associated Macrophages

TNFa Tumor Necrosis Factor Alpha

TSP-1 Thrombospondin 1

WT Wild-type

ZEB-1 zinc finger Eboxbinding homeobox 1

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Biography

I was born in Mexico then a couple of years later I moved to El Paso, TX. I enrolled at The University of Texas at El Paso (UTEP) to pursue my bachelor's degree in biology. Before graduating I began volunteering in a lab under the mentorship of Dr. Giulio Francia, who inspired me to pursue my graduate degree with an emphasis in Cancer Biology. I developed *in vivo* models that allow for greater reproduction in mice of aspects of late stage breast cancer in cancer patients (e.g. through orthotopic implantation- and the subsequent development of metastatic disease. In addition, by the use of intracranial and intracardiac injection techniques. I was able to generate breast cancer models of metastasis to the brain and other sites and soon after I then began to use these models to test metronomic chemotherapy regimens. Currently, I am working on one of the specific aims of my dissertation which is to determine if drug resistance is depends on the organ where metastases have seeded. These results, together with the models of late stage disease, may eventually help to tailor the most effective treatments for individual breast cancer patients.