

An examination of the pharmacology of HER2 inhibitor
responses in cellular models of breast cancer.

A thesis submitted for the degree of PhD

By Fiona O'Neill B.Sc. M.Sc.

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The work in this thesis was carried out under the
supervision of

Dr Robert O'Connor and Dr Sinéad Aherne

National Institute for Cellular Biotechnology

School of Nursing and Human Sciences

Dublin City University

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Abbreviations:

AHR/ARNT	Aryl hydrocarbon receptor/hypoxia inducible factor 1 beta
ALDH3A2	Alcohol dehydrogenase
ATCC	American type culture collection
BGA	Between Group Analysis
CCND1	Cyclin D1
CDKN1B	cyclin dependent kinase inhibitor 1B
CIA	Co-inertia Analysis
CISH	Chromogenic <i>in situ</i> hybridisation
CML	Chronic myeloid leukaemia
C _t	Comparative threshold
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
ER	Estrogen receptor
FCS	Fetal calf serum
FDA	Food and drug administration
FFPE	Formalin fixed paraffin embedded tissue
FISH	Fluorescent <i>in situ</i> hybridisation
FOXO3a	Forkhead box protein 3
GSK	GlaxoSmithKline
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
HMEC	Human mammary epithelial cells
HR	Hormone receptor
IC ₅₀	50% inhibitory concentration
IC ₇₀	70% inhibitory concentration

IHC	Immunohistochemistry
MDR	Multidrug resistance
NR3C1	nuclear receptor sub family 3 group C member 1
NSC	Non-symmetric correspondence analyses
NSCLC	Non small cell lung cancer
OLF1	Olfactory neuron-specific factor
PAX3	Paired box gene 3
PAX9	Paired box gene 9
PBS	Phosphate buffered saline
PDX	Patient derived xenograft
PICS	Protease inhibitor cocktail
PIK3C3	phosphoinositide 3 kinase class 3
PR	Progesterone receptor
PSSM	Position specific scoring matrix
RAR	Retinoic acid receptor
RB1CC1	RB1 coiled-coil protein 1
RQ	Relative expression
SCID	Severe combined immune-deficient
SD	Standard deviation
SNP	Single nucleotide-polymorphism
STR	Short tandem repeat
STR	Short tandem repeat profile
TF	Transcription factor
TFBS	Transcription factor binding site
TKI	Tyrosine kinase inhibitor
UHP	Ultra high purity
VDR/RXR	vitamin D receptor/retinoid X receptor

WHO World Health Organisation

ZNF143 Zinc finger protein 143

Abstract

So-called “targeted therapeutics”, agents with tumour-selective action, promise to revolutionise treatment of specific malignancies; however, realisation of the therapeutic promise of such drugs requires new methods of rapidly distinguishing patients who will derive treatment benefit. Tyrosine Kinase Inhibitors (TKIs), a group of small molecule therapeutics, inhibit specific aspects of the phosphorylation-mediated intracellular signalling pathways commonly altered in cancer. Overexpression of one such pathway, initiated by the HER2 growth factor receptor, occurs commonly in breast cancer. Lapatinib, a HER2 TKI, has been used in combinations with other cancer drugs for treating HER2 overexpressing breast cancer. The aim of this study was to evaluate gene expression changes in response to these targeted therapies to examine their ability to predict treatment response.

In this thesis, microarray data from lapatinib-treated drug sensitive breast cancer cell lines was interrogated using an emerging bioinformatic technique, Co-inertia analysis (CIA). Using this technique, 512 genes were found to be altered in a specific response to lapatinib treatment in the cell lines. 27 gene targets were chosen for more detailed analysis using Taqman RT-PCR, of which five showed predictive response in a broader panel of breast cancer cell lines treated with lapatinib.

Expression of the five genes was further examined in response to other HER2 targeted therapies and the analysis indicated that the gene expression changes remained consistent with these other treatments, demonstrating a more broadly representative anti-HER2 response pattern. An *in vivo* study sought to evaluate these gene expression responses in a more *in vivo*-relevant scenario and found that they were also conserved in this model.

Our research indicates that there are commonalities among the gene expression response to HER2-targeting therapeutics in responsive cells which may extrapolate to HER2-amplified patient tumours and more broadly suggests that characterisation of gene changes shortly after treatment may provide a valuable rapid predictor of inhibitor response, potentially guiding a more specific use of such agents by identifying patients that will benefit from these therapies.

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1 . Introduction

1.1 Cancer

1.1.1 History

Commonly thought to be one single malady, cancer is in fact a highly complex group of diseases. It can originate and affect any organ and system in the body and affects men and women of all ages. In the developed world it is one of the biggest killers and it is becoming more prevalent in the developing world. Of the 7.6 million deaths in 2008 associated with cancer, 4.8 million of those occurred in developing countries [1]. In 2012, the number of deaths associated with cancer in Ireland was 8544. Of this number 30% were neoplasms of the gastrointestinal tract (e.g. colon cancer), excluding the stomach, 21% were trachea, bronchus and lung cancers, 17% were genitourinary and 8% were breast neoplasms [2]. In the last decade or so our knowledge of the molecular origins and implications of cancer has grown significantly and this has successfully translated into improved treatment options and patient outcome[3].

Early detection of cancer in any form can reduce the need for extensive treatment. Typically, once located, a solid tumour is surgically removed and, if it determined that it has not spread to other part of the body, a patient may not have to undergo chemotherapy or radiation. In some cases, especially breast cancer, clinical experience suggests that adjuvant treatment with radiation and/or chemotherapy may additionally ensure that no undetected errant cancer cells have survived. However, if it found that the cancer has spread or is more locally advanced a more intense treatment approach is indicated with toxic chemotherapy being the mainstay of disease management.

On its own or in combination with radiation and or surgery, application of relatively non-specific cytotoxic chemotherapy drugs has been a mainstay in the treatment and management of advanced malignancies (where the disease is a major burden in the body and/or has spread) for several decades and this is discussed in more detail in section 1.2. However more recently, treatment agents have started to be more targeted to the molecular drivers of the cancer, making them more specific. As examples, the treatment of breast cancer and chronic myeloid leukaemia (CML) are good illustrations of success of this approach of so-called “molecularly targeted therapy”. Overall survival of CML, in the chronic phase has improved from 15% pre-introduction of imatinib (a tyrosine kinase inhibitor) to as high as 87% since the introduction of the targeted therapy [4]. Molecular aberrations, such as over expression of the HER2 growth factor receptor and expression of the BCR/ABL transgene, have been successfully targeted with agents such as trastuzumab and gefitinib respectively. Breast cancer has become a particular focus for treatment individualisation and application of targeted therapeutics with notable success in increased survival, complemented by increased early detection and disease management. From initial studies carried out using trastuzumab in combination with first-line chemotherapy, data showed an increase in survival from 20.3 months with chemotherapy alone to 25.1 months with chemotherapy combined with trastuzumab [5].

1.1.2 Breast Cancer

According to the World Health Organisation (WHO), breast cancer accounts for 16% of all cancers that affect women globally. Nationally, the Irish Cancer Society estimates that 1 in 10 Irish women will develop breast cancer at some stage in their life and it accounted for 29% (excluding non-melanoma skin cancer) of the number of cancer diagnoses in Ireland between 1995 and 2007 in women [6]. The disease is most commonly diagnosed in women over the age of 50 but can affect women of any age. Pathological classification of this cancer, undertaken to provide predictive diagnostic information, is based on a number of aspects such as, tumour size, tumour progression, nodal spread and receptor status e.g. estrogen receptor (ER), progesterone status (PR) and human epidermal growth factor 2 (HER2). Classification of these clinical parameters will generally dictate the course of treatment to be recommended to the patient. Breast cancer is often detected by self-examination or routine mammographic screening. Tumour classification/staging can be completed using a number of methods. One such method is the TNM classification system. The tumour stage is assessed based on three criteria; tumour size (T), whether nearby lymph nodes are involved (N) and the presence or absence of metastatic tumours at other sites (M) [7]. Later stage breast cancers are often associated with a reduced chance of survival and the type of treatment offered may change. In early stage disease treatment will often be multimodal involving surgery followed by radiation, endocrine therapy (for hormone-positive malignancies) and/or adjuvant chemotherapy (aimed at eradicating any small undetected tumour deposits in the body). Chemotherapy, often simply called “chemo” involves treatment with cytotoxic drugs designed to bring about cell cycle arrest and/or apoptotic tumour cell

death [8] However, the application of these agents is limited by their toxicity to normal cells and the development of drug resistance. The toxicity to normal cells can result in adverse effects such as hair loss [9], extreme fatigue [10], depression of the immune system and vomiting [11]. Hence there is need for more personalised medicine allowing treatment and dosage to be tailored to a particular patient's tumour type, thus improving response to treatment and limiting patient exposure to unnecessary drugs.

As breast cancer treatment has evolved over the last decades, it was recognised that there were many inconsistencies in the outcome of breast cancer patients when pathological classification alone was employed for prediction and prognosis. Hence a lot of research effort has gone into improved molecular subclassification of tumour type to give better prognostic information for the patient and their clinician, with more recent research being used to predict the most efficacious treatments for specific molecular subtypes of cancer [12]. For example, using microarray analysis and hierarchal clustering analysis of over 100 patient tumour samples, Sorlie *et al.*, [13] evaluated the different subtypes occurring in breast cancer patients. The microarray analysis consisted of examination of the expression of 534 genes. (Figure 1.1)

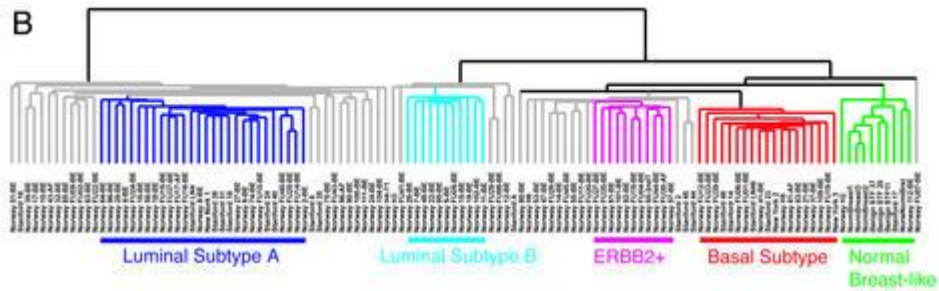


Figure 1.1: Hierarchical clustering of subtypes of breast cancer from Sorlie *et al* 2003. The tumour samples were analysed using microarray analysis which consisted of 534 genes. The microarray analysis identified four major cancer subtypes, which have their own individual characteristics and expected prognosis and the normal breast like tissue. The four cancer subtypes identified were luminal subtype A (blue), luminal subtype B (turquoise), ERBB2+ (pink) and basal subtype (red).

Their analysis indicated that there were four major subtypes within the tumour sample population which they termed luminal type A, luminal type B, ERBB2 (HER2) positive and basal. Each of the subtypes has their own individual characteristics that give general indications as to patient prognosis and what treatment type will often be most effective. Luminal type A and luminal type B are both considered to be ER positive, with subtype A found to be low grade with subtype B tending to be more high grade. As both subtypes express ER, they are treated with hormone inhibitor therapies (endocrine therapies) such as tamoxifen or aromatase inhibitors (usually in post-menopausal women), with subtype B requiring additional chemotherapy [14]. The basal subtype is also referred to as triple negative as it does not overexpress HER2, ER or PR receptors. This sub group of cancer has been associated with poor prognosis [15, 16]. While many patients have been shown to respond to chemotherapy treatment, the use of newer targeted therapies in the treatment of this form of breast cancer has been very limited due to the lack of specific targets [17, 18].

The last subtype ERBB2/HER2-overexpressing has been shown to be one of the most suitable for targeted therapies, and this is discussed in detail below.

1.1.3 Role of HER2 in Breast Cancer

Aberrations in the epidermal growth factor (EGFR) family of proteins have been identified as being important in breast cancer. One particular member of this growth factor receptor family, HER2/ ERBB2, has been found to be particularly important in breast cancer biology, in a subset of patients [19]. Although research is now trying to tease out the role and relevance of the three remaining members of this receptor group, EGFR, HER3 and HER4. The EGFR family are tyrosine kinase receptors, a class of transmembrane receptors that can bind and phosphorylate with other receptors within the family to activate a kinase-signalling pathway within the cell. Activation of the functional component of these receptors requires that the Src Homology 2(SH2) domain of the receptors must undergo phosphorylation. The SH2 regions are conserved domains first identified within the Src oncoprotein [20]. The ability to enzymatically phosphorylate target substrates results from dimerisation and auto-phosphorylation of the C terminal tyrosine residue, induced by ligand binding to the extracellular domain of the receptor.

Signal transduction within the cells therefore requires receptor dimerisation [21]. While homodimerisation can take place, a number of combinations of functional heterodimers have been identified since the first dimerisation was suggested [22, 23]. The combination of receptors in the homo/heterodimer(s) will dictate which

signalling pathway is activated, with some dimers having inherent signalling activity and others requiring ligand binding for activation [24].

However, these receptors do have some limitations. The kinase domain of HER3 is inactive and as a result the protein can only signal through heterodimerisation partners [25]. HER2 has no known ligands and therefore acts as a signal transducer by heterodimerising with EGFR, HER3 and HER4 [26].

HER2-positive breast cancer, in which the HER2 receptor is overexpressed, generally through amplification of the *HER2* gene, is found in approximately 20-30% of human breast cancers [27] and has been associated with poorer prognosis and outcome [19, 28]. This alteration has been associated with the increase of tumour invasiveness by the down-regulation of α -4 integrin via the p38MAPK pathway[29]. It is this poor responsiveness, the aggressive nature of HER2+ breast cancers and the relatively high incidence of the target that has made this growth factor receptor a good therapeutic target. In addition, HER2-overexpressing breast tumours tend to become dependent on this aberration, a process which has been termed “oncogene addiction” hence inhibition also represents an effective treatment[30].

1.1.4 Cancer Biomarkers

The use of biomarkers has become increasingly important in the diagnosis and treatment of many malignancies including breast cancer. A biomarker has been described as a cellular, biochemical or molecular alteration that can be measured in a biological medium, e.g. blood or tissue. These alterations can be evaluated as an indicator of pharmacological response to therapy or a pathogenic process [31, 32]. Biomarkers can be classified as prognostic, predictive or diagnostic. Prognostic markers can be used to evaluate how a disease will progress in a patient regardless of their treatment. Predictive markers can be used to determine the effects that a treatment will have on a patient's disease and diagnostic markers can be used to investigate the presence of a disease.

1.1.4.1 Biomarkers in breast cancer

In the diagnosis of breast cancer, the evaluation of the molecular markers PR, ER and HER2 are routinely used in order to determine the most suitable treatment regime [33]. There are currently only two FDA-approved methods to determine that HER2 overexpression is present, immuno-histochemical analysis (IHC) and fluorescence in-situ hybridisation (FISH) [34].

Recent developments in the field which utilise molecular-based assays to classify the different subtypes of cancer are moving away from the anatomical/pathological classification of the malignancy and have been focussed on giving more predictive

and prognostic information, thereby promising to allow physicians identify the most suitable treatment options available. Many of the assays that will be described below have both prognostic and predictive applications although it will likely be several years before their full utility is characterised and their use becomes the international standard of care.

The Breast BioClassifier or PAM50 is an RT-PCR assay which utilises analysis of the expression of 50 genes and 5 control genes to determine which subset of breast cancer the patient samples belong to [35, 36]. In the prognostic setting this assay evaluates the risk of recurrence or the likelihood of relapse following 5 years in patients based on the expression of these genes [36-38]. In the predictive setting, this assay has been utilised in the RxPonder trial to determine the most suitable course of treatment for ER positive, node positive patients, whether it be chemotherapy or hormone therapy [37].

The *oncotype* DX assay which examines the expression of a 21 gene signature is used in the prognostic setting to evaluate the likelihood of disease recurrence in women with estrogen receptor (ER) positive only breast cancer [39]. In the TAILORx clinical trial, which was devised by the National Cancer Institute (NCI), the *oncotype* DX gene signature was used to predict if adjuvant chemotherapy treatment should be indicated in ER-positive, node negative breast cancer patients. [40]. The test uses recurrence scores to predict which patient will likely need additional chemotherapy added to their hormonal treatment. This recurrence score is calculate by normalising the expression of the 21 genes to the expression of the 5 reference genes that are also screened for in the assay [41]. The resultant recurrence score is then segregated into 3 groups which dictates treatment option, scores lower

than 16 (hormonal treatment alone), scores 16 to 25(randomised arm) and scores greater than 26 (hormonal treatment in combination with chemotherapy) [41].

MammaPrint is a 70 gene expression-based assay that is manufactured by Agendia BV. This microarray based assay which uses fresh mRNA from patient resections has been utilised to determine the risk of metastasis from a breast cancer tumour. This assay is being utilised in the MINDACT clinical trial which aims to predict the appropriate therapy option for patients suffering from lymph node negative, ER positive or negative breast cancer [42]. Using the MammaPrint assay in combination with standard clinicopathologic prognostic factors (tumour grade, ER status and HER2 status), patients are assigned high or low risk status. Patients with a high risk status as determined by the MammaPrint assay and clinicopathological factors are treated with chemotherapy, patients with a low risk status from both assessments are not treated and are monitored and patients that show a difference between the two tests are given a randomised treatment. In patients that are ER+ that present with a high risk from both the MammaPrint and the clinicopathologic prognostic factors, hormonal therapy (tamoxifen) is prescribed in combination with chemotherapeutic agents. In patients that present as low risk, while no chemotherapy is given they do undergo a course of hormonal therapy. This is the same for patients that are enrolled in the randomised arm [43].

The three assays that have been described above all use gene expression signatures to evaluate how a disease is expected to develop (prognosis) and also have been used to predict what patients would benefit from treatment with chemotherapeutic agents. However, none of these assays has the capability to determine if the patient will respond to the treatment that they will receive. In the case of HER2 positive patients who receive HER2 targeted therapies such as the monoclonal antibody trastuzumab

or the tyrosine kinase inhibitor lapatinib, it is estimated that as many as 50% of patients will develop resistance to the therapy within 12-36 months [27]. The cost of these therapies as well as the undue burden that they can cause to patients has resulted in regulatory agencies such as the FDA promoting the development of companion diagnostics that can be used by clinicians to determine whether or not patients are responding to treatment.

1.2 Cancer Treatment

1.2.1 Conventional treatment

Conventional treatment of cancer can include combinations of surgery, radiation and/or chemotherapy, depending on a large number of variables including patient age, anatomical location and molecular and pathological characteristics of the tumour.

Surgery has proven to be one of the most successful methods for tumour eradication when the tumour is localised. and can be used in combination with chemotherapy, radiation and targeted drug therapies [44]. Radiation treatment uses ionising radiation to target and treat solid tumours, many of which have been detected at an early stage. By targeting the nuclear DNA of the cancer cell and disrupting it, radiotherapy is thought to work primarily by interrupting the cell cycle process and thereby killing the cancer cell [45].

1.2.2 Cancer chemotherapy

Chemotherapy is the use of cytotoxic chemical agents that have the ability to damage cancer cells, where successful this action disrupts growth of the cancer cell and can result in cessation of cell growth and/or (preferably) tumour cell death [46]. As a neo-adjuvant treatment, chemotherapy can be used, much like radiation to shrink tumours prior to surgery. As an adjuvant therapy it can be used to eliminate any cancer cells that may have spread from the primary tumour site and hence target

metastasis that might otherwise occur at a later date [47]. As with other cancer treatments, chemotherapy is also used in combination with radiation and it is not uncommon for a patient to be on regimens containing multiple agents. [48].

Chemotherapy has been proven to be a useful therapeutic tool in the treatment of metastatic cancers. As radiation tends to damage surrounding tissue, it is generally employed only in localised disease, while chemotherapy can disseminate throughout the bloodstream to multiple potential sites in the body. Chemotherapy can be administered by oral dosage or, more usually, intravenously for delivery throughout the bloodstream [46].

The cytotoxic actions of chemotherapy drugs are not specific to cancer cells, healthy cells can also be affected as a result of this, and hence, many patients suffer adverse effects. Fatigue is the most commonly reported side effect associated with chemotherapy treatment. Alopecia (hair loss), nausea, vomiting, diarrhoea, low blood cell counts, cardiotoxicity, fertility and sterility issues and neurotoxicity are also common side effects. In addition to these, patients undergoing chemotherapy can become immunosuppressed leaving them susceptible to opportunistic infections. This effect is due to chemotherapeutic agents causing damage to the bone marrow, which is responsible for the production of many of the immune cells that the body needs to fight infection, such as T-cell, B-cells, and dendritic cells [44].

Chemotherapy drugs are classified depending on what stage of DNA synthesis they are capable of interfering with.

Alkylating agents, such as cyclophosphamide, are used in the treatment of some breast cancers. These drugs add an alkyl group which damages cellular DNA. Platinum agents, e.g. cisplatin, damage DNA with the addition of a platinum adduct.

These cytotoxic agents are used in the treatment of many malignancies, including lung and ovarian cancer.

Antimetabolites are responsible for the inhibition of pyrimidine and purine metabolites production which are required for DNA and RNA synthesis (a vital component of cell growth and division) [44]. Methotrexate and 5-fluorouracil are two agents that fall into the antimetabolite category. They are employed in many forms of cancer, for example, in CMF (cyclophosphamide, methotrexate, 5-fluorouracil) combination therapy which has been used successfully in the treatment of node positive breast cancer [48].

Anthracyclines or anti-tumour antibiotics such as doxorubicin or epirubicin are again used in several forms of cancer including breast cancer. These agents generally function by an intercalation interaction with DNA [49].

Antimitotic agents, for example taxanes such as paclitaxel, target spindle formation in mitosis, thereby having a major impact on tumour cell growth. Paclitaxel is used in many malignancies including lung, ovarian and breast cancer treatment [44].

1.2.3 Targeted therapies

The use of targeted therapies has come to the forefront of oncology in the past decade. Monoclonal antibodies and tyrosine kinase inhibitors are currently being used for the treatment of a number of receptor and pathway alteration-driven forms of cancer. The Philadelphia chromosome (BCR-ABL) has proven to be extremely vulnerable to targeting with specific TKIs [50]. The introduction of imatinib into the

portfolio of drugs employed for the treatment of chronic myeloid leukaemia has been extremely successful, with one study indicating that, since its introduction, imatinib has improved the 8 year survival rate to 87% from a 20% pre-imatinib rate [4]. Following the success of imatinib, additional targeted therapies have also been developed for the treatment of CML, such as sunitinib and dasatinib. The epidermal growth factor receptor (EGFR) is another commonly expressed cell surface protein on cancer cells that has also been successfully targeted using TKI. Overexpression of this receptor has been found commonly in lung cancer and, as a result, gefitinib, an EGFR inhibitor, has been used in the treatment of EGFR-overexpressing lung and breast cancer [51, 52].

1.2.3.1 Targeted therapies for HER2 positive breast cancer

Since the identification of frequent overexpression of HER2 (especially in breast cancer) in the 1980s, HER2 receptor-based inhibitor strategies have proven to be a poster child for the applicability and clinical of molecularly targeted therapies. The monoclonal antibody trastuzumab was developed by Genentech and has been used extensively in HER2+ metastatic breast cancer patients, both as a mono-therapy and in combination with chemotherapeutic agents. In addition to monoclonal antibodies, tyrosine kinase inhibitors have also been developed that bind specifically to the HER2 kinase domain. Lapatinib, along with second generation agents, such as afatinib and neratinib have been used in a large number of clinical trials. Figure 1.2 illustrates the different binding domains of the epidermal growth factor family that are targeted by the molecularly targeted therapies discussed in this section.

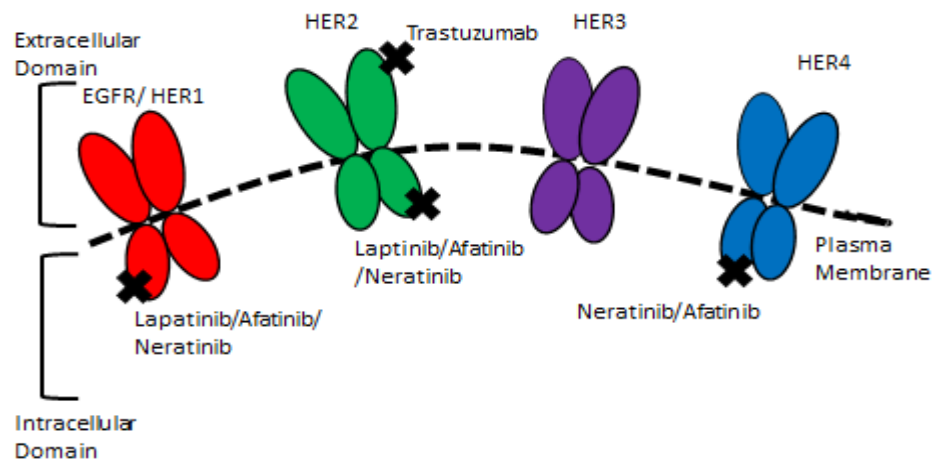


Figure 1.2: Schematic representing the binding site of the target therapies trastuzumab, lapatinib, afatinib and neratinib.

1.2.3.1.1 Trastuzumab

Trastuzumab was the first inhibitor developed to target HER2. The recombinant humanised monoclonal antibody was generated by Genentech and works by preventing activation of downstream pathways such as RAS-MAPK and PI3K-AKT-mTOR by the antibody binding to the extracellular membrane of the domain of the HER2 receptor. By inhibiting these downstream pathways, susceptible cancer cells are unable to proliferate [5]. A large number of clinical trials have demonstrated that the drug is a clinically active and tolerated therapy in metastatic HER2-overexpressing breast cancer patients who have undergone extensive prior treatment [53]. Administered intravenously, patients are typically given a loading dose of

4mg/kg followed by a weekly infusion of 2mg/kg until the patient has progressed or responded to the therapy [54]. Cardiac toxicity is one of the main adverse effects attributed to trastuzumab. [55]. With the cost of treatment per patient estimated to be approximately €24,000 per year, the risk of cardiac toxicity and the incidence of intrinsic and acquired resistance, it has become increasingly important to clinicians to be able to determine which patients will benefit most significantly from this drug.

A number of clinical trials have been undertaken to evaluate the synergistic effects of combining trastuzumab with other treatments. Kaufman *et al.*, [56] evaluated that the combination of trastuzumab with anastrozole, an aromatase inhibitor.; When compared with anastrozole alone in HER2 positive, hormone positive metastatic breast cancer patients there was an increase in the activity of the combination treatment. Piccart-Gebhat *et al.*, [57] and Romond *et al.*, [58] have also provided results that indicate that combining trastuzumab with adjuvant chemotherapy has reduced the risk of relapse and death quite substantially.

While trastuzumab has been shown to be extremely effective in the treatment of metastatic breast cancer, clinical experience, especially in advanced disease, has found that some patients don't respond to treatment and a large number of patients develop resistance to the monoclonal antibody treatment [59]. In an attempt to overcome the development of this resistance, TDM1, a trastuzumab-maytansine 1 conjugate has been developed and evaluated pre clinically and is now successfully in a number of clinical trials [60-62]. In combining the cytotoxic drug maytansine with the HER2 specific antibody trastuzumab, TDM1 ensures that the toxin is delivered directly to tumour cells [63].

1.2.3.1.2 Lapatinib

Lapatinib, a dual kinase inhibitor developed by GlaxoSmithKline, targets both HER2 and EGFR receptors[64]. By inhibiting the tyrosine kinase domains of the receptors, lapatinib prevents activation of important pro-cancer pathways such as Erk/MAPK (extracellular-signal-regulated kinase/mitogen-activated protein kinase) and PI3K (Phosphatidylinositol 3-kinases) which have vital roles in cell proliferation and survival [64, 65]. Lapatinib is currently approved for the second line treatment of metastatic breast cancer when used in combination with the chemotherapeutic agent capecitabine, a pro-drug that is enzymatically converted to 5-fluorouracil in the tumour [66]. Due to their complementary mechanisms of action, a number of studies have been undertaken to evaluate the synergistic effects of combining trastuzumab with lapatinib for the treatment of HER2 positive metastatic breast cancer [63, 67-69]. As a result of these studies, this treatment regime has been approved for patients suffering from HER2 positive, HR negative metastatic breast cancer by the European Medicines Agency (EMA) [70]. It has also been approved in combination with the aromatase inhibitor, letrozole, in HER2 positive, hormone positive metastatic breast cancer patients, with the combination increasing progression free survival as well as clinical benefit rate [71].

There have been some studies to evaluate the potential use of lapatinib in non-breast malignancies such as HER2 positive gastric cancer [72]. These studies were based upon results that indicate the response of this subtype of gastric cancer to trastuzumab [73, 74] .

The typical dosage of lapatinib is 1250mg taken daily, orally. The TKI is usually taken for 21 days at a time with the next 7 days off. It is also recommended that the agent be taken one hour before or after food [75]. Adverse effects associated with lapatinib treatment include, cardio-toxicity, however, the risk is thought to be lesser than that associated with trastuzumab treatment. Additional adverse effects include diarrhoea, hepatic and skin toxicity [64].

1.2.3.1.3 Afatinib

Afatinib is also a tyrosine kinase dual inhibitor of HER2 and EGFR[76]. This agent, developed by Boehringer Ingelheim, has been shown to irreversibly inhibit the HER2 and EGFR receptors[77]. The drug has been shown to be more potent than lapatinib and, as a result, a dose of 50mg per day has been identified as being effective in comparison to the 1250mg twice daily that is required for lapatinib [78, 79]. Clinical trial data has indicated that afatinib may have activity in metastatic breast cancer patients that have developed resistance to trastuzumab[80] Due to inhibition of the EGFR receptor, the inhibitor has been proven to be effective in the treatment of non-small cell lung cancer and lung adenocarcinomas and a number of phase 2 and 3 clinical trials have been undertaken studying these malignancies. The results of these trials have indicated that in patients who have developed resistance to a number of first line treatments such as gefitinib and erlotinib, treatment with afatinib may be beneficial [81, 82]. Bouche, O *et al.*, [83]have also suggested that there may be some evidence to support the use of the agent in the treatment of

colorectal cancer [83]. As with lapatinib there are some notable adverse reactions with the use of afatinib, in particular diarrhoea, rash and fatigue [84].

1.2.3.1.4 Neratinib

Neratinib (HKI-272), a dual kinase inhibitor, developed by Pfizer, targets both HER2 and EGFR [85]. By irreversibly binding to the HER2 and EGFR receptors, this TKI reduces auto-phosphorylation within the cell which in turn prevents the activation of downstream pathways [86, 87]. Phase 1 clinical trials within patients with solid tumours indicated that the most suitable dose of neratinib was between 240 and 320mg per day. Doses greater than this resulted in dose-related toxicities, most predominately diarrhoea [88]. Initial phase 1 trials also indicated that treatment of patients with neratinib that had been pre-treated with trastuzumab, anthracyclines or taxanes showed therapeutic potential and warranted further studies [87]. Due to dose related adverse effects that has been associated with the drug, the number of phase 2 trials have been limited in metastatic breast cancer [89-91]. As with afatinib, due to its ability to target EGFR, neratinib has also been investigated in the treatment of NSCLC [92]. Phase 1 [87] and phase 2 [88] trials have been limited and have shown disappointing results, with little to no response rate evident in the study.

1.3 Resistance to targeted therapies

Resistance to targeted therapies has been the Achilles heel of the successful application of these emerging agents. The resistance can be as a result of pre-existing mutations/ alterations in the drug target (intrinsic) or induced following drug treatment (acquired) [93].

Acquired resistance, i.e. resistance evident in patients who were initially responsive and have been treated typically a year to 18 months, has become a major issue for clinicians treating patients with targeted therapies, in particular trastuzumab and lapatinib. While the mechanisms by which this resistance occurs have not yet been fully characterised, a large number of studies have provided potential hypotheses. Liu *et al.*, [94] have evaluated the activation of AXL, a member of the receptor tyrosine kinase subfamily, in BT474 cell lines with acquired resistance. The cell line was exposed to lapatinib over an extended period of time in order to induce the acquired resistance phenotype. Using AXL gene targeting siRNA, AXL expression was decreased in the BT474 lapatinib-resistant cell line, restoring sensitivity to the targeted therapy. While this initial data provides strong supporting evidence to examine the expression of AXL in patients, as of yet there has been no clinical evaluation of this finding. Xia *et al.*, [95] investigated the role of estrogen receptor signalling and its involvement in the development of acquired resistance. Similar to Liu *et al.*, the BT474 parental cell lines were cultured continuously for an extended period of time in the presence of lapatinib in order to establish an acquired resistance model. Transfection with siRNA targeting the estrogen receptor indicated that estrogen signalling was increased following treatment with lapatinib in the BT474 lapatinib sensitive cell line. In the resistant cell line it was identified that cell survival

was no longer being driven by the HER2 pathway but a co-dependence of the HER2 and ER pathways combined. This finding was confirmed using tumour biopsies from patients undergoing lapatinib treatment. It was hypothesised that acquired resistance could be avoided by inhibiting both the HER2 and ER pathways simultaneously during treatment. In addition, Eichhorn *et al.*, [96] established that mutation in the PI3K pathway leads to resistance to lapatinib *in vitro*. The mutations identified, loss of function of PTEN or activating mutation in PIK3CA, drove the resistance to lapatinib. However the resistance phenotype was reversed following treatment with a PI3K inhibitor developed by Novartis known as BEZ235 [97].

Through the evaluation of all of these studies it has become clear that there are a number of different mechanisms that can be responsible for the induction of acquired resistance in patients undergoing treatment with targeted therapies. Further studies will have to be completed in order to determine what pathways are involved and what methods can be used in order to overcome them.

1.4 Aims of project

Combining a number of bioinformatics, *in vitro* and *in vivo* techniques the aims of this project were to:

- Identify differentially expressed genes which might act as possible indicators of response to HER2 drug treatment using analysis of a microarray data set derived from a panel of cell lines treated with lapatinib
- Validate the relevance of these genes of interest in a more diverse panel of breast cancer cell lines with varying HER2 expression and in response to a broader range of therapeutics
- Evaluate the relevance of these genes in a model of acquired lapatinib resistance
- Assess whether the alterations in specific genes identified in the cell line models transpose to an *in vivo* cell line-derived tumour xenograft

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2. Materials and Methods

2.1. Cell Culture and reagents

2.1.1. Cell Culture Solutions

The following solutions were prepared as needed. Phosphate buffered saline (PBS) was prepared by dissolving 1 tablet (Oxoid, Basingstoke, Hampshire, England) in 100mL of ultra-high purity (UHP) water and autoclaved. Sodium acetate buffer was prepared by dissolving sodium acetate (Sigma, St. Louis, MO, USA) to a concentration of 0.1M with 0.1% (v/v) triton-X-100 and then pH to 5.5. Phosphate substrate was prepared dissolving p-nitrophenol phosphate to a concentration of 10mM in the previously described 0.1M sodium acetate. 1N NaOH was prepared by dissolving 40g/L of NaOH (Sigma, St. Louis, MO, USA) in UHP water. EDTA was prepared by dissolving 1% w/v EDTA (Sigma) in UHP and was then autoclaved. 1X trypsin was prepared by combining 50mL of 10X trypsin (Gibco by Life technologies, Grand Island, NY 14072, USA) and 10mL of 1% EDTA solution to 440mL of sterile PBS.

2.1.2. Cell Culture Equipment

The following equipment was used throughout the course of this research. 30mL sterile universal containers (Ramboldi Ltd, Limassol, Cyprus), 96-well plates (Corning, Costar, Sigma Aldrich, St.Louis, MO), T25cm² and T75cm² vented flasks (Corning, Costar, Sigma Aldrich, St. Louis, MO) and cryovials (Greiner Bio-One GmbH, Frickenhausen, Germany) were used in cell culture.

2.1.3. Cells culture and aseptic techniques

All cell culture work was carried out in a class II laminar airflow cabinet, Holten LaminAir Maxisafe. All experiments involving cytotoxic compounds were conducted in a cytoguard laminar airflow cabinet. Before and after use the laminar airflow cabinet was cleaned with 70% v/v industrial methylated spirits (IMS). Any items brought into the cabinet were also swabbed with 70% IMS. Only one cell line was used in the laminar airflow cabinet at a time and upon completion of work with any given cell line, the laminar airflow cabinet was allowed to clear for at least 15 minutes before further use. This was to eliminate any possibility of cross-contamination between cell lines. The cabinets were cleaned weekly with industrial disinfectants (Virkon, Antech International, P0550) and operation validated by annual inspection by a certified contractor.

2.1.4. Monitoring of sterility of cell culture solutions

Sterility testing was performed on all cell culture media and related culturing solutions. Samples of prepared basal media were incubated at 37°C for a period of seven days. This ensured that no bacterial or fungal contamination was present in the media.

2.1.5. Sub-culturing of cell lines

The waste cell culture medium was removed from the tissue culture flask to a sterile bottle. The flask was rinsed with 1mL of 1X trypsin solution to ensure the removal of any residual media. 2mL of 1x trypsin was then added to the flask and incubated at 37°C for the required period of time until all cells were detached from the inside surface of the culture flask. The trypsin was deactivated with an equal volume of complete media. The cell suspension was removed from the flask and placed in a sterile universal container and centrifuged at 200g for 5 minutes. The supernatant was then discarded from the universal and the pellet was suspended in complete medium. A cell count was performed using a haemocytometer. An aliquot of cells was then used to re-seed a flask at the required density with fresh media. All cell lines used in the course of this research are described in table 2.1.

2.1.5.1. Sub-culturing of Human Mammary Epithelial Cells (HMEC)

As cultured cells derived from normal mammary epithelium, the culturing of the HMEC (Human mammary epithelial cells) (CC-2551, Lonza) was different to that of the immortalised cell lines used within this project. The preparation of the media was completed 5-7 days prior to thawing of cells. The singlequots kit (CC-4136, Lonza) which contained cytokines, growth factors and supplements were added to the MEBM (Mammary epithelial basal media, CC-3151, Lonza) and the media underwent sterility checks as outlined in section 2.1.4.

30 minutes prior to addition of cells, 1mL/5cm² of media was added to a T25cm² vented flask and returned to the incubator. Cells were seeded at a recommended density of 2500 cells/cm². The flask was gently rocked to ensure dispersion of the cells. The media was changed the day after thawing and every other day thereafter.

To sub-culture these cells, the Lonza ReagentPack (CC-5034, Lonza) was recommended for use. Waste media was removed from the flask and HEPES-buffered saline was first used to wash the cells. Following this, trypsin/EDTA solution was added to the flask and the cells were returned to the incubator in order to allow the cells to detach from the flask. Trypsin neutralizing solution was used to stop the trypsin action and to wash all detached cells from the flask. Harvested cells were centrifuged at 220g for 5 minutes to pellet them. Once again the cells were re-suspended at a density of 2500 cells/cm². Cells were re-suspended in T75cm² vented flasks. According to the manufacturer, these cells are capable of 18 doubling and have a doubling time of approximately 18 hours.

2.1.6. Cryopreservation of cells

Cells for cryopreservation were harvested in the log phase of growth. Cell pellets were resuspended in a suitable volume of FCS (PAA, GE Healthcare BioScience Corp, New Jersey, USA). An equal volume of a 10-20% DMSO/FCS solution was added drop-wise to the cell suspension. A total volume of 1mL of this suspension was then placed in a cryovial. These vials were then placed in the vapour phase of a liquid nitrogen container, which was equivalent to a temperature of -80°C.

After a period of three hours, vials were removed from the vapour phase and transferred to the liquid phase for storage (-196°C).

2.1.7. Thawing of cryopreserved cells

A volume of 5mL of fresh warmed growth media was added to a sterile universal. The cryopreserved cells were removed from the liquid nitrogen tank and thawed rapidly at 37°C. The cells were removed from the vials and transferred to the aliquoted media. The resulting cell suspension was centrifuged at 200g for 5 minutes. The supernatant was removed and the pellet resuspended in fresh culture medium. Thawed cells were then added to a T25cm² tissue culture flask with a suitable volume of fresh growth media.

2.1.8. Mycoplasma testing

Cell lines were tested for possible mycoplasma contamination quarterly in house by Mr Michael Henry according to the SOP 007-01 using the in-direct test.

Table 2.1: Description of breast cancer cell lines, characteristics and basal media components

Cell Line	Characteristics	Basal Media	Source
BT474	HER2-overexpressing, ER positive	Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 2% L-Glutamine and 1% Sodium Pyruvate	NICB through the ATCC
SKBR3	HER2-overexpressing, ER negative	RPMI 1640 medium supplemented with 10% fetal bovine serum	NICB
EFM 192a	HER2-overexpressing ER positive	RPMI 1640 medium supplemented with 10% fetal bovine serum	NICB
HCC1954	HER2-overexpressing, ER negative	RPMI 1640 medium supplemented with 10% fetal bovine serum	NICB
MDAMB453	HER2-overexpressing, ER negative	RPMI 1640 medium supplemented with 10% fetal bovine serum	NICB
MDAMB231	Triple negative, ER negative	RPMI 1640 medium supplemented with 10% fetal bovine serum	NICB
HMEC-Mammary Epithelial Cells	Normal breast tissue	MEGM BulletKit (CC-3150) containing MEBM (CC3151) and the SingleQuots (CC4136)	Lonza Bioresearch, Basel, Switzerland
SKBR3-L	HER2-overexpressing acquired lapatinib resistance	RPMI 1640 medium supplemented with 10% fetal bovine serum	NICB, generated by Fiona O'Neill as part of this thesis

All cell lines were cultured at 37°C in a 5% CO₂/95% humidified air incubator.

The chemical compounds listed in Table 2.2 were prepared as stock solutions in dimethyl sulfoxide (Sigma Aldrich, St Louis, MO, USA) with the exception of trastuzumab and epirubicin which were provided already in solution and not in powder form as the other were.

Table 2.2: Source and stock concentrations of chemotherapy drugs and inhibitors in this project

Drug Name	Source	Stock Concentration
Lapatinib	Sequoia Chemicals Pangbourne, RG8 7AP, United Kingdom	10mM
Neratinib	Sequoia Chemicals	10mM
Afatinib	Sequoia Chemicals	10mM
5'DFUR	Sigma	10nM
Gefitinib	Sequoia Chemicals	10mM
Dasatinib	Sequoia Chemicals	10mM
Trastuzumab	Roche, Basel, Switzerland	21mg/ml
Epirubicin	Pfizer Inc. 235 East 42nd Street, New York, NY 10017	21mg/mL

2.2. Proliferation Assay

Proliferation was measured using an acid phosphatase assay [1]. This colorimetric assay uses *p*-nitrophenyl phosphate to determine the percentage of live cells following treatment with a therapy. This percentage is expressed as the IC₅₀ which is the inhibitory concentration in which 50% of the biological activity of the cell lines is inhibited. 1×10^3 cells/well were seeded in 96-well plates with the exception of the BT474 cells which were seeded at 5×10^3 cells/well. Plates were incubated overnight at 37°C followed by the addition of drug at appropriate concentrations and incubated for a further 5 days until wells were 80% to 90% confluent. All media was removed and the wells were washed twice with PBS (Sigma). 10mM paranitrophenol phosphate substrate (Sigma-Aldrich) in 0.1M sodium acetate buffer with 0.1% Triton X (Sigma) pH 5.5 was added to each well and incubated at 37°C to 1 hour. 50µL of 1 M NaOH was added and the absorbance was read at 405 nm with a 620nm reference wavelength using the Synergy HT plate reader (BioTek, Winooski, Vermont, USA).

2.3. Establishment of Lapatinib-Resistant Cell Lines

The lapatinib-sensitive, HER2+ cell line, SKBR3 was chosen to be conditioned in lapatinib. A 96 well plate dose response proliferation assay was completed in order to determine the IC₇₀ concentration for conditioning. This toxicological value was employed as it was felt to be more appropriate for developing resistant lines, consistent with Sheikh *et al.*, [2] who had also been developing a lapatinib resistant

cell line (of pancreatic origin). Duplicate flasks of the cell line were grown until 65-70% confluent at which time they were treated with 250nM lapatinib. The cells were treated with the lapatinib twice a week for 6 months. After two months the dose of lapatinib was increased to 300nM and again to 350nM two months later. Proliferation assays were performed at regular intervals in order monitor the alteration in response to the drug. Cells were considered to be resistant when an IC₅₀ value of 1µM or greater was achieved.

2.4. Genomic DNA Isolation

Using the Promega Wizard SV genomic DNA purification system, genomic DNA was isolated for parental and lapatinib-resistant variants of the SKBR3 breast cancer cell line. Briefly, a cell suspension containing 2×10^6 cells was spun down and re-suspended in 150µL of cell lysis buffer. This sample lysate was then transferred to a Wizard SV mini-column. The column was centrifuged for 1 minute at 13,000g. The eluent was removed. 650µL of wash solution containing 95% of ethanol was added to the column and once again was centrifuged for 1 minute at 13,000g. This step was repeated a further 3 times. The eluent was discarded following each spin. To elute the DNA, 250µL of nuclease free water containing 2µL of RNase, which removes any co-purified RNA, was added to each column and allowed to incubate for 2 minutes at room temperature. The column was then centrifuged for 1 minute at 13,000g. The isolated genomic DNA was stored in labelled tubes at -20°C until it was required for DNA fingerprinting analysis. The DNA was quantified using the

Nanodrop (ND-1000 spectrophotometer, Thermo Scientific) and a DNA purity with an OD 260/280 ratio of 1.8 was required for the DNA fingerprinting.

2.5. DNA fingerprinting of the SKBR3 parental and acquired resistance cell lines

The DNA fingerprinting of the parental and resistant variants of the SKBR3 cell line was carried out by the lab of Dr. Michael Crowley in the University of Alabama at Birmingham. The method used looked at STR profiling of 9 polymorphic regions. Briefly, the lab used the ABI Identifiler® Kit (Life Technologies) to map the DNA fingerprint of the cell lines. The markers that were present in this kit include those as used by the ATCC which included Amelogenin, CSF1PO, D135317 and TH01. Using gene amplification technologies, the genomic DNA was mapped. The mapped genomic DNA was then analysed using Gene-mapper software, and the genomic fingerprint was compared to that provided by the ATCC.

2.6. RNA Extraction

RNA extraction was achieved using the Qiagen RNeasy mini Kit (Qiagen). Briefly, cells were grown until confluent in a T75cm² cell culture flask. Media was removed and cells were trypsinised, centrifuged at 8000 x g and a pellet formed. All media was removed from the pellet as it can interfere with the lysis buffer. One volume of lysis buffer per number of pelleted cells was added to the cells, in this case 600µL,

and mixed gently. Cells were passed gently through a 19 gauge needle to induce lysis. One volume of 70% ethanol (600 μ L) was then added to the cells and 700 μ L of the cell suspension was added to the column and were centrifuged for 15 seconds at 8000g. This step was repeated with the remainder of the sample. Flow through was discarded following each spin. Following this 350 μ L of the RW1 buffer provided was then added to the column and was spun for 15 seconds at 8000g. The Qiagen DNase incubation mix was prepared. 70 μ L of RDD buffer and 10 μ L of the DNase 1 stock were mixed together per sample with additional volume prepared for pipetting error. The incubation mix was placed directly on the column membrane and was left for 15 minutes at room temperature. 350 μ L of the RW1 buffer was added to the column and it was centrifuged for 15 seconds at 8000g. 500 μ L of RPE buffer was added to the column and it was further centrifuged for 15 seconds at 8000g. This step was repeated and the column centrifuged for 2 minutes at 8000g. To dry, the column was then added to a new collection tube and centrifuged at full speed for 1 minute. The column was transferred to a 1.5mL collection tube. 40 μ L of nuclease free water was added and it was centrifuged for 1 minute at 8000 x g. This was repeated once more. The extracted RNA was quantified using a Nanodrop (ND-1000 spectrophotometer, Thermo Scientific). The RNA was stored in labelled tubes at -80°C until analysed.

2.7. Homogenisation of animal tissue

To extract RNA from mouse tumour tissue, a homogenisation step was required prior to the addition of one volume of 70% ethanol in the extraction method above. Two methods of tissue homogenisation were optimised for the animal tissue.

2.7.1. Disruption and homogenisation using a rotor-stator homogeniser

30mg of tissue was placed in 2mL non-stick nuclease-free tubes which contained 1 5mm diameter stainless steel bead. 600 μ L of lysis buffer from the Qiagen RNeasy kit containing 1% β -mercaptoethanol was added to the tube. Samples were placed in the Qiagen tissuelyser, which was then operated at 20-30 Hz until the tissue was fully disrupted. The steel bead was removed from the tube and disposed of and the sample was centrifuged at full speed for 3 minutes. The supernatant was removed and retained at -80°C until the RNA extraction could be completed.

2.7.2. Disruption and homogenisation using a pestle and mortar and QiaShredder

30mg of tissue was placed in a pestle and mortar, to which a small volume of liquid nitrogen was added. The sample was crushed until a fine powder formed. Once all

the tissue was broken down, the liquid nitrogen was allowed to evaporate without allowing the tissue to thaw. The tissue in its powder form was then scraped into a 2mL non-stick nuclease-free tube containing 600 μ L of lysis buffer from the Qiagen RNeasy kit containing 1% β -mercaptoethanol. The powder was dissolved in the buffer. The suspension was then centrifuged at full speed for 3 minutes. The supernatant was removed and retained at -80°C until the RNA extraction could be completed.

2.8. Reverse Transcription (RT) Reaction

To synthesise cDNA, the extracted RNA was diluted to a concentration of 2 μ g. A high capacity cDNA reverse transcription master mix (Applied Biosystems) was prepared containing 2 μ L of 10X RT Buffer, 0.8 μ L of 25X dNTP, 2 μ L of 10X RT primers, 1 μ L of Multiscribe Reverse transcriptase, 1 μ L RNase inhibitor and 3.2 μ L nuclease free water per reaction. 10 μ L of the master mix was added to 10 μ L of the 2 μ g RNA solution. The solution was pipetted gently to mix and briefly centrifuged to remove any bubbles. The tubes were labelled and were placed in the G storm thermo cycler (Model GS1, Somerton Biotechnology Centre, Somerset, UK). The thermo cycler conditions were 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. The resulting cDNA was stored at -20°C in labelled tubes.

2.9. Taqman Real Time PCR

Taqman real time PCR was used for the mRNA quantification in this study. Real-time RT PCR monitors the progress of the reaction as it occurs and only requires a few nanograms of target RNA in order for the amplification reaction to take place. The reactions are characterised by the point during the cycle when the amplification of a target is first detected. This detection occurs when the quencher dye that is found in the 3' end of the probe is separated from the fluorescent dye which is found in the 5' end of the probe due to cleaving of the probe with the target. The higher the copy number of the target, the sooner a fluorescent signal will be observed. The value at which this increase is seen is known as the threshold cycle (C_t). It is defined as the fractional cycle number at which the fluorescence passes the fixed threshold[3].

2.9.1. Taqman Array Fast 96-well plate

RNA was extracted and cDNA synthesised as per section 2.3 and 2.4. Taqman Real Time PCR analysis was performed using Taqman Array Fast 96-well plate (Applied Biosystems), which was pre-prepared containing assays for our 28 targets and 4 endogenous controls. The experiment was prepared by diluting the cDNA to a concentration of 40ng/well. A total cDNA volume of 5 μ L is required per well. 5 μ L/well of Taqman fast universal PCR master mix (2x), NO AmpErase UNG (Applied Biosystems) was combined with the diluted cDNA and 10 μ L of the solution was added to each of the wells on the Taqman plate. Plates were vortexed to remove any bubbles and ensure that the solutions were mixed. The thermocycler

conditions were as follows: hold at 95°C for 20 seconds, followed by melt at 95°C for 3 seconds and anneal/extend at 60°C for 30 seconds for 40 cycles on a 7900 fast real-time PCR instrument (Applied Biosystems).

2.9.2. Individual Taqman Gene expression assays

Additional Taqman RT PCR reactions were performed using individual gene expression assays.

The assay was prepared by diluting the cDNA to a concentration of 80ng/well (20ng/μL) in dH₂O. A total cDNA volume of 4μL is required per well. For each assay a master mix was prepared containing 1μL of the taqman assay, 10μL of Taqman fast universal PCR master mix (2x), NO AmpErase UNG (Applied Biosystems) and 5μL of dH₂O was required for each well. 16μL of the assay and master mix solution was added to each well and 4μL of the 80ng/well cDNA solution in dH₂O. Plates were vortexed to remove any bubbles and ensure that the solutions were mixed. The thermocycler conditions were as follows: hold at 95°C for 20 seconds, followed by melt at 95°C for 3 seconds and anneal/extend at 60°C for 30 seconds for 40 cycles on a 7900 fast real-time PCR instrument (Applied Biosystems).

Table 2.3 Individual Taqman Gene expression assays

Gene Target	Species detected	Assay ID
ALDH3A2	Human	Hs00166066_m1
CCND1	Human, Murine	Hs00277039_m1
CDKN1B	Human	Hs00153277_m1
ERBB3	Human	Hs00951444_m1
FOXO3a	Human	Hs04195365_s1
FOXO3a	Human, Murine	Hs00818121_m1
FOXO3a	Murine	Mm00490673_m1
GAPDH	Human	Hs99999905_m1
GAPDH	Murine	Mm99999915_g1
NR3C1	Human, Murine	Hs00230818_m1
NR3C1	Human	Hs00230813_m1
PIK3C3	Human, Murine	Hs00176908_m1
RB1CC1	Human, Murine	Hs01089010_m1
RB1CC1	Human	Hs00207547_m1

2.9.3. Relative gene expression analysis using the Comparative C_T method

Relative expression values were calculated using the comparative threshold cycle (C_t) method [3]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the endogenous control for all experiments. The cycle threshold (C_t) indicates the cycle number by which the amount of amplified target reaches a fixed threshold. The C_t data for GAPDH was used to create ΔC_t values [$\Delta C_t = C_t$ (target gene) - C_t (GAPDH)]. $\Delta\Delta C_t$ values were calculated by subtracting ΔC_t of the calibrator (untreated controls) from the ΔC_t value of each drug-treated sample. Relative quantification (RQ) values were calculated using the equation:

$$2^{-\Delta\Delta C_t}$$

Genes with a fold change ± 2 in the drug-treated BT474 and SKBR3 cell lines were deemed to be differentially expressed.

Standard deviations (SD) were calculated on the mean of the $\Delta\Delta C_t$ value, which was based on triplicate replicate measurements of the relative expression of each gene. Error bars on each graph represent the SD of each estimate. This mean $\Delta\Delta C_t$ value was then used in the calculation of the RQ value using the equation above. This method for calculating the error bars was used in lieu of using the RQ_{max} and RQ_{min} values, which are which is sometimes employed in related analysis.

2.10. Protein Extraction

Whole cell lysis buffer was prepared by combining 7M urea (Sigma), 4% w/v CHAPS (Sigma), 1% Triton X (Sigma) and 1x PICS (Protease Inhibitor Cocktail) (Roche Diagnostics, Mannheim, Germany) in UHP water. This lysis buffer was aliquotted into 1mL eppendorfs and stored at -20°C. In order to extract the protein from the cells, the cells are first trypsinised and pelleted. Once a pellet had been formed following centrifugation, 100µL of the lysis buffer was added to the cell pellet and was pipetted gently. The cell suspension and lysis mixture was left on ice for approximately 1 hour, with the eppendorf being vortexed every 20 minutes. Following an hour on ice, the cell suspension was centrifuged at 3000 x g at 4°C for 15 minutes. The supernatant was removed and stored in labelled tubes at -20°C.

2.11. Protein Quantification

Protein concentrations were quantified using a Bradford Assay. Bovine Serum Albumin standards were prepared from a stock concentration of 2mg/mL, with concentrations per well from 0.312µg to 10µg prepared. To ensure that the sample concentrations were within the standard curve, one sample was chosen and diluted down by various factors. These dilution factors were then examined to make sure that they were within the standard curve. If dilutions were required, all samples were diluted. All samples and standards were left on ice. To each plate, 5µL of either standards or sample were added to each well. To this, 245µL of Bradford reagent

was added to each well. The plate was then left in the dark to develop and the plates, once developed, were read at 595nm on the BioTek Plate reader.

2.12. Immunoblotting

Protein (30 μ g) was electrophoretically resolved on 7.5% (Lonza, 59501) denaturing polyacrylamine gels. The resolved proteins were then transferred to nitrocellulose membranes (Invitrogen, IB3010-01) using the iBlot transfer system (Invitrogen, IB1001). Protein transfer was visually confirmed using Ponceau S staining (Sigma, P7170). Membranes were blocked with 2.5% skimmed milk powder in PBS and incubated overnight at 4°C with primary antibodies. Antibody dilutions and sources can be found in Table 2.4. Proteins were visualised using horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Sigma – see Table 2.4). and ECL Prime reagent (GE Healthcare, RPN 2232). Membranes were washed with 0.1% PBS-tween 3 times for 10 minutes each, both prior to and following incubation with secondary antibodies. Following the final washing, the membranes were exposed to ECL Prime reagent (GE Healthcare, RPN 2232) in the dark room and the membrane was exposed to autoradiographic film (Kodak,) for various times (from 10 seconds to 30 minutes depending on the signal). The exposed autoradiographic film was developed for 3 minutes in developer (Kodak, LX-24). They were then washed in water and transferred to fixative (Kodak FX-40) for 5 minutes. The film was then washed with water and allowed to dry at room temperature.

Table 2.4 List of primary antibodies.

Protein of interest	Primary Antibody	Primary Antibody Dilution	Source	Secondary Antibody	Source	Secondary Antibody Dilution
CCND1	#2922s	1/1000	Cell Signalling Technology (CST)	Rabbit	Sigma Aldrich	1/10,000
ERBB3	#4754s	1/1000	CST	Rabbit	Sigma Aldrich	1/10,000
FOXO3a	#2497s	1/1000	CST	Rabbit	Sigma Aldrich	1/10,000
NR3C1	#7437s	1/1000	CST	Rabbit	Sigma Aldrich	1/10,000
RB1CC1	17250-1-AP	1/1500	ProteinTech	Rabbit	Sigma Aldrich	1/10,000
β -actin		1/10,000	Sigma	Mouse	Sigma	1/10,000

2.13. Development of cell line-derived tumours *in vivo* (xenografts)

2.13.1. Mice

28-35 day old CB17/lcr-*Prkdc*^{scid}/Crl mice were used under the guidelines of the Irish Department of Health and procedures approved by the research ethics committee of Dublin City University, Dublin 9. CB17/lcr-*Prkdc*^{scid}/Crl mice were purchased from Charles River (Charles River International Inc., Wilmington, MA). The immunodeficiency of these animals is as a result of the inhibition to produce B and T lymphocytes. This deficiency provides the mice with a wide tolerance to implanted foreign tissues and tumours, which makes them an ideal model for research.

2.13.2. Cell line suspension preparation

BT474 cells were grown until 70% confluent in T75cm² vented flasks. A cell count was completed and the cells were re-suspended at a density of 5×10^6 per 100µL in serum free Dulbeccos modified Eagles Medium (DMEM). To this cell- and serum-free media suspension was added a 1:1 volume of matrigel (BD, Franklin Lakes, NJ, USA 07417). Cell and matrigel suspension was stored on ice until needed.

2.13.3. Estrogen implantation and tumour development

BT474 cells are estrogen dependent. In order to support the growth of the cells *in vivo*, 72mg estrogen pellets (Innovative technology, Sarasota, FL, USA) were implanted in the shoulder region of the mice. 200µL of the cell lines and matrigel suspension was subcutaneously injected into the flank of the mice. Mice were monitored daily and once tumours were observed they were measured using a digital calliper. The equation used to evaluate the tumour volume was;

$$\frac{H(\text{height of tumour}) \times W(\text{width of tumour}) \times D(\text{depth of tumour})}{2} = \text{volume of tumour (mm}^3\text{)}$$

2.13.4. Tumour graft implantation

Once tumours had developed following the injection of the cell line into the subcutaneous layer of the skin, they were removed and placed into serum free media. 24 hours prior to implantation with the tumours, mice were implanted with 72mg estrogen pellets. The tumour was cut into approximately 2 x 2 x 2mm³ sections and dipped in matrigel before they were inserted subcutaneously in a small incision in the flank of mice. The incision was sealed using a wound clip, which was removed 10 days post-surgery.

2.13.5. Vehicle and Drug preparation

The vehicle used to administer the drug suspension to the mice was prepared by dissolving 0.5% hydroxyl-propyl-methyl-cellulose (Sigma-Aldrich, St Louis, MO, USA) and 0.1% Tween 80 (Sigma-Aldrich, St Louis, MO, USA) in UHP water.

Lapatinib ditosylate (Sequoia Chemicals) was administered at a dose of 200mg/kg. 100 μ L of drug was given to each mouse by oral gavage.

Animals were monitored daily during the treatment with either the vehicle or 200mg/kg lapatinib. They were weighed prior to the treatment and visually observed post treatment to ensure that there were no complications.

2.13.6. Drug Quantification in Plasma samples using LC-MS

Lapatinib was quantified according to [4] and the analysis was carried out by Dr. Sandra Roche, NICB, DCU.

2.13.6.1. Liquid-liquid extraction procedure

100 μ L of 500ng/mL internal standard was added, to 10 μ L of plasma. Dasatinib 500ng/mL was used as the internal standard for lapatinib. Also 200 μ L of 1M ammonium formate pH 3.5 buffer and 1.6mL of extraction solvent *t*BME/ACN (3:1) w/v was added to the plasma. The extraction tubes were vortexed and mixed on a blood tube mixer for 15mins. The samples were centrifuged at 3200g for 5 minutes; the organic layer was removed with a glass pasteur pipette and 1.1mL of solvent was

transferred to conical bottomed glass LC autosampler vials. The vials were evaporated to dryness using a Genevac EZ-2 evaporator at ambient temperature, without light. The samples were reconstituted in 40 μ L of acetonitrile with 20 μ L injected automatically by the autosampler

2.13.6.2. Preparation of standard curve

10 μ L of plasma was added to an extraction tube, with the addition of 100 μ L of 500ng/mL internal standard and 100 μ L of analyte varying in concentration from 1ng/mL to 1000ng/mL. Samples were extracted as outlined in section 2.13.6.1. Each concentration point was extracted in triplicate. Samples were analysed by LC-MS/MS at intervals during the sample run time.

2.13.6.3. System standard preparation

A 10mL solution of lapatinib 100ng/mL and 100ng/mL dasatinib was prepared in acetonitrile and divided into 200 μ L aliquots. Aliquots were stored at -20 $^{\circ}$ C. A system standard was analysed before each run to verify the instrument was operating as normal. During the analysis of a batch of samples, system standards and mobile phase blanks were run at intervals to identify any potential instrumentation errors.

2.13.6.4. Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved using a Hyperclone BDS C18 column (150mm×2.0mm i.d., 3µm particle size) with a SecurityGuard C18 guard column (4mm×3.0mm i.d.) both from Phenomenex, UK. An optimised mixture of acetonitrile and 10mM ammonium formate pH 4 (54:46, v/v) was used as mobile phase, at a flow rate of 0.2mL/min. The column temperature was maintained at 20⁰C and the temperature of the autosampler was maintained 4⁰C. The complete chromatographic run time of each sample was 10min, which separated dasatinib and lapatinib from each other with retention times 2.3 and 5.1 minutes respectively. Peaks were quantified using Agilent Masshunter Software.

The mass spectrometer was operated using an ESI source in the positive ion detection mode. The ionisation temperature was 350⁰C, gas flow rate was 11L/min and nebulizer pressure was 50psi. Nitrogen was used as the ionisation source gas and ultrapure nitrogen, the collision cell gas.

Analysis was performed using MRM (multiple reaction monitoring) mode with the following transitions: m/z 581→m/z 365 for lapatinib, and m/z 488→m/z (231 and 401) for dasatinib, with a dwell time of 200ms.

Quantification was based on the integrated peak area as determined by the Masshunter Quantification Analysis software which quantitates the peak areas of the MRM transitions of each analyte.

2.14. References

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3. Gene expression changes in response to lapatinib

3.1 Introduction

Cancers originating from cells of the breast are among the most common female malignancies [1]. Breast cancer is a very heterogeneous malignancy and there has been extensive research conducted aimed at better subcategorising breast cancer into subtypes of the disease to better understand the implications of this heterogeneity in treatment response and survival [2]. Over-expression of HER2, defines one such subtype that has been identified [3]. Due to the downstream effects of activation of the HER2 signalling pathway, HER2 over-expression is associated with more aggressive, often treatment resistant disease with patients often presenting with the malignancy at a lower median age than the general breast cancer population. [4, 5]. Multimodality therapy (surgery and/or drug treatment with radiation therapy) is the mainstay of general treatment. However, treatment options have moved towards a more targeted approach. Current targeted therapies available for this breast cancer subtype include the monoclonal antibody trastuzumab and the dual tyrosine kinase inhibitor lapatinib. The adverse effects associated with these types of therapies are generally less severe than those of traditional chemotherapies as they target cancer cells more specifically [6].

Tyrosine kinases are a group of enzymes that play a critical role in the signalling cascades of the cell. The function of these enzymes is typically coupled to, and moderated by ligand binding (receptor) components, and receptor-coupled tyrosine kinases are involved in the phosphorylation of tyrosine receptors in targeted proteins. Many important receptor-coupled tyrosine kinases are located in the cell membrane and proteins are activated by the binding of ligands to their extracellular domain. HER2 and EGFR (epidermal growth factor receptor) are two such examples of

growth factor receptors which can homodimerise or heterodimerise with other members of the human epidermal growth factor receptor family, which, in turn, activates their tyrosine kinase moiety. The activated tyrosine kinases have critical roles in cell signalling processes such as cell proliferation and growth [7, 8]. Tyrosine kinase inhibitors (TKIs) prevent the activation of these tyrosine kinases thus inhibiting the activation of the pathways that would promote tumour cell growth and proliferation.

Despite the wide application of HER2 testing in breast cancer to stratify patients for HER2 targeting treatment, a significant proportion of HER2-positive patients do not respond to HER2-targeted therapy [9]. In recent studies performed using lapatinib as a monotherapy, in combination with capecitabine and also with trastuzumab, clinical benefit response rates were found to range from 12.4% with lapatinib alone, 22% in combination with capecitabine and 24.7% for the combination of lapatinib with trastuzumab [10-12]. When one factors in the enormous financial cost of such medicines, there is huge need to develop methods to identify patients who will specifically benefit from treatment. We have therefore sought to use cellular models to examine and identify the gene expression changes which might be characteristic of response to treatment with lapatinib. The longer term hope being that if such gene expression changes were characteristic of human tumour response to lapatinib, they could form the basis of a diagnostic capable of very rapidly identifying patients who are responding to treatment from those who are not.

3.2 Materials and Methods

In summary, microarray data on a panel of responsive and non-response cell lines in response to lapatinib completed by GSK was analysed using the Co-inertia Analysis (CIA) technique as described in detail in section 3.3.1-4. All bioinformatic analysis was completed by Dr. Stephen Madden.

The sensitivity of the BT474, SKBR3, EFM192a, HCC1954, MDAMB453 and MDAMB231 cell lines to lapatinib was determined by measuring IC_{50} values using proliferation assays as described in section 2.2. Taqman RT-PCR was used to examine the expression of a panel of 19 genes and 9 transcription factors identified as potentially being altered in cells responding to lapatinib in a panel of cell lines (Table 3.4). These cell lines were cultured until 70% confluent at which point they underwent treatment with $1\mu\text{M}$ lapatinib for 12 hours. These optimal treatment conditions were determined based on the bioinformatics analysis undertaken (section 3.6). Following this treatment, RNA was extracted from the cells using the Qiagen RNeasy extraction kit (section 2.2). As described in sections 2.8 and 2.9 the extracted RNA underwent reverse transcription followed by Taqman RT-PCR to evaluate the gene expression changes in the cells following treatment with the drug. Using the comparative cycle threshold method, the relative expression of these genes was examined.

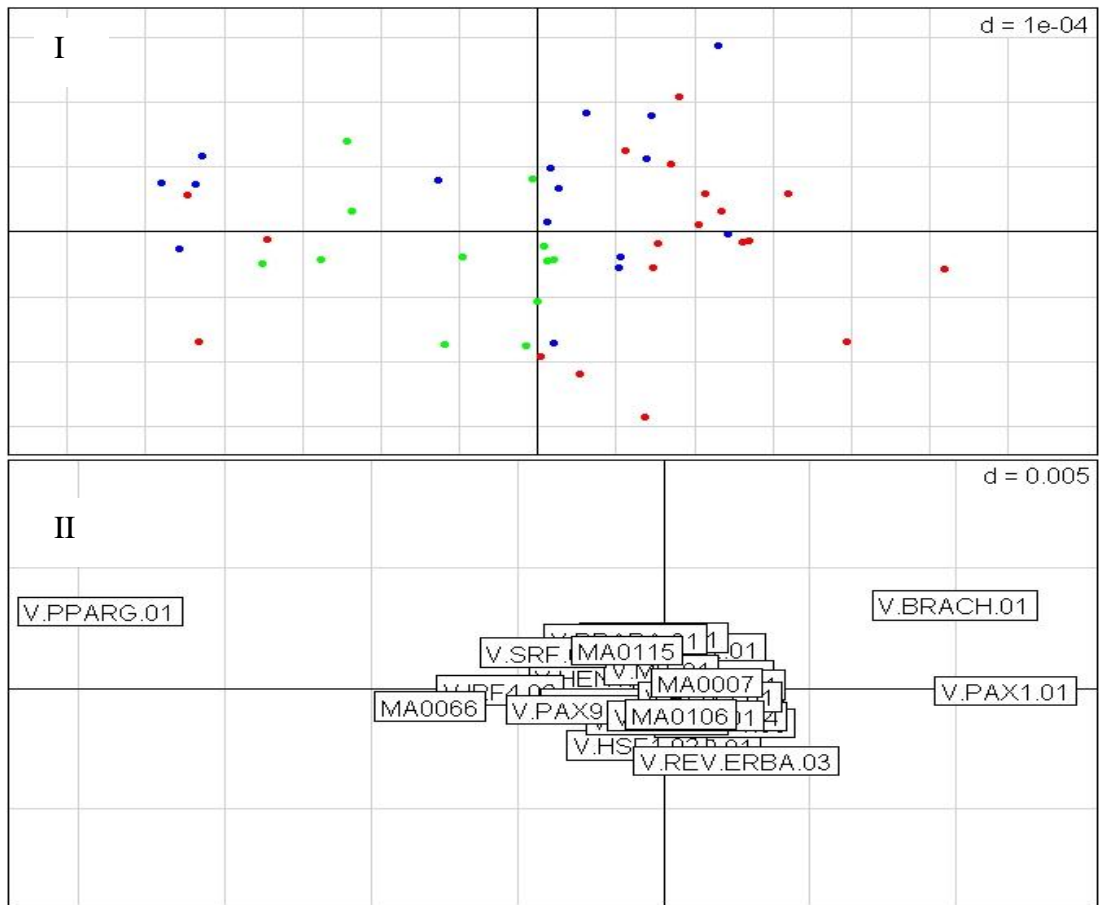
3.3 Results

3.3.1 GSK microarray data set

Microarray data provided by GlaxoSmithKline (GSK) formed the back bone for this study. Completed and published by Hedge *et al.* [13], the microarray analysis consisted of gene expression data from 4 cell lines; BT474, SKBR3, T47D and MDAMB468, which were treated with varying concentrations of lapatinib at different time points. The analysis was conducted using the Affymetrix human genome HG U133A arrays containing more than 22,000 probe sets. RNA was extracted from four independent biological replicates for each cell line condition. An array for each of the following conditions was completed on each of the four cell lines; 0.1% DMSO-treated cells at 0, 2, 6, 12 and 24 hours (5 arrays), 0.1 μ M lapatinib-treated cells at 2, 6, 12 and 24 hours (4 arrays) and 1 μ M lapatinib treated cells at 2, 6 and 12 hours (3 arrays). When completed in each of the four independent replicates, there were a total of 48 arrays per cell line. When completing the bioinformatics analysis of this data set, the data associated with T47D and MDAMB468 cell lines were not taken into consideration. The hypothesis of the experiment was to initially identify genes that were differentially regulated in response to lapatinib. As T47D and MDAMB468 cell lines do not overexpress the HER2 receptor and as such are not responsive to the drug, the gene expression analysis from these cell lines would not contribute to evaluating differentially expressed genes in response to the HER2 targeted therapy lapatinib. Figure 3.1 illustrates the unsupervised CIA that was undertaken on the microarray expression data for the MDAMB468 and T47D cell lines. This analysis indicated that there was

no clear clustering of the replicate samples when the samples were labelled according to the treatment they received. Figure 3.2 and figure 3.3 illustrate the clustering that was evident in the BT474 and SKBR3 cell line data when the data for these cell lines was labelled in the same fashion. While this analysis indicated that there were no consistent gene expression changes in the MDAMB468 and T47D cell lines in response to lapatinib and as such the data from these cell lines would not be useful to identify genes indicative of response to the drug, it did highlight 15 gene targets that were found to be differently expressed in all four cell lines. These genes were disregarded from all further analysis as they would not have informed the objective of the analysis which was to evaluate genes differently expressed in responsive cell lines.

A



B

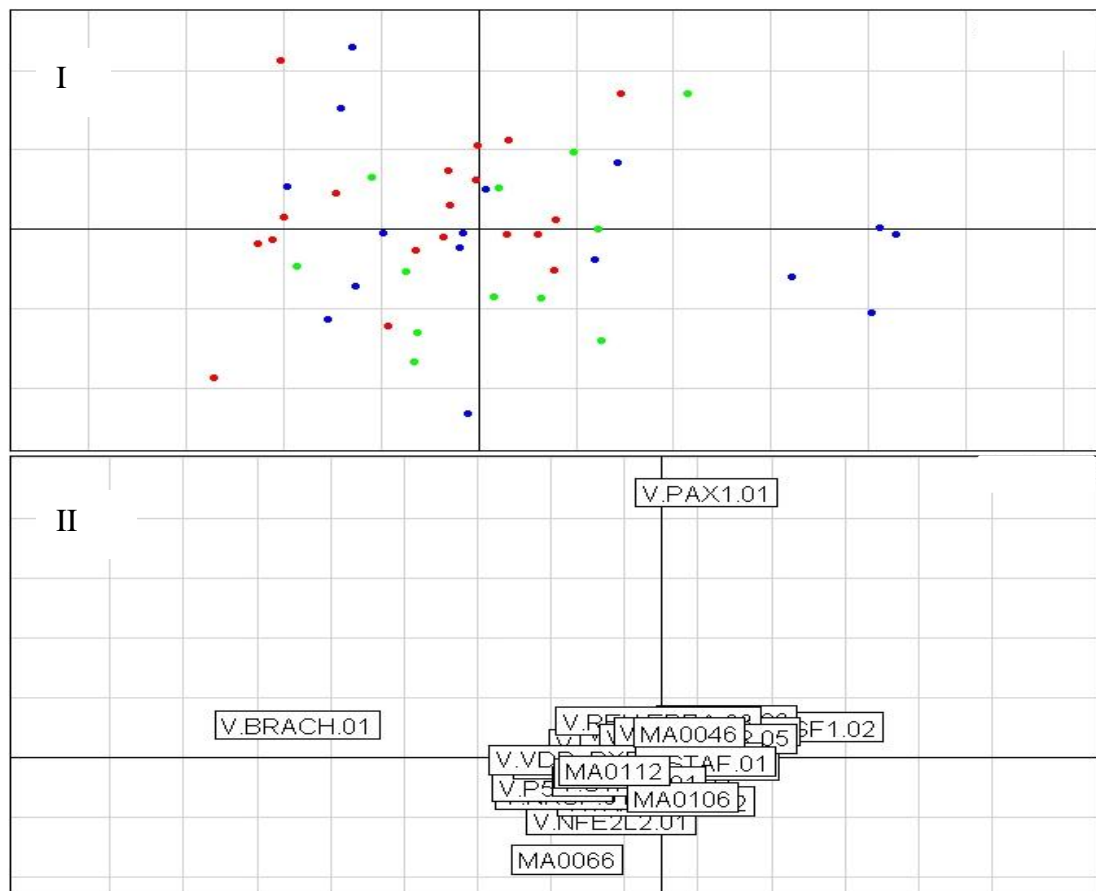


Figure 3.1: Plot of the correlation of axes 1 (horizontal) and 3 (vertical) of the unsupervised CIA for (A) MDAMB468 and (B) T47D cell line data. A gene/TFBS (transcription factor binding site) frequency table produced with a position specific scoring matrix (PSSM) threshold of 0.8 was used. (I) shows the projection of the cell line samples. There is no clear split in the 0.1% DMSO-treated samples (red), the 0.1 μ M-lapatinib treated samples (blue) or the 1 μ M lapatinib treated samples (green) in either cell line. (II) Shows the projection of the TFBS motifs. The position of the individual TFBS in (II) had a direct relationship with the genes in the same position in (I). The TFBS in (II) was predicted to regulate the expression of the genes situated at the same position in (I).

3.3.2 Co-Inertia analysis of BT474 and SKBR3 cell lines

There are a number of data mining and bioinformatics techniques that have the capability to identify differentially expressed genes from microarray data, such as canonical correlation analysis or canonical correspondence analysis. While these techniques could have been used, CIA (Co-inertia analysis) was applied in this study, as this emerging technique has previously been shown to be capable of correlating gene expression data with databases containing target predictions such as miRNA or transcription factor binding sites [14, 15]. This ability to correlate TFBS with predicted gene targets was used to guide the selection of genes for validation using Taqman RT PCR. Without this guidance from the TFBS, the gene expression data could have been evaluated using gene lists generated by between group analysis (BGA), examining the fold changes of the genes from the microarray data or clustering trends [16, 17].

CIA is a multi-variant data analysis technique that allows for the identification of trends in gene expression across multiple datasets of the same samples. CIA is flexible and is a suitable approach for unifying data to identify patterns and trends from data that contain the same sample. CIA analysis provides simple representation of the data and is robust and efficient in its application to large datasets [18, 19].

The use of the CIA multi-variant technique in this study was the first time this method was applied to breast cancer data. This analysis was used as a data exploration technique on the microarray data set and allowed incorporation of time series data at low and high drug concentrations from lapatinib-sensitive HER2-positive cell lines. This analysis, described in detail in sections 3.3.3 and 3.3.4,

yielded a list of 512 genes differentially regulated in response to the lapatinib treatment in the BT474 and SKBR3 cell lines. The six comparisons described in table 3.1, which incorporated all data from the two lapatinib responsive cell lines at all of the time points following treatment (2 hours, 6 hours, 12 hours and 24 hours) and treatment conditions (0.1% DMSO, 0.1 μ M and 1 μ M lapatinib), were utilised in the generation of the list of differentially regulated genes. The description of these comparisons was suggested by the initial unsupervised analysis. The 512 genes were found to be differentially expressed across all six comparisons and the direction of their changes was also consistent across all of the comparisons. This list of genes whose expression was altered can be found in appendix 4. In addition to this, the analysis suggested the optimal treatment conditions for the molecular validation study that was completed using qRT-PCR. These conditions were chosen based on the separation of data points observed in figure 3.2 and 3.3. These conditions were a 12 hour treatment of 1 μ M lapatinib.

Table 3.1: The six comparisons of the gene expression data which were determined by the CIA (Group 1 vs Group 2)

Comparison Number	Cell Line	Group 1	Group 1 Microarray Number	Group 2	Group 2 Microarray Number
1	BT474	6hr & 12hr 1 μ M lapatinib	8	all remaining samples	40
2	BT474	6hr & 12hr 1 μ M lapatinib	8	6hr & 12hr 0.1% DMSO	8
3	SKBR3	6hr & 12 hr 0.1 and 1 μ M lapatinib	16	all remaining samples	32
4	SKBR3	6hr & 12 hr 0.1 and 1 μ M lapatinib	16	remaining samples less 24hr 0.1 μ M lapatinib	28
5	SKBR3	12hr 0.1 and 1 μ M lapatinib	8	all remaining samples	32
6	SKBR3	12hr 0.1 and 1 μ M lapatinib	8	remaining samples less 24hr 0.1 μ M lapatinib	28

Furthermore, to identifying significantly differentially expressed genes, this CIA multivariate statistical technique was used to link transcription factor binding site target predictions and gene expression data in order to identify transcription factors (TFs) associated with the cellular response to lapatinib [15, 20]. CIA allowed us to identify commonality between the expression of the genes and the TFs that are predicted to target these genes. It also provided a ranked list of TFs predicted to be associated with the cellular response to lapatinib.

To complete the CIA analysis, the microarray expression data underwent two methods of analysis. Un-supervised analysis, which assumes that there is no prior knowledge of the sample data, allowed for the clustering of groups of similar samples e.g. the same treatment concentration or time exposed to drug. This form of analysis is not driven by any hypothesis, it is used to evaluate the clustering of samples that behave similarly. Supervised analysis evaluated the number of differentially expressed genes in response to the drug treatment. Supervised analysis requires a hypothesis to be in place to apply to the data. The hypothesis that genes would be differentially expressed following exposure to a targeted therapy for a period of time formed the basis of our supervised analysis. Thus the unsupervised step allowed for data exploration and the identification of interesting trends or splits in the data as a result of the lapatinib treatment and trends in the predicted TFBS (transcription factor binding sites) frequency tables. The supervised analysis allowed us to identify which TFs were specifically associated with the response to lapatinib and ranked them in order of importance. This ranked list aided in the selection of the genes that were chosen for validation using Taqman RT-PCR, as the majority of the genes selected were predicted to be regulated by one of the eight ranked TFs (table 3.4).

3.3.3 Unsupervised Co-Inertia Analysis identified clear and consistent gene expression changes in the BT474 and SKBR3 cell lines

In analysing the data from the responsive cell lines (BT474 and SKBR3), CIA was employed to simultaneously analyse mRNA expression levels arising from the arrays with the detail on transcription factor binding sites (TFBS) in the promoters of the genes in the data. Unsupervised CIA was applied to the data from the 48 microarrays for each of the BT474 and SKBR3 cell lines and the associated gene/TFBS frequency tables and was used to identify underlying trends in the data in each of the cell lines. The TFBS tables were generated from information known about 1236 known TFBS from three publications[21-23] and predicted TFBSs for ~17,000 genes. The ultimate aim of this analytical approach was to identify the transcription factors (TF) responsible for the gene expression change trends in the data which would therefore also identify the differentially regulated genes these TFs were predicted to target. The results of the unsupervised CIA of the BT474 and SKBR3 cell lines are shown in figures 3.2 and 3.3 respectively. CIA was used to combine two linked datasets (two sets of measurements on the same objects) and perform two simultaneous non-symmetric correspondence analyses (NSC) and identify the axes that are maximally co-variant [19, 20]. The use of an ordination method such as NSC allowed us to summarise the data in a low dimensional space. In this case, the two linked datasets were normalised gene expression data from the lapatinib-treated cell lines and TFBS information for the same genes.

3.3.3.1 Unsupervised co-inertia analysis of the BT474 cell line identifies a separation of 6- and 12-hour 1 μ M lapatinib-treated samples from those treated for 24 hours

Axes one and three of the CIA for BT474 are plotted in figure 3.2. This allowed us to evaluate the response in the BT474 cell line to the 1 μ M lapatinib treatment at various time points. Axes one and three were chosen as they represent the dominant split within the data. The samples are represented as different colours based on treatment. The samples at 6 hours and 12 hours post treatment with 1 μ M lapatinib (green) clearly separate from those treated with 0.1% DMSO (red) with 0.1 μ M lapatinib (blue) and those 2 hours post treatment with 1 μ M lapatinib (green). With the exception of the 2 hour 0.1 μ M treatment, all 1 μ M treated samples form a clear separation from all of the other samples. However, no difference was observed in the data from the 0.1 μ M lapatinib-treated cells and 0.1% DMSO-treated cells. This suggested a dosage-dependant response in that a data separation only occurred between the data from control samples and the high dose lapatinib samples, with the exception of one outlier on the far right of the plot. The lack of sample separation at 2 hours post treatment with 1 μ M lapatinib suggested that the gene expression effects of the drug were not yet apparent at this time point. These observations guided our choice of comparisons for both the supervised CIA and the differential gene expression analysis which are summarised in Table 3.1.

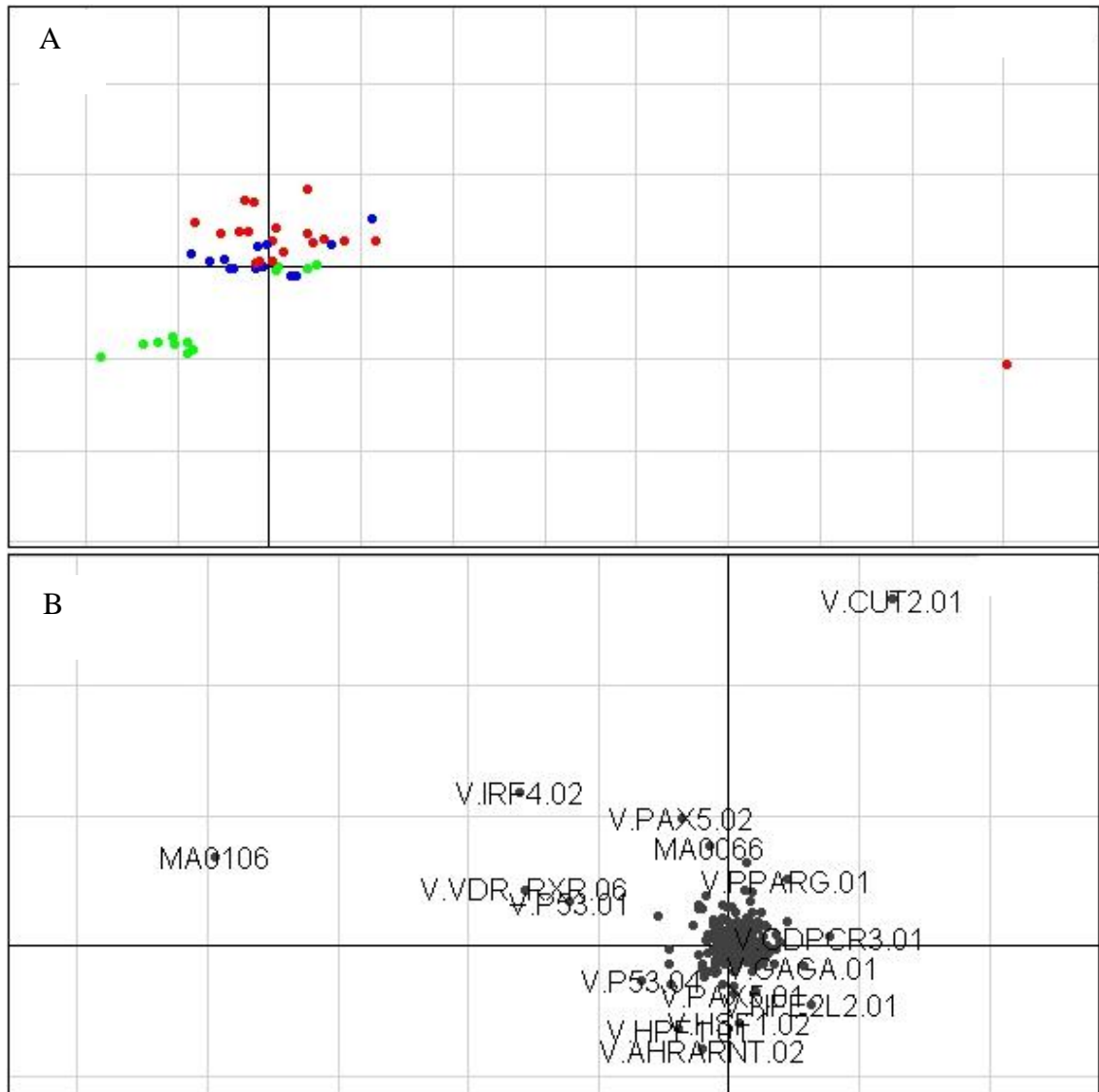


Figure 3.2 Plot of the correlation of axes 1 (horizontal) and 3 (vertical) of the unsupervised CIA for BT474 cell line data. A gene/TFBS frequency table produced with a position specific scoring matrix (PSSM) threshold of 0.8 was used. (A) shows the projection of the cell line samples. The 0.1% DMSO-treated samples (red), the 0.1 μ M-lapatinib treated samples (blue) are split from the 1 μ M lapatinib treated samples (green), the exception being the four 1 μ M lapatinib treated samples at 2 hours post treatment. (B) Shows the projection of the TFBS motifs. The position of the individual TFBS in (B) had a direct relationship with the genes in the same position in (A). The TFBS in (B) was predicted to regulate the expression of the genes situated at the same position in (A).

Figure 3.2B shows the TF motifs associated with the genes separating the data. The most extreme motifs along each axis are labelled and named. Those motifs furthest from the origin in the same orientation as the split of interest shown in figure 3.2A are most associated with that split. V.AHRARNT.02 was the motif most associated with the separation of 1 μ M lapatinib-treated cells from the other samples and therefore was the motif most associated with the response to lapatinib. This was the motif for the agonist-activated heterodimer AHR/ARNT (Aryl hydrocarbon receptor/Arnt (hypoxia inducible factor 1 beta)) which directly associates with the estrogen receptors ER-alpha and ER-beta in ER-positive breast cancer, although its function in HER2-positive breast cancers is not well characterised[24].

These results indicated that the BT474 gene expression response was dose-dependent.

3.3.3.2 Unsupervised co-inertia analysis of the SKBR3 cell line identifies a separation of 6- and 12-hour 0.1 μ M and 1 μ M lapatinib treatment samples from the 24-hour 0.1 μ M lapatinib treatment samples.

Figure 3.3A shows axes one and two of the CIA for the SKBR3 cells. The samples are labelled as before based on treatment. There was a clear split between the 0.1 μ M (blue) and 1 μ M (green) lapatinib-treated cells at 6 and 12 hours post treatment from the 0.1% DMSO treated cells (red), with the exception of one outlier. As with the BT474 cell line, there was no separation at 2 hours post treatment with 0.1 μ M and 1 μ M lapatinib suggesting that the effects of the drug are not yet apparent at this time point in both cell lines. However, in this cell line the split occurred at both the 0.1 μ M and 1 μ M lapatinib dosages. Again, as with the BT474 data, these analyses were used to guide our comparisons for the supervised CIA and the gene expression analysis (Table 3.1).

The TF motifs associated with this split in the data are in the same orientation relative to the origin as the genes separating the data in Figure 3.3B. These include the VDR/RXR heterodimer (V.VDR_RXR.06, vitamin D receptor/retinoid X receptor). The expression of the VDR/RXR heterodimer has been previously associated with numerous cancers, including breast cancer. It has been suggested that its expression of this heterodimer may indicate a patient population that may response better to adjuvant therapy[25].

These results indicated that the SKBR3 gene expression response was also a time-dependent response.

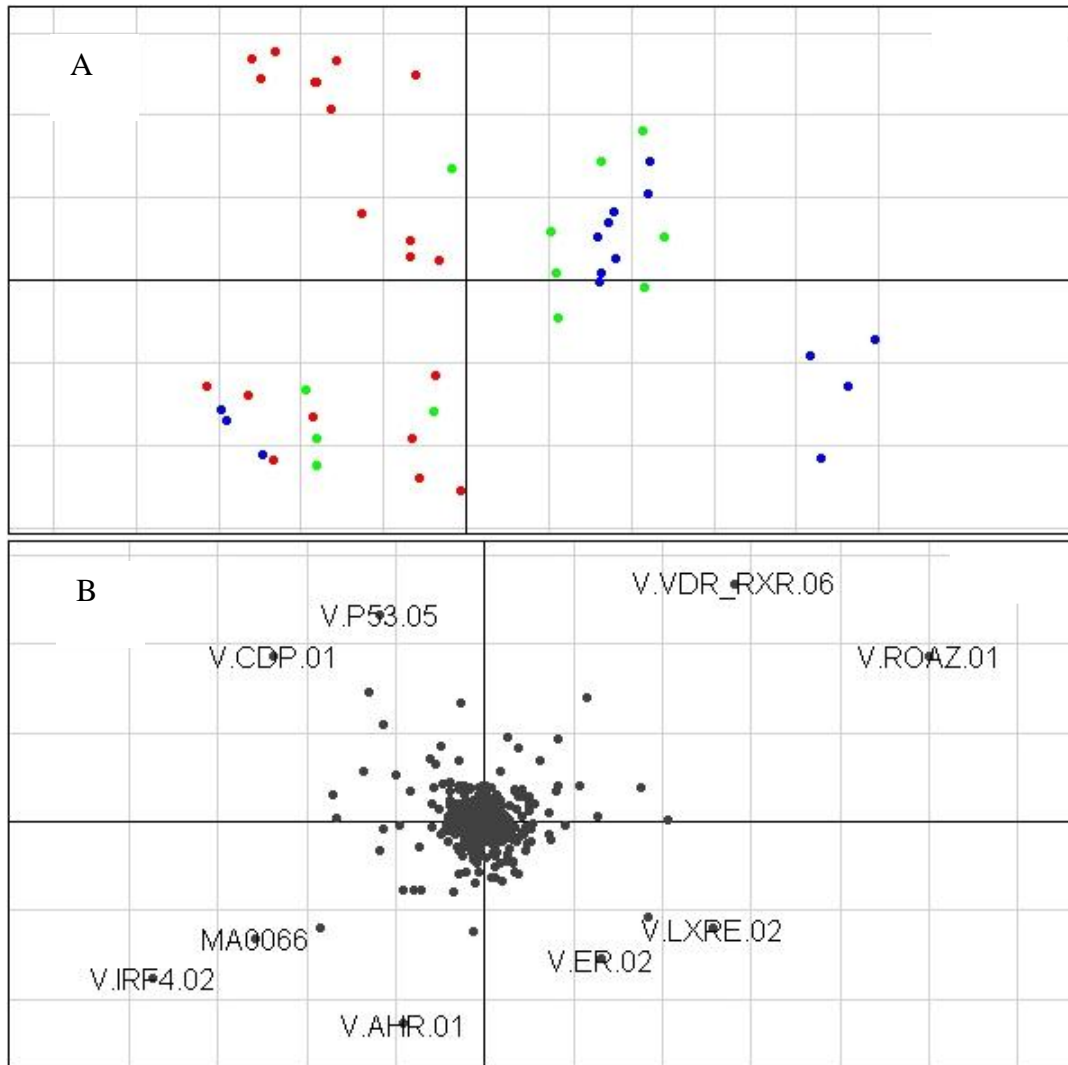


Figure 3.3: Plot of the correlation of axes 1 (horizontal) and 2 (vertical) of the unsupervised CIA for SKBR3 cell line data. A gene/TFBS frequency table produced with a PSSM (position specific spacing matrix) threshold of 0.8 was used. The PSSM gives the log-odds score for finding a particular motifs in a target sequence. (A) shows the projection of the cell line samples. The 0.1% DMSO-treated samples (red), are split from the 0.1 μ M lapatinib-treated samples (blue) and the 1 μ M lapatinib-treated samples (green), the exception being the eight 0.1 μ M lapatinib and 1 μ M lapatinib-treated samples at 2 hours post treatment. (B) Shows the projection of the TFBS motifs. . The position of the individual TFBS in (B) had a direct relationship with the genes in the same position in (A). The TFBS in (B) was predicted to regulate the expression of the genes situated at the same position in (A).

3.3.4 Supervised CIA identifies 8 putative transcription factors associated with the response to lapatinib

In order to systematically identify the TFBSs specifically associated with the response to lapatinib in the two responsive cell lines, we performed a supervised analysis of the data, combining CIA and Between Group Analysis (BGA). BGA coordinates the expression data from sets of grouped microarray samples described in table 3.1 [17]. CIA was performed twice in the BT474 dataset and four times in the SKBR3 dataset to perform all comparisons described in table 3.1. The description of these comparisons was provided by the most distinct separation of the data in the unsupervised analysis (sections 3.3.4 and 3.3.5). This resulted in six ranked lists of TFBS associated with a response to lapatinib treatment. The 8 transcription factor motifs (representing 8 individual transcription factors) which were consistently ranked highly across the six comparisons are displayed in Table 3.2. From these motifs we can infer the transcription factors which are most important in driving the response to lapatinib in these cell lines.

Table 3.2: The ranked list of TFs associated with the response of BT474 and SKBR3 to lapatinib.

TF	Motif ID	Description
RAR	V.RAR_RXR.02	Retinoic acid receptor
RXR	V.RAR_RXR.02	Retinoid X receptor
ARNT	V.AHRARNT.02	hypoxia inducible factor 1 beta
AHR	V.AHRARNT.02	Aryl hydrocarbon receptor
ZNF143	V.STAF.02	Zinc finger protein 143
PAX9	V.PAX9.01	Paired box gene 9
OLF1	V.OLF1.01	Olfactory neuron-specific factor
PAX3	V.PAX3.01	Paired box gene 3

3.4 Selection of breast cancer cell line panel

The analysis of the microarrays outlined in the previous sections was undertaken in SKBR3 and BT474 breast cancer cell lines. Both of these cell lines are HER2 overexpressing and have been previously shown to be responsive to lapatinib[3]. Proliferation assays were performed on these cell lines to determine their sensitivity to lapatinib. In order to examine if this sensitivity corresponds to the magnitude of the differential gene expression, a further four cell lines known to have different levels of lapatinib sensitivity were also analysed using the methods outlined in section 2.2 (Table 3.3). The additional cell lines chosen were the lapatinib-sensitive HER2over-expressing EFM192A and HCC1954, the lapatinib-insensitive HER2 over-expressing MDAMB453 and the lapatinib-insensitive triple negative MDAMB231. A triple negative cell line is one that does not expression the oestrogen receptor (ER), progesterone receptor (PR) nor overexpress HER2. For our purposes we defined an insensitive cell line as one demonstrating a drug IC_{50} greater than $1\mu M$ [3].

Table 3.3: Lapatinib IC₅₀ values for the panel of breast cancer cell lines employed in this programme n=3 (biological replicates).

	Cell Line Name	IC₅₀ ± SD (µM)
Lapatinib-Sensitive Cell Lines	BT474	0.036 ± 0.015
	SKBR3	0.080 ± 0.017
	EFM 192A	0.193 ± 0.067
	HCC1954	0.416 ± 0.180
Lapatinib-Insensitive Cell Line	MDAMB453	6.08 ± 0.83
Triple Negative Cell Line	MDAMB231	7.46 ± 0.10

3.5 Determination of appropriate endogenous control genes

An endogenous gene control was required for this analysis as it was necessary for the calculation of the relative expression of the genes in response to lapatinib [26]. For a particular gene to be useful as endogenous control it should be expressed at a constant level across different sample types and should not be affected by experimental treatments [27, 28]. The C_t values of each of the evaluated genes were normalised to the C_t value of the endogenous control to provide the ΔC_t value of that gene.

Four endogenous control genes (*GAPDH*, *GUSB*, *18S* and *HPRT1*) were examined for their suitability and were quantitated using Taqman RT-PCR. These endogenous controls are commonly employed in the literature [28, 29]. In order to evaluate which would be most suitable in the comparative threshold analysis, the C_t values of the four candidate genes in the BT474 and SKBR3 cells lines (untreated and treated with 1 μ M lapatinib in triplicate) were examined to evaluate the variability of the expression of the putative control genes. The standard deviations (SD) of the triplicate untreated and treated C_t values of both cell lines at both conditions were calculated (Figure 3.3). The SDs resulting from these analyses were as follows; *18s* = 1.14, *GAPDH* = 0.92, *GUSB* = 0.77 and *HRPT* = 1.23. The SD for *18s* and *HRPT* were deemed to be too high so they were eliminated as endogenous control candidates. In choosing between *GAPDH* and *GUSB*, with the SD values being quite similar, *GAPDH* was chosen over *GUSB* for use as an endogenous control in further studies as it demonstrated a higher level of expression with C_t values of ~18, while the *GUSB* had C_t levels ~26. *GAPDH* is also a very well established control in the literature [30, 31].

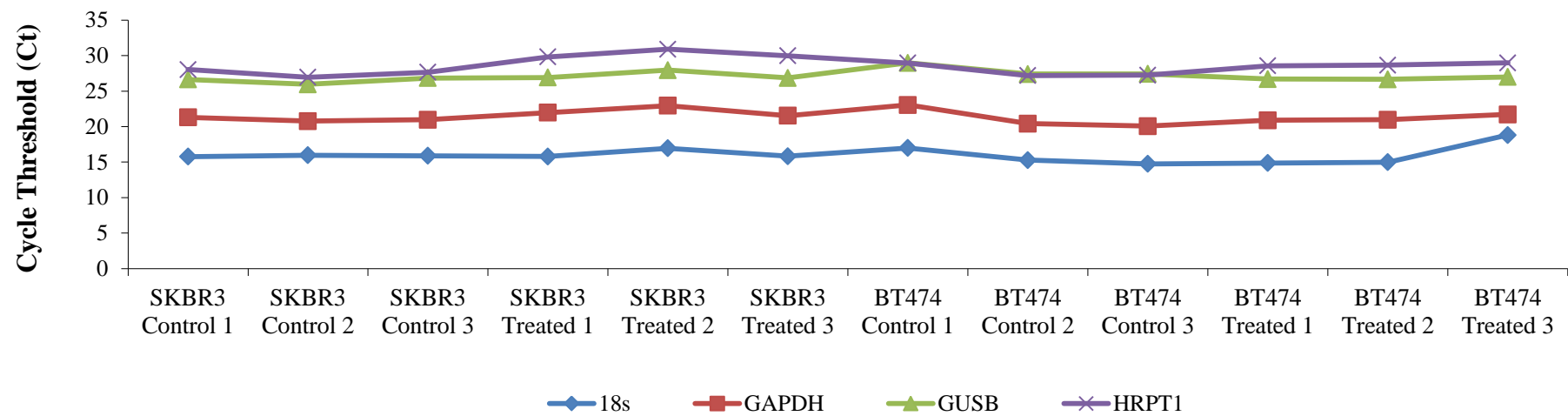


Figure 3.4: Determination of endogenous controls in SKBR3 and BT474 cell lines. Triplicate (untreated and treated with 1 μ M lapatinib for 12 hours) C_t values were evaluated and the standard deviation values were calculated. Utilising a combination of both of these values *GAPDH* was determined to be the most suitable endogenous control.

3.6 Expression of eight transcription factors in response to lapatinib

Taqman RT-PCR analysis was performed to validate the transcription factor expression in lapatinib-treated BT474 and SKBR3 breast cancer cell lines. Treatment with 1 μ M lapatinib for 12 hours was chosen as the optimal condition for treating the cells based on the separations seen in CIA analysis (Figures 3.2 and 3.3). In addition, 1 μ M of lapatinib is a clinically relevant concentration [32]. These two cell lines are highly sensitive to lapatinib with IC₅₀ values of 0.036 μ M \pm 0.015 μ M and 0.080 μ M \pm 0.017 μ M respectively (table 3-1) [3]. Four additional cell lines were also chosen based on their sensitivity to lapatinib (EFM192A, HCC1954, MDAMB453 and MDAMB231). Their IC₅₀ values are shown in Table 3.3.

The expression levels of eight TFs described in table 3.2 were assessed. Six of the eight transcription factors were found to be expressed following 1 μ M 12hr lapatinib treatment relative to untreated controls (Figure 3.5). C_t values for these TFs were all determined to have values between 27 and 29. Although these TFs were not identified as significantly differentially expressed in the microarray analysis, they were clearly dysregulated in these cell lines, as predicted by CIA. Two of the predicted transcription factors (*PAX3* and *OLFI*) were not expressed (data not shown). While the expression of the TFs did not follow an obvious pattern, there were some distinct trends. For example, all of the TFs were up-regulated following treatment in the most lapatinib-sensitive cell line (BT474) and nearly all were down-regulated following lapatinib treatment in the most lapatinib-insensitive cell line

(MDAMB453). In addition, ARNT was up-regulated following lapatinib treatment in all lines, apart from MDAMB231, the triple negative cell line.

It should be noted that even the smallest changes in the expression of any of the TFs could have had some major effects. This would be due to the key role that these TFs play in a large number of pathways so as such large changes in the expression of these genes would not be expected.

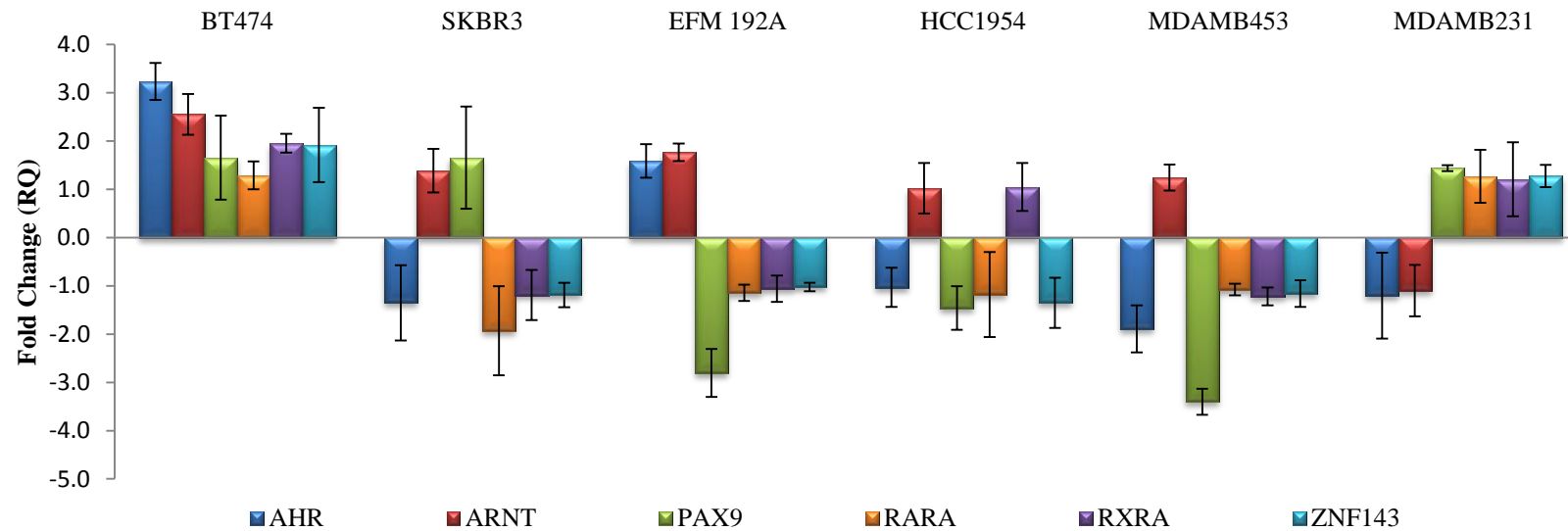


Figure 3.5: The expression of transcription factors (TFs) was evaluated in the six cell line panel following 1 μ M lapatinib for 12 hours and are ordered left to right in order of importance from the ranked list generated from the CIA (table 3.2). Although these TFs were not identified as significantly differentially expressed in the microarray analysis, they are clearly dysregulated in these cell lines as predicted by the CIA. The cell lines are represented in order of lapatinib sensitivity, with BT474 being the most sensitive and MDAMB231 being the triple negative insensitive cell line. The analysis was completed in triplicate the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

3.7 Expression of differentially expressed genes in response to lapatinib

From the panel of 512 genes of interest arising from the original CIA analysis, a subset of 19 genes was selected for more detailed validity analysis. The genes were selected on the basis of the following criteria; a large alteration in expression following treatment, the expression of the gene was predicted to be regulated by one or more of the ranked TFs or the gene was known to play an important role in some aspect of cancer biology. (Table 3.4). As with the TFs, the 19 genes (*CCND1*, *ERBB3*, *FOXO3a*, *NR3C1*, *RB1CC1*, *ALDH3A2*, *CDKN1B*, *PIK3C3*, *AKT1*, *BID*, *E2F3*, *eIF4E*, *FKBP4*, *MAPK9*, *PARP2*, *PSMD13*, *SLC29A1*, *TFPT* and *CBFA2T2*) were first analysed for expression in BT474 and SKBR3 cells that had been treated with 1 μ M lapatinib for 12 hours using untreated cells as a control (Figure 3.5). Table 3.4 summarises the basic characteristics of these genes of interest. The analysis was first completed in these two cell lines as they were the cell lines used by Hedge *et al.*, [13] in the microarray analysis. Eight of the 19 genes (*CCND1*, *ERBB3*, *FOXO3a*, *NR3C1*, *RB1CC1*, *ALDH3A2*, *CDKN1B* and *PIK3C3*) were found to be differentially expressed with an RQ value of $\geq \pm 2$ in both the BT474 and SKBR3 cell lines. The remaining 11 genes (*AKT1*, *BID*, *E2F3*, *eIF4E*, *FKBP4*, *MAPK9*, *PARP2*, *PSMD13*, *SLC29A1*, *TFPT* and *CBFA2T2*), were also found to be differentially expressed, however, the alterations in gene expression level did not occur in both of the cell lines and hence we did not conduct any further analysis of them.

Table 3.4 : List of genes chosen for Taqman RT PCR validation.

Gene Symbol	Gene Name	Key	Known Gene Function	Specific TF that gene is predicted to be a target of
CCND1	Cyclin D1	^{1,3} ₊	regulates the cell-cycle during G(1)/S transition [33]	AHR/ARNT, PAX9,RAR/RXR
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	^{2,3} ₊	Binds and is activated by neuregulins [34]	OLF-1
FOXO3a	Forkhead box protein O3	^{1,3} ₊	Downstream target of PIK3/AKT pathway which are associated with cellular differentiation, metabolism, tumorigenesis [35]	RAR/RXR
NR3C1	nuclear receptor sub family 3 group C member 1	^{2,3} ₊	Regulated transcription of the NR3C1[36]	AHR/ARNT, PAX3
RB1CC1	RB1 inducible coiled coil protein 1	³ ₊	Inhibition of G1-S progression [37]	ZNF143
ALDH3A2	aldehyde dehydrogenase 3 family	² ₊	role in the detoxification of	

	member 2a		aldehydes generated by alcohol metabolism and lipid peroxidation [38]	
CDKN1B	cyclin dependent kindase inhibitor 1B	²⁺	Regulation of cell cycle progression [39]	
PIK3C3	phosphoinositide 3 kinase class 3	²⁺	mediates formation of phosphatidylinositol 3-phosphate [40]	
AKT1	v-akt murine thymoma viral oncogene homolog 1	³	Tumour angiogenesis[41]	RAR/RXR
BID	BH3 interacting domain	²	important component of death receptor-mediated caspase activation [42]	
E2F3	E2F transcription factor 3	^{1,3}	control the cell cycle-dependent expression of genes that are essential for cellular proliferation [43]	AHR/ARNT, OLF-1, PAX9, ZNF143
eIF4E	eukaryotic translation initiation factor 4e	³	role in cancer initiation and progression [44]	PAX3, RAR/RXR
FKBP4	Fk506 binding protein 4	^{2,3}	regulates progesterone activity [45]	ZNF143

MAPK9	mitogne-activated protein kinase 9	²	cell proliferation, differentiation, migration, transformation and programmed cell death [46]	
PARP2	poly (ADP-ribose) polymerase 2	²	DNA-break sensing and signaling[47]	
PSMD13	proteasome 26S subunit non-ATPase 13	²	processing of class I MHC peptides[48]	
SLC29A1	solute carrier family 29 member 1	²	mediates the cellular uptake of nucleosides from the surrounding medium [49]	
TFPT	TCF3 (E2A) fusion partner	^{2,3}	promotes cell differentiation [50]	ZNF143
CBFA2T2	core-binding factor, runt domain, alpha subunit 2; translocated to, 2	²	promoting leukemogenesis [51]	

¹ denotes a highly differentially regulated gene (± 2 fold across all 6 supervised CIA comparisons) ² denotes a gene that has been previously shown to be associated with cancer or was a known drug target, ³ denotes a gene was predicted to be targeted by one or more of the transcription factors. Those in bold were found to be consistently dysregulated in response to lapatinib in all 6 of the cell lines. + denotes that the gene was found to be differentially expressed in BT474 and SKBR3 cell lines but not in the additional four cell lines also tested.

For further validation, expression levels of *CCND1*, *ERBB3*, *FOXO3*, *NR3C1*, *RB1CC1*, *ALDH3A2*, *CDKN1B* and *PIK3C3* genes were measured in two additional lapatinib-sensitive cell lines; EFM192A and HCC1954. These genes were chosen for further validation as they exhibited RQ values that were greater than 2 in both the BT474 and SKBR3 cell lines. Both of these cell lines are HER2-overexpressing and have varying sensitivities to lapatinib, with IC_{50} values of $0.193\mu\text{M} \pm 0.067\mu\text{M}$ and $0.417\mu\text{M} \pm 0.18\mu\text{M}$, respectively (Table 3.3). Two lapatinib-insensitive cell lines were also analysed, MDAMB453 and MDAMB231.

In the lapatinib-sensitive cell lines (BT474, SKBR3, EFM192A and HCC1954), five of the eight genes (*RB1CC1*, *FOXO3a*, *NR3C1*, *ERBB3* and *CCND1*) analysed showed differential expression post lapatinib treatment and are highlighted in bold in table 3.4. The relative expression of three genes that did not follow this trend (*PIK3C3*, *ALDH3A2* and *CDKN1B*) is shown in figure 3.7. It should be noted however, that this expression trend was evident in the BT474 and SKBR3 cell lines but not in the other four cell lines. Thus these genes would not be considered as robust indicators of response to the lapatinib.

Figure 3.6 shows the expression of *RB1CC1*, *FOXO3A*, *NR3C1*, *ERBB3* & *CCND1* in the four lapatinib-sensitive cell lines and clearly demonstrates a correlation between the degree of sensitivity of the cell line to lapatinib and the magnitude of gene expression change. BT474 was the most lapatinib-sensitive cell line and has the highest expression values for the four up-regulated genes. As the cell lines become less sensitive to lapatinib, the magnitude of gene expression decreased. In contrast, in the lapatinib-insensitive cell lines, MDAMB453 and MDAMB231, the differential

expression of these genes was seen to be down regulated following lapatinib exposure.

In the case of *CCND1*, the differential gene expression pattern followed a largely proportional response across the various cell lines. In the lapatinib-sensitive cell lines expression of this gene was found to be strongly down-regulated following the 12hr treatment. The magnitude of this down-regulation was reduced as the cells became more lapatinib-insensitive (Figure 3.6).

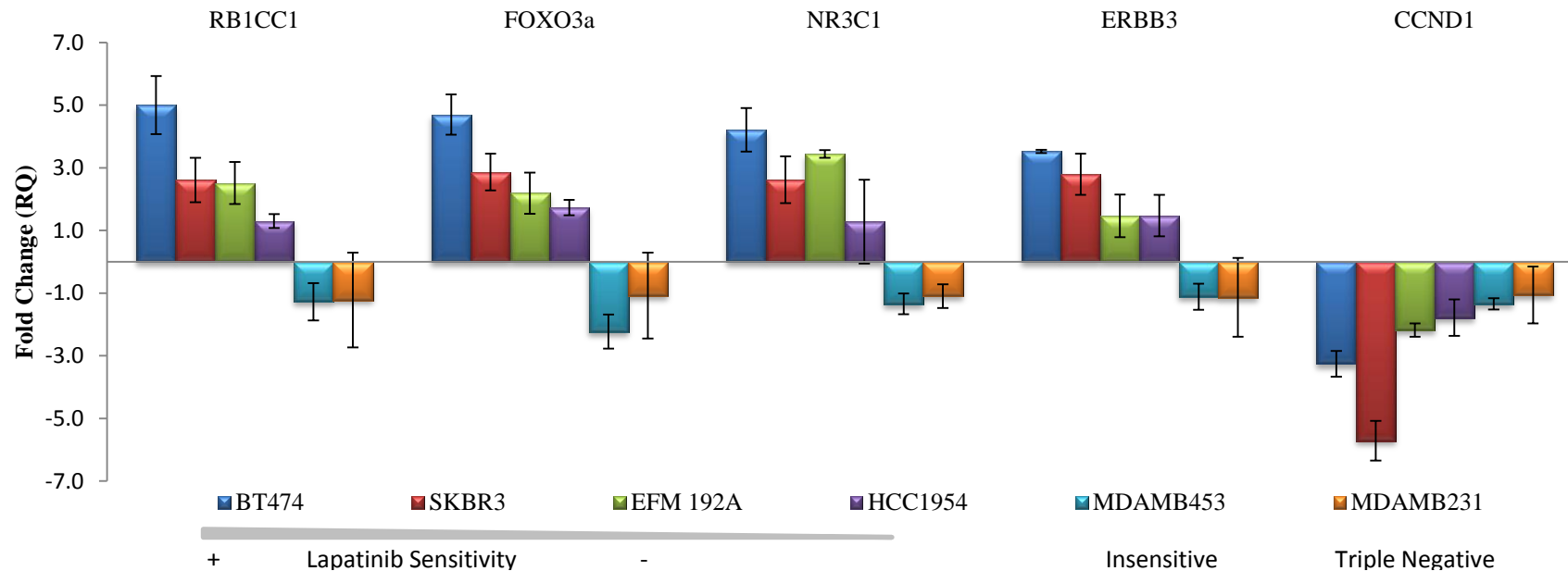


Figure 3.6: The differential expression of five genes was examined following 1µM lapatinib treatment for 12 hours in a panel of six breast cancer cell lines with varying degrees of lapatinib sensitivity. It was established that the degree of dysregulation of the genes in each of the cell lines was proportional to the response to lapatinib. The cell lines are represented in order of sensitivity to lapatinib, with BT474 being the most sensitive and MDAMB231 being the least. The analysis was completed in triplicate the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

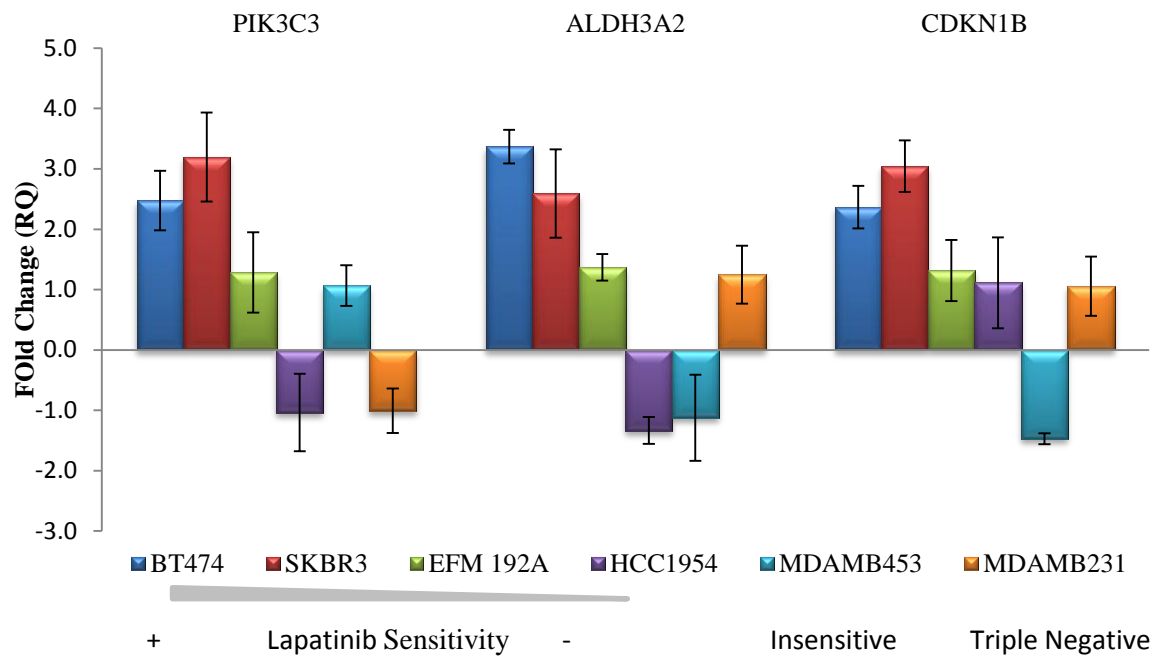


Figure 3.7: The differential expression of three genes was examined following 1µM lapatinib treatment for 12 hours in a panel of six breast cancer cell lines with varying degrees of lapatinib sensitivity. There were some differences in the expression pattern of these three genes when compared with the five genes examined in figure 3.6, in particular in the HCC1954 cell lines, The cell lines are represented in order of sensitivity to lapatinib, with BT474 being the most sensitive and MDAMB231 being the least. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

3.8 Comparison of pre-seeded TaqMan plates vs individual TaqMan assays.

For the initial validation of the bioinformatic predictions from the CIA (Section 3.7) custom pre-seeded Taqman RT-PCR plates were used for the analysis. These custom pre-seeded Taqman RT-PCR plates are manufactured with the selected genes assays lyophilised to the wells of the 96 well plates. All cell lines were examined in biological triplicate using these plates. In using these custom pre-seeded plates, it was possible to validate the expression of a larger number of genes using a small concentration of target material. When further analysis on a sub-set of eight genes was required, individual gene assays were utilised. These individual assays were employed as they were more versatile and cost effective for the smaller number of gene targets. The cell lines were examined in both biological and technical triplicate using the individual assays. These individual assays were prepared by the addition of the Taqman universal master mix with the individual assay and the sample cDNA. For the pre-seeded plates the Taqman universal master mix in combination with the cDNA for the sample was added to each well as the assay was already present in the well.

In figure 3.8 the fold changes calculated from the custom pre-seeded Taqman plates and the individual assays have been compared. The trend that was exhibited in the initial analysis with the pre-seeded plates remained evident in the analysis completed with the individual assays. However, in the BT474 cell lines, there was a large increase in the magnitude of expression of *RB1CC1*, *FOXO3a* and *PIK3C3* using the individual assays.

No published data could be found which could explain the difference that was observed. It is possible that there was some evaporation of the assays which were lyophilised on to the pre-seeded plates. There was no difference in the C_t values for the endogenous controls between the pre-seeded plates and the individual assays with all of them showing values between 21 and 22. However, there was some variation in the C_t values of the target genes which account for the changes in magnitude of gene expression. For all further gene expression analysis, the individual assays were utilised as they were more suitable for the smaller number of genes being validated.

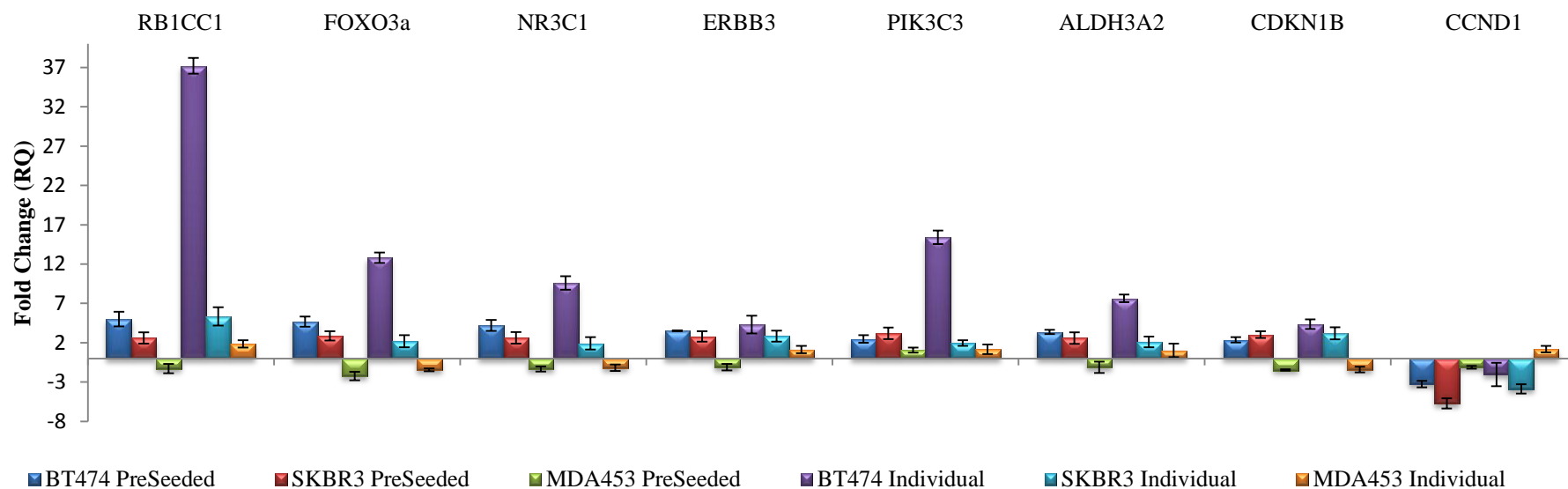


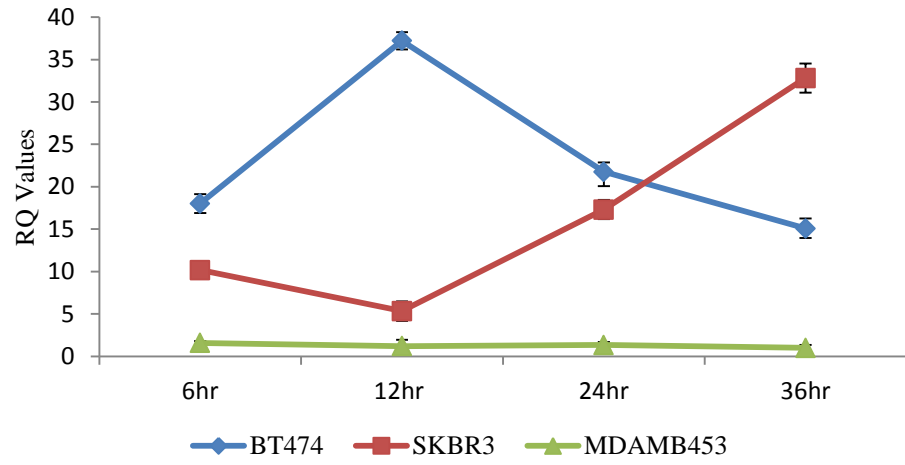
Figure 3.8: Differential gene expression of significant genes using both preseeded custom Taqman plates and individual gene assays. Analysis was completed on all six cell lines and the cells lines were treated with 1 μ M lapatinib for 12 hours. The preseeded custom taqman plates were used for the initial validation of the 27 targets and the individual gene assays were used for all other analysis completed following the selection of the gene targets. The main difference between the two is exhibited, particularly, in the BT474 cell lines which showed higher fold change values for all of the gene targets using the individual assays. The analysis was completed in triplicate the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

3.9 Evaluation of the lapatinib gene expression profile in a sub-panel of lapatinib-treated breast cancer cell lines over time

In order to establish if the altered expression of the genes that had been shown in figure 3.6 and figure 3.7 was stable over a period of time, lapatinib-induced gene expression changes over different durations of time were quantitated. Three cell lines were chosen for this study; BT474 and SKBR3, the most lapatinib-sensitive cell lines and MDAMB453, the HER2-overexpressing lapatinib-insensitive model. The cells were treated for 6hrs, 24hrs and 36hrs with 1 μ M lapatinib. The 12hr treated cells were also re-analysed using the individual Taqman RT-PCR assays. The expression of the five genes (*RB1CC1*, *FOXO3a*, *NR3C1*, *ERBB3* and *CCND1*) in the lapatinib-treated cell lines were compared with those of the untreated cell lines, see figure 3.9.

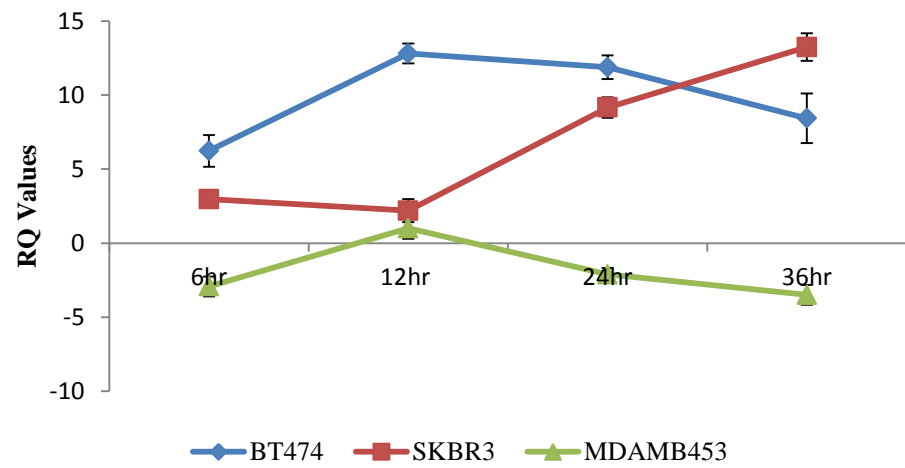
A

RB1CC1



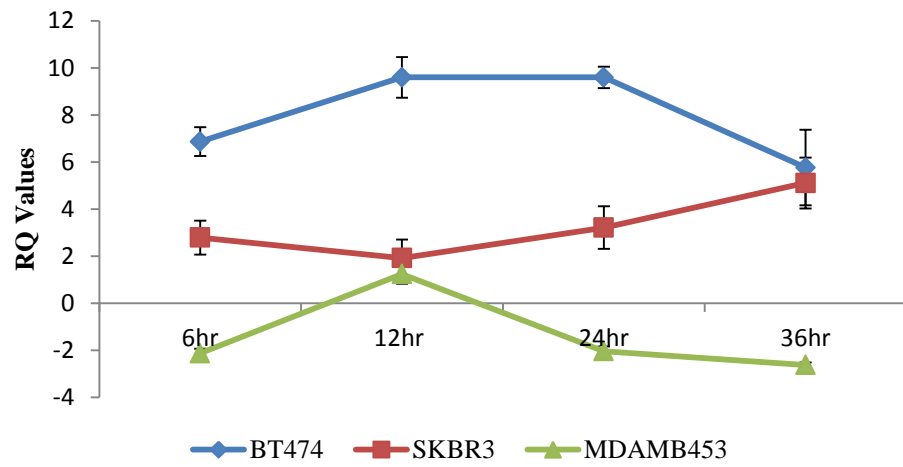
B

FOXO3a



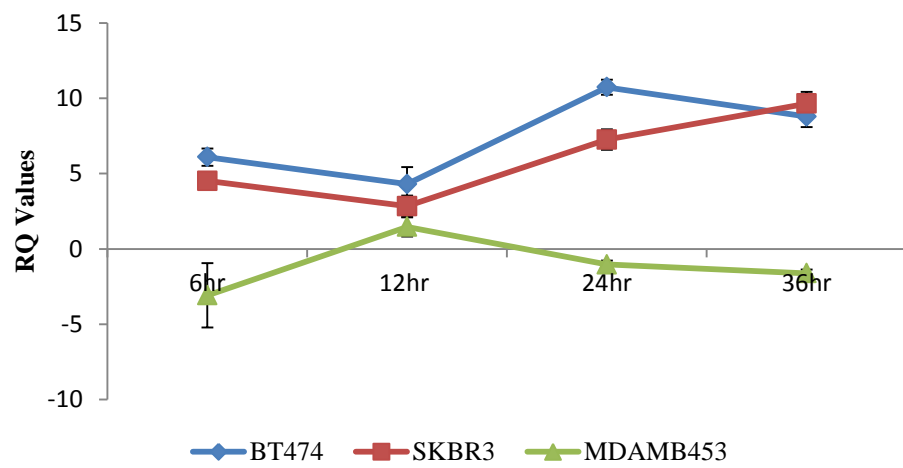
C

NR3C1



D

ERBB3



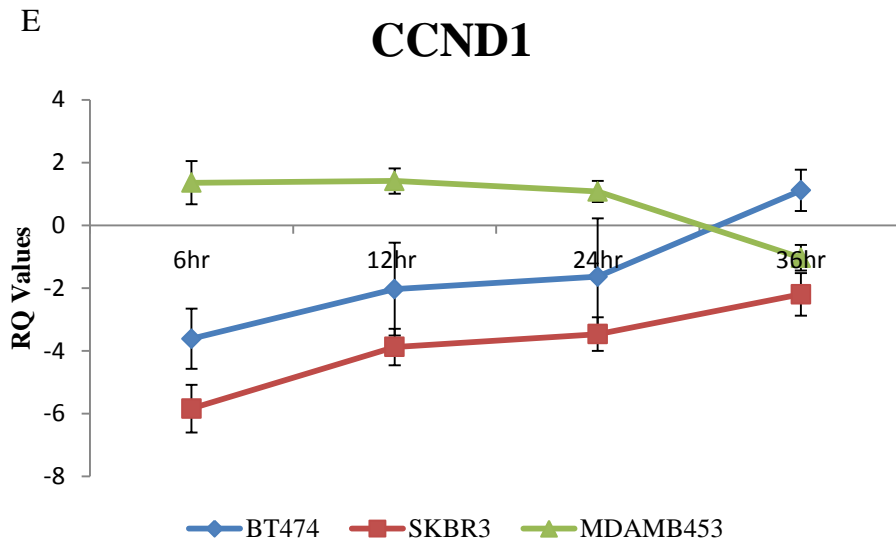


Figure 3.9: Analysis of (A) *RB1CC1*, (B) *FOXO3a*, (C) *NR3C1*, (D) *ERBB3* and (E) *CCND1* in BT474, SKBR3, and MDAMB453 over 4 different time points. Cell lines were treated with 1 μ M lapatinib for 6, 12, 24 and 36 hours. This analysis was undertaken to determine how robust the differential expression of the genes were over a time course. The analysis indicated that the expression of the genes remained consistent to a time point of 36 hours. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

In figure 3.9, the alteration in the relative expression of each gene has been graphed independently showing the three cell lines that were tested over the 36 hour time period. The expression of *RBICC1* (figure 3.9 A) in the lapatinib-insensitive cell line MDAMB453 was found to be unchanged over the 36 hour time period. In comparison, the lapatinib-sensitive cell lines BT474 and SKBR3 demonstrated considerable up-regulation of *RBICC1* in response to the lapatinib treatment over the 36 hours.

FOXO3a (figure 3.9 B) expression in the MDAMB453 cell line was found to be down-regulated in response to lapatinib. The lapatinib-sensitive BT474 cells showed a slight decrease in *FOXO3a* expression following the 12 hour treatment but the expression remained consistently up-regulated. As with the expression of *RBICC1*, in the SKBR3 cells, the expression of *FOXO3a* increased after the 12 hour time point, and at 36 hours the expression was higher in the SKBR3 cell lines than it was in the BT474 cells.

NR3C1 (figure 3.9 C) expression slightly increased in the MDAMB453 cell line from 6 to 12 hours, after which its expression became down regulated in the remaining time points. The expression in the lapatinib-sensitive cell lines showed a slight increase after 12 hours in the SKBR3 and a higher level of expression in the BT474, with a decrease at 36 hours.

The expression of *ERBB3* (figure 3.9 D) in the MDAMB453 cell lines was shown to be predominately down regulated. In the SKBR3 and BT474 cell lines, The expression of the gene, showed a constant up-regulation across all the time points.

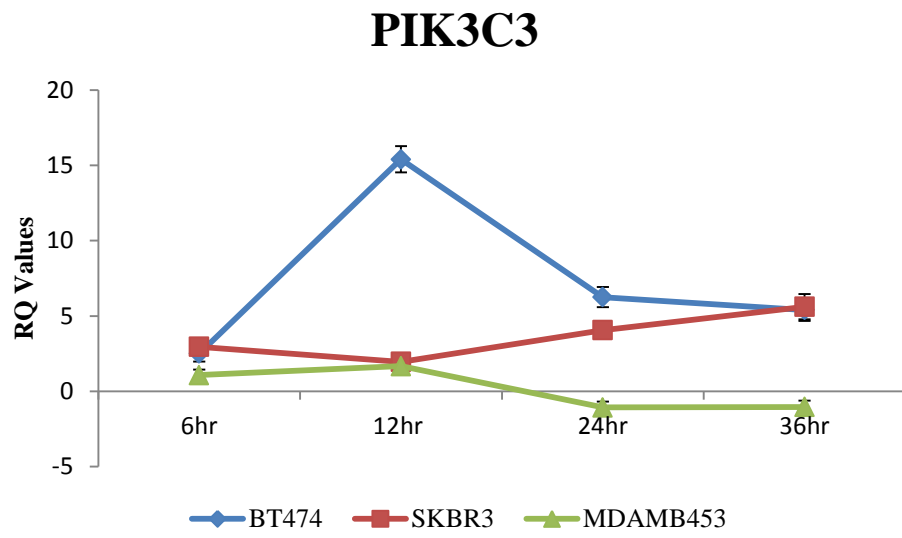
The last of the genes, *CCND1* (figure 3.9 E), was shown to be unchanged in response to the lapatinib treatment over the time course. The response was

considered unchanged as it had a fold change less than two. As was found in the initial analysis of these genes, there was a down-regulation in the expression of this gene in the lapatinib-sensitive cell lines. The trend remained consistent over the time points.

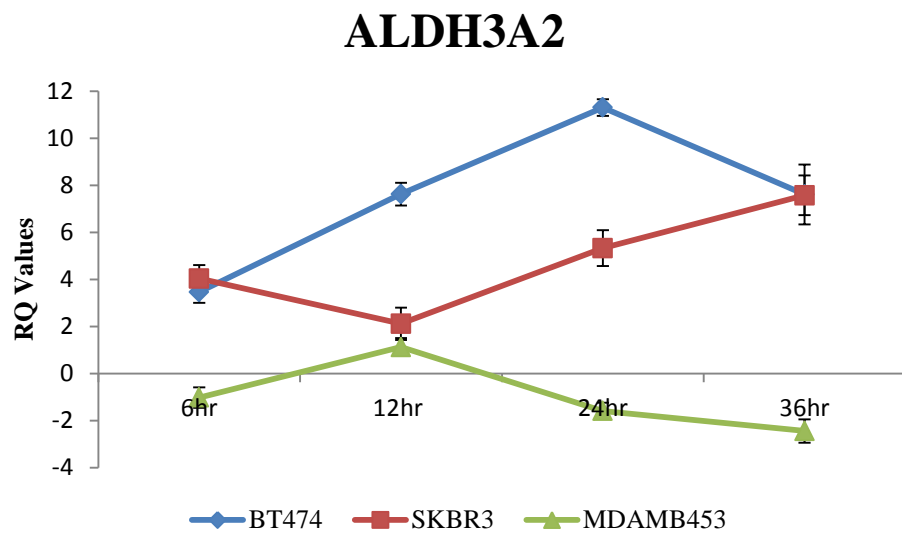
A comparison of the data from all of the different time points indicated that the expression of the genes was reasonably constant over the 36 hours of examination and that difference in the expression levels between the lapatinib-insensitive cell lines MDAMB453 and the lapatinib-sensitive BT474 and SKBR3 remained recognisable. All RQ values can be found in appendix 2.

As shown in section 3.7 it was determined that while the genes *PIK3C3*, *ALDH3A2* and *CDKN1B*, were interesting in response to lapatinib in BT474 and SKBR3 cell lines, they did not follow the same trend of proportional response to lapatinib that the remaining five genes did when the panel of cell lines was expanded. The relative expression changes of these genes over the 36 hour time period did follow similar patterns to those observed in the other five genes (Figure 3.10 A-C). In *PIK3C3*, there was a continued up-regulation in the lapatinib sensitive cell lines and a down-regulation in the MDAMB453 cell line. This trend was evident also in relation to the expression profile for the *ALDH3A2*. In the expression profile for *CDKN1B*, the gap between the expression of the gene in the lapatinib-sensitive and insensitive cell lines was most evident. The expression of the gene in the MDAMB453 cell line remained stable over the 36 hours. In the BT474 and SKBR3 cell lines, the expression was stable over the four time points and has at a similar level to each other.

A



B



C

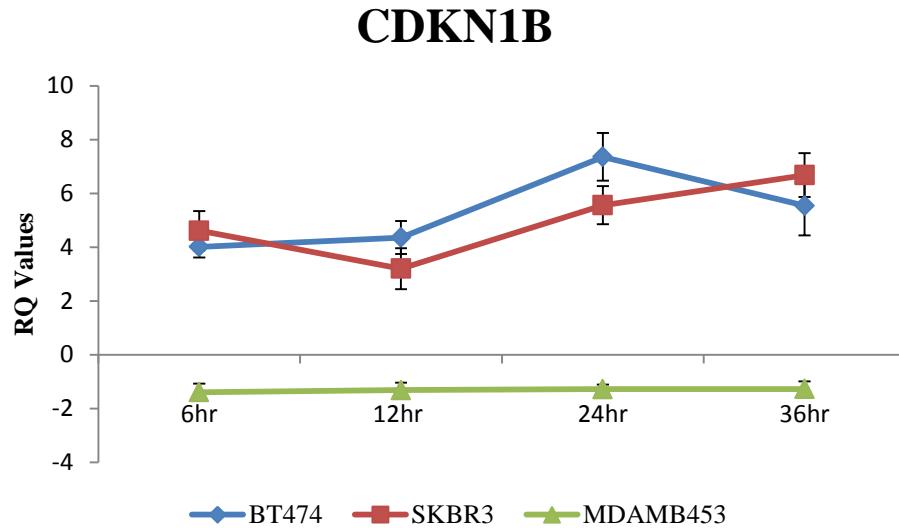


Figure 3.10 A-C: Analysis of (A) *PIK3C3*, (B) *ALDH3A2* and (C) *CDKN1B* in BT474, SKBR3, and MDAMB453 over 4 different time points. Cell lines were treated with 1 μ M lapatinib for 6, 12, 24 and 36 hours. This analysis was undertaken to determine how robust the differential expression of the genes were over a time course. The analysis indicated that the expression of the genes remained consistent to a time point of 36 hours. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

3.10 Evaluation of differential gene expression changes in normal breast tissue cells in comparison to the panel of breast cancer cell lines.

If the findings of this study were to be applied to a real cancer treatment scenario to evaluate the gene expression changes in response to drug treatment, samples would be required for both pre and post treatment. To translate this method to the clinic would therefore require biopsy samples to be taken pre and post treatment from the tumour site. The tumour material that would be taken during these biopsies would not necessarily be pure tumour. As a result of this, it was necessary to establish if normal breast tissue would have an impact on the gene expression pattern that had been identified.

To investigate this, human mammary epithelial cells (HMEC) isolated from donor normal breast tissue were purchased and cultured as described in section 2.1.5.1. RNA was extracted from the cells pre and post 12 hour lapatinib treatment (1 μ M). Cells were treated with the lapatinib so as to evaluate if it was possible to identify the gene expression pattern pre and post lapatinib treatment.

Figure 3.11 shows that when the gene expression changes were examined in the HMEC cells pre and post 1 μ M lapatinib treatment for 12 hours, there was no evidence of lapatinib-induced differential expression of the genes in these cells. This result indicated that the normal breast cells did not express the pattern observed in the cancer cell lines in response to lapatinib, indicating that the gene expression changes are specific to tumour tissue. It also indicated that inclusion of normal tissue in the examination of tumour biopsies could have an impact on the gene expression analysis as contamination of the tumour tissue with normal tissue could reduce the

signal of the gene in the analysis. Such interference could lead to an inaccurate result of no change in gene expression.

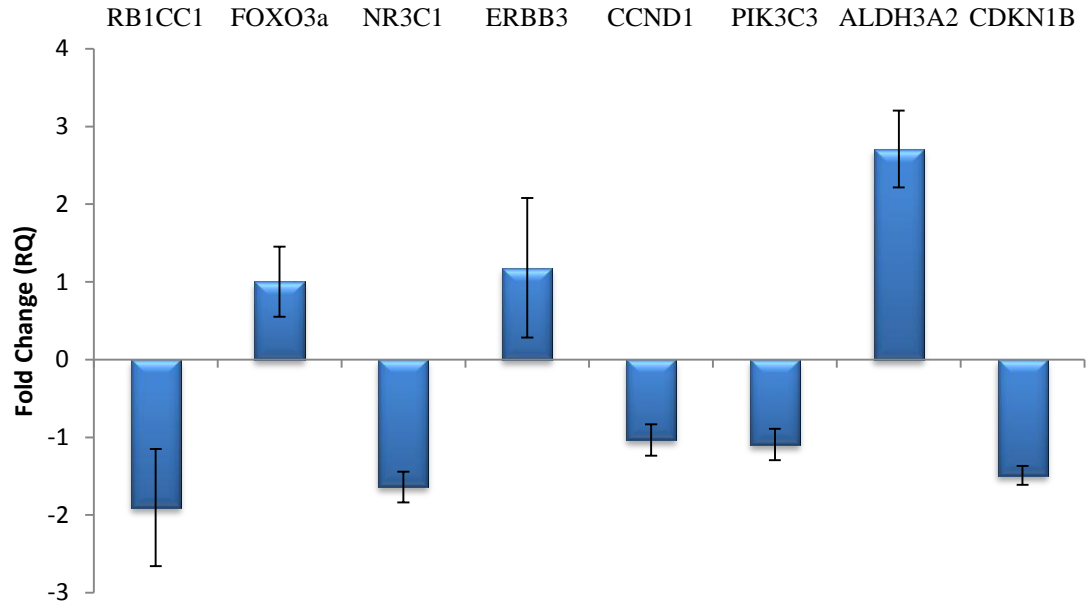


Figure 3.11: In order to establish if there would be any interference in the gene expression pattern from normal breast tissue in the event that biopsy samples were taken, the expression of the genes was examined following 1 μ M lapatinib treatment for 12 hours in human mammary epithelial cells (HMEC). The analysis indicated that there was no distinct pattern evident in the gene expression in the normal breast cells following the lapatinib treatment. However as some of the genes did indicate a fold change greater than 2, it is possible that the inclusion of normal tissue in the examination of tumour biopsies may reduce the gene signal from the tumour material leading to an inaccurate result. The analysis was completed in triplicate the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

Table 3.5 represents the gene expression changes between untreated and treated HMEC cells with untreated and treated breast cancer cell lines. To determine the relative expression of the genes, in the untreated comparison, the RNA expression values from the untreated HMEC cells were used as the calibrator sample. In the treated comparison, the lapatinib-treated HMEC cells were used as the calibrator sample. These comparisons, breast cancer cell lines versus HMEC cells pre and post lapatinib treatment, were used to evaluate the tumour specificity of the findings and evaluate if the gene expression pattern could potentially be identified using a heterogeneous biopsy material from a patient which could likely contain both normal breast and tumour tissue.

Table 3.5 and figure 3.12 A +B shows the differences in the expression of the genes between the HMEC cells and the sub-panel of breast cancer cell lines. The gene changes were examined both pre and post 1µM lapatinib treatment. The relative gene expression change for a number of the genes was quite high in the untreated comparison. The expression of *ERBB3* was exceptionally high, with RQ values of >200 in comparison to the expression of *RBICC1*, which has RQ values between two and eight across the three cell lines. The expression of *NR3C1* was greater in the MDAMB453 cell line compared to the BT474 and SKBR3, which indicated that there are differences in the expression of the genes in the different cell lines. With the exception of *ERBB3*, MDAMB453 showed a higher relative expression of the genes in the untreated comparison. In the expression of *ERBB3* the MDAMB453 cell lines had a lower level of relative expression than the BT474 and SKBR3 when related to the expression in the HMEC cells. It should be noted that the C_t values for the endogenous control for the HMEC cells both untreated and treated were shown

to be stable with values ~ 17-18 which is comparable to those seen in the breast cancer cell lines.

Table 3.5: Relative gene expression comparisons between HMEC cells and the sub-panel of HER2 over-expressing breast cancer cell lines.

	HMEC untreated vs Cancer untreated RQ values			HMEC Treated vs Cancer Treated RQ values		
	BT474	SKBR3	MDAMB453	BT474	SKBR3	MDAMB453
RB1CC1	2.0	2.1	8.9	19.2	7.8	13.7
FOXO3a	4.4	1.1	14.9	20.6	2.5	3.9
NR3C1	-1.0	1.8	199.7	6.8	5.7	30.9
ERBB3	276.3	247.8	178.9	823.9	456.2	1193.9
CCND1	18.9	10.2	57.4	5.6	1.9	44.3
PIK3C3	9.1	1.8	37.7	24.7	5.1	23.7
ALDH3A2	8.9	5.0	92.7	11.2	3.4	22.9
CDKN1B	29.6	9.9	84.0	104.5	43.2	124.6

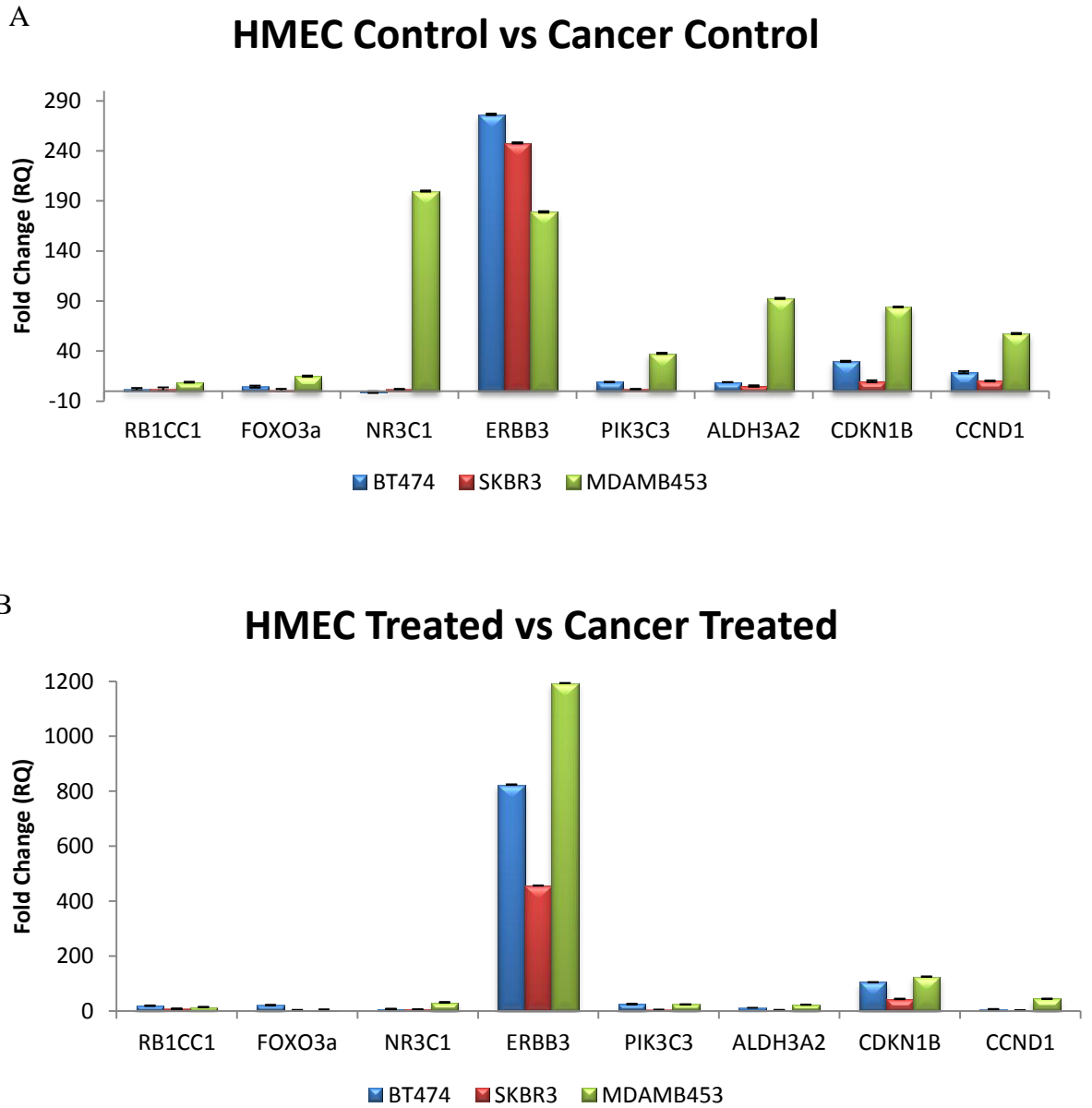


Figure 3.12: To determine if there was any distinct pattern emerging from the comparison of the HMEC cells and three of the breast cancer cell lines, gene expression analysis was completed both before (A) and after (B) 1µM 12 hour lapatinib treatment. While expression changes were clearly evident, there were no distinct patterns that could be used to evaluate response to lapatinib using the different cell types. This analysis indicated that the optimal method to evaluate the gene expression changes in response lapatinib treatment would be pre and post treatment tumour samples. The analysis was completed in triplicate the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

Following treatment with lapatinib, the RQ values for the genes in the MDAMB453 cell line were seen to be reduced compared to RQ values exhibited in the untreated cell line data with the exception of the values for *RB1CC1*, *ERBB3* and *CDKN1B*. The *ERBB3* response also differed following treatment with lapatinib in the BT474 and SKBR3 cell lines. There was a large increase in the RQ values, with values ranging from 200-276 to 456-1193. The largest rise in the expression value is in the MDAMB453 cell line. In the SKBR3 cell line there was a sharp increase in expression from ERBB3 to 823 following treatment. Biologically, these large RQ values make sense. HMEC cells have been shown not to overexpress HER2 [52] and the co-expression of HER2 and ERBB3 is frequently observed [53] which would indicate that the HMEC cells would not over-express ERBB3 either. Studies have indicated that there is an increase in the expression of *ERBB3* in breast cancer following lapatinib treatment [54]. This observation would support the large relative expression (RQ) value for *ERBB3* that was seen following lapatinib treatment in the breast cancer cell lines in comparison to HMEC cells.

Unlike the data shown in figures 3.6 and 3.7, while there were changes evident, there was no distinct pattern emerging from the data that could be used to evaluate a response to lapatinib using the different cell types. These results indicate that the optimal method to evaluate the gene expression changes in response to lapatinib treatment would require pre and post treatment tumour samples.

3.11 Discussion

CIA, a multi-variant bioinformatics technique was used to identify a number of genes and TFs associated with the cellular response to lapatinib. In applying these methods there was no requirement for the filtering of gene expression data or the generation of gene lists or clusters that can be necessary in other bioinformatics methods such as gene ontology. This technique utilised transcription factor binding site information in combination with the genes that these TFs putatively regulate. This was the first time that this technique had been applied to a breast cancer dataset in response to targeted treatments. The technique has also been employed to other linked data sets, for example, to link geographic locations and species composition in ecology [20]. Jeffery *et al.*, [15] provided examples of how the CIA technique can be utilised in determining transcriptional pathways associated with differences between benign and metastatic samples in prostate cancer. Using this technique we identified a panel of 512 gene targets of interest which was reduced to 19 genes and 8 TFs that were associated with lapatinib response in BT474 and SKBR3 cell lines. The 19 genes and 8 transcription factors were selected for more detailed qPCR analyses based on varying combinations of the following criteria; (i) the magnitude of response to lapatinib, (ii) whether the selected genes were predicted targets of the 8 TFs, (iii) the involvement of the gene in important oncogenic processes (determined from functional annotation using the literature mining analysis software Pathway Studio Enterprise (Ariadne Genomics)). Genes were manually selected on the basis of meeting two or more of these criteria. This panel included both known and novel markers of lapatinib response and represents a cohort of markers for predicting both the response and the cellular sensitivity to lapatinib. The gene expression pattern was

evaluated in a panel of breast cancer cell lines that had varying sensitivities to lapatinib but also differing HER2 over-expression status. There have been a number of recent publications that have found a correlation between pTEN/AKT/PI3K pathway activation and the response of the patient to either trastuzumab or lapatinib. The consensus is that patient with low pTEN expression would suggest trastuzumab resistance but sensitivity to lapatinib [55-57]. These studies have provided a valuable insight into intrinsic resistance in the HER2 target models. The changes in PTEN and AKT expression have been well characterised and published by a large number of preclinical and clinical studies. However, there have been limited studies completed on molecular gene targets that are expressed in response to lapatinib or other targeted therapies.

From the panel of 19 genes that were molecularly validated, five were found to be differentially regulated in a lapatinib sensitive manner with changes in the expression of these genes correlated directly with the lapatinib sensitivity of the cell line being examined (figure 3.6). These five genes included known lapatinib response genes such as *FOXO3A* and *CCND1*, as well as novel genes such as, *RB1CC1* and *NR3C1*. In addition to these five genes, *PIK3C3*, *ALDH3A2* and *CDKN1B* were also found to be differentially expressed in response to lapatinib in BT474 and SKBR3 cell lines, however, they did not provide as compelling a response in the remaining four cell lines (EFM192a, HCC1954, MDAMB453 and MDAMB231) and so were not further investigated.

Putative candidate regulators (TFs) of this lapatinib response were also identified, none of which have been previously studied in lapatinib-treated cells. It is important to note that none of these TFs were associated with the lapatinib response through conventional microarray differential expression and their prioritisation here was only

achieved via the novel use of the CIA method in this breast cancer dataset. CIA was not restricted to a specific gene list but rather used the entire microarray data as input. CIA is therefore not limited to arbitrary cut-offs which may exclude important TFs of interest.

The expression of 6 TFs, *AHR*, *ARNT*, *RXR*, *RAR*, *PAX9* and *ZNF143* were found to be altered across all the cell lines in response to lapatinib treatment. These TFs are putative regulators of the cellular response to lapatinib and are predicted to target a number of the significantly differentially regulated genes. The expression of these TFs does not follow a set pattern but do follow some distinct trends as mentioned above in section 3.6. All of these TFs have been previously demonstrated to play important roles in cancer, although their function in HER2-positive breast cancer is unclear. The *AHR/ARNT* heterodimer has been implicated as having importance in ER positive breast cancer and has been shown to directly associate with estrogen receptors ER-alpha and ER-beta[24, 58, 59]. Retinoids targeting the *RXR/RAR* heterodimer have marked effects on cellular processes such as proliferation and apoptosis and this has been shown both *in vivo* and *in vitro* in breast cancer models [60]. The *RARA* receptor has also been recently identified as being co-amplified with HER2 in some breast cancers [61]. While being known oncogenes, *PAX9* and *ZNF143* have not been extensively studied in breast cancer [62, 63]. The remaining two TFs that were identified through the CIA were OLF-1 and PAX3. Although the TFs were present in the microarray probes and were identified as being involved in the regulation of a number of the genes, it was not possible to validate them using Taqman RT PCR. It was possible that the signal emitted from the expression of the TF was not strong enough to be detected using the Taqman probes.

Utilising transcription factor genes or indeed the TFs themselves as potential biomarkers could prove to be very difficult in practice. A small change in the expression of a transcription factor can have a much larger an effect on the expression of a gene that it regulates. Also, as this study has indicated, it can be difficult to measure these changes using microarray or Taqman RTPCR methods. In addition, TF genes are not suitable as potential drug targets as, even if other practical hurdles were overcome, a single transcription factor can be responsible for the regulation of a number of different genes, inhibiting its expression could have an impact on a number of different pathways and cell functions.

In the case of five of the genes identified *FOXO3A*, *CDKN1B*, *CCND1*, *RB1CC1* and *NR3C1*, in the panel of six cell lines their expression correlated broadly with sensitivity of each cell line to lapatinib. Four of the genes; *FOXO3A*, *CDKN1B*, *RB1CC1* and *NR3C1* were found to be up-regulated in response to lapatinib. The results indicate that the more sensitive that the cell line is to lapatinib, as determined using proliferation assays, the greater the magnitude of up-regulation of the four genes. In contrast to the findings with the sensitive cell models, these genes were found to be downregulated in the remaining two lapatinib-insensitive cell lines (MDAMB453 and MDAMB231). In the case of *CCND1*, the expression of *CCND1* became less down-regulated as the level of lapatinib sensitivity decreased.

All five of the genes have been previously demonstrated to have importance in cancer. Increased RB1 inducible coiled-coil 1 (*RB1CC1*) expression has been shown to be associated with improved long term survival of breast cancer patients and has been found to have a role in the inhibition of G1/S progression and proliferation in breast cancer cell lines [37, 64]. Genetic variations to a non-coding *BclII* restriction fragment length polymorphism in *NR3C1*, a glucocorticoid receptor, have been

associated with poor response to treatment in multiple myeloma samples [65]. Up-regulation of *ERBB3* (HER3) has been connected with invasive breast carcinomas and also drug resistance in some HER2 over-expressing cancers [66, 67]. *FOXO3A* and *CCND1* have been demonstrated to be important in both breast cancer and the lapatinib response [13, 68]. *FOXO3A* and *CCND1* were both shown by [13] to be differently expressed following treatment with lapatinib. This group reported up-regulation of *FOXO3A* in both BT474 and SKBR3 and also a down-regulation of *CCND1* in the same cell lines following lapatinib treatment. These results are consistent with the results obtained by our study. It should be noted that *CDKN1B* was also differentially expressed in response to lapatinib in our study although its dysregulation did not correlate with lapatinib sensitivity. The authors identified that these three genes all played roles in the regulation of the AKT pathway, both positive and negative. They noted that the down regulation of *CCND1* and that the up-regulation of *CDKN1B* in response to lapatinib could be as a result of a *FOXO3A*-dependent mechanism, which promotes lapatinib-induced apoptosis. However, they did not examine the expression of these genes in other lapatinib sensitive cell lines nor did they observe that the expression of these genes correlated with the sensitivity of the cell lines to lapatinib. They also observed additional changes in response to genes associated with a number of cellular processes such as glycolysis and cell cycle regulation.

Of the five lapatinib responsive genes, lapatinib associated alterations in *FOXO3A* and *CCND1* have been previously described in lapatinib-treated BT474 and SKBR3 cell lines by the group that generated the original microarray dataset [13]. However, the inclusion of the additional 4 cell lines allowed us to examine expression of the five differentially expressed genes in the context of cell lines with varying

sensitivities to lapatinib. The up-regulation of *RBICC1* and *NR3C1* in response to lapatinib has not been previously observed, while only limited work has been performed on *ERBB3*, *FOXO3A* and *CCND1* in this setting [69, 70].

In evaluating the expression of these genes over a period of 36 hours we sought to establish if the relative expression changes in our genes of interest were stable over this period. There was an increase in the expression of *RBICC1*, *NR3C1*, *FOXO3a* and *ERBB3* over the additional time points in the lapatinib-sensitive BT474 and SKBR3 cell lines. For *CCND1*, the relative expression of the gene remained down regulated in the lapatinib sensitive cell lines. All of the genes remain unchanged in the MDAMB453 lapatinib in-sensitive cell line. Examination of the gene expression changes at a number of different time points indicated that these changes are steady for more than 24 hours. This finding could have an impact on the translation of this work to patient samples. Pre and post treatment samples would be required to complete the analysis and these results indicated that the post treatment sample could be taken a number of days following treatment.

We also sought to examine any alterations in our gene expression profile in normal breast cells in comparison to the breast cancer cell lines, BT474, SKBR3 and MDAMB453. Evaluation of the lapatinib gene expression profile response in the HMEC cells provided evidence that the profile exhibited was not seen in normal, non-HER2 over-expressing cells. This indicated that in order to evaluate the response, analysis would have to be run on relatively pure cancer cell samples. This finding was further strengthened by the data looking at the relative expression of the genes between the HMEC cells and a sub panel of breast cancer cell lines. While there were changes evident in both the untreated and the lapatinib-treated cells compared to the HMEC cells, only the lapatinib-responsive cells demonstrated

distinct gene expression patterns that could be used to determine response to the therapy. These results also indicated that there would be implications in the presence of normal tissue in tumour samples that would be analysed. Too much normal tissue with the tumour tissue could confound the results generated from the gene expression analysis. It should be noted, however, that this analysis has some impactful limitations. In order for cancer cell lines to continue to proliferate indefinitely, cell cycle pathways involved in replicative senescence and apoptosis are disturbed which results in the cells continuing to divide and multiply. This results in an immortal cell line that can continue to proliferate indefinitely [71]. The HMEC cells, as a primary cell source, would not have undergone any of these changes and, as such, can only be cultured for a finite period of time [72] (as indicated which by the limited doubling times recommended by the supplier, in this case 18 doublings). It is therefore important to note that the pathways associated with replicative senescence in both of the cell lines and the HMEC cells would be extremely different, which could have an impact on the expression of genes that are associated with cell cycle progression, proliferation and cell death. These distinct differences between the two cell types would explain some of the large differential expression changes that were observed in this analysis.

In using the CIA method, a number of genes and TFs associated with the cellular response to lapatinib were identified. This was the first time that this technique has been applied to a dataset derived from drug-treated breast cancer cells. CIA allowed for the integration of two data sets, the transcription factors binding sites and the gene expression data. Using this method it was possible to identify TF that were involved in the regulation of a number of the genes without them being differentially expressed on the microarray. The methods utilised in this study represent a novel

route to identify putative response biomarkers or targets for therapeutic intervention to increase treatment efficacy. The Taqman RT PCR methods that were applied to this study were fast and specific and provided relative expression of the genes. Although initial validation of the 19 genes and 8 TFs were completed using custom pre-seeded Taqman 96 well plate, all further examination of target genes was completed using individual assays. These preseeded plates, while advantageous for the initial validation, did not have the flexibility that the individual assays provided. Another advantage of the Taqman RT-PCR method was the small amount of sample material that was necessary. The maximum concentration of RNA necessary to complete this type of analysis is as low as 2µg. Although the maximum concentration of RNA (2µg) was used for this analysis, which resulted in the cDNA generated needing considerable dilution, analysis of the dilutions employed suggests that it would be possible to perform the RT PCR with as little as 20ng of RNA. In needing only a small amount of sample material it would allow for additional analysis to be completed and/or allow for further gene expression analysis to be completed.

While all of this work has been undertaken in cell lines, in order for this to move forward and potentially be tested in the clinic, a number of challenges first need to be overcome. Firstly, as all of the gene expression changes have been identified by relating transcription of specific genes before and after treatment hence, multiple samples over a relatively short duration of time from the same patients would be needed. For this to progress further, it will also be necessary to determine how many days after treatment had started that the second biopsy should be taken to provide the most suitable tumour sample, i.e. the time at which relative changes are clearest. Our cell line data does suggest though that this is likely to be a reasonably large

window of several days, particularly when some of the pharmacokinetic differences in drug delivery between cell models and real human tumours, and the differences in human tumour growth rates are considered. The Taqman method itself, although it does not require a large amount of material to be analysed, would require a trained person to process the samples correctly without incorporating any genomic DNA contamination which can have an impact on the analysis.

This initial study has delivered extensive preclinical data that can be further investigated. The five genes identified have the potential to represent a strong panel of biomarkers of response to lapatinib and potentially additional targeted therapies used for the treatment of HER2 positive breast cancer.

3.12 References

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4. Gene expression changes in response to a panel of targeted therapies

4.1 Introduction

While chemotherapeutic drugs, such as anthracyclines, are still routinely used in breast cancer treatment, in recent years a more molecularly-informed approach has been taken with the introduction of targeted therapies. Tyrosine kinase receptors have proven to be successful targets for inhibition. Lapatinib was one of the first HER2-targeting tyrosine kinase inhibitors (TKI) to be used in the clinic. It is approved in combination with capecitabine for the treatment of metastatic breast cancer that is no longer responding to trastuzumab [1]. Trastuzumab is a humanised monoclonal antibody that binds to the extracellular membrane of the HER2 receptor, preventing activation of the receptor and interrupting the cell cycle progression [2]. More recently the small molecule TKI therapeutic arsenal has seen the addition of newer agents such as, afatinib and neratinib.

Afatinib is an irreversible EGFR/HER2 inhibitor developed by Boehringer Ingelheim [3] currently being clinically evaluated in non-small cell lung cancer(NSCLC). The aniline-quinazoline structure of the inhibitor has the potential to irreversibly bind to the EGFR and HER2 receptors, which in turn prevents activation of the kinase domain [3-5].

Similar to afatinib, neratinib is also an irreversible inhibitor of the EGFR and HER2 receptors. Developed by Wyeth, this small molecule drug also inhibits the HER4 receptor [6]. Neratinib interferes with phosphorylation by binding to the cytoplasmic domain of the receptors resulting in the inhibition of downstream phosphorylation of substrates. This inhibition in turn has an effect on the cells ability to proliferate and

can ensure that the cell arrests at the correct cell cycle transition to ensure cell death occurs [7, 8].

TKIs have also been employed, with some specific successes, in the treatment of other forms of cancer by targeting different tyrosine kinase receptors. Gefitinib, an EGFR inhibitor, has been used in the treatment of lung cancer patients with EGFR mutations or overexpression [9]. Dasatinib, a BCR/ABL inhibitor, has been used in the treatment of chronic myeloid leukaemia in patients that are not responding to imatinib [10].

In order to assess if the panel of lapatinib response gene expression changes identified in chapter 3 were exclusive to the agent alone, were also associated with other HER2 targeting therapies or were non-specific, we examined the gene expression changes associated with a number of drug treatments. To characterise the breadth and specificity of the response we employed three groups of agents, 1) a panel of HER2-targeted therapies, which included, afatinib, neratinib, trastuzumab and, the clinically-relevant combination of lapatinib in combination with capecitabine [11, 12];. 2) non-HER2 TKI therapeutics, gefitinib and dasatinib, and 3) the apoptosis-inducing cytotoxic anthracycline, epirubicin. All agents were employed at therapeutically relevant concentrations.

4.2 Materials and Methods

Similar to the initial lapatinib experiments (Section 3.7), BT474, SKBR3 and MDAMB453 breast cancer cell lines were exposed to clinically relevant doses of tyrosine kinase inhibitors neratinib (150nM for 12 hours), afatinib (150nM for 12 hours), dasatinib (1 μ M for 12 hours) and gefitinib (1 μ M for 12hours), monoclonal antibody trastuzumab, (150nM for 12 hours) and the combination of lapatinib and capecitabine (1 μ M and 20 μ M for 12 hours). In addition to this, the response to an anthracycline chemotherapeutic was evaluated using epirubicin (25nM for 12 hours). RNA was extracted and the differential expression of targeted genes was determined, using Taqman RT-PCR, and compared to the relative gene changes in response to lapatinib alone.

In the analysis of this data the trend of the relative differential expression changes in response to the drugs when compared with lapatinib in the three cell lines were examined to establish if the response of the cells to these additional targeted therapies resulted in alterations in the expression of the same genes.

Results

4.3 Comparison of gene expression changes between lapatinib and other TKIs

Following the identification of the panel of response genes from section 3.7 evident after 12 hours of 1 μ M lapatinib treatment, the gene expression responses associated with additional TKI drugs were examined to establish if these gene changes are unique to a lapatinib response or more broadly characteristic of other drug responses.

4.3.1 Neratinib

Neratinib, an irreversible tyrosine kinase inhibitor that targets HER2, HER4 and EGFR [13], is more potent than lapatinib (Table 4.1). BT474 (HER2+, lapatinib-sensitive), SKBR3 (HER2+, lapatinib-sensitive) and MDAMB453 (HER2+, lapatinib-insensitive) cell lines were treated with a relevant dose of neratinib for 12 hours. In this case the dose chosen for the treatment was 150nM. This dose was based on knowledge of the C_{max} (peak plasma concentration) of the drug [14].

Table 4.1: IC₅₀ values for BT474, SKBR3 and MDAMB453 for lapatinib and neratinib. n=3

Cell Lines	Lapatinib IC ₅₀ (μ M \pm SD)	Neratinib IC ₅₀ (μ M \pm SD)
BT474	0.036 \pm 0.015	0.0019 \pm 0.0005
SKBR3	0.08 \pm 0.02	0.0023 \pm 0.0001
MDAMB453	6.08 \pm 0.83	0.820 \pm 0.140

The differential expression of the five genes characteristic of the lapatinib response, *RB1CC1*, *FOXO3a*, *NR3C1*, *ERBB3* and *CCND1* (section 3.7), was examined following treatment with 150nM neratinib and compared to expression changes in response to lapatinib. This data is presented in figure (A) and demonstrated that the gene expression pattern was similar in response to both drug treatments.

As had been previously seen with the lapatinib findings, in the expression comparison for *RB1CC1*, the magnitude of differential up-regulation seen in the BT474 and SKBR3 cell lines was not present in the MDAMB453. While the gene was distinctly up-regulated in the lapatinib sensitive cell lines, the relative expression (RQ) of the gene in the MDAMB453 cell line was less than 2 and as such was considered unchanged in response to the neratinib treatment. The other genes, *FOXO3*, *NR3C1*, *ERBB3* were also found to be differentially up-regulated in response to neratinib, similar to the response that was observed when the cells were treated with lapatinib in the BT474 and SKBR3 cell lines. It should be noted that the magnitudinal changes in the relative response to neratinib were higher than the changes exhibited in response to lapatinib. This increase in the gene response to neratinib treatment may be as a result of the irreversible inhibitory action of neratinib as opposed to the reversible inhibition provided by lapatinib treatment. It should be noted that the C_t values for the endogenous control *GAPDH* following treatment with both lapatinib and neratinib were equivalent in all the cell lines. This indicated that although different doses of drug were used, there was no difference in the expression in the control gene. For *CCND1*, the lapatinib-sensitive cell lines responded with a downregulation of expression of this gene in a similar fashion in response to neratinib as they did in response to lapatinib as did the lapatinib insensitive MDAMB453.

In relation to the relative gene expression changes in the remaining three genes, *PIK3C3*, *ALDH32* and *CDKN1B*, shown in (figure 4.1B), while the genes were up-regulated in the lapatinib-sensitive cell lines BT474 and SKBR3 in response to neratinib, which follows the trend seen in response to lapatinib, they were found to be unchanged in the lapatinib-insensitive cell line MDAMB453 in response to neratinib. All RQ and standard deviation values can be found in appendix 2.

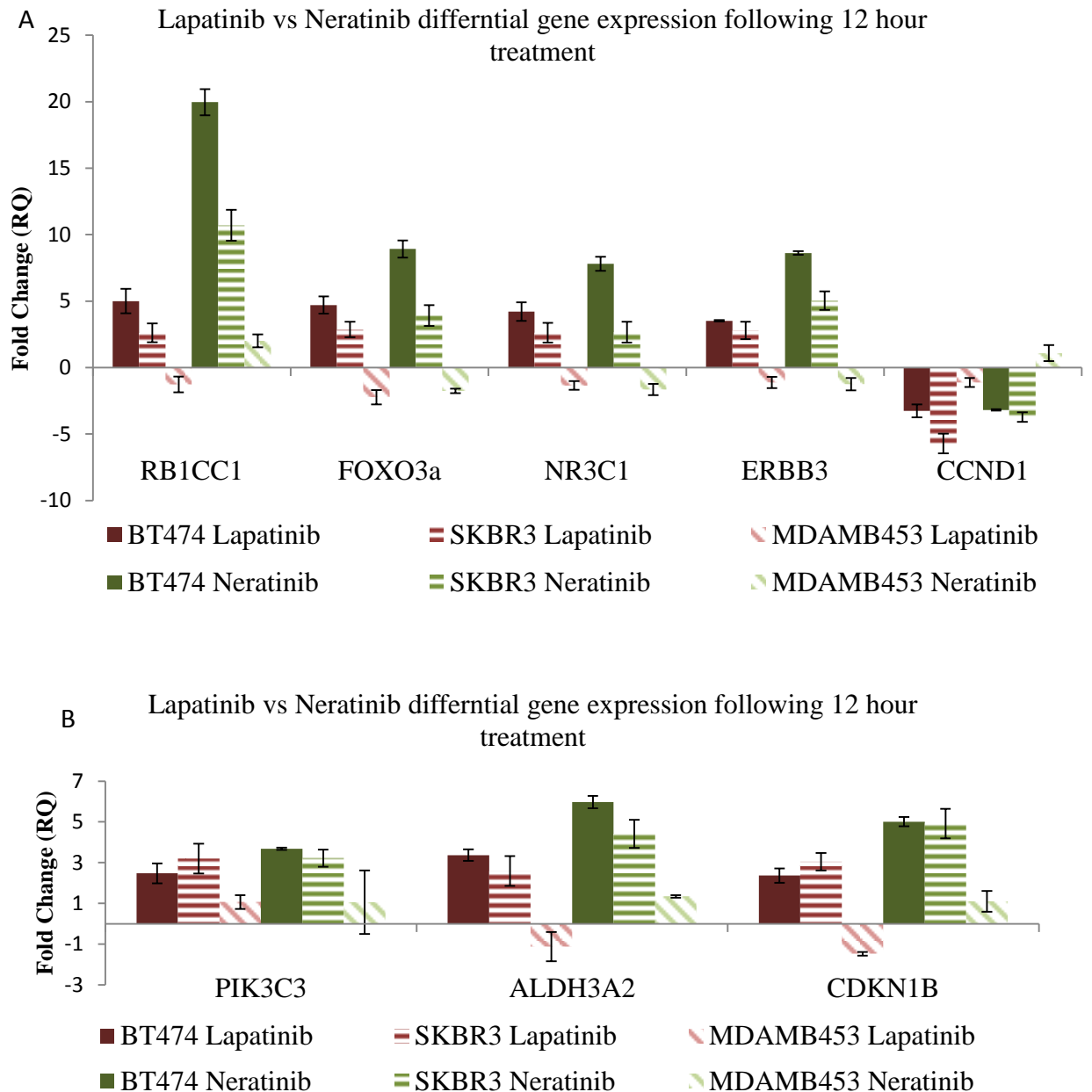


Figure 4.1(A&B) Mean relative differential gene expression changes in BT474, SKBR3 and MDAMB453 cell lines in response to lapatinib (1 μ M for 12 hours) and neratinib (150nM for 12 hours) treatment. Similar to lapatinib, neratinib is a HER2 targeted TKI that irreversibly inhibits HER2 signalling. The gene expression changes in response to lapatinib were conserved when examined in response to the neratinib treatment with some increase in the magnitude of the fold change in response to neratinib. This result indicated that the gene expression pattern that had been identified was not specific to lapatinib response but may also be an indicator of additional HER2 TKI response. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

4.3.2 Afatinib

Afatinib, an irreversible tyrosine inhibitor, which inhibits EGFR, HER2 and HER4 [15] is more potent than lapatinib (Table 4.2.) BT474, SKBR3 and MDAMB453 cell lines were treated with afatinib for 12 hours. In this case the dose chosen for the treatment was 150nM.

Table 4.2: IC₅₀ values for BT474, SKBR3 and MDAMB453 for lapatinib and afatinib.

Cell Lines	Lapatinib IC ₅₀ (μM± SD)	Afatinib IC ₅₀ (μM± SD)
BT474	0.036 ± 0.015	0.0032 ± 0.008
SKBR3	0.08 ± 0.02	0.0075 ± 0.005
MDAMB453	6.08 ± 0.83	1.59 ± 0.18

As illustrated in figure 4.2 A, it should be first noted that the genes that were found to be up-regulated in response to lapatinib were also found to be up-regulated in response to afatinib. Interestingly, the magnitude of expression of these 4 up-regulated genes in response to afatinib was far greater than the magnitude of expression of the genes in response to lapatinib in the BT474 cell lines, the most sensitive of the HER2-overexpressing cell lines. Afatinib was slightly less potent than neratinib in our cell lines models (tables 4.1 and 4.2) with IC₅₀ values differing in all cell lines, e.g. for BT474 IC₅₀ value for afatinib was 0.0032μM ± 0.0001 and for neratinib was 0.0019μM ± 0.0005. In spite of this observation, the gene

expression changes in response to afatinib had a higher magnitude of expression than in response to neratinib. It should also be noted, as with the neratinib treatment, the C_t value of the endogenous control gene *GAPDH* for the afatinib treatment was equivalent to that of the lapatinib and the neratinib treatment.

RB1CC1 expression in response to afatinib was shown to be similar to that following the neratinib treatment (Figure 4.1A and 4.2A). The lapatinib-sensitive cell lines BT474 and SKBR3 showed an increase in the magnitude of expression of the gene in response to neratinib, while the relative expression of the gene in the MDAMB453 cell line was less than 2 which was considered unchanged in response to the drug. (Figure 4.2 A) The expression of the *FOXO3a* gene in BT474 cell lines was shown to be highly differentially up-regulated in response to afatinib. The MDAMB453 cell line demonstrated down-regulation of this gene in response to afatinib treatment and, although the SKBR3 cell line exhibited up-regulation of the *FOXO3a* gene, the magnitude of the expression change was lower than that shown in response to lapatinib.

NR3C1 and *ERBB3* relative gene expression changes displayed similar expression patterns for all three cell lines in response to afatinib. There was a high level of up-regulation in the BT474 cell line, slightly lower level of expression in the SKBR3 and, while the genes were not shown to be down regulated in response to the drug in the MDAMB453 cells, there was no up-regulation shown either. The expression pattern for *CCND1* displayed in response to afatinib (Figure 4.2 A) was very similar to that seen in response to neratinib (Figure 4.1A). The lapatinib-sensitive cell lines responded in a similar profile as they did in response to lapatinib. Interestingly in the lapatinib-insensitive cell line MDAMB 453, the gene became up-regulated in

response to afatinib. All RQ and standard deviation values can be found in appendix 2.

For the remaining three genes, (figure 4.2 B) *PIK3C3*, *ALDH3A2* and *CDKN1B*, it was once again evident that the expression profile showed a higher magnitude of expression in the BT474 cell line in response to afatinib than lapatinib. For *PIK3C3*, there was very little difference in the level of expression between the SKBR3 and MDAMB453 cell lines. For *ALDH3A2* and *CDKN1B*, the trend shown in response to lapatinib in the three cell lines was similar to that shown in response to afatinib.

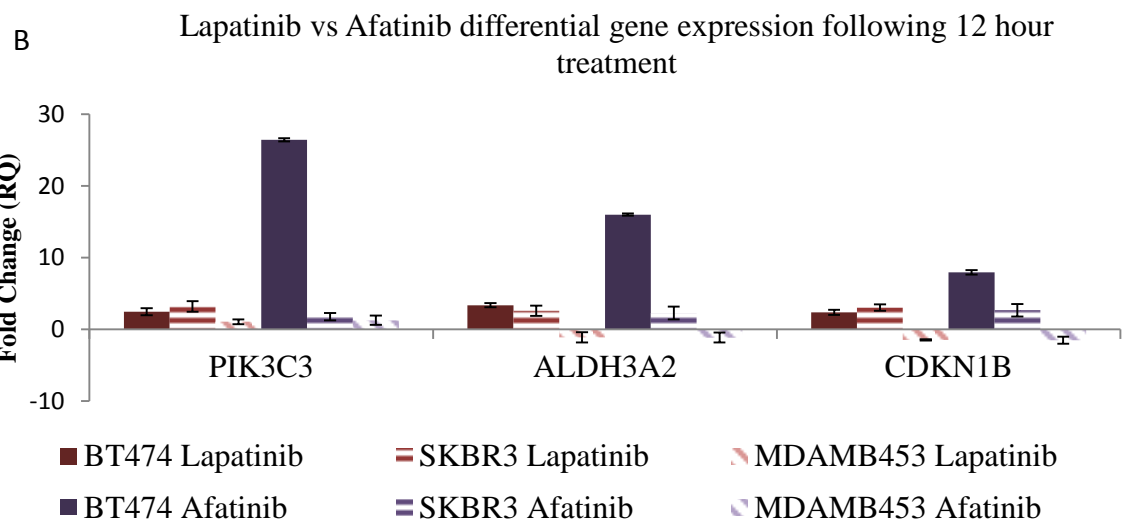
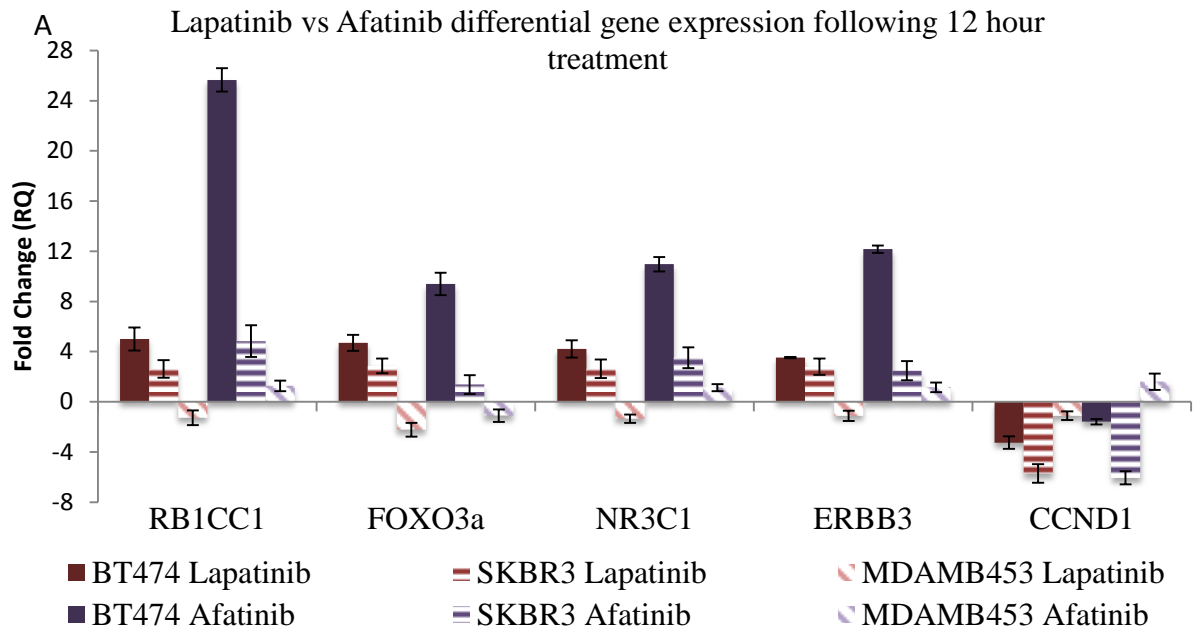


Figure 4.2(A&B) Mean relative differential gene expression changes in BT474, SKBR3 and MDAMB453 cell lines in response to lapatinib (1 μ M for 12 hours) and afatinib (150nM for 12 hours) treatment. Similar to neratinib, afatinib is a HER2 targeted TKI, that irreversibly inhibits HER2 signalling. The gene expression changes in response to lapatinib were conserved when examined in response to the afatinib treatment with some increase in the magnitude of the fold change in response to afatinib. This result indicated that the gene expression pattern that had been identified was not specific to lapatinib response but may also be an indicator of additional HER2 TKI response. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

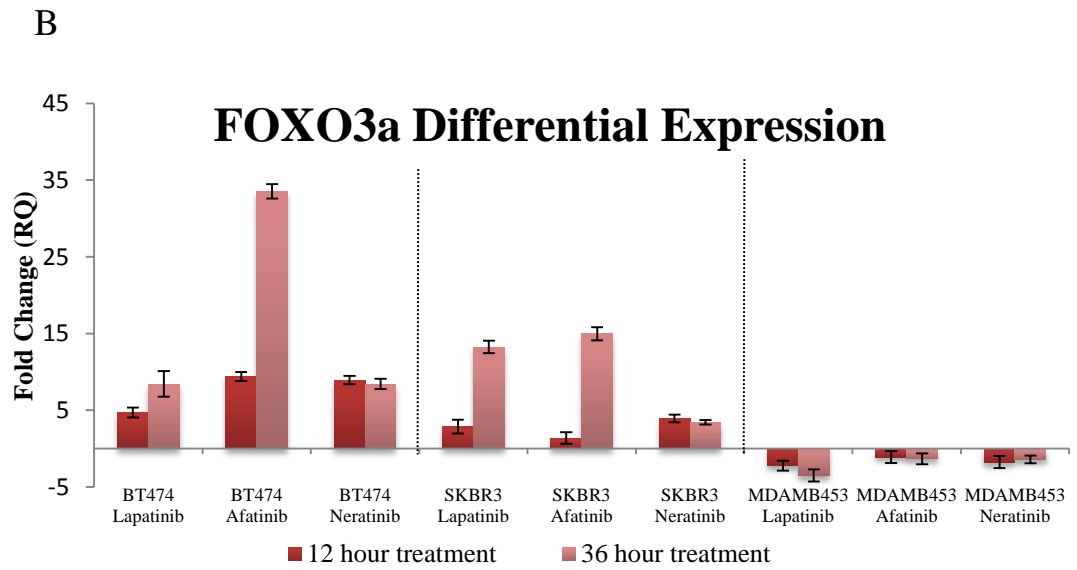
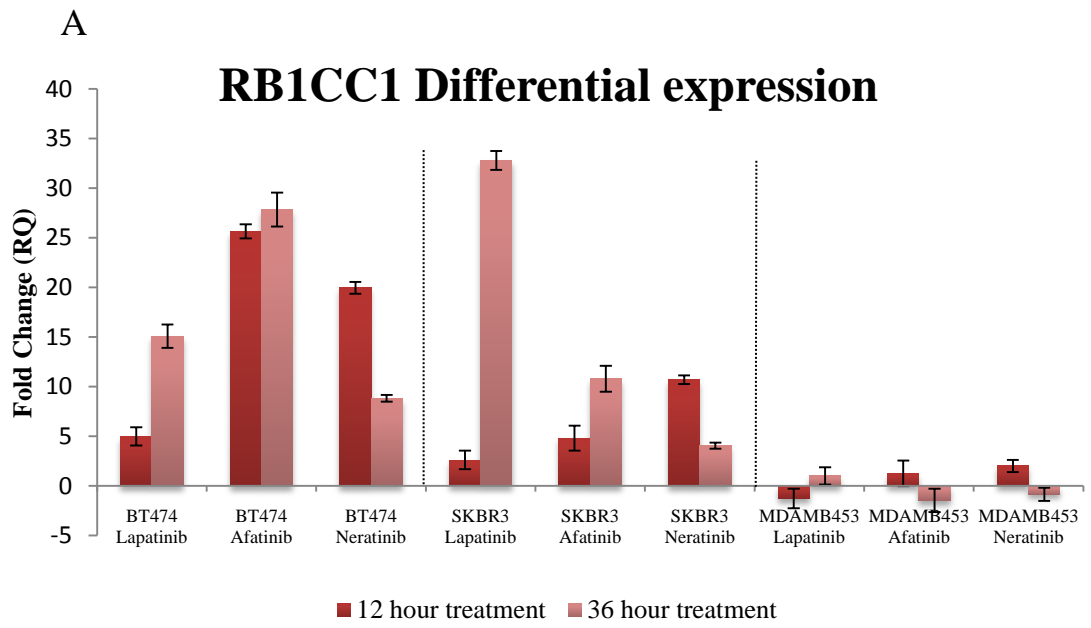
4.3.3 Differential expression of genes in response to lapatinib, neratinib and afatinib examined at 36 hours

To examine if the gene expression changes found at the 12 hour time point were specific to that duration of drug exposure or were more temporally robust, the cell lines were treated with the three HER2 TKIs for a period of 36 hours. As before, the differential gene expression changes were assessed using Taqman RT-PCR. The differential expression changes were compared to the 12 hour treatment gene changes.

For *RBICCI*, *FOXO3a*, *NR3C1* and *ERBB3* in the lapatinib- and afatinib-treated cells there was an increase in the magnitude of up-regulation in the BT474 and SKBR3 cell lines 36 hours post treatment, while in the MDAMB453 cell line the expression of the genes remained unchanged or slightly more down-regulated in response to the treatments (Figure 4.3 A-E). In the neratinib-treated cell lines, the same trend was evident in the BT474 and SKBR3 cell results, with a large increase in gene expression post treatment, albeit the extent of this increase varied somewhat over the time course of the experiment. As with the other treatments, in the MDAMB453 cells the gene expression levels remained unchanged or down-regulated 36 hour post treatment.

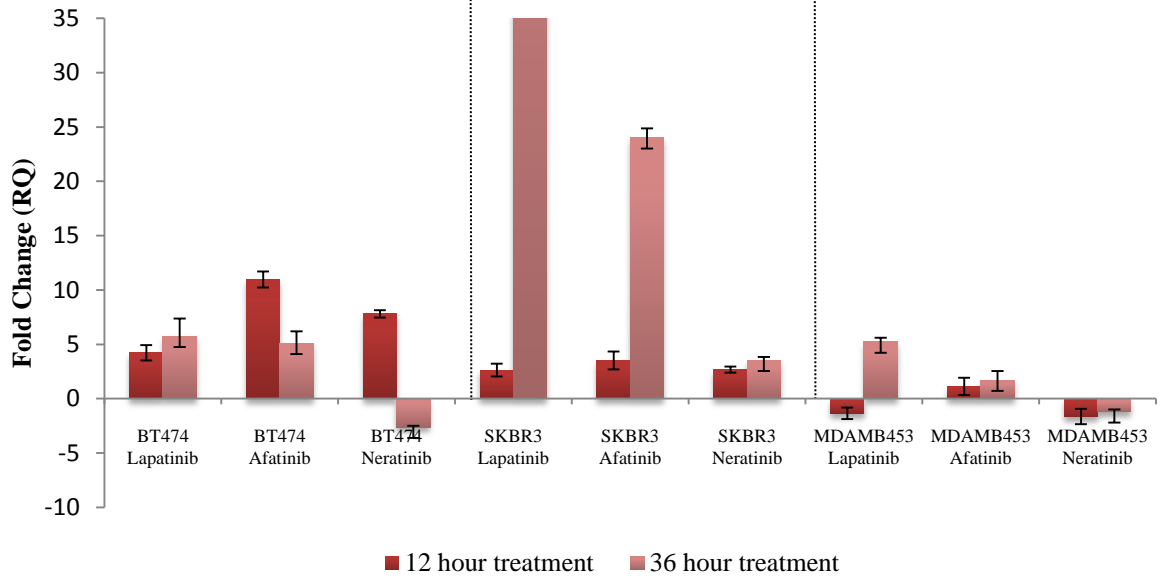
Expression of the *CCND1* gene in the lapatinib-treated BT474 and the SKBR3 cell lines continued to be down-regulated or showed no change in expression 36 hour post treatment. In the MDAMB453 cells the gene expression remained unchanged in response to the 36 hour drug treatment. RQ values that were less than 2 were considered unchanged. For the afatinib and neratinib-treated BT474 and SKBR3 cell

lines the gene expression changes remained down regulated 36 hour post treatment of the drugs. As was the case with the other four genes, the expression pattern remained largely unchanged between treated and untreated cells (either drug) in the MDAMB453 cells. All RQ and standard deviation values can be found in appendix 2.



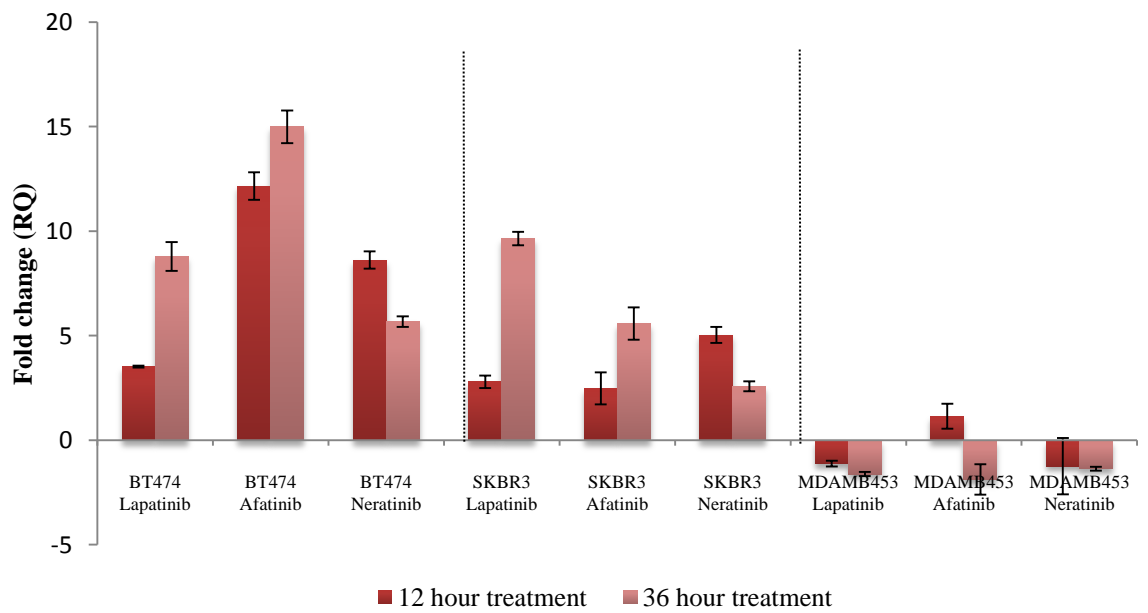
C

NR3C1 Differential expression



D

ERBB3 Differential expression



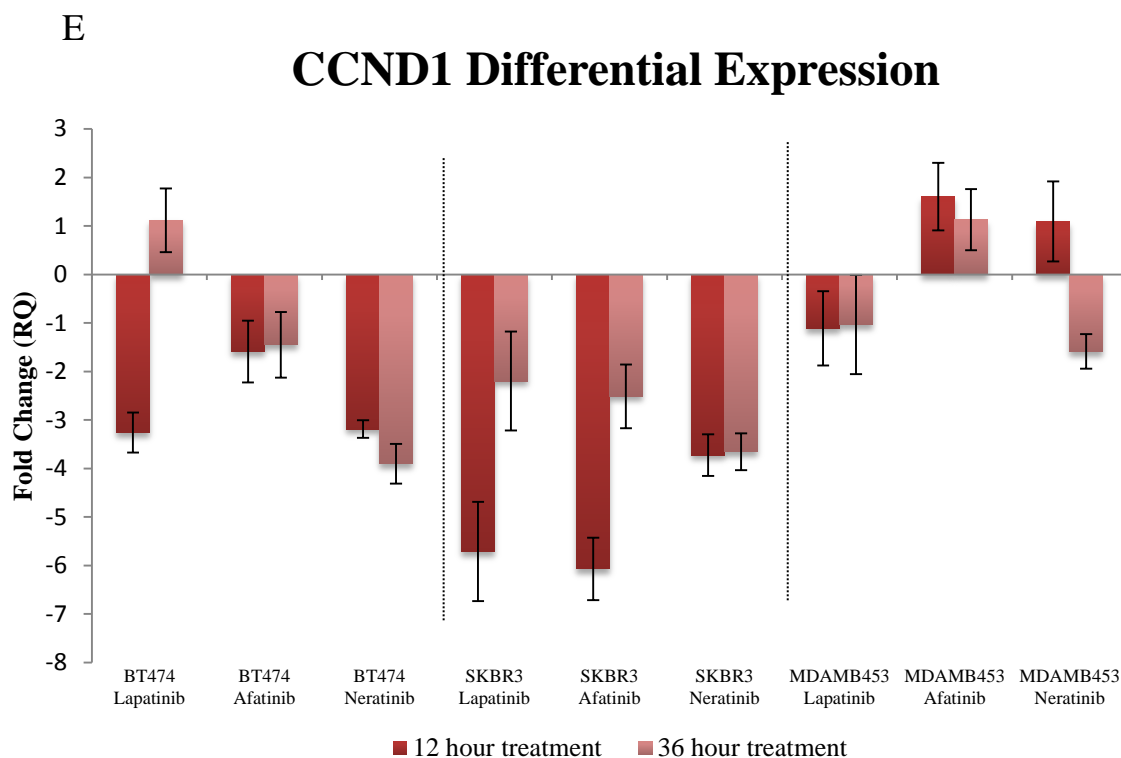


Figure 4.3 (A-E): Differential gene expression changes in the 5 genes of interest following 12 and 36 hour treatment with 1 μ M lapatinib, 150nM afatinib and 150nM neratinib. The figures show that the following 36 hours treatment the trend that was exhibited following the initial 12 hour treatment remained and thus indicates that the genes are robustly changed following longer drug treatments. This result supports the analysis that was completed in chapter 3 which indicated that the differential gene expression was robust following a 36 hour treatment with 1 μ M lapatinib treatment. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

4.3.4 Western Blot analysis of proteins after 36 hours of drug treatment

The corresponding proteins of the five main genes of interest were assessed through immunoblotting (section 2.12). This was undertaken in order to evaluate if the genes of interest are expressed at the protein level following 36 hour treatment with lapatinib, afatinib and neratinib.

Lysates were processed following a 36 hour treatment with 1 μ M lapatinib, 150nM afatinib and 150nM neratinib. β -actin acted as endogenous and loading control for the analysis. Figure 4.4 illustrates the expression of the proteins following the drug treatments.

CCND1 protein expression exhibited some changes in the BT474 and SKBR3 cell lines; however, there was no distinct trend or pattern visible. NR3C1 demonstrated a limited expression in the cell lines, in particular the MDAMB453 cell line which displayed no expression following treatment with the panel of targeted therapies. FOXO3a protein was detectable across the cell lines and in the different treatment samples; however, it was not possible to discern any specific trends in expression of the protein between samples. RB1CC1 protein levels were detectable; however, the expression change that was seen at the mRNA level was not evident at the protein level. Finally, HER3 was detectable in all of the samples across the cell line panel, however, once again it was not possible to identify specific trends between samples.

The protein changes that were exhibited in response to the 36 hour treatment with lapatinib, afatinib and neratinib did not appear to correlate in any way with the gene expression changes that were previously described in figure 4.3 A-E, indicating that

the changes at the gene transcription levels did not correspond to changes at the translational level of the protein products of the gene.

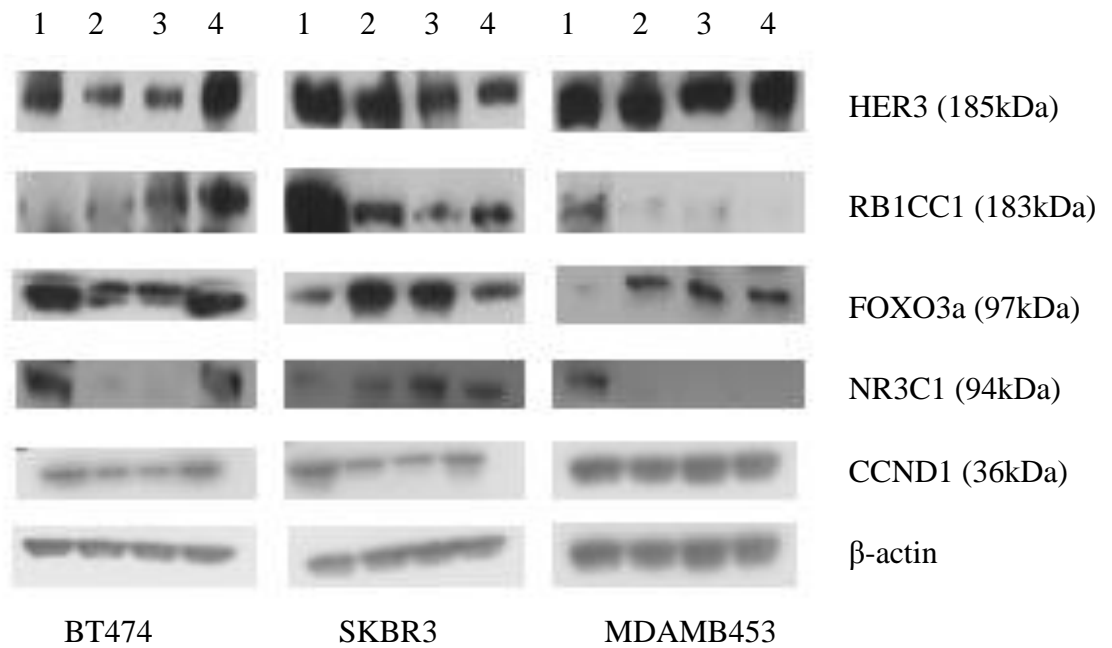


Figure 4.4: Western blot analysis of corresponding proteins to the genes of interest. These Western blots represent a single replicate for each cell line (BT474, SKBR3 and MDAMB453) and drug treatment (36 hour treatments). Lane one of each cell lines represents the control (untreated) samples. Lane two represents the 36 hour 1 μM lapatinib treatment. Lane three represents the 36 hour 150 nM afatinib treatment and lane four represents the 36 hour 150 nM neratinib treatment. This analysis indicated that the expression of the corresponding proteins of the genes of interest did not correlate in response to the drug treatments in the same manner in which the genes did. This result indicated that examining the protein expression of the gene genes of interest would not provide relevant drug response information.

4.3.5 Gene expression changes in response to treatment with lapatinib in combination with capecitabine

Currently the US FDA licence lapatinib to be used in combination with the cytotoxic chemotherapeutic agent, capecitabine, in the second line treatment of metastatic breast cancer [1]. Capecitabine is a pro-drug and is converted in humans (but not *in vitro*) to 5-fluorouracil in the tumour, which, in turn, inhibits DNA synthesis and slows the growth of the tumour cells [16]. Prior to conversion to 5-fluorouracil, the drug is converted into a number of precursors. The major precursor is 5'-deoxy-5-fluorouridine (5'DFUR) and it was this active agent that the cells were treated with for 12 hours in combination with lapatinib (1 μ M). The dose of 5'DFUR that was used was 20 μ M.

Figure 4.5A and 4.5B illustrated the gene expression profile of the three cell lines in response to lapatinib in combination with 5'DFUR (capecitabine active agent) in comparison to the cell lines treated with lapatinib alone. The addition of capecitabine in the treatment of the cell lines did not impact prominently the expression profile that was exhibited by lapatinib alone treatment.

The main differences that were seen between the two treatments were an increase in the magnitude of expression of the four up-regulated genes (*RB1CC1*, *NR3C1*, *ERBB3* and *FOXO3a*) in the BT474 cell lines with the lapatinib plus capecitabine treatment when compared with lapatinib alone. In addition to this, there was a slight reduction in the amount of increased expression of *FOXO3a* in the SKBR3 cell lines in response to the combination treatment. Figure 4.6B also indicated that the combination treatment resulted in an increase in the expression of *PIK3C3*,

ALDH3A2 and *CDKN1B* in the BT474 cell lines, while the amount of increased expression of the three genes in the SKBR3 cell line was slightly reduced in the combination treated cells.

These results indicated that the inclusion of the capecitabine to the cell treatment did not particularly impact the gene expression response pattern which was evident with lapatinib alone. This observation has significant implications, as lapatinib has been shown to be modestly successful as a mono-therapy and is much more commonly used as part of the 2nd line capecitabine combination treatment [17]. All RQ and standard deviation values can be found in appendix 2.

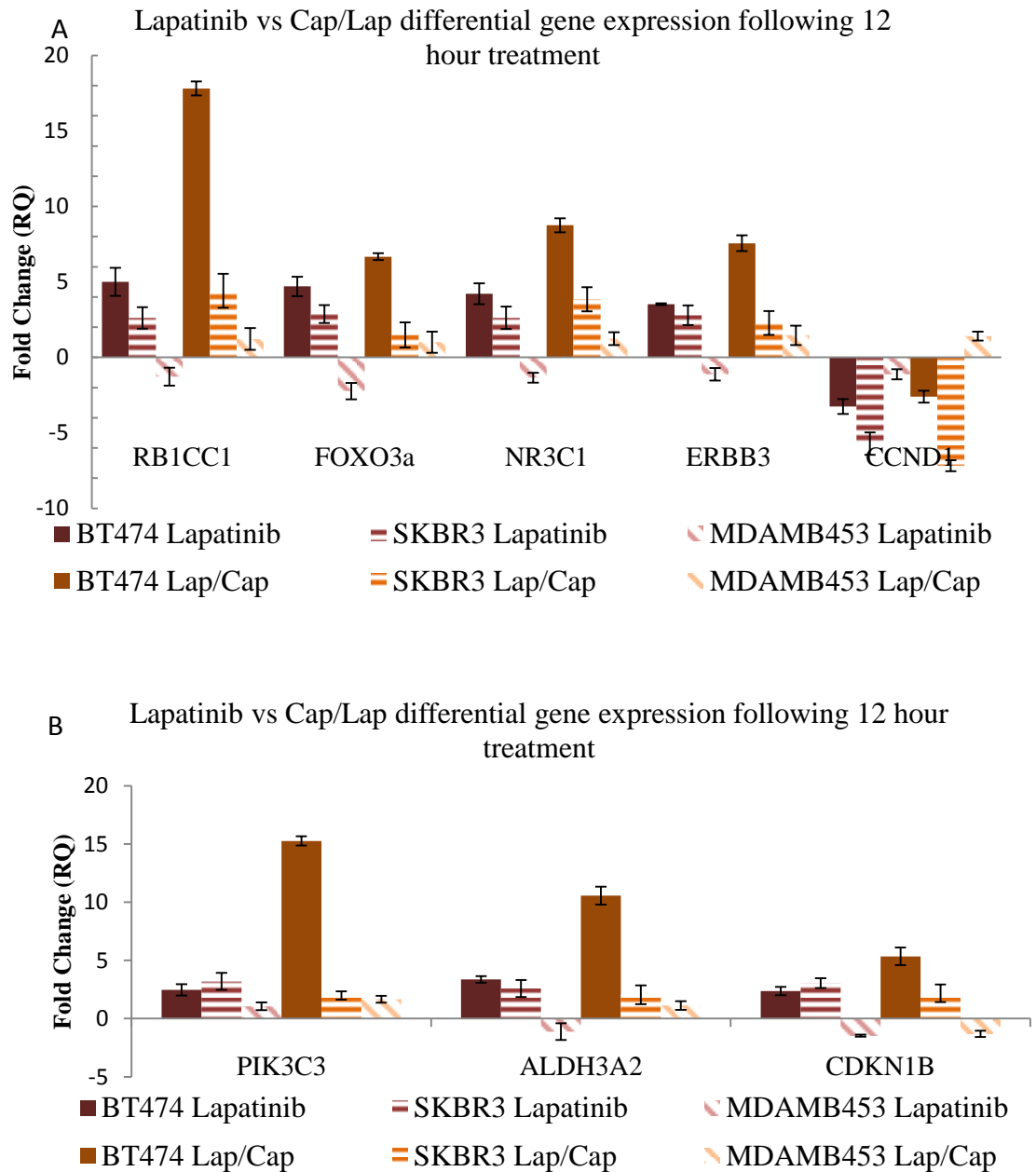


Figure 4.5(A&B): Mean relative differential gene expression in BT474, SKBR3 and MDAMB453 cell lines in response to lapatinib (1 μ M for 12 hours) and lapatinib in combination with capecitabine. This analysis was undertaken to evaluate the effect capecitabine could have on the gene expression pattern. Lapatinib is licenced in combination with capecitabine as a second line treatment of metastatic breast cancer. The gene expression changes in response to lapatinib were conserved when examined in response to the lapatinib and capecitabine combination treatment. These results indicate that the inclusion of capecitabine in the treatment regime did not interfere with the gene expression pattern that has been seen in response lapatinib alone. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

4.3.6 Gene expression changes in response to trastuzumab

Trastuzumab is a monoclonal antibody that targets the HER2 pathway. It was the first therapeutic developed for the treatment of HER2 over-expressing breast cancer. As with the other drug treatments that were carried out, a relevant dose of trastuzumab, 150nM was used to treat BT474, SKBR3 and MDAMB453 cell lines. This concentration represented the typical pharmacokinetic trough concentration (C_{\min}) that has been reported from patient trials [18] .

Figure 4.6A and 4.6B illustrated the gene expression response following treatment with trastuzumab in comparison to the gene expression response following lapatinib treatment. The results indicated that treatment with trastuzumab generates the same gene expression changes in *RB1CC1*, *FOXO3a*, *NR3C1* and *ERBB3* as was evident with lapatinib treatment in all cell lines. The magnitude of expression in the cell lines between both treatments was also equivalent to that seen in response to lapatinib with the exception of the relative expression of the *FOXO3a* gene in the SKBR3 cells which was found to be below 2 and as a result was considered unchanged in response to the treatment. In the case of *CCND1*, expression of the gene was found to be up-regulated following the trastuzumab treatment in the BT474 and MDAMD453 cell lines.

These results indicated that the expression pattern that had been identified in response to lapatinib in chapter 3 is not specific to TKI treatment in the HER2 setting but could also be used to provide response information for HER2 targeting monoclonal antibodies. All RQ and standard deviation values can be found in appendix 2.

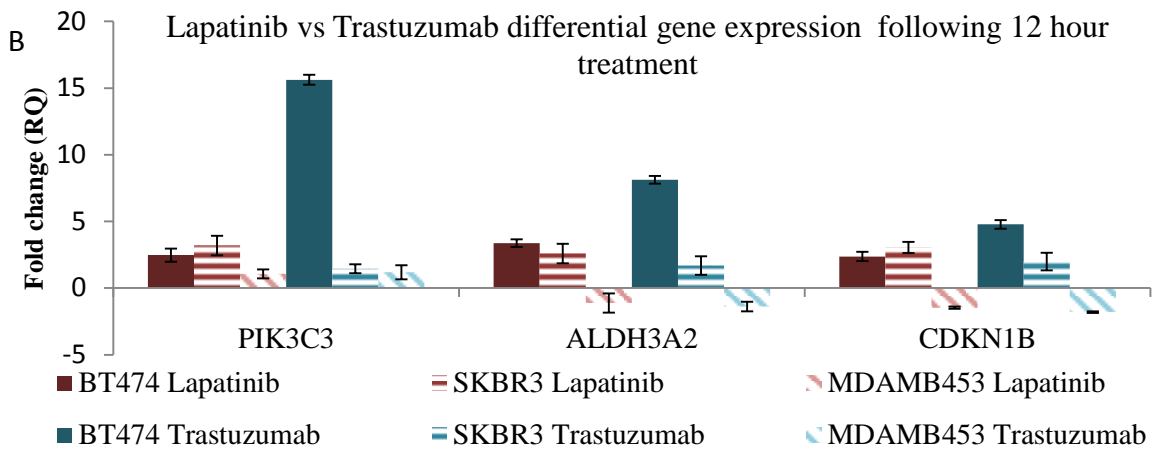
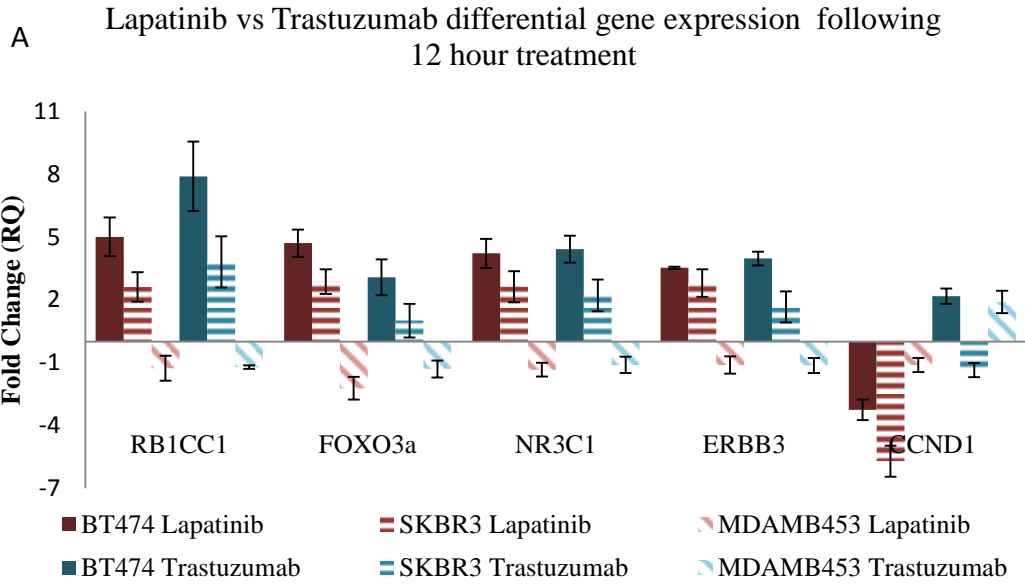


Figure 4-6(A&B): Mean relative differential gene expression in BT474, SKBR3 and MDAMB453 cell lines in response to lapatinib (1 μ M for 12 hours) and trastuzumab (150nM for 12 hours). Trastuzumab is a monoclonal antibody that was one of the first targeted therapies produced for the inhibition of HER2 and is widely used. This analysis was undertaken to determine if the gene expression pattern that had been identified in response to the HER2 targeting TKIs would also be evident in response to other forms of HER2 targeted therapies, such as monoclonal antibodies. The gene expression changes in response to lapatinib were conserved when examined in response to the trastuzumab treatment. This result indicated that the gene expression pattern identified can also be utilised to determine response to other forms of HER2 targeted therapies and not limited to TKIs. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

4.3.7 Gene expression changes in response to dasatinib

Dasatinib, a TKI that targets the BCR/ABL transgene product as well as Src kinase, but not HER2+ or EGFR, was used to examine if the gene changes that had been exhibited in response to lapatinib, neratinib and afatinib were also evident with other non-HER2-targeting tyrosine kinase inhibitors. Following a 12 hour treatment with 1 μ M dasatinib, the expression pattern of the genes of interest was examined in BT474, SKBR3 and MDAMB453 cell lines. This dose was selected based on the IC₅₀ values reported in the literature.

The dasatinib gene expression pattern from the three cell lines was compared to that of the response to lapatinib. Changes in *RB1CC1*, *NR3C1* and *CDKN1B* relative expression levels showed a similar pattern in response to both lapatinib and dasatinib (Figure 4.7).

However, in the case of *FOXO3a*, *ERBB3*, *PIK3C3* and *ALDH3A2*, the gene expression changes exhibited following dasatinib treatment in the three cell lines were different to those exhibited in response to lapatinib (Figure 4.7). In BT474, the genes were seen to be up-regulated, while, in SKBR3 and MDAMB453 the genes were shown to be down-regulated. It should be noted that magnitude of the expression in the MDAMB453 in response to dasatinib treatment was greater than that seen in response to lapatinib.

For *CCND1*, the profile exhibited in response to dasatinib was the opposite of the profile that was found in response to lapatinib. None of the *CCND1* expression changes were of a magnitude that would be considered significant in the cell lines treated with dasatinib.

Examination of the gene expression changes of *RB1CC1*, *ERBB3*, *NR3C1*, *FOXO3a* and *CCND1* in the panel of cell lines in response to dasatinib treatment indicated that the gene expression profile seen in response to lapatinib, afatinib and neratinib was not visible when the HER2 pathway was not being targeted. All RQ and standard deviation values can be found in appendix 2.

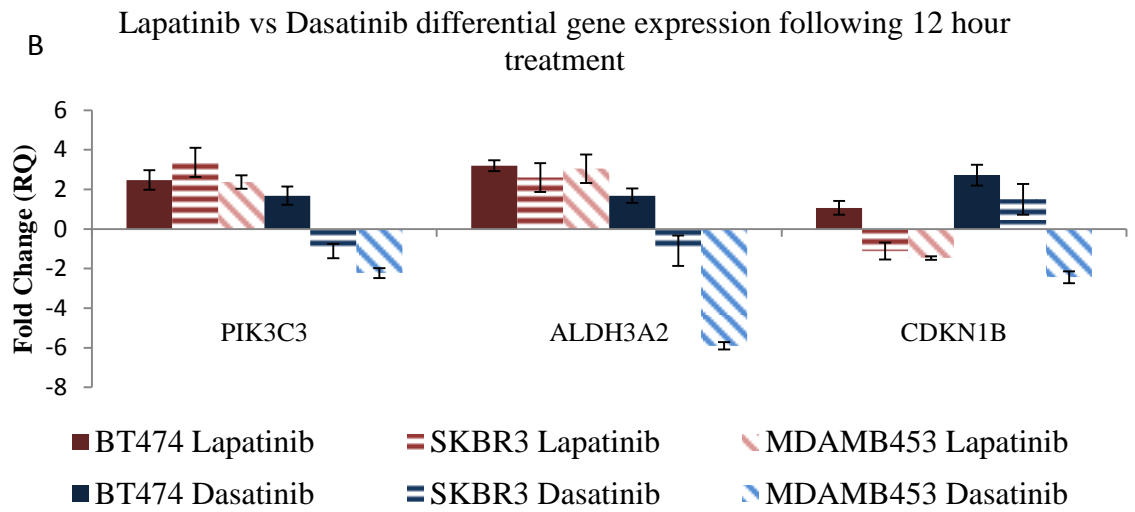
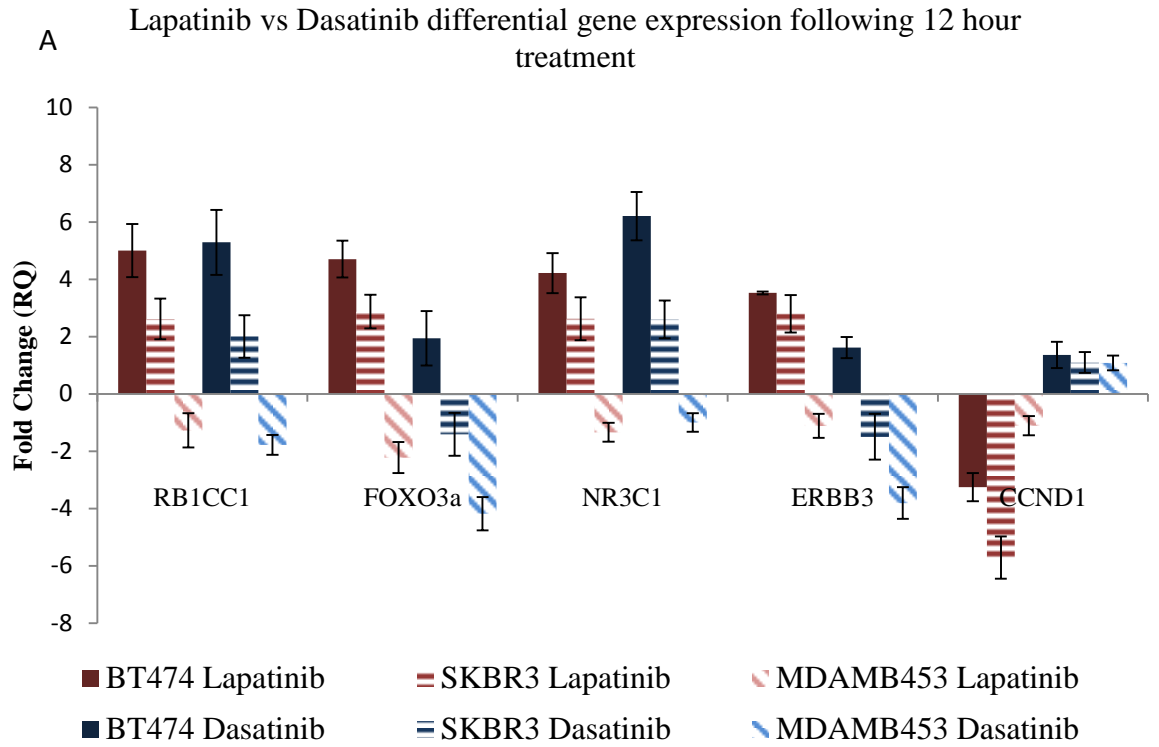


Figure 4.7(A&B): Mean relative differential gene expression in BT474, SKBR3 and MDAMB453 cell lines in response to lapatinib (1 μ M for 12 hours) and dasatinib (1 μ M for 12 hours) treatments. The dasatinib treatment was undertaken to evaluate if the gene expression pattern evident in response to HER2 inhibition was visible in response to other TKI treatments. The gene expression changes in response to the lapatinib treatment were not conserved when examined in response to the dasatinib treatment indicating that the response was specific to HER2 TKI. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

4.3.8 Gene expression changes in response to gefitinib

The HER2-targeting TKIs (lapatinib, afatinib and neratinib) that were employed in the previous work were all also EGFR inhibitors. In order to determine if the gene expression pattern that had been identified was associated with the disruption of the EGFR pathway, or specific to HER2 inhibition, cell lines were treated with 1 μ M gefitinib. Gefitinib is a TKI that targets the EGFR receptor kinase and interrupts signalling through the EGFR pathway. The BT474 and SKBR3 cell lines express low levels of EGFR [19] and are sensitive to the drug [20]. However, the MDAMB453 cells do not express EGFR [19] and are insensitive to the drug [21]. The sensitivity of these cells to gefitinib is important as the gene expression pattern that we have identified is based on the responsiveness of the cells to lapatinib. When the differential expression of the cells following gefitinib treatment was compared to that of the lapatinib treatment, it was evident that even though the cells had a similar sensitivity to gefitinib as they did to lapatinib, the gene expression pattern was different.

Gefitinib treatment at 1 μ M resulted in a completely different expression pattern within the cell lines following 12 hour treatment. With the exception of the *RB1CC1* expression in the BT474 and SKBR3 cells and the *FOXO3a* expression in the BT474 cells, the relative expression of all of the remaining genes was either down-regulated or there was no change post treatment. These results, combined with those following dasatinib treatment, indicated that unless the HER2 pathway of the cell line was being directly inhibited or targeted, the gene expression pattern that was seen in response to lapatinib was not evident.

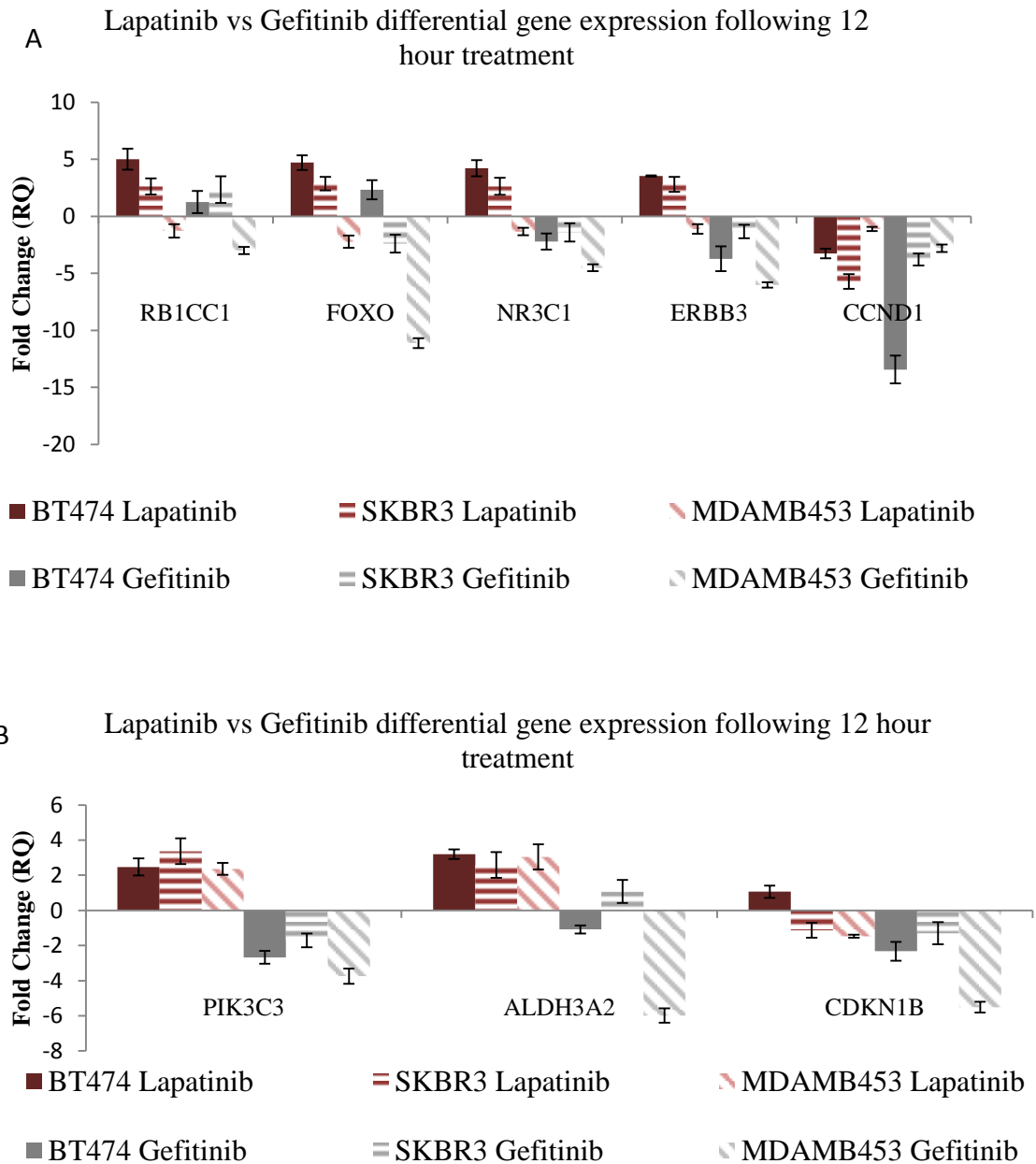


Figure 4.8(A&B) Mean relative differential gene expression in BT474, SKBR3 and MDAMB453 cell lines in response to lapatinib (1 μ M for 12 hours) and gefitinib (1 μ M for 12 hours) treatments. Gefitinib inhibits EGFR expression, which lapatinib, neratinib and afatinib also have been shown to inhibit, as well as HER2. This analysis was undertaken to evaluate if the gene expression pattern was evident when EGFR expression was targeted. The gene expression changes in response to lapatinib were not conserved when examined in response to the gefitinib treatment again indicating that the gene expression pattern was specific to HER2 inhibition. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

4.3.9 Gene expression changes in response to epirubicin

Epirubicin is an anthracycline cytotoxic chemotherapeutic used in the management of many cancers, including breast and ovarian cancer [22], which has been shown to stimulate apoptosis in cancer cells through inhibition of the topoisomerase II enzyme[23].

By examining the gene expression changes in response to this chemotherapeutic drug (Figure 4.9A and 4.9B), we were able to establish that once again the profile exhibited by the cells post treatment with the HER2 targeted agents was as a result of the specific inhibition of the HER2 pathway.

As with the gefitinib treatment, the relative gene expression change exhibited after epirubicin treatment was completely different to that post lapatinib treatment. In all cases the genes of interest were found to be unchanged or differential down-regulated in response to the epirubicin treatment.

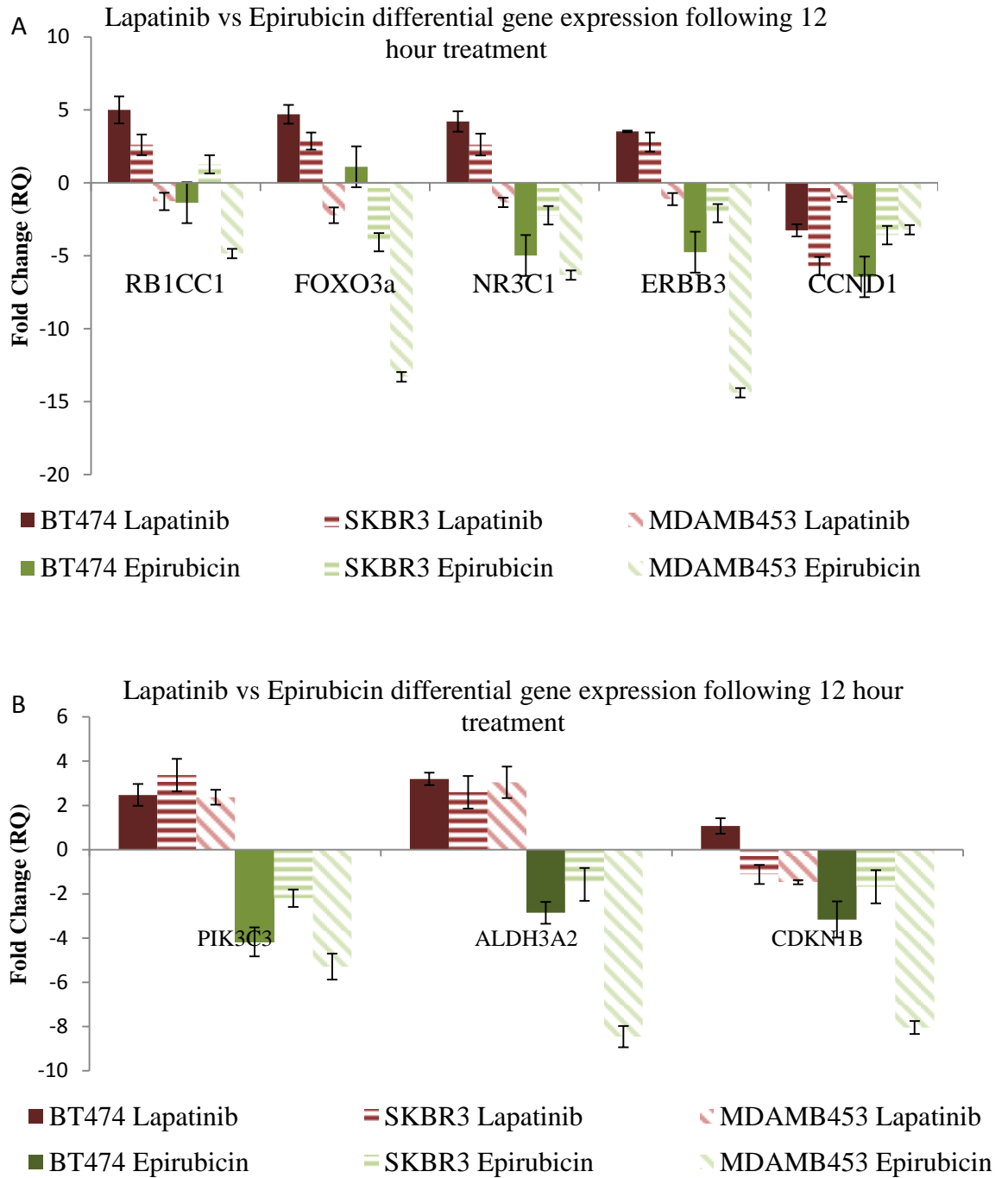


Figure 4.9(A&B): Mean relative differential gene expression in BT474, SKBR3 and MDAMB453 cell lines in response to lapatinib (1 μ M for 12 hours) and epirubicin (25nM for 12 hours) treatment. This analysis was used to further demonstrate that the gene expression pattern that was initially seen in response to lapatinib and further more with afatinib, neratinib and trastuzumab was only evident when the HER2 receptor was being targeted. The gene expression changes in response to lapatinib were not conserved when examined in response to the epirubicin treatment. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

4.4 Discussion

In this study, we aimed to further examine the significance of our prior finding (section 3.7) of a characteristic five gene expression response pattern to lapatinib treatment. To do this we characterised the impact of two other HER2-targeting TKIs, afatinib and neratinib, on these genes changes, and the durability of this response over different time points. In addition, we assessed the gene changes in response to two further approved treatments for HER2-positive breast cancer; trastuzumab, and lapatinib in combination with capecitabine. Finally, to evaluate how HER2-centric the changes were, we interrogated gene expression changes in response to the EGFR inhibitor, gefitinib, the BCR/ABL and Src inhibitor, dasatinib, and the anthracycline agent epirubicin [24]. BT474, SKBR3 and MDAMB453 cell lines were treated with 150nM afatinib and neratinib for 12 hours and the gene expression analysed using RT-PCR. In line with the previously reported lapatinib treatment finding, in our panel of five genes (section 3.7), four *RB1CC1*, *NR3C1*, *FOXO3A* and *ERBB3* were also up-regulated in response to other HER2 inhibitor treatments. The magnitude of the expression of these genes was correlated with the sensitivity of cells to the drug. *CCND1* was shown to be down-regulated in response to the drug treatment, again consistent with the previously published lapatinib data.

Even in patients who over-express targetable molecular changes in cancer, we cannot predict who will respond, hence there is an urgent clinical and pharmaco-economically driven need for an effective diagnostic assay for targeted therapies that would have the ability to determine if a patient is responding to the treatment during the early stages of the treatment regime. Currently over-expression of HER2 is the only available indicator for use of HER2 targeting drugs in patients.

However, not all patients that over express HER2 will respond to treatment with a HER2 targeting therapeutic. It has been estimated that as many as 50% of patients that overexpress HER2 do not respond to targeted therapies [25] either due to an innate/ *de novo* resistance or an acquired resistance to the treatment. There have been some limited studies evaluating expression of the cell proliferation marker, Ki67, following lapatinib treatment to evaluate if it could be employed as a predictive marker for treatment response [26]. Expression of this protein can be detected during the active phases of the cell cycle but is not evident during the rest phase G₀ [27]. Decensi *et al.*, [26] found that in patients with ER-negative tumours, Ki67 expression was reduced by as much as 34% in comparison to the placebo arm of their study. In patients with ER-positive tumours, the expression of Ki67 was reduced by approximately 12%. In measuring the level of expression of this protein, using IHC, it may be possible to determine if the cells are continuing to proliferate or if they are undergoing apoptosis, regardless of the treatment being employed [28]. In addition to the clinical drivers for early prediction of response, these agents are also extremely costly so hence there is a clear pharmacoeconomic imperative to know, as early on in a treatment regime, if a patient is responding and in so doing potentially save the responsible health service significant money. If the patient is not responding to a treatment, continuing on the regime could cause unnecessary toxicity without potential efficacy and at significant financial cost. The similarity of our findings among the HER2-targeting treatments of the panel of cell lines, with trastuzumab, and the combination treatment of lapatinib with capecitabine, further strengthens the hypothesis that this gene expression pattern is indicative of the HER2 pathway being inhibited. Both of these additional HER2-targeting treatments provided similar expression patterns at 12 hours post treatment. It should also be noted that despite

the addition of the chemotherapeutic agent capecitabine to the lapatinib treatment, the gene expression profile remained evident.

To examine if the gene changes are stable over a longer period of time, the cell lines were treated for 36 hours with the 1 μ M lapatinib, 150nM afatinib and 150nM neratinib. The trends that were exhibited 12 hour post treatment were also seen 36 hour post treatment. These results provide a strong indicator that expression changes in this panel of genes is a good and robust representation of responsiveness not only to lapatinib but also afatinib and neratinib.

An examination of the expression of proteins corresponding to the genes of interest was also undertaken. In examining the corresponding proteins of the genes previously examined, we aimed to determine if these proteins would be altered following 36 hours treatment with lapatinib, afatinib and neratinib. If a treatment induced a corresponding protein expression response, this might indicate the possibility that the drug response could be monitored via protein-detecting assays, a generally cheaper and more straightforward analytical process. If protein alterations were detected and such proteins were part of the tumour secretome, it might additionally be possible to measure response using by quantification of the level of these proteins circulating from the tumour cell. Following evaluation of the protein expression in response to the three targeted treatments, there was no correlation in the expression of the proteins and their corresponding genes. This lack of correlation is not uncommon, as other studies have established that correlation between the expression of proteins and their corresponding gene can be as low as 40% due to the large number of processes that must be undertaken between transcription and translation [29, 30]. The lack of correlation could also be attributed to a possible transient nature of expression of these proteins and the inherent variations in protein

half-life and stability [31] . It is possible that for some of the genes that were being examined, the levels of the proteins which would have correlated with the expression of the corresponding gene may have been better examined at shorter time points such as 12 or 24 hours. These earlier time points may have allowed for a better representation of the changes in the protein levels in response to the treatment. The blots that were represented in section 4.3.4 may have been over saturated with protein expression following a 36 hour treatment of the cells with the different drug treatments. Additional time point analysis would be necessary to examine this.

To evaluate if this gene panel was only responsive to HER2-targeted therapies, the panel of cell lines (BT474, SKBR3 and MDAMB453) were also treated with 1 μ M gefitinib. Gefitinib is a EGFR inhibitor that is used in the treatment of non-small cell lung cancer [32]. The panel of cell lines examined have a variable level of EGFR expression. MDAMB453 do not express any EGFR [19] with BT474 expressing low levels [33] and SKBR3 expressing intermediate levels [19]. BT474 and SKBR3 are both sensitive to gefitinib[34]. The trend that was observed in response to gefitinib did not correlate with that shown in response to the HER2-targeting TKIs, giving a strong indication that this gene expression trend is associated with response to HER2 and not EGFR inhibition. Cells were also treated with 1 μ M dasatinib, a BCR/ABL and src inhibitor and 25nM epirubicin for 12 hours. Acting as control treatments, the observation that there was no similarities in the gene expression exhibited following these treatments, allows us to assume that it is the inhibition of the HER2 pathway that gives rise to this and not the induction of apoptosis using unspecific targeted or chemotherapeutic agents.

Although all of the genes in this panel have been reported to have roles in breast cancer [24], there have been no reports of expression changes in *NR3C1* and

RB1CC1 genes in response to afatinib, neratinib or gefitinib. *FOXO3A* expression changes have not been reported to change in response to neratinib or afatinib. However, there are a small number of publications that have indicated that gefitinib can target *FOXO3A* and thereby mediate cell cycle arrest and apoptosis in breast cancer [21, 35, 36]. *ERBB3* has not been studied in the context of neratinib treatment and very limited information regarding the effects of afatinib on the expression of this gene is available [3].

CCND1 expression changes have not been reported for cellular responses to neratinib or afatinib, however, there is some data in the literature demonstrating that treatment with gefitinib can result in down-regulation of *CCND1* which supports our finding [37, 38].

In conclusion, by exposing the three breast cancer cell lines, BT474, SKBR3 and MDAMB453 to an array of targeted and chemotherapeutic agents, it has been determined that the gene expression profile, that had been observed in response to lapatinib, was also observed in response to other HER2 specific agents. This profile was not evident following the treatment of the cell lines with non-HER2 specific agents. Therefore, this profile has the potential to be used not only in determining response to lapatinib but also afatinib, neratinib and trastuzumab and most likely all HER2-targeting agents. The advantage to having one test that could be applied to the identification of treatment efficacy with a number of established and validated therapies is that the same diagnostic assay could be used globally where the treatment regime may differ due to the clinicians preferred course of therapy. As of yet there has been no validated or approved assay or biomarkers developed or identified. However, it should be noted that pharmaceutical companies and the FDA have realised the economic benefits of having such assays and are looking for

evidence of such diagnostics as part of the regulatory registration submission for such agents [39].

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**5. Differential gene expression
changes associated with targeted
therapy treatment in the lapatinib
resistant SKBR3-L cell line.**

5.1 Introduction

Development of resistance to treatment with targeted therapeutic agents in tumours is a significant challenge in the efficacious use of such agents. For example, acquired resistance to lapatinib, i.e. breast cancer which initially responds to therapy but then recurs during ongoing treatment, has proved to be a hurdle that has yet to be overcome or, indeed, fully understood [1-3]. In many cases, patients who initially respond to targeted therapies such as lapatinib or trastuzumab combinations with chemotherapy will relapse within a year of commencing treatment [4-7]. Development of a mechanistic understanding of the molecular causes of such resistance could clearly contribute to formulation of resistance circumvention strategies and generation of cell lines resistant to targeted agents, such as lapatinib, may provide simple models with which to explore and better understand resistance phenomena.

5.2 Material and methods

5.2.1 Materials

The SKBR3 cell line was cultured as set out in section 2.1.4.

The SKBR3-L cell line was developed as outlined in section 2.3. Briefly, SKBR3 cells were treated with increasing doses of lapatinib over a period of six months and proliferation assays (section 2.2) were used periodically to evaluate the level of resistance that was being developed by the cell line. Once the cell line had reached an IC_{50} of $>1\mu\text{M}$, the cell line was considered to be resistant and the treatment was ceased. The parental cell line has an IC_{50} value of $0.08\ \mu\text{M} \pm 0.017$. IC_{50} values

were also determined for afatinib and neratinib in these cell lines. After such an extensive period in culture, DNA fingerprinting was undertaken, as described in section 2.4 and 2.5., to ensure that the cell line had not been contaminated and maintained a genotype consistent with the original SKBR3 parental line.

5.2.2 Methods

In order to examine the expression of the genes of interest identified from chapters 3 and 4, in a lapatinib resistance setting, the lapatinib-resistant SKBR3 cells (termed SKBR3-L) were treated with 1 μ M lapatinib, 150nM afatinib or 150nM neratinib. The differential gene expression of the five genes was determined using Taqman RT-PCR, as described in sections 2.6, 2.8 and 2.9. The three remaining genes were not evaluated in this cell line study as they were not considered as distinct, robust indicators of response to the targeted therapies. The results were evaluated to determine if the acquired lapatinib resistance of the cell lines had an impact on the differential expression of the genes.

5.3 Results

5.3.1 Evaluation of differential gene expression changes in SKBR3-L cell line.

SKBR3 are a routinely used HER2 over-expressing lapatinib-sensitive cell line. They have proven successful for the development of acquired lapatinib resistance cell line models [8]. It was determined that their IC_{50} values were $0.08 \pm 0.017\mu\text{M}$ for lapatinib, $0.0075 \pm 0.005\mu\text{M}$ for afatinib and $0.0023 \pm 0.0001 \mu\text{M}$ for neratinib (Table 5.2). Following six months of continuous treatment with increasing concentrations of lapatinib, starting with 250nM twice weekly for approximately 1 month before being increased to 305nM, the resistance was tested using proliferation assays. Triplicate assays determined that the cell lines had an IC_{50} which was greater than $1\mu\text{M}$. The IC_{50} values were determined to be $>1\mu\text{M}$ for lapatinib, $0.130 \pm 0.045\mu\text{M}$ for afatinib and $0.083 \pm 0.038\mu\text{M}$ for neratinib in the SKBR3-L cell line.

For DNA fingerprinting analysis, the short tandem repeat (STR) profile of the SKBR3-L cells was compared to that provided by the American type culture collection (ATCC) for the SKBR3 parental cells to confirm that there were no differences in our resistant model of the cell line. Short tandem repeat profiles are used to compare specific loci on DNA from different samples. *Amelogenin* is a gene used for gender determination while the remaining eight are core STR loci that are used by the ATCC to discriminate human cell lines [9]. Gender is determined by detecting different sizes and locations of two gene products, *AMEX* and *AMEY* on the sex chromosome [10]. Table 5.1 details the STR profile of SKBR3 as published on the ATCC website and that of SKBR3-L profile that was generated following the fingerprinting analysis.

Table 5.1 STR profile for the SKBR3 parental cell line from the ATCC and the profile generated from the DNA fingerprinting carried out on the SKBR3-L cell line

ATCC STR Profile for SKBR3	SKBR3-L STR Profile from DNA fingerprinting
Amelogenin: X	Amelogenin: X
CSF1PO: 12	CSF1PO: 12
D13S317: 11,12	D13S317: 11,12
D16S539: 9	D16S539: 9
D5S818: 9,12	D5S818: 9,12
D7S820: 9,12	D7S820: 9
THO1: 8,9	THO1: 8,9
TPOX: 8,11	TPOX: 8,11
vWA: 17	vWA: 17

5.3.2 Eradication of mycoplasma contamination in SKBR3-L cell line using plasmocin.

During routine mycoplasma testing that was performed quarterly on all cell lines used (section 2.1.8) it was found that the SKBR3-L cell line had become infected with mycoplasma. Due to the time and materials involved in the development of this resistant cell line, the decision was taken to treat the cell line in an attempt to eradicate the mycoplasma infection using Plasmocin (Invivogen, Toulouse, France) [11]. This treatment works by combining two bactericidal components. One component interferes with bacterial ribosome translation which has an impact on the protein synthesis machinery. The other component interferes with bacterial DNA replication. Both of these targets are found in mycoplasma and bacterial cells, however, they are not found in eukaryotic cells. This treatment generally has a high

success rate with one study indicating that it had a 65% success rate in the elimination of mycoplasma in the panel of cell lines used [11]. The anti-mycoplasma treatment was used as recommended for 2 weeks at 25µg/mL.

Following completion of the recommended treatment, the cells were tested for the presence of mycoplasma 14, 21 and 28 days post Plasmocin treatment. Using both direct and indirect methods (Section 2.1.8.1 and 2.1.8.2) it was determined that the cells were mycoplasma free. At this point the cells were also re-tested to confirm that the cells remained resistant to lapatinib and it was found that they were.

Table 5.2 details the response of the SKBR3-L cell line to lapatinib, afatinib and neratinib. The second column indicates that the acquired lapatinib resistance resulted in decreased sensitivity of the cell line to afatinib and neratinib.

Table 5.2 IC₅₀ values comparing SKBR3 and SKBR3-L for lapatinib, afatinib and neratinib.

	SKBR3 (Lapatinib sensitive)	SKBR3-L (acquired lapatinib resistance)
	IC ₅₀ (µM±SD)	IC ₅₀ (µM ±SD)
Lapatinib	0.08 ± 0.017	>1
Afatinib	0.0075 ± 0.005	0.130 ± 0.045
Neratinib	0.0023 ± 0.00008	0.083 ± 0.038

N=3

Figure 5.1 showed that the treatment with Plasmocin had an impact on the expression of the five genes in the untreated parental SKBR3 cell line. Following

treatment with the Plasmocin for 2 weeks at the recommended dose of 25 μ g/mL, it was shown that the expression of the genes was differentially down-regulated following the treatment. Examination of the C_t values for the endogenous control *GAPDH* indicated that the expression of this gene was equivalent across all treatments in the cell lines.

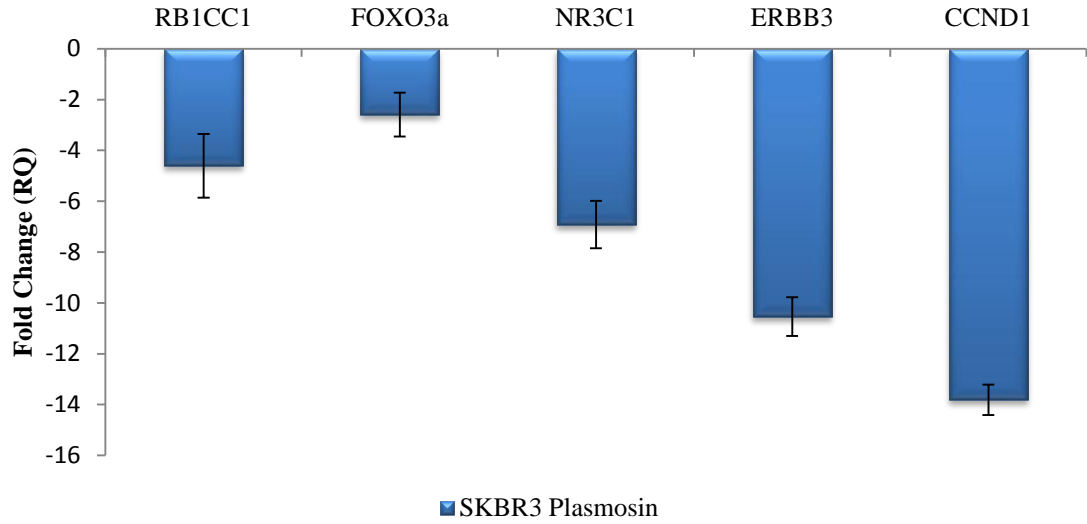


Figure 5.1: The mean fold changes in expression of the five gene expression pattern. This data was generated by comparing the gene expression values for plasmocin-treated SKBR3 parental cells with the corresponding values from untreated SKBR3 parental cells. The parental cell line was treated with the recommended treatment of Plasmocin of 25 μ g/mL for 2 weeks. The plasmocin treatment resulted in differential downregulation of all of the genes in the SKBR3 parental cells, which indicated that the agent had an effect on the expression of these genes. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

5.3.3 Gene expression changes in SKBR3-L cell line in response to targeted therapies

SKBR3-L cells were treated with 1 μ M lapatinib, 150nM afatinib and 150nM neratinib for 12 hours. These treatments were completed in order to determine if the acquired resistance of the cell line to lapatinib would have an impact on the expression pattern that had been previously described in chapter 3 and 4 in response to lapatinib, afatinib and neratinib. Taqman RT-PCR was completed. When comparing the expression of the genes in the SKBR3-L and the SKBR3-L cells treated with lapatinib, afatinib and neratinib, the relative expression was shown to be unchanged (as indicated by a fold change less than 2) in response to the treatment (Figure 5.2). The ΔC_t values from the untreated parental SKBR3 cell line were used as the calibrator in the calculation of the RQ value (Figure 5.3). These results indicated that the changes established in chapters 3 and 4 were only present in sensitive cell lines and not those with acquired resistance to lapatinib. This strengthens the hypothesis that the pattern was indicative of the response in sensitive cell lines. Analysis of the gene expression pattern in further acquired resistant cell lines would need to be completed in order to evaluate if the gene changes are not just as a result of a cell lines specific response.

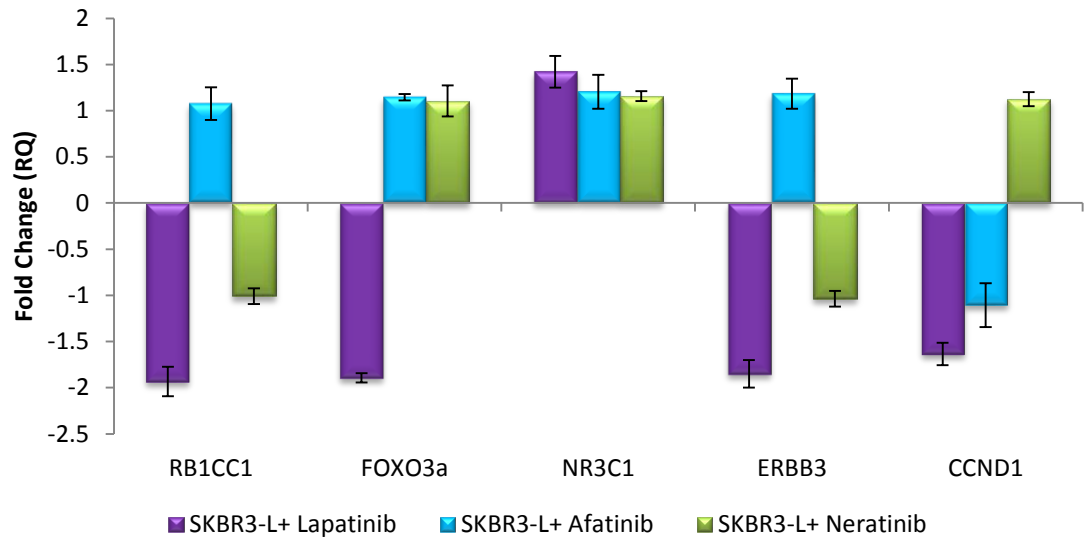


Figure 5.2: Gene expression changes from SKBR3-L cells treated with 12 hour treatments of $1\mu\text{M}$ lapatinib, 150nM afatinib and 150nM neratinib respectively with all data normalised by division of the relevant untreated SKBR3-L gene expression values. All cells in this experiment had previously been treated with plasmocin. The results indicated that the acquired resistance of the SKBR3-L cells to lapatinib impacted the gene expression pattern that had been seen in the parental cell lines response to the same targeted treatments as seen in chapter 4. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

Plasmocin treatment of the SKBR3-L was necessary to attempt to eliminate a mycoplasma contamination on the cell lines. To establish if the Plasmocin treatment itself had an impact on the gene expression within the cell lines, Plasmocin treatment of the parental SKBR3 cell line was undertaken. The parental cells were treated with the same recommended dose of 25µg/mL for 2 weeks. Following treatment the cells were allowed to recover and RNA was extracted and Taqman RT-PCR was completed. The ΔC_t values of the Plasmocin treated SKBR3 parental cell lines were used as a calibrator in the calculation of the RQ values of the SKBR3-L cells following treatment with the panel of TKIs. (Figure 5.3)

Chapters three and four illustrated that differing treatments resulted in variability in the magnitude of the differential expression of the genes in the same cell lines. In the SKBR3-L cell lines, this was not the case. The magnitude of the gene expression remained consistent regardless of the four conditions that were compared. The four conditions were the SKBR3-L cells alone, the SKBR3-L cell line following 12 hour 1µM lapatinib, 150nM afatinib and 150nM neratinib. Irrespective of the calibrator sample that was used to calculate the RQ values, whether it was SKBR3 untreated or SKBR3 treated with Plasmocin, this trend remained.

The change in the calibrator from SKBR3 control not treated with Plasmocin to SKBR3 control treated with Plasmocin did have an impact on the quantitation of the expression of the genes. In the analysis of the RQ values when the calibrator sample was the SKBR3 untreated cell lines, there was noteworthy down-regulation in the relative expression of *NR3C1*, *ERBB3* and *CCND1* genes in response to all of the conditions (Figure 5.3). *RB1CC1* expression was shown to be slightly down-regulated with RQ values of between two and three. *FOXO3a* expression was shown

to be unchanged in all of the cell treatments. When the calibrator was switched to utilise the SKBR3 plasmocin-treated gene expression results, the relative expression of the genes was unchanged. This was defined by a fold change less than 2 in response to the generation of acquired resistance to lapatinib or the treatment of the acquired resistant cell line with lapatinib, afatinib or neratinib (Figure 5.4).

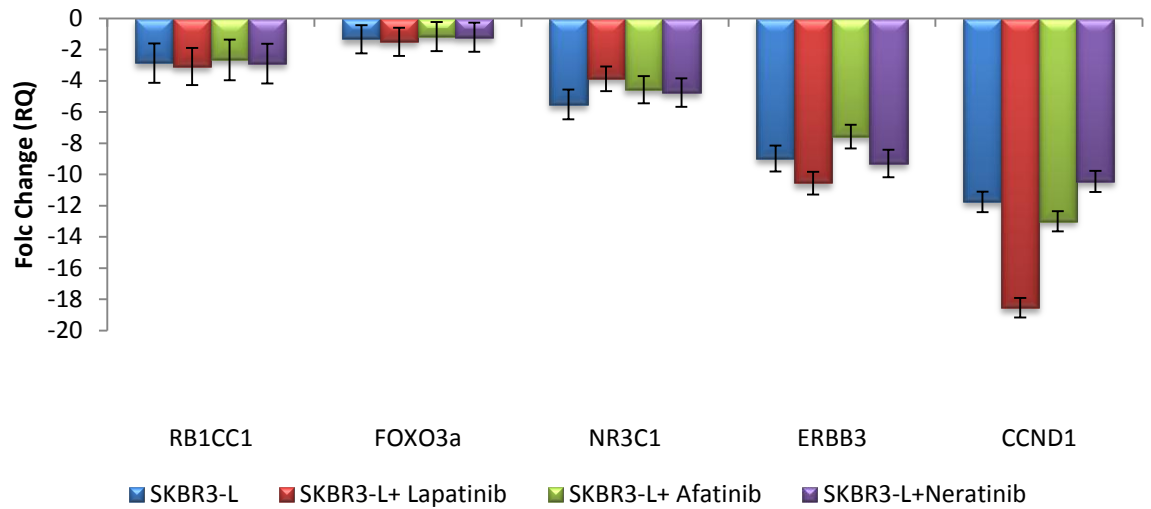


Figure 5.3: Relative gene expression changes in SKBR3-L cells (previously treated with Plasmocin) which were then further treated with 1 μ M lapatinib, 150nM afatinib and 150nM neratinib for 12 hours. The control sample was untreated SKBR3 parental cells. The results indicated that the acquired resistance of the SKBR3-L cells resulted in a different expression pattern than that seen in the parental cell lines in response to the targeted treatments which are described in chapter 3. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

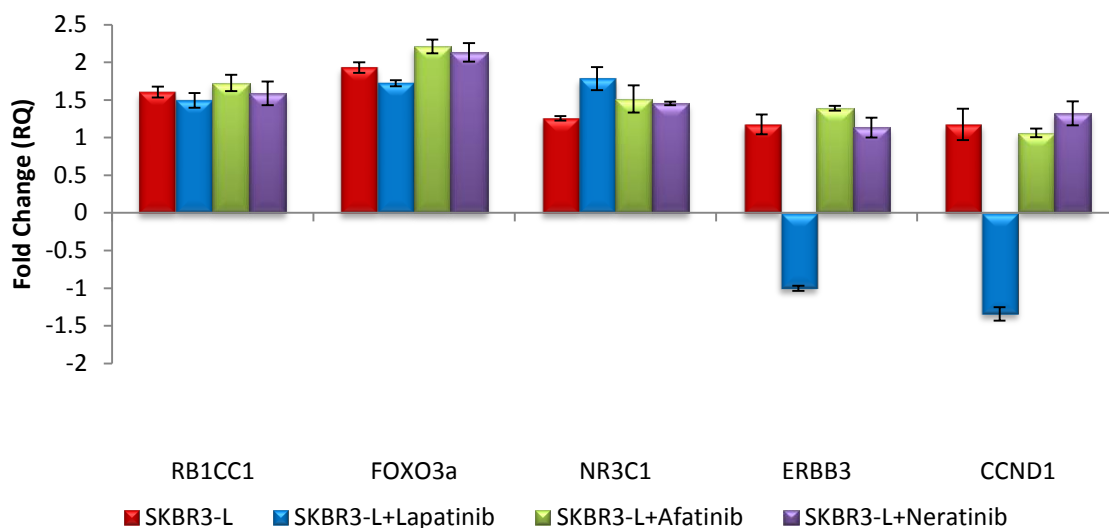


Figure 5.4: Relative gene expression changes in SKBR3-L cells (previously treated with Plasmocin) which were then further treated with 1 μ M lapatinib, 150nM afatinib and 150nM neratinib for 12 hours. The control sample was plasmocin-treated SKBR3 parental cells. The results indicated that the acquired resistance of the SKBR3-L cells resulted in a different expression pattern than that seen in the parental cell lines in response to the targeted treatments which are described in chapter 3. The results indicated that the acquired resistance of the SKBR3-L cells resulted in a different gene expression pattern than that seen in the parental cell lines in response to the targeted treatments. It also indicated that the plasmocin treatment has an impact on the gene expression pattern as it results in differences in the gene expression changes that were exhibited in figure 5.3. The analysis was completed in triplicate, the error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

5.4 Discussion

While the development and analysis of *in vitro* models of resistance has been shown to be helpful in understanding mechanisms of resistance and in therapy regime development, some concerns have more recently been raised as to whether they provide a realistic picture of what occurs *in vivo*. Gillet *et al.*, 2011 [12] have provided evidence that suggests that characterisation experiments carried out on both *in vitro* cell-based models and tumour samples resulted in very different outcomes. In particular they looked at the expression of the Multi Drug Resistance (MDR) transcriptome using RT-PCR assay technology. The MDR transcriptome is a well characterised and extensively researched group of 380 genes associated with aspects of MDR. The study investigated the expression of genes associated with this mechanism in six different cancer types (ovarian, glioblastoma, colorectal, breast, metastatic melanoma and leukaemia) using the NCI-60 [13] cell line panel and clinically relevant samples. One of the outcomes of the study indicated that the cell lines had been highly selected for expression of a number of genes that were associated with MDR. They suggested that this expression was as a result of the cells adapting to their “new” environment [14]. It also highlighted that the cell culture models did not reflect what clinical samples did in relation to the MDR gene expression patterns.

While this study does show some limitations to the applicability of *in vitro* cell models of resistance, until a more easily developed and cultured model is available, it will continue to be utilised by researchers to identify the mechanism by which acquired resistance is established [15].

Summarising the gene changes in chapter three and four, where the cell lines were sensitive to the targeted therapies, there was an increase in the differential expression of *RB1CC1*, *FOXO3a*, *NR3C1* and *ERBB3*, while *CCND1* was shown to be differentially down-regulated in response to the HER2 targeted treatments. When the expression of these five genes was evaluated in response to lapatinib, afatinib and neratinib in the lapatinib-resistant, SKBR3-L, cell line, the trend that was exhibited in responsive cell lines was not evident at all. However, it should be taken in to account that the Plasmocin treatment that was applied to the SKBR3-L cells to eliminate a mycoplasma contamination may have impacted the cells response to the drugs. This was supported by the analysis of the parental cell line and the expression of the genes before and after the treatment with Plasmocin. Mycoplasma contamination has been shown to have an impact on the expression of different genes, eg oncogenes and tumour suppressor genes [16] however, there is evidence to support our assumption that once cells have been treated with plasmocin or other anti-mycoplasma treatment, the function of these genes can be restored [17, 18].

Gene expression changes associated with lapatinib resistance have not been extensively researched. Using qRT-PCR methodology, Penzvalto *et al.* [19] have identified three genes associated with resistance to lapatinib. The three genes were *FURIN*, *ME1* and *TMOD3*. There are no published reports linking *NR3C1* and *RB1CC1* with lapatinib resistance. Depression of *FOXO3a* expression increased ER transcriptional activity and increased localisation of *ERBB3* have all been proposed as potential mechanisms of acquired resistance to lapatinib [20-22]. As an important oncogene and regulator of cell cycle progression *CCND1* and a number of the genes single nucleotide-polymorphisms (SNPs) has recently been associated with clinical response to lapatinib in combination with capecitabine [23]. Any dysregulation of

the gene might possibly have a negative impact on the response of the patient to the therapy.

A limitation of this study is that only one cell line model was used. The mycoplasma contamination would also have had an impact on the results. In order to fully understand the effect of acquired resistance on the expression of the five genes indicative of response to HER2 targeting therapies, multiple cell lines would need to be treated in order to develop acquired resistance to lapatinib and also possibly additional targeted therapies such as afatinib or neratinib. As a back-up, multiple flasks would need to be treated individually with identical treatments so that in the event of a contamination there would be another flask that is undergoing treatment. Routine mycoplasma testing would also need to be completed.

However, these preliminary results do give an indication that the gene expression pattern that was exhibited in the responsive cell lines is not evident in the acquired resistant SKBR3-L cells following treatment with lapatinib, afatinib and neratinib.

The fact that there was a difference between the expression pattern, even in this one responsive cell line and its acquired resistance counterpart, strengthens the hypothesis that this panel of five genes provide a strong marker of response to HER2 targeted therapies in sensitive cell lines undergoing a treatment with such agents.

5.5 References

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**6. *In vivo* evaluation of tumour gene
expression in response to targeted
therapies**

6.1 Introduction

While *in vitro* analysis has proven to be extremely useful in the cancer research setting, it has been clearly established that the use of these immortal cell lines does not give a fully clinically relevant picture in regards to changes in protein or gene expression [1] and as such has significant limitations. Cancer cell lines are typically grown in a monolayer or in suspension and cultured in plastic flasks. Some argue this can cause changes that would not occur in an *in vivo* setting and that these changes could have varied effects on the targets that are being examined. By using *in vivo* models, where tumours are cultured in immune compromised animals, such as nude mice or severe combined immune-deficient mice (SCID mice), researchers are able to determine if the micro-environment of the animal would have an impact, either negatively or positively, on the results that they have found in the *in vitro* setting [2]. Nude and SCID mice are immune compromised by their inability to generate enough T lymphocytes, in the case of nudes or the inability to produce any B or T lymphocytes which are vital in mounting an immune response, as is the case for SCID [3]. It is due to these immune alterations that allow for the implanted “foreign” cells to grow and develop the xenograft models. Xenografts in these immune-compromised mice can be generated in a number of ways. Methods include injection of a cell line, either sub-cutaneous or intra-peritoneal injection, or implantation of a sample of patient material. Once these cell lines or patient-derived materials have developed tumours, they can be passaged into further animals. Such tumour models much more closely imitate human tumours in key ways since they have a blood supply and are three dimensional but do, however, have some limitations. Murine stroma can infiltrate the human tumour where the characteristics

of the malignancy can be affected by the murine micro-environment; the blood supply to the tumour is supplied by the murine host which can have different characteristics to humans in terms of hormones, growth factors etc, [4]. There have been some studies that have indicated that the pharmacological response seen in the mice models does not always correspond to that seen in humans [5,6].

Cognisant of such limitations, we sought to advance our *in vitro* findings with a pilot *in vivo* examination. This pilot *in vivo* study aimed to determine if the gene changes that were described in chapter 3 in response to lapatinib treatment remained when examined in tumours formed from the BT474 cell line in SCID mice.

6.2 Materials and methods

As laid out in more detail in section 2.13, the following methods were used in the process of the *in vivo* study.

The cell based tumours were developed by sub-cutaneous implantation of the BT474 cell lines in eight SCID mice. Once tumours had developed in these animals, three were sacrificed and the tumours were used for implantation into 14 SCID mice, for further growth. Three of the remaining animals underwent treatment with either a vehicle or lapatinib at a concentration of 200mg/kg. Following five days treatment, the animals were sacrificed and the tumours were removed for further analysis using qRT-PCR.

The 14 mice implanted with tumours from the donor mice were allowed to develop tumours. Animals were monitored daily and once the tumours were palpable, they were measured twice weekly to determine the tumour volume. There were three treatment conditions in the tumour implanted group of mice, (1) vehicle (0.5% hydroxyl-propyl-methyl-cellulose/0.1% Tween 80), (2) one day lapatinib treatment with 200mg/kg and (3) five day treatment with 200mg/kg. Once the treatment was completed, the tumour, as well as organs tissue and skin were snap frozen for analysis with qRT-PCR.

6.3 Results

BT474 cells were selected for this study as they had been used successfully in a large number of *in vivo* studies. They were also the most lapatinib-sensitive cell lines that were in the panel that was used in previous experiments.

Figure 6.1 provides a flow chart of the course of the experiment and the number of animals used in each arm.

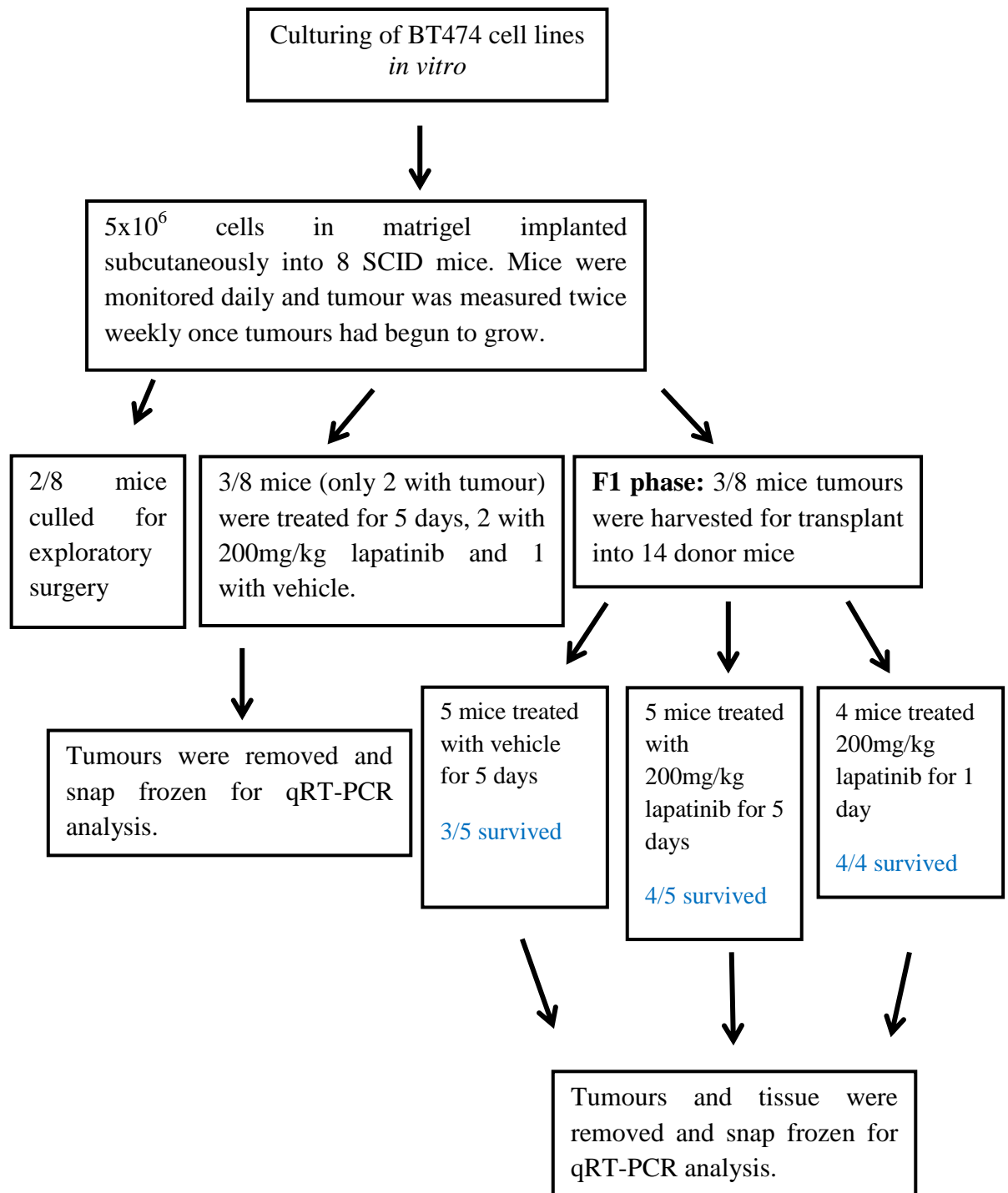


Figure 6.1: Schematic of *in vivo* experiment. Outline of the steps involved in the generation of the cell-line derived tumours and tumour xenografts used to evaluate the gene expression pattern identified in chapter 3 in response to lapatinib.

6.3.1 Generation of the cell line and tumour-derived xenografts in SCID mice

The outcome for all of the animals that were treated in the study is detailed in table 6.1 and 6.2. It was necessary to cull two animals in order to determine the progress of the tumours.

Table 6.1: Assignment of animals used for the development of BT474 cell line derived tumours.

Animal number n=8	Outcome during investigation
2/8	Culled for exploratory surgery
3/8	Used for tumour donation for the F2 phase of the study. Tumours had been growing for 35 days at point of removal for implantation
3/8	Of the 3 animals, 2 of them had developed tumours. 1 of the tumoured mice and the mouse with no tumour were treated with 200mg/kg of lapatinib for 5 days The remaining mouse with a tumour was treated with the vehicle for 5 days.

Table 6.2: Assignment of animals used in the development of tumour derived xenografts.

Animal Number n=14	Donor Mouse	Treatment	Animals survived to end of experiment
5/14	Donor 3 and 1 sample from donor 2	1 day 200mg/kg	4/5
5/14	Donor 2	5 days vehicle	3/5
4/14	Donor 1	5 days 200mg/kg	4/4

Figure 6.2 shows images of the tumour-derived xenograft models for the 11 remaining animals of F2 phase of the study. It should be noted that the size of the tumours was quite small, at the time of dissection they were no more than 100mm³. For comparison, in an additional study carried out by the group, tumours were developed using a DLKP-variant lung cancer cell line. After 30 days of growth these tumours typically reached a volume of approximately 1500mm³ (data not shown). The tumours were derived in the same mouse model (SCID) and the same cell line concentration was implanted in this study. These cells did not require estrogen for proliferation so the mice were not implanted with estrogen pellets prior to cell implantation. The presence of increased vasculature in the BT474-implanted mice is visible in figure 6.2, in particular in images C, I and J (highlighted with yellow arrows). This vasculature was a sign that the implanted tumour sections had begun to proliferate. In a number of the images slightly enlarged lymph nodes are also visible, in particular, images A, D, H, I and J. Once these enlarged lymph nodes were visualised during dissection, they were also removed and snap frozen in liquid nitrogen for qRT-PCR analysis. It was hoped that analysis of these nodes would give an indication if cells from the implanted tumour had travelled to the lymph system of the mice and as a result could form potential metastasis.

Another issue that may have impacted the size of the tumours was the formation of bladder stones in animals as a result of the estrogen pellets that were implanted to maintain the growth of the tumours. BT474 cells are estrogen dependent and in order for them to continue to grow *in vivo* estrogen pellets were implanted into the shoulders of the animals 24 hours prior to the sub-cutaneous injection of cells or the implantation of a tumour fragment. This is a standard approach for the generation and maintenance of such tumours [7, 8]. In F1 phase of the study, which examined

the growth of tumours derived from the cell line, approximately 19-21 days following the implant of the cells, deterioration in the health of the animals was noticed. They had begun to lose weight and there was a hardening around the bladder area. During exploratory surgery on two of the animals, a build-up of a significant volume of a hardened white crystalline material was found in the bladders of the animals (Figure 6.3). On a detailed examination of the literature in this field, this adverse effect was also identified by a number of other groups using estrogen dependent cell line-derived tumours [8, 9]. The bladder sac had also increased in size. Following discovery of this adverse effect of the estrogen tablets, the animals were given sub-cutaneous injections of sterile saline in an attempt to delay the build-up of this material in phase 2 of the study through increased flushing of the bladder. The animals were also closely monitored for any signs of distress as well as palpated daily for any hardening in the region of the bladder.

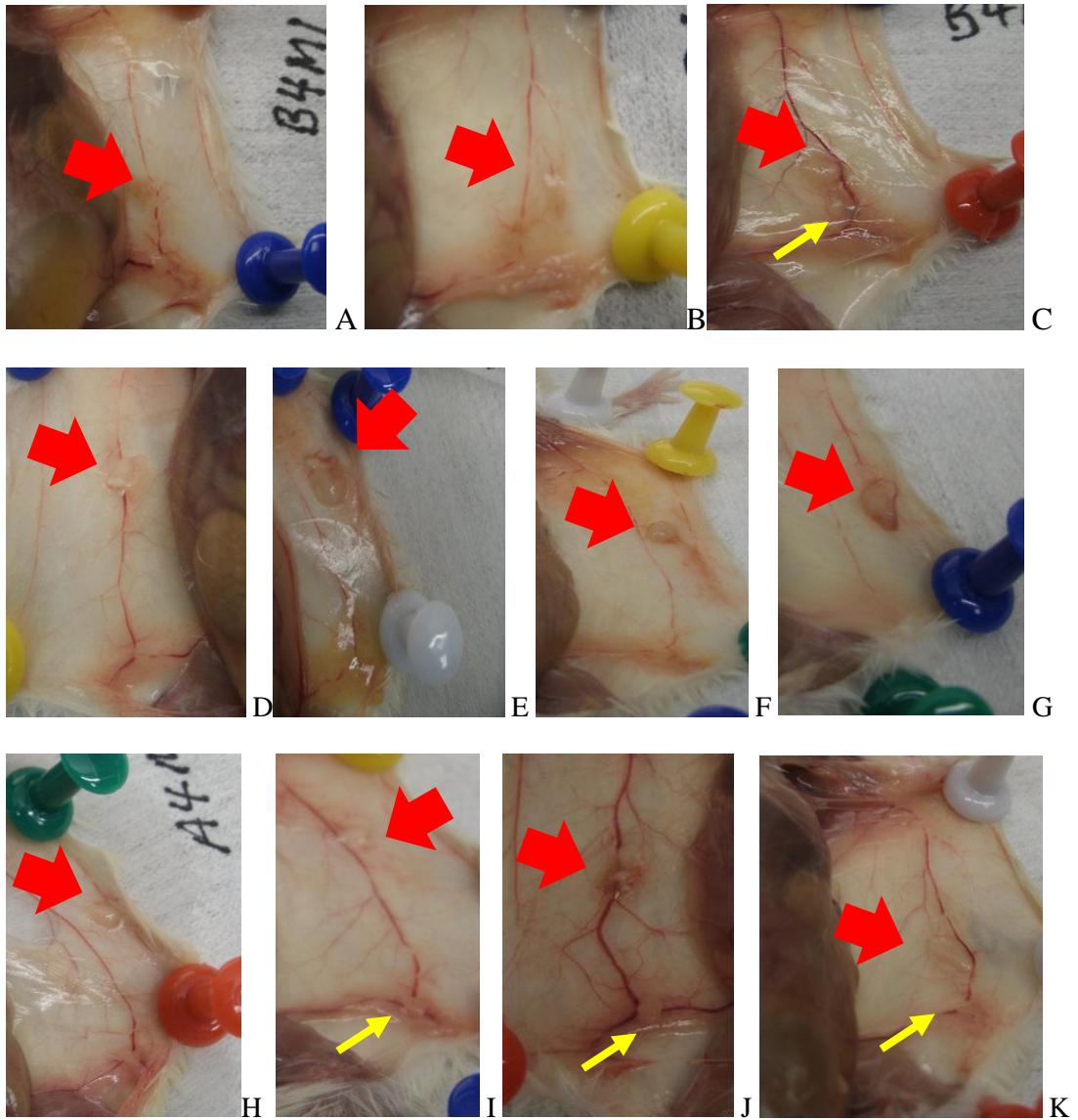


Figure 6.2: Location of BT474-derived tumours following subcutaneous implantation in the surviving animals following treatment with either vehicle, lapatinib 200mg/kg for 1 day or lapatinib 200mg/kg for 5 days. Images A-C indicate the tumours that were present in the mice that were treated with the vehicle, images D-G indicate the tumours that were present in the mice treated with lapatinib 200mg/kg for 1 day and images H-K indicate the tumours present that were present in the mice treated with lapatinib 200mg/kg for 5 days. The red arrow indicated the location of the implanted tumour. Increased vasculature is clearly visible around the tumour and the adjacent lymph node (highlighted by the yellow arrows).

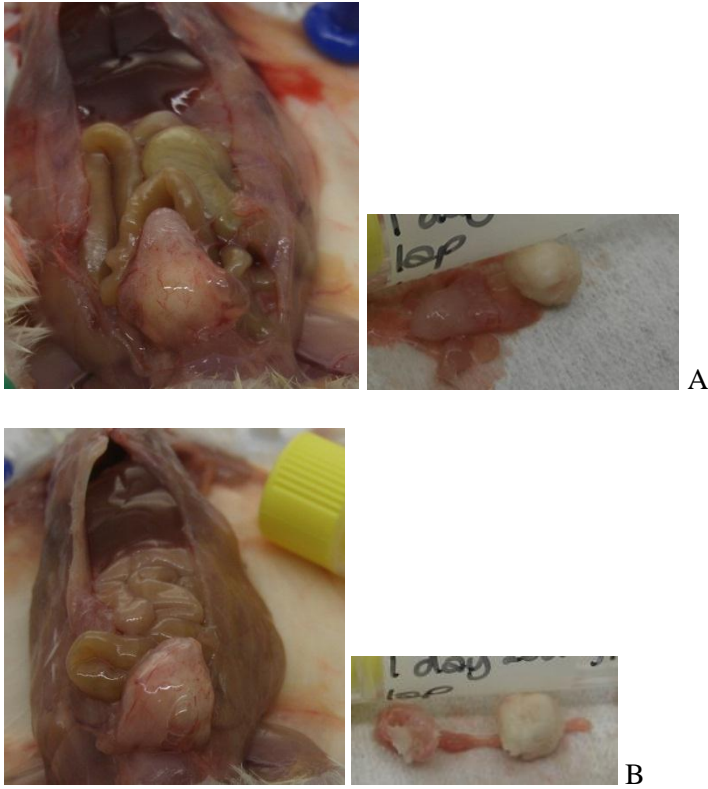


Figure 6.3: *In situ* visualisation of enlarged bladders (and the dissected bladders) that occurred following supplementation of animals with estrogen pellets to support the growth of the tumours. A white crystalline substance was found to have been deposited in the bladders of the mice approximately 19-21 days following the implantation of the estrogen tablets. The extent of the build-up was not known until an exploratory surgery was performed on the two of the animals affected which are shown in A and B. The only external indication that there was an issue was a swollen abdomen on the affected animals and the solid mass could be felt when the area surrounding the bladder was palpated.

6.3.2 Isolation of RNA and Taqman RT-PCR of snap frozen tumour samples

Two methods of RNA extraction from the tumour and tissue were optimised (homogenisation using a mortar and pestle in combination with the Qiagen Qiashtredder or the Qiagen automated Tissue Lyser) as outlined in section 2.7. These two methods are routinely used for the homogenisation of tumour samples for analysis [10]. Following RNA extraction using either method of tissue homogenisation, the RNA concentration in each sample was evaluated using a nanodrop. Two tumour samples were used for the optimisation of the two methods. When the extraction process was completed using the tissue lyser method in the autolyser for 20 minutes, it was evident that the tumour sample was not fully homogenised. Although the automated tissue lyser would have allowed for 12 samples to be homogenised at once, for the purpose of this study, the tumour and tissue samples were homogenised using liquid nitrogen in a mortar and pestle in combination with the Qiagen Qiashtredder method as it ensured that all of the tissue was homogenised and it was also a faster method to use.

Once the samples were homogenised they underwent extraction using the Qiagen RNeasy extraction kit that had been used for all of the *in vitro* cell line samples. The samples were quantified using the nanodrop in order to evaluate concentration of RNA for the reverse transcription reactions. It was necessary to concentrate the samples using the Maxi dry vacuum concentration system. All samples were concentrated so as to prevent any bias in the quality of the samples. Table 6.2 outlines the average ng/ μ L concentration of RNA found for each of the tumour, lymph node, lung and skin samples from each of the treatment groups. Each sample was quantified twice and the standard deviations are also reported. It should be noted

that the samples for the tumour from mouse 2 and mouse 3 from the 5 day lapatinib treated group did not contain an accurately quantifiable concentration of RNA as concentrations below 10ng/ μ L measured by the nanodrop were not considered to be reliable.

A number of internal controls were utilised throughout this analysis. On each 96 well Taqman plate there were 3 controls; no RT, no template control (NTC) and a pooled control. The no RT control was prepared at the same time as the RNA was undergoing reverse transcription. It contained no multiscribe reverse transcriptase. The no RT control was used to determine that there was no genomic DNA contamination in the RNA. DNase treatment during the RNA extraction protocol and correct handling of the samples would also aid in reducing genomic DNA contamination. The NTC control contained a gene assay and the universal Taqman mastermix but no target cDNA and was used to determine if there was any contamination of the reagents used in the qRT-PCR. The final control was the analysis of a pooled sample. Once RNA quantification was completed, 1 μ L of each of the RNA samples was pooled together and it underwent reverse transcription. A gene assay was chosen at random and this assay was analysed on each plate using the pooled sample. For the purpose of this study, *FOXO3a* was used for this control. As expected, on all of the plates, it was expected that this pooled sample had the same C_t value. This control exhibited a C_t value of ~26 on all plates tested indicating that there was no intra-plate variability and that the machine was working comparably on different days and different plates.

Table 6.3: Quantification of extracted RNA from tumour, lung and skin samples

Sample ID	ng/μL	\pm SD
F2 samples		
Tumour vehicle m1	29.09	0.099
Tumour vehicle m4	32.63	0.163
Tumour vehicle m5	25.115	0.318
Skin vehicle m1	106.95	0.085
Skin vehicle m4	137.09	2.659
Skin vehicle m5	47.86	0.156
Lung vehicle m1	298.38	2.036
Lung vehicle m4	204.54	1.259
Lung vehicle m5	255.92	0.969
Tumour 1 day m1	63.40	0.247
Tumour 1 day m2	45.29	0.127
Tumour 1 day m3	67.07	0.057
Tumour 1 day m4	52.63	1.499
Tumour 5 day m2 #	-----	-----
Tumour 5 day m3 #	-----	-----
Tumour 5 day m4	46.5	0.849
Tumour 5 day m5	25.31	0.495
Lymph node 5 day m2	101.05	3.521
Lymph node 5 day m3	89.29	0.325
Lymph node 5 day m4	144.05	0.933
Lymph node 5 day m5	135.6	0.714
Skin 5 day m2	89.83	1.322
Skin 5 day m3	139.68	0.269
Skin 5 day m4	109.5	0.820
Lung 5 day m2	171.28	2.206
Lung 5 day m3	393.84	2.652
Lung 5 day m4	161.69	1.421
Lung 5 day m5	208.71	0.403
Exploratory animal Samples		
Cell derived tumour vehicle	14.82	0.078
Cell derived tumour 5 day	10.30	0.219

indicates that the RNA from these two samples could not be accurately determined due to the concentration being too low. n= 2

As depicted in figure 6.1, the F1 phase of the *in vivo* study involved the subcutaneous implantation of 5×10^6 BT474 cells per mouse in eight SCID mice. Of these eight animals three were used as donors for arm 2 of the study, two were sacrificed for investigation purposes and the remaining three animals were treated as a pilot to give experience of the treatment schedule and demonstrate that sampling would be possible and likely to generate viable data post treatment in the second arm of the study. Unfortunately, of the three remaining animals, only two of them had developed tumours. Two of the mice (one of which had no tumour growth) were treated with 200mg/kg of lapatinib for 5 days and the remaining mouse was treated with vehicle alone for 5 days. Following sacrifice, the tumours were analysed using qRT-PCR. Of the eight genes determined to be differentially expressed in response to lapatinib in the panel of cell lines in chapter 3, five of them; *RB1CC1*, *FOXO3a*, *NR3C1*, *ERBB3* and *CCND1* provided a more compelling response pattern in response to lapatinib in chapter 4. It was these five that were evaluated in the *in vivo* setting.

6.3.3 Gene expression changes in response to lapatinib in cell line derived tumours

The tumour cells that were injected sub-cutaneously in the mice, were anticipated to be supported by the murine stroma [4, 6, 11]. As the cells that were injected were derived from a human tumour it was expected that the expression profile of these would be maintained. Of the five genes that we had previously examined in the *in vitro* experiments, it was found that only the primer for *ERBB3* was human specific, while the remaining four primers employed had the ability to detect both human and mouse variants of the gene. In order to determine if there was any infiltration of the murine stroma into the human cell, human specific assays for the genes were sourced. There was no human specific assay for *CCND1* available; however for all other genes, human specific assays were purchased. The endogenous control assay for *GAPDH* was also determined to be human specific. The assays that were not human specific, would have been expected to detect expression of the gene in question in both the human and murine tissue. A mouse specific assay for both *FOXO3a* and *GAPDH* were also purchased so as to determine the expression of these genes in the mouse tissues. It was expected that there would be no differential expression of these genes following treatment with lapatinib, as the mouse tissue should not respond to lapatinib treatment.

From the *in vitro* work that was performed in chapters three and four, the pattern that had been observed from the cell lines showed an increase in the differential expression of the *RB1CC1(H/M)*, *FOXO3a(H/M)*, *NR3C1(H/M)* and *ERBB3(H)* genes and a decrease in the differential expression of *CCND1(H/M)* in response to lapatinib. This pattern was not evident in the single cell line-derived tumour in this

pilot experiment. As shown in figure 6.4. *FOXO3a(H/M)*, *CCND1(H/M)* and *FOXO3a(H)* showed no change in the expression of the genes of interest between the 5 day 200mg/kg lapatinib (single tumour) and the vehicle alone treatments (single tumour). The genes were considered unchanged if the RQ value was less than 2. Decreases in gene expression following the treatment were seen in *RB1CC1(H/M)*, *NR3C1(H/M)*, *ERBB3(H)*, *RB1CC1 (H)*, *NR3C1(H)* and *FOXO3a (M)*. The C_t values for the *GAPDH (H)* endogenous control were equivalent to the values expressed in the cell line *in vitro* analysis with values of 20 and 22 for the untreated and 5 day lapatinib treated animals respectively. As there was only one replicate of each treatment these results could not be conclusively analysed but the experiment did prove that sufficient high quality RNA could be generated to permit accurate gene expression measurement.

BT474 cell line-derived tumours 5 day lapatinib treatment 200mg/kg

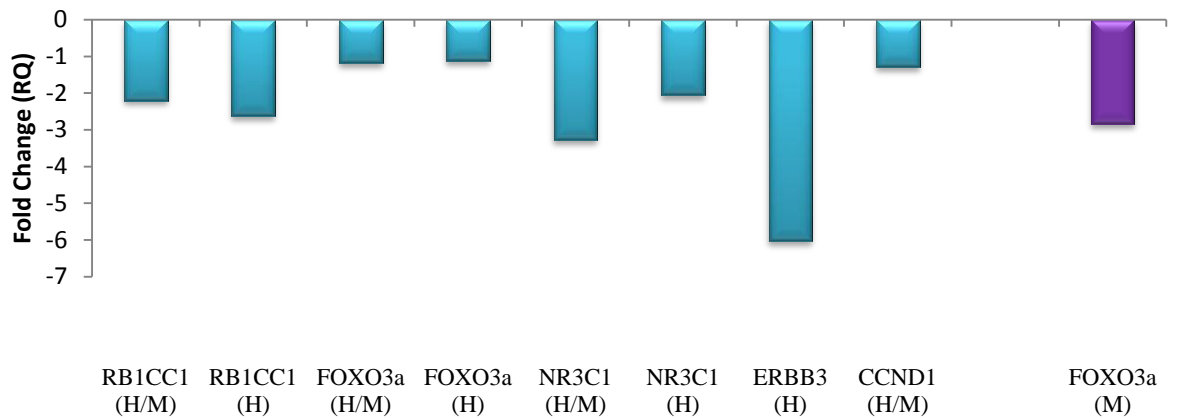


Figure 6.4: A summary of the changes in gene expression in a BT474 cell line xenograft tumour from an animal treated with lapatinib (200mg/kg) for 5 days, when compared to the gene expression data from a non-drug treated tumour. (H) indicates the human specific gene assay. (M) indicates the murine specific assay. (H/M) indicates that this assay detects both human and murine gene.. The control animal was treated with vehicle ((0.5% hydroxyl-propyl-methyl-cellulose/0.1% Tween 80) for 5 days, the tumour was then excised and expression of the relevant genes calculated. The test animal was treated with 200mg/kg lapatinib in the same vehicle for 5 days. Both treatments were administered using oral gavage. There was no correlation between the *in vitro* pattern that was discussed in chapter 3 and the *in vivo* pattern from this pilot study. n=1

6.3.4 Gene expression changes in response to lapatinib in tumour derived xenografts

As outlined in figure 6.1, the F2 phase of the study examined gene expression changes in tumour-derived xenograft models. 14 animals were implanted with fragments from tumours derived from the BT474 cell lines in arm 1. Once split into three groups, the tumours were allowed to develop and two groups were then treated with 200mg/kg of lapatinib for one or five days with the third group treated with vehicle (0.5% hydroxyl-propyl-methyl-cellulose/0.1% Tween 80) alone for 5 days.

Figure 6.5 shows the differential gene expression changes following one and five days 200mg/kg lapatinib treatment. It should be noted that not all of the animals in each group of the experiment showed expression of the genes and that the graphs below show a mean expression change from those animals that did. Table 6.4 outlines the number of individual C_t values for each gene at each treatment conditions that were included in the calculation of the relative expression of the genes (Individual C_t values can be found in appendix 3).

Table 6.4: Number of individual C_t values for each gene used in the RQ calculation

	Number of Ct values for each gene used in the RQ calculation		
	Vehicle /3	1 Day/4	5 Day/4
RB1CC1(H/M)	2	3	3
FOXO3a (H/M)	3	4	4
NR3C1(H/M)	2	ND	ND
ERBB3(H)	2	3	3
CCND1(H/M)	3	4	4
RB1CC1 (H)	2	3	2
FOXO3a (H)	2	2	4
NR3C1 (H)	2	4	2
GAPDH (H)	2	4	4
FOXO3a (M)	3	4	4
GAPDH (M)	3	4	4

All genes showed a differential upregulation following 1 day of treatment with 200mg/kg lapatinib with the exception of NR3C1(H/M) where no expression was detected in any sample. *FOXO3a(H/M)*, *CCND1(H/M)* and *NR3C1 (H)* showed the highest levels of differential expression. The upregulation of *CCND1(H/M)* was unexpected as it has been downregulated in response to lapatinib in all of the other studies completed. The calibrator sample for all of the RQ calculations was the 5 day vehicle treatment.

In the *in vitro* experiments, *RB1CC1(H/M)*, *FOXO3a (H/M)*, *NR3C1(H/M)*, *ERBB3(H)* were found to be up-regulated in response to lapatinib and *CCND1(H/M)* was found to be down-regulated in response to lapatinib in the BT474 cell line. In comparison in the *in vivo* experiment, all of the genes with the exception of *NR3C1(H/M)* which was not expressed in the 1 day lapatinib treated xenografts, were all differentially up-regulated in response to lapatinib.

Figure 6.5A showed the differential expression of the genes in response to 1 day treatment of lapatinib. There was no RQ value for *NR3C1* determined as there were no C_t values detected for the gene in the treated animals. It is possible that no signal could be detected using this probe due to stromal contamination of the samples. This could possibly be evidenced by the finding that the *FOXO3a(H/M)* assay showed a higher level of expression than that of the human specific assay. The C_t values for the *FOXO3a (H/M)* were slightly lower than that of the human specific assay (Appendix 3) which could be attributed to the higher differential expression for the non-specific assay. There was also some biological variability in the C_t values for the *FOXO3a (H)* vehicle control group as well as the 1 and 5 day lapatinib treatment groups which could account for some of the discrepancies in the RQ values exhibited. Interestingly, the mouse specific gene was also shown to be differently expressed. There was a high magnitude of up-regulation in the *CCND1(H/M)* and the *NR3C1(H)*. The *CCND1(H/M)* up-regulation was an unexpected result as down-regulation of the gene in response to lapatinib has been identified by a number of studies [12, 13].

Figure 6.5B shows the expression of the panel of genes in response to 5 days treatment of lapatinib. As with the 1 day lapatinib treatment, all of the genes were shown to be up-regulated. However, in comparison to the 1 day lapatinib treatment, there were some differences in the expression of the genes following the 5 day treatment. There was a reduction in the magnitude of *FOXO3a(H/M)*, *CCND1(H/M)* and *NR3C1(H)*. The levels of *RB1CC1 (H/M)*, *ERBB3(H)*, and *FOXO3a(H)* remained the same. A relative expression for the *NR3C1(H/M)* assay was evaluated, however, there was only one animal in the 5 day lapatinib treatment group that

expressed detectable levels of the gene, so no standard deviation value could be evaluated.

All C_t values can be found in appendix 3.

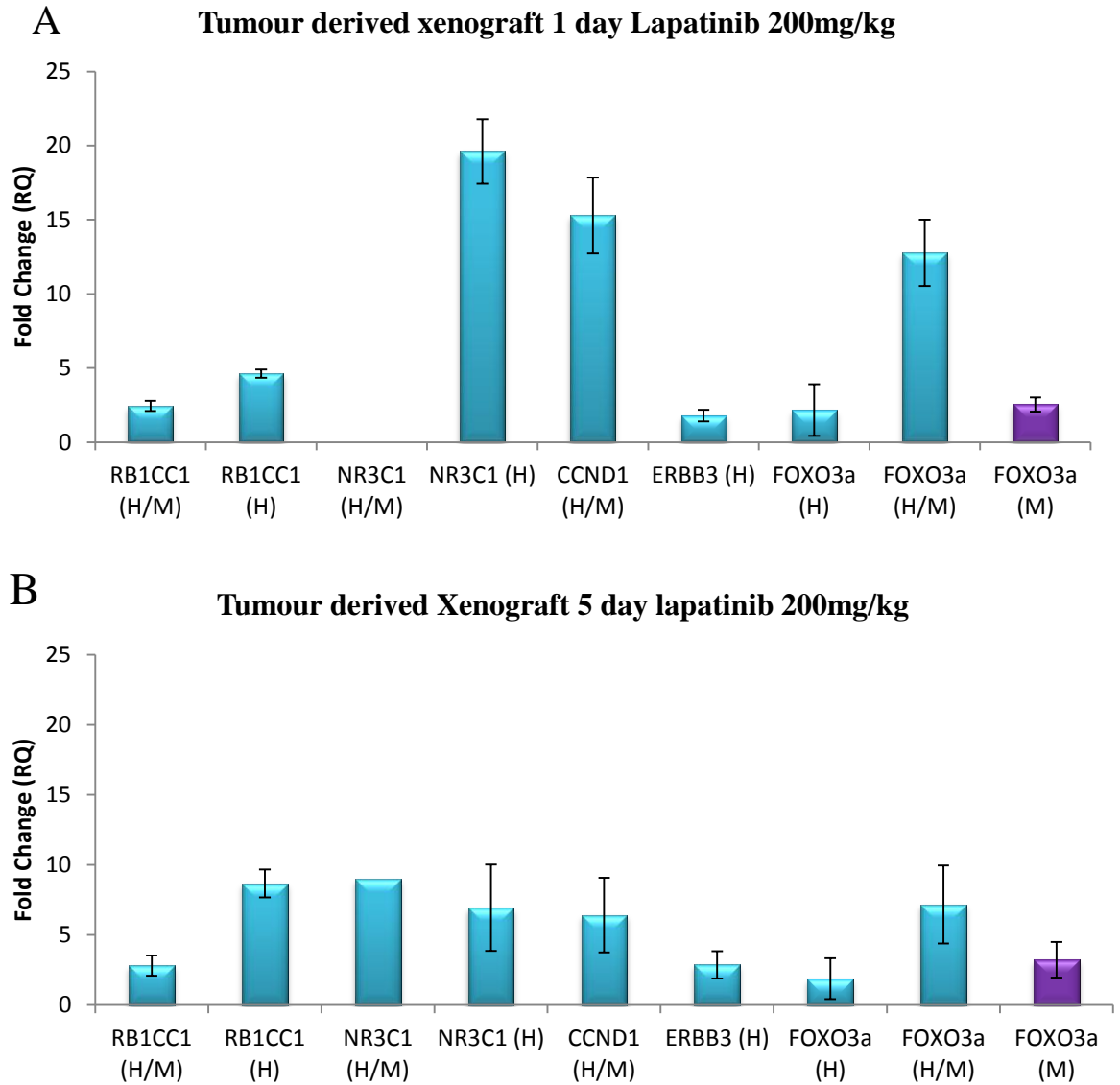


Figure 6.5: Relative changes in mean differential expression of the panel of genes following 1 and 5 days lapatinib treatment in tumour derived xenografts. The expression of the genes was evaluated relative to the expression of the same genes in the vehicle treated animals. The n values (number of biological replicates) for each gene expression measurement can be found in table 6.4. (H) indicates the human specific gene assay, (M) indicates the murine specific assay (H/M) indicates that this assay detects both human and murine gene. Control animals were treated with vehicle, (0.5% hydroxyl-propyl-methyl-cellulose/0.1% Tween 80) for 5 days. Test group (A) was treated with 200mg/kg lapatinib suspended in vehicle for 1 day (A) and group (B) treated with the same agent for 5 days. Treatments were administered using oral gavage. The error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

6.3.5 Gene expression changes in response to lapatinib in lymph-nodes, lung and skin

In addition to the tumours, the lymph-nodes, lung and skin tissue of the vehicle and 5 day lapatinib treated mice were assessed for the gene expression pattern. The lymph-nodes were examined as it was observed that in a number of the mice examined that the lymph-nodes were enlarged (Figure 6.6). The lung tissue was examined, as it is a normal tissue, and as such the genes should not be differentially expressed. The skin was evaluated as a known adverse effect of lapatinib treatment in humans is skin irritation [14-16] and it has been observed in some studies, that patients who develop a rash/skin irritation have an increase in life expectancy [17].

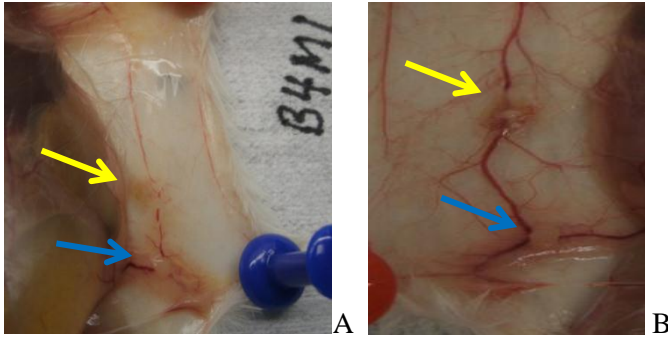


Figure 6.6: Images illustrating the enlarged lymph-nodes that were associated with the tumour implantation that were observed in the vehicle(A) and 5 day lapatinib (B)-treated mice. The blue arrow highlights the enlarged lymph-node. The yellow arrow indicates the implanted tumour.

Following extraction of the RNA from the tissue samples (concentration of RNA can be found in table 6.3), Taqman RT-PCR was completed on the samples. In all of the samples, it was not possible to evaluate the relative expression of the following genes, *RB1CC1(H/M)*, *FOXO3a(H/M)*, *NR3C1(H/M)*, *ERBB3(H)*, *CCND1(H/M)*, *RB1CC1(H)*, *FOXO3a(H)* and *NR3C1(H)* as no C_t values for these genes were detected in the samples, with the exception of *FOXO3a(H/M)* and *CCND1(H/M)* (C_t values can be found in appendix 3). There was also no detectable C_t value for the *GAPDH(H)* which is used to normalise the expression of the gene in the Livak method[18].

The murine specific *FOXO3a* and *GAPDH* did, however, have detectable C_t values and the relative gene expression was calculated. Figure 6.7 illustrated that following the treatment of lapatinib for 5 days, the gene was considered to be unchanged in response to the lapatinib treatment in the three tissues as they had an RQ value that was below 2.

***FOXO3a* (M) expression in different tissues following 5 day lapatinib treatment**

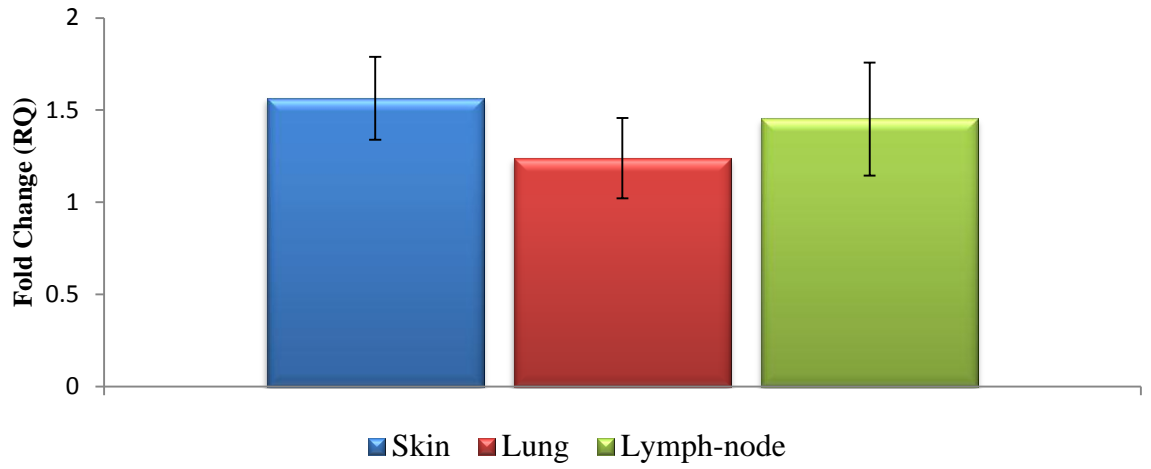


Figure 6.7: Changes in mean relative murine *FOXO 3a* gene expression in response to five days of lapatinib treatment from mouse skin, lung and lymph-nodes tissues in comparison to untreated tissue for the same regions. The error bars represent the standard deviation of the mean $\Delta\Delta C_t$ value.

6.4 Lapatinib quantification in plasma using LC-MS.

In order to evaluate the level of drug that was present in the animals system, drug quantification was completed. Dr Sandra Roche completed the analysis. Blood samples were taken from the animals before they were sacrificed and the plasma was separated from the whole blood by centrifugation for 15 minutes at 2000g. The plasma was then stored at -80°C until ready for analysis. There were no samples collected for two mice (C4M3 or C4M4 day lapatinib treatment) as blood could not be sampled from the animal. The method was completed as set out in section 2.13.6.

Duplicate technical samples were taken from each of the plasma samples, with the exception of the 1 day lapatinib treated mouse 4 as there was insufficient plasma volume, and were analysed. Table 6.5 shows the lapatinib concentrations that were determined in the plasma samples. There are no standard deviations as these concentrations were determined based on only two readings.

Table 6.5: Lapatinib plasma concentration as determined using LC-MS.

Treatment	Mouse ID	Plasma Concentration (ng/mL)	±SD (n=2)
Vehicle	B4M1	below limit of detection	n/a
Vehicle	B4M4	below limit of detection	n/a
Vehicle	B4M5	below limit of detection	n/a
1 day Lapatinib	A4M2	106.44	9.18
1 day Lapatinib	A4M3	5556.50	2331.04
1 day Lapatinib	A4M4	42.13	n/a *
1 day Lapatinib	A4M5	33.63	6.11
5 day Lapatinib	C4M2	51.39	3.53
5 day Lapatinib	C4M3	98.61	7.44

*indicates that there was only one replicate analysed so no SD could be determined

The plasma concentrations of lapatinib in the vehicle samples were below the limit of detection, which was to be expected as no lapatinib was given to the animals. In the 1 day lapatinib treated A4M3 there was extremely high level of lapatinib in the plasma, with a concentration that was >5000ng/mL. This was an unexpected result and could not be explained. The two technical readings of these samples both showed high level of lapatinib in the plasma. It is possible that there was some form of sample handling error that could have resulted in the reading of a high level of lapatinib in this plasma sample. In addition to this it is also possible that the blood sample did not have sufficient anticoagulant in the tube during collection and as a result there was some clotting of the blood sample. The remaining samples all had levels that were between 33 (A4M5) and 106 (A4M2) ng/mL.

6.5 Discussion

The use of xenograft models has gained importance in particularly in the clinical trial setting[19]. In the course of this *in vivo* study, two approaches were employed, a cell line-derived tumour as well as the implantation of a tumour fragment from a donor mouse into a recipient mouse.

The BT474 cell line that was selected to generate the cell line-derived tumour had been successfully used in previous HER2+ tumour xenograft experiments in the literature. [20-23]. As the most lapatinib sensitive cell line in our breast cancer cell lines panel from the *in vitro* studies from chapter 3 and 4, it was deemed the most suitable for the *in vivo* investigation. In culture, the cell line has a tendency to be slow growing and can take a number of weeks to become confluent following thawing [24]. When the cells were implanted it was discovered that the tumours derived from these cells were also slow growing as, after 23 days, the tumours had only reached a volume of approximately 100mm³. This tumour volume was in line with what other BT474 *in vivo* studies determined at this time point [25, 26]. In comparison to an unrelated study that used the same method this tumour volume was quite small as at the same time point, the volume of tumours derived from the DLKP cell line had reached over 400-500mm³. In addition to the small tumour size, the mice suffered an unusual and potentially life threatening reaction to the estrogen pellets that were implanted in order to maintain the growth of the estrogen-dependent BT474 cells.

The pellets were implanted 24 hours prior to the cells to allow the mice to recover from the anaesthesia needed for this procedure prior to tumour cell implantation. During daily monitoring it was discovered that a number of the mice had significant

hardening in the bladder region, approximately 20 days post implantation of the pellet and, upon closer observation, it was noticed that they were not producing normal volumes of urine. It was also observed that the urine that was produced was extremely dark in colour and was causing staining of the fur. It was decided that an exploratory surgery would be undertaken in order to determine the cause of these symptoms. Upon dissection of the chosen mouse, it was discovered that the bladder has become blocked with a large deposit of crystalline material that was preventing the animal from urinating (Figure 6.3). This adverse effect had not been described by the producer of the pellet, however, when a detailed literature search was performed it was found that this effect has been observed by a number of other researchers [8, 27-29]. Kang *et al.*, [9] described, that by using lower doses of the estrogen pellet that the number of deaths that they had observed could be reduced but that the lowest dose of the estrogen pellet that was available still resulted in some deaths. They also described similar build ups in the bladder, which coincide with the timeline that the mice within our study exhibited these adverse effects.

In conjugation with these adverse estrogen effects and the slow growing nature of the tumours, it was not possible to leave the animals any more than 35 days post implantation with the cell line before they were sacrificed for use in the tumour-derived model or before the 5 day lapatinib treatment could be initiated. If the animals were left any longer, it was feared that this could cause undue suffering and could also possibly result in them succumbing to death from the bladder stones and the loss of the experimental material. The saline treatment that was given to the animals, while it may have provided the animals with increased hydration, did not prevent or slow down the crystalline build-up that was found in the bladders. As the experiment progressed, it was hypothesised that prolonged use of this palliative

method might also possibly cause increased harm to the animals as it was increasing their fluid intake, which they did not have the ability to adequately relieve due to the bladder blockage.

The gene expression pattern that was determined for the cell line-derived tumours was evaluated using RNA extracted from a tumour from a vehicle treated mouse and a tumour from a 5 day lapatinib treated mouse. From the cell line analysis, it was expected that we would see an up-regulation in expression of *RB1CC1(H/M)*, *FOXO3a(H/M)*, *NR3C1(H/M)* and *ERBB3(H)* and a down-regulation in the expression of *CCND1(H/M)* in the lapatinib treated tumours. However, in this initial pilot with only material from one control and one test animal this pattern was not evident. The anticipated down-regulation of *CCND1(H/M)* was evident but all of the remaining genes were also all down-regulated. There are a number of rationales for why this may have been the case. It is possible that the tumours did not respond to the treatment and if that was the case, the response that was evident would coincide with profile of non-responding cell lines to lapatinib treatment. This non-response could have been due to drug preparation issues or possibly due to issues with the absorption of the drug. Due to the small size of the tumour, it was not possible to use some of the material to evaluate the levels of the drug in the tumour using LC-MS as described in section 6.4. This analysis would have allowed us to determine that there was sufficient drug present in the tumour to affect gene expression. All previous analysis was done on triplicate cell line sample measurements for each gene; this analysis was only completed on one tumour sample and hence the finding could be subject to significant inaccuracy. In addition to the five assays that were used in the *in vitro* experiments, three human specific assays for *RB1CC1(H)*, *FOXO3a(H)* and *NR3C1(H)* and one mouse specific assay *FOXO3a (M)* were also employed in the

analysis. These human specific assays were used in the hope of distinguishing tumour (human cells) from murine gene expression, and thereby help evaluate the tumour specificity of lapatinib-induced gene expression changes

The probes used throughout the analysis were either human specific, murine specific or cross-reactive for both species. The original Taqman probes that were used in the *in vitro* analysis were (unintentionally) a mixture of human specific and cross-reactive probes. Human specific probes were employed for the *in vivo* analysis to help evaluate the effect of potential murine stromal contamination of the tumour material. The species specific probes, although targeting the same genes as the cross-reactive probes, target a different region of the gene sequence that has not been conserved in the different species. The targeting of the different region could account for some of the variability that was exhibited in the gene expression in the tumour derived xenograft samples following lapatinib treatments.

Another objective of this arm of the study was to determine if the mice could cope with the dose of lapatinib they would receive for 5 days. The half-life of lapatinib has been determined to be approximately 24 hours and a steady state level of lapatinib occurred after five to seven days in human patients [30, 31]. It was these aspects of the pharmacology of lapatinib that determined the time points in the lapatinib treatment of 1 and 5 days. 200mg/kg was the maximum drug dose administered. There have been a number of *in vivo* studies where varying doses of lapatinib have been administered [23, 32-35]. Had there been sufficient animals to use, it would have been ideal to conduct a dose range experiment on a number of different animals to evaluate any dose dependent correlations in the gene findings. However, due to time and experimental limitations our dose regime was based on previous literature. A dose of 200mg/kg was selected as literature suggested it would

provide a sufficient dose of the drug to infiltrate the tumour while being of limited toxicity to the mice over durations longer than we proposed to use. It was hypothesised that exposure of the tumours to this drug level for the durations chosen (particularly for the five day duration) should be roughly consistent with the maximal durations previously evaluated *in vitro* and result in maximal gene expression changes.

The xenograft study, involved 14 mice that underwent treatment with the vehicle alone, lapatinib for 1 day or lapatinib for 5 days. As the estrogen pellets were also required in F2 phase of the study, where the tumour fragments from donor mice were transplanted into recipient mice, sub cutaneous injections of saline were given to the animals from approximately day 10 post implantation in order to encourage urine production, in the hope that extra fluid delivery would “flush” the bladder, preventing or delaying development of the bladder issues. Unfortunately all of the mice within each of the three treatment groups (vehicle, 1 day lapatinib treatment and 5 day lapatinib) developed the same bladder issues as those in the F1 phase of the study. As a result of this, the animals were treated with either vehicle or 1 or 5 day 200mg/kg of lapatinib using oral gavage before the tumours had sufficient time to develop into larger tumours. All genes showed a differential upregulation following 1 day of treatment with 200mg/kg lapatinib with the exception of *NR3C1(H/M)* where no expression was detected in any sample. *FOXO3a(H/M)*, *CCND1(H/M)* and *NR3C1 (H)* had the highest levels of differential expression. The upregulation of *CCND1(H/M)* was unexpected as it has been downregulated in response to lapatinib in all of the other studies completed. When examining the differential expression of the genes following 5 days treatment with lapatinib, there

was a change in the level of expression when compared with the values from the 1 day lapatinib treated tumours.

There was a significant biological variation between the C_t values for the genes between each mouse which accounts for the large standard deviation values that are present (appendix 3). The variation in the tumour response could be explained by differences in the tumour section that was implanted into the mice. The tumour that was generated by injecting BT474 cells sub-cutaneously into the mouse, was cut up in order to be transplanted into the donor animals. It is possible that there were differences in the characteristics of the different sections such as the relative level of murine stroma infiltration or a concentrated pocket of cells that could explain the biological variation. The size of the tumours could also have had an impact on the tumour response. The tumours that developed were quite small in size in comparison to the tumour that developed following implantation with the BT474 cells. As a result of the small size it is possible that there was limited amount of human tissue from which RNA could have been extracted. When the tumours were being dissected from the mice there were small amounts of murine tissue extracted simultaneously. This murine tissue contamination could have reduced the signal for the human specific probes.

LC-MS/MS analysis was employed to measure the concentration of lapatinib in plasma samples from the animals. Measurements of the drug (table 6.5) indicated that there were measureable levels of the drug in the animals system following the oral gavage administration. In order to confirm that sufficient levels of the drug were present in the tumour, this analysis would need to be completed on tumour samples following treatment. Unfortunately this was not possible due to the small size of the tumours. It was believed that the levels measured in the blood indicated that

sufficient levels were present, that the tumour had internalised the drug to affect gene expression within the cancer cells.

Examination of the gene expression changes in the lymph-nodes, the lung and the skin following 5 day lapatinib treatment was completed to determine if there was any similarity of the expression pattern in other animal tissue samples. As anticipated, it was not possible to determine the relative expression of the genes, including the endogenous control, *GAPDH*, that were targeted by the human specific assays in these murine tissues.. It was possible to evaluate C_t values for the *FOXO3a(H/M)* and *CCND1(H/M)* but as there as there was no *GAPDH* C_t values, it was not possible to generate relative expression data. C_t values for the murine specific *FOXO3a* and *GAPDH* were detected and hence the relative expression of the murine gene variants was measurable. In all of the tissue samples the gene expression was determined to be unchanged after lapatinib treatment as the RQ values were below 2 indicating that this normal tissue was not responsive to the circulating drug.

It was promising to see that it was possible to evaluate the gene expression changes in both the cell line and tumour-derived xenograft models and with proportionately small amounts of tumour material. In order to fully examine the gene changes in this *in vivo* setting it would be critical to repeat this experiment with a number of changes. At the initial implantation and generation of the tumours, it would be important to monitor and possibly reduce the dose of the estrogen that is required for the experiment or else use a non-estrogen-dependent HER2+ model for the xenografts. Kang *et al.*, [9] has shown that low dose estrogen can still maintain and support growth of the receptor-dependent cell lines and can extend the life expectancy of the animals. It has also been shown that introducing the estrogen in the water could be a plausible way to ensure dosing of the animals at a low dose

[36]. It has also been shown that it may not be necessary to use estrogen to maintain the tumour growth [37]. The strain of mice that was used may also be important to look at. Although SCID mice have been evaluated as being more immune-compromised than nudes, they are not the only suitable model that could be used. They have been shown to have a tendency to develop lymphomas, which could impact results of the study [38]. Nude mice have been shown to be comparable for the generation of xenograft models [36, 39, 40]. Nude mice tend to have some other practical limitations though as they have been shown to not support metastasis studies [41], while SCID mice appear to be more supportive of metastasis studies [3]. A slightly larger cohort of animals would also need to be used for each treatment group, giving tighter statistical findings to the results and also that biological variation could be examined more. It would also be important to blind the study and RNA analysis. Blinding the study would mean that animals were transplanted with tumour sections from differing sources. If this was to be completed in human studies, it would involve treating tumour samples from different patients under the same treatment conditions and determining the mean relative gene expression.

It was not possible to detect a signal for the endogenous control in the lymph-node, lung and skin tissue samples. There were also biological variations in the C_t values that were determined for the tumour samples. It would be necessary to optimise the expression of a more suitable endogenous control for any further study. Further control genes such as TFRC, ABL and PSMC4 as evaluated by McNeal, R.E *et al.*, [42] may be more suitable, however, optimisation would have to be undertaken.

In principle, this study indicated that the experimental approach is appropriate for the evaluations we were attempting. Tumours were developed using both breast cancer cell lines and also secondary tumour implants derived from those cell lines. The

mouse strain that was utilised, SCID, successfully supported tumour culture, with nearly a 100% tumour growth success rate. It was also evident that the mice could tolerate the dose and treatment regime of the targeted therapy lapatinib. Successful RNA extraction and Taqman RT-PCR analysis were completed on the tumour material. Inclusion of a patient derived xenograft (PDX) model could also provide interesting data as it provides a more realistic representation of a human tumour due having been less modified by culture and since such tumours typically demonstrate a presence of human stromal tissue. Further *in vivo* studies would be required in order to fully examine the effect of the lapatinib on the gene expression pattern; however the preliminary results described here are a strong foundation to build additional studies on. These results do show a consistency with the initial premise of the experiment, which was to evaluate if the gene pattern identified *in vitro* can be assed in an *in vivo* model.

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7. Discussion

7.1 Discussion

The aim of this project was to employ bioinformatics and *in vitro* and *in vivo* techniques to understand the molecular effect of targeted therapies on a panel of breast cancer cell lines. By investigating molecular gene expression changes, it was hoped to identify potential markers of response to a panel of HER2 targeted therapies. Development of novel targeted drugs is a major focus for the pharmaceutical industry; however, there is enormous need to identify a means to characterise which patients will benefit from treatment, thereby making such agents more useful and cost effective. It was hoped that if we could identify markers of response this might be part of a potential route to ensure that only patients that will benefit from these expensive targeted therapies will receive the treatment.

Extensive bioinformatic interrogation of microarray data from two HER2-positive lapatinib-sensitive cell lines treated with varying concentrations of lapatinib resulted in a list of 512 genes which were significantly altered in response to the drug. This list was generated using the Co-inertia analysis (CIA) technique, a multi-variant data analysis method that allows for the identification of trends or co-relationships in gene expression across multiple datasets which contain the same samples. This was the first time the analysis method had been applied to breast cancer cell lines or cellular drug response data. From this list of 512 targets, a subset of 19 genes was examined using RT-PCR with five genes being shown to be differentially expressed in response to lapatinib and their magnitude of expression change was proportional to the sensitivity of the cell lines to the targeted drug [1]. When these five genes (*RB1CC1*, *ERBB3*, *NR3C1*, *FOXO3a* and *CCND1*) were examined in response to additional HER2 targeted therapies (afatinib, neratinib and trastuzumab), they

showed a similar gene expression pattern, indicating that the pattern identified has the potential to predict not only response to lapatinib but also, more broadly, HER2 targeting therapies [2]. The gene expression pattern was not present when it was evaluated in a cell line (SKBR3-L) that has developed acquired resistance to lapatinib. When combined with our findings from a series of intrinsically sensitive and resistant cell models, this result suggested that the gene expression pattern has the potential for differentiating cell lines that are responsive, insensitive or have become resistant to lapatinib. Finally, when evaluated in a pilot *in vivo* study, the gene expression pattern was also largely evident in a xenograft model following treatment with lapatinib for both 1 and 5 days.

The results generated from this project suggest that measuring gene expression changes in tumours treated with HER2-targeting drugs may have the potential to be utilised as a rapid, sensitive predictive marker of response to such therapies. More generally, such an approach might potentially be adaptable to examination of the drug response of tumours to various drug treatments. In using this approach to determine response to therapies, it could be possible to avoid treating patients with expensive drugs that they will receive no benefit from and which could cause potentially serious side effects without any likely benefit. This could greatly improve overall treatment efficiency and efficacy.

7.2 Current state of the art in predictive cancer diagnostics

7.2.1 Diagnostically Predictive Microarray technologies

In examining the currently available prognostic or predictive biomarker approaches, there is clear diversity in the methodologies that have applied using each specific technology.

Microarray technology has been utilised by a number of different groups that have identified gene expression patterns that can be used as either prognostic or predictive indicators of patient response. Oncotype DX, which involves a 21 gene signature, has been used in a prognostic manner to identify patients that are at risk of a recurrence of disease[3]. In the TAILOR X clinical trial the Oncotype gene expression signature was utilised to identify patients that would benefit from the inclusion of chemotherapy in their treatment regime[4]. MammaPrint uses a similar methodology [5] and in their approach a 70 gene signature has been used to predict patients that were likely to suffer from metastasis from their original tumour. This assay is employed in the MINDACT trial, as with the TAILOR X trial, to predict which patients would benefit, from the inclusion of chemotherapy in their treatment[6]. Of interest, it has been suggested that the number of genes in both of these tests could be reduced as more detailed analysis has indicated that several do not make major contributions to the final therapeutic assessment and clearly in reducing the number of genes analysed there is potential to reduce the cost of the test [7].

7.2.2 Diagnostically predictive gene mutation tests

Examination of tumoural genetic mutations has also come to forefront of attempts to predict patients that will respond to certain chemotherapeutic or targeted therapies. The MassARRAY platform developed by Sequenom has been used to identify specific gene mutations that have been validated to predict to response to certain therapies. In combining PCR amplification steps with mass spectrometry (MALDI-TOF) detection, the MassARRAY analyser 4 system has the capability of high throughput analysis of a large number of patient samples. In utilising this technology the following studies were able to determine the most suitable treatment regimens for patients based on the presence of gene mutations. Brevet, M *et al.*, [8] utilised the MassARRAY technology to evaluate the presence of EGFR mutations in order to predict who would have an improved overall survival following erlotinib treatment, an EGFR inhibitor. Parker WT *et al.*, [9] determined that identification of BCR-ABL gene mutations following failure to respond to imatinib treatment in leukaemia patients, could predict which second line treatment would be the most suitable. Su, K *et al.*, [10] used the MassARRAY technology to identify the presence of the T790M mutation in lung cancer. Presence of this mutation has been associated with resistance to the EGFR inhibitors erlotinib and gefitinib. Identifying the presence of this mutation prior to or soon after beginning treatment could guide a more suitable treatment regime.

With the advances in sequencing technologies, the ability to characterise a patient's genomic tumour signature (including mutations etc.) more directly has become much easier and cheaper [11]. Next generation sequencing has proven to be a valuable tool in the deep sequencing of the tumour genome [12]. This high throughput technology

has the capability to sequence hundreds if not thousands of DNA sequences concurrently, cutting down on the time necessary to undertake this type of analysis [13]. It has the ability to be utilised in a number of studies, including SNP (small nucleotide polymorphisms), gene insertion and mutation, and copy number mutation analyses [11, 14]. While the technology has vastly improved in the last decade, there is still more work to be done before such technology can form the basis of a routine diagnostic and deep sequencing of a cancer genome results in an extremely rich data set, which requires expert analysis or would not be clinically useful [15]. That being said, this technique has the capability to identify small somatic gene mutations that could have huge clinical implications in relation to suitable treatment options [16]. RNA-sequencing, also known as RNA-seq utilises next generation sequencing technologies and applies them to sequencing RNA transcripts. In using this method, researchers can investigate alternative gene splicing [17], post-translational changes, changes in gene expression [18], ribosomal changes [19] and examine different RNA populations such as miRNA and tRNA.

7.2.3 *Ex vivo* diagnostic approaches

In addition to these molecular biological approaches to evaluate tumour response, an *ex-vivo* methodology has also been developed to assess a patients response to different therapies[20]. Precision Therapeutics Inc. have developed ChemoFX to evaluate the response of patient samples to possible treatment regimens at varying drug concentrations using core biopsy samples [21]. Briefly, a primary culture is prepared from the patient biopsy by mincing the samples, which are then seeded to a culture flask with the correct medium and cultured until confluent. The cells are then

seeded to a 384 well plate and incubated for 72 hours with the relevant drug treatments. Survival fractions are calculated using untreated cells as controls and dose response curves are generated to evaluate the response index of the treatments. While this method was initially developed for the evaluation of the sensitivity of gynaecological cancers such as ovarian [22, 23] and cervical [24] cancer, this assay has also been applied to a number of breast cancer studies[25-28].

7.2.4 Sample preparation challenges

When examining the different approaches for assessing predictive biomarkers, a common challenge that seems to affect all researchers is the quality of the samples on which the test will be performed. This challenge also relates to the validation and optimisation that is required in the preclinical evaluation of these markers. Historically FFPE tissue samples are the most common archival samples available, however, recent studies have indicated that this method of preservation is not conducive to the high quality RNA extraction necessary to evaluate mRNA and gene expression [29-31]. While there can be practical challenges with gene expression analyses from this type of material, it should be noted that while mRNA can be degraded in FFPE tissues, miRNA is generally not as impacted and as such archival material can be useful in miRNA studies.[32] While flash frozen samples would be more suitable for this type of analysis, the capability of hospitals to complete this method of preservation and store the collect samples is extremely restricted. In the Sequenom and Oncotype DX methodologies described in preceding paragraphs, FFPE is the recommended source material, while fresh tissue samples are preferred for the Mammaprint and ChemoFX methods. The Sequenom method is a DNA

based technology and as DNA is not as sensitive to degradation, FFPE tissues can be used successfully in this analysis.

7.2.5 Regulatory relevance of companion diagnostics

The drive to provide diagnostic tests for response to therapy has been continually increasing. The FDA have recently issued a directive, 21CFR809, calling for all pharmaceutical companies to incorporate companion diagnostics into their clinical and regulatory strategies[33] . Biomarker analysis and diagnostic development will now be required in conjunction with the pre-clinical and clinical trials with drug development [34]. However, it is not just regulatory bodies that are calling for companion diagnostics, national health services are also looking for tests that will indicate which patients will benefit from targeted therapy treatments. The National Centre for Pharmacoeconomics (NCPE) in Ireland and the National Institute of Health and Care Excellence (NICE) in the UK are responsible for determining if a drug should be made available to patients. Most of newly developed targeted therapies are expensive and have been seen to work in only a limited subset of potential patients. Without a companion diagnostic to determine which patients will benefit from treatment with these costly drugs, many of these therapies are not recommended to be made available for patients. In Ireland, the NCPE recently announced that it would not support the use of pertuzumab for treatment in HER2 positive metastatic breast cancer patients due to cost. The monoclonal antibody has proven to be extremely successful in clinical trials when combined with trastuzumab [35]. In the UK, NICE announced that it would not support the use of lapatinib in patients with HER2 positive metastatic breast cancer. Without the ability to evaluate

who will benefit from treatment with the TKI, they determined that it was not cost effective to recommend the treatment [36]. These are just two examples of how, due to inability to determine benefit from treatment, successful therapies are not being utilised to their full capacity.

In the area of HER2-targeted therapies, there are currently a limited number of approved companion diagnostics and none of these can be used to evaluate tumour response. The approved kits use either immunohistochemistry (IHC), fluorescent *in situ* hybridisation (FISH) or chromogenic *in situ* hybridisation (CISH) to evaluate HER2 status and expression [37]. However, only a subset of all HER2 overexpressing patients will respond to HER2 targeting therapies. More broadly in the larger cancer field, utilisation of cancer gene expression changes as potential markers of response has been applied to a limited number of experimental studies. Wang, XD *et al.*, [38] identified potential predictive gene expression markers of response to dasatinib which correlated with sensitivity of the cell lines used in their study. A similar approach, where gene expression changes were evaluated before and immediately after the start of treatment, was used by Modlich, O *et al.*, [39]. This group sought to determine if a comparison of gene changes before and after treatment could predict response to neoadjuvant chemotherapy regimens in patients with primary breast cancer. Their initial analysis indicated that this approach could be utilised for this purpose but that additional studies would need to be completed in order to fully evaluate which genes would be most suitable as markers.

7.2.6 Context of this work to the predictive diagnostic field

The evaluation of gene expression changes has been used by a number of the methods described above; however, none of the commercial kits evaluate gene changes after a treatment has been given. All of the methods described above employ gene expression changes to determine if a patient should be given a treatment or not, but do not evaluate if the patient should remain on the treatment already started. The gene expression pattern described in this thesis has the potential to be utilised in determining if a patient should continue to respond to a HER2-targeting treatment that they have recently been given or not. For most clinicians the only indicator for a patient to be given one of the HER2 targeted therapies is that they demonstrate amplification of the HER2 gene. However, as indicated in previous chapters summarising the literature and the *in vitro* results described in chapters 3 and 4, the presence of HER2 is not necessarily an indicator of ultimate patient response to therapy. In our models this is illustrated, for example, with our use of the MDAMB453 cell line which over-expressed HER2 but has an innate resistance to the effects of lapatinib.

While this preliminary work has both demonstrated that there are clear alterations in the expression of a specific set of genes in response to HER2 inhibitor treatment in sensitive cells and possibly more broadly that this concept might therefore be adaptable to other agents, further research will be needed to take this to the stage of being a useful diagnostic. In addition, while the findings of our *in vivo* study support our general hypothesis, there were practical challenges in our first experiment in this area. Overcoming the estrogen-related bladder issues and thereby generating a larger amount of tumour material for evaluation in larger groups of animals will make for a

much clearer and more therapeutically-relevant understanding of our findings. Functional validation of our genes of interest will confirm their role in the cellular HER2 inhibitor response. It is also possible that there may be more suitable gene targets from the original list of 512 genes described, in particular from the 19 genes which were more closely examined in chapter 3. The roles of PIK3C3, CDKN1B and ALDH3A2 may warrant examination in parallel with our five gene response in any future *in vivo* study to determine if they may provide additional response information. A patient-derived xenograft, which has much closer architecture and biological characteristics to that of a human tumour, would also help to assess the relevance and significance of the gene expression changes described.

One of the challenges that will have to be assessed in order to turn these gene changes into a useful diagnostic will be how the tumour samples will be taken and processed in a conventional patient/clinical context. As described in chapter 3, the minimum amount of RNA that is necessary to complete this type of analysis is as low as 20ng. A needle biopsy of a patient tumour would provide sufficient tumour material; however, processing of the sample will likely be very important. As the analysis is RNA-based, preventing contamination with genomic DNA and RNase degradation will be vital to ensuring that the quality of the sample remains at as high a standard as possible so that these contaminations do not interfere with the outcome of the analysis. So too will be our ability to utilise just tumour material, as our research has clearly shown that the response will not be evident in non-tumour material and a gene expression change signal could therefore be “crowded out” by RNA from non-tumour material. The capacity to use an automated processing step would remove some of the opportunity for these types of contamination to enter the samples. It appears likely that it will be necessary to complete a second biopsy of the

patient tumour within approximately one week of the treatment regime beginning which could cause some issues. In addition to patient discomfort and practical challenges with resampling from the same area, intra-tumour heterogeneity could have an impact on the analysis so it would be important that the second biopsy be taken close to the first one. It is conceivable that a second biopsy might not be required if the primary material could be kept alive and treated with clinically relevant concentrations of the test agent. Sampling of the gene expression before and after treatment would then be possible without having to go back to the patient and the associated inherent challenges of that approach. Further *in vivo* investigation could help to remedy some of these challenges and fine tune the optimal approach. A suitable gene analysis platform would also need to be chosen. As there may only be five genes in the expression pattern, use of microarray technology and next generation sequencing would be an inefficient use of these platform types as well as being costly.

In conclusion, there is an enormous imperative on biomedical science to identify diagnostics which predict or very rapidly measure tumour response in a patient if we are to improve the pharmacoeconomic viability of the emerging raft of new cancer pharmaceuticals and improve the selectivity of cancer treatment for our community. The work outlined in this thesis has identified a gene pattern that is predictive of response to HER2-targeted therapies in cell line models and a cell line derived xenograft. Using a multi-disciplinary approach which incorporated an emerging bioinformatic technique and molecular biological methods, the gene expression targets were successfully interrogated in a panel of cell lines with varying sensitivity to lapatinib and also in response to a number of different anti-HER2 therapies. By examining the genes in cells with varying sensitivities, we were able to determine if

the sensitivity of the cells to the treatment had an impact on the gene expression response, and hence whether the extent of alterations in our gene expression response could be used to predict the ultimate response of cells. In these HER2-expressing cell line models, lapatinib, neratinib, afatinib and trastuzumab treatment generated a characteristic and specific gene expression response, proportionate to the sensitivity of the cell lines to the HER2 inhibitor. Characterisation of the induced changes in expression levels of these genes may therefore give a valuable, very early predictor of the likely extent and specificity of tumour HER2 inhibitor response in patients, potentially guiding more specific use of these agents. Adoption of such an approach in the investigation of other therapeutics may also point the way for a whole new route and rapid methodology to assess treatment response.

7.3 Future Work

- In order to functionally validate the gene targets, an siRNA study should be completed. By examining the toxicological impact of knocking down the genes in the cell line sub panel (BT474, SKBR3 and MDAMB453) the causative or associative role of the genes in response to lapatinib will be more apparent. This relevance and toxicological impact might also be examined and assessed by looking at induced alterations in the expression of genes (and their associated proteins) and even the targets of those genes downstream of the siRNA gene target.. A proteomic study using mass spectrometry methodology should be able to identify any major changes in the levels of such proteins. These roles should also be evaluated in the cell line panel in response to the additional HER2 targeted therapies (afatinib, neratinib and trastuzumab). This functional study will help to indicate if sensitivity of the cell lines to the targeted therapies has any impact on the roles of the genes in their response.
- It would be informative to investigate the relevance of our gene expression pattern in HER2 over-expressing cell lines with acquired resistance to additional targeted therapies such as afatinib, neratinib or trastuzumab.
- To further inform the *in vivo* results generated in this thesis the following adaptations of the described *in vivo* study (chapter 6) should be undertaken. Throughout the course of the experiment, the presence of the estrogen pellets resulted in bladder issues developing in a large percentage of the animals and this greatly impacted on our need to prematurely excise and analyse the tumours when it would have been preferable to leave them develop to a

larger size. In order to determine if estrogen supplementation is necessary to support the growth of the tumours and if implantation was the most suitable administration method, a further study in which one group would not be given any estrogen supplementation, while another group would be given estrogen but in their water will be necessary. The *in vitro* identification of the gene expression pattern has indicated that this response pattern is indicative of response to not just lapatinib treatment but also additional HER2 targeted therapies. To evaluate that this observation translates to the *in vivo* setting, study evaluating gene expression response to these additional therapies should be carried out. Evaluation of the gene pattern in a HER2 positive patient-derived xenograft (PDX) should further inform the results that have already been generated in this study. A PDX better represents the molecular characteristics of the original tumour and reproduces more effectively the architecture of the primary tumour compared to cell line derived xenografts.

7.4 References:

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8. Appendix

Appendix 1: Relative Expression (RQ) and Standard Deviation (SD) Values for breast cancer cell lines in response to lapatinib. (n=3)

12 Hours Lapatinib Preseeded Plates (Figures 3.6 and 3.7)												
	BT474		SKBR3		EFM192A		HCC1954		MDAMB453		MDAMB231	
	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD
RB1CC1	5.002	0.925	2.612	0.707	2.516	0.671	1.303	0.223	-1.273	0.595	-1.219	1.512
FOXO3a	4.702	0.644	2.869	0.588	2.189	0.660	1.729	0.245	-2.226	0.543	-1.078	1.373
NR3C1	4.215	0.699	2.624	0.746	3.446	0.123	1.285	1.337	-1.342	0.330	-1.097	0.378
ERBB3	3.524	0.051	2.794	0.659	1.466	0.681	1.477	0.665	-1.118	0.416	-1.132	1.260
PIK3C3	2.474	0.491	3.198	0.737	1.286	0.666	-1.036	0.642	1.066	0.338	-1.007	0.370
ALDH3A2	3.370	0.279	2.593	0.732	1.369	0.220	-1.333	0.225	-1.123	0.715	1.246	0.481
CDKN1B	2.366	0.351	3.044	0.428	1.316	0.504	1.113	0.753	-1.471	0.091	1.058	0.490
CCND1	-3.256	0.412	-5.711	0.637	-2.180	0.217	-1.781	0.583	-1.341	0.182	-1.060	0.906

	6 hour Lapatinib Treatment (Figure 3.9)						12 hour Individual Assay Lapatinib Treatment (Figure 3.8)					
	BT474		SKBR3		MDAMB453		BT474		SKBR3		MDAMB453	
	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD
RB1CC1	18.021	1.119	10.172	0.773	1.592	0.218	37.214	1.007	5.339	1.162	1.221	0.724
FOXO3a	6.235	1.069	2.974	0.304	-2.924	0.675	12.809	0.666	2.194	0.773	1.007	0.708
NR3C1	6.871	0.613	2.791	0.726	-2.122	0.193	9.601	0.869	1.925	0.780	1.244	0.418
ERBB3	6.096	0.576	4.518	0.478	-3.081	2.139	4.305	1.125	2.836	0.701	1.462	0.641
PIK3C3	2.510	0.543	2.955	0.140	1.073	0.366	15.407	0.875	1.954	0.361	1.669	0.300
ALDH3A2	3.468	0.456	4.040	0.571	-1.018	0.439	7.632	0.482	2.118	0.684	1.127	0.378
CDKN1B	4.016	0.402	4.615	0.728	-1.387	0.317	4.362	0.612	3.205	0.762	-1.311	0.273
CCND1	-3.614	0.959	-5.839	0.758	1.360	0.689	-2.031	1.478	-3.875	0.584	1.415	0.404

	24 hour Lapatinib Treatment (Figure 3.9)						36 hour Lapatinib Treatment (Figure 3.9)					
	BT474		SKBR3		MDAMB453		BT474		SKBR3		MDAMB453	
	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD
RB1CC1	21.757	1.110	17.309	1.141	1.339	0.351	15.088	1.191	32.812	1.701	1.004	0.331
FOXO3a	11.884	0.794	9.169	0.689	-2.113	0.448	8.433	1.681	13.244	0.929	-3.492	0.665
NR3C1	9.603	0.458	3.216	0.901	-2.034	0.200	5.765	1.604	5.115	1.082	-2.618	0.109
ERBB3	10.730	0.500	7.260	0.681	-1.036	0.271	8.789	0.693	9.645	0.781	-1.619	0.251
PIK3C3	6.253	0.665	4.064	0.345	-1.072	0.380	5.406	0.732	5.615	0.839	-1.035	0.413
ALDH3A2	11.307	0.355	5.334	0.758	-1.584	0.345	7.614	1.267	7.578	0.840	-2.436	0.495
CDKN1B	7.358	0.887	5.565	0.707	-1.274	0.172	5.543	1.107	6.683	0.816	-1.268	0.279
CCND1	-1.637	1.857	-3.465	0.535	1.082	0.335	1.117	0.655	-2.197	0.678	-1.032	0.408

RQ values for remaining 11 genes not used for further evaluation.

	BT474	SKBR3	EFM192A	HCC1954	MDAMB453	MDAMB231
AKT1	-1.454	-1.580	-1.567	1.013	1.091	1.289
BID	-1.212	-2.017	-2.367	1.219	-1.288	-1.108
E2F3	-1.697	-3.013	-2.574	-1.006	1.025	1.192
EIF4E	-1.218	-1.802	1.028	1.001	-1.038	1.003
FKBP4	-1.155	-1.664	-1.759	-1.207	1.297	1.456
MAPK9	-1.301	-1.478	-1.212	-1.124	-1.289	1.202
PARP2	1.359	-1.316	-1.238	-1.355	1.000	-1.050
PSMD13	1.159	1.362	-1.369	-1.453	-1.199	1.228
SLC29A1	1.265	-2.071	-1.365	-1.812	-1.269	-1.014
TFPT	-1.086	1.362	-1.728	-1.443	-1.268	1.062
CBFA2T2	1.850	2.812	1.101	1.135	1.024	1.366

Appendix 2: Relative Expression (RQ) and Standard Deviation (SD) Values for breast cancer cell lines in response to additional treatments (n=3)

	12 Hour Neratinib Treatment (Figure 4.1)						36 Hour Neratinib Treatment (Figure 4.3)					
	BT474		SKBR3		MDAMB 453		BT474		SKBR3		MDAMB 453	
	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD
RB1CC1	19.971	0.986	10.704	1.293	2.014	0.610	8.827	0.879	4.058	1.185	-0.844	0.667
FOXO3a	8.924	0.647	3.916	0.786	-1.752	0.777	8.416	0.791	3.444	0.713	-1.402	0.506
NR3C1	7.803	0.528	2.664	0.807	-1.645	0.712	5.225	0.371	1.707	0.834	-1.198	0.207
ERBB3	8.619	0.142	5.040	0.602	-1.240	1.343	5.669	0.102	2.574	0.730	-1.369	0.092
PIK3C3	3.684	0.055	3.221	0.417	1.055	1.560	2.194	0.256	1.215	0.448	-2.233	0.264
ALDH3A2	5.975	0.301	4.409	0.691	1.342	0.067	4.145	0.106	1.643	0.814	-1.228	0.379
CDKN1B	5.011	0.230	4.919	0.721	1.098	0.516	2.771	0.301	1.413	1.034	-0.967	0.408
CCND1	-3.189	0.767	-3.725	0.695	1.091	0.825	-3.903	1.026	-3.657	0.631	-1.585	0.357

	12 Hour Afatinib Treatment (Figure 4.2)						36 Hour Afatinib Treatment (Figure 4.3)					
	BT474		SKBR3		MDAMB 453		BT474		SKBR3		MDAMB 453	
	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD
RB1CC1	25.653	0.929	4.827	1.262	1.266	0.423	27.855	0.955	10.790	1.301	-1.454	0.309
FOXO3a	9.394	0.900	1.367	0.758	-1.104	0.496	33.545	0.823	14.978	0.863	-1.320	0.291
NR3C1	10.966	0.583	3.512	0.838	1.129	0.278	87.956	0.631	24.008	0.864	3.540	0.283
ERBB3	12.165	0.297	2.481	0.769	1.147	0.382	14.999	0.324	5.581	0.779	-1.875	0.235
PIK3C3	26.396	0.218	1.760	0.522	1.280	0.650	6.054	0.374	2.652	0.381	-1.836	0.299
ALDH3A2	15.982	0.162	2.275	0.885	-1.144	0.689	26.450	0.231	7.645	0.681	-2.716	0.385
CDKN1B	7.955	0.324	2.675	0.868	-1.513	0.484	9.270	0.481	4.993	0.789	-1.497	0.245
CCND1	-1.589	1.020	-6.070	0.645	1.606	0.429	-1.452	1.017	-2.512	0.656	1.130	0.379

	12 Hour Lapatinib with Capecitabine(Figure 4.5)						12 Hour trastuzumab Treatment (Figure 4.6)					
	BT474		SKBR3		MDAMB 453		BT474		SKBR3		MDAMB 453	
	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD
RB1CC1	17.814	0.462	4.412	1.129	1.221	0.724	7.895	1.660	3.810	1.219	-1.217	0.079
FOXO3a	6.673	0.232	1.483	0.829	1.007	0.708	3.077	0.856	1.001	0.796	-1.306	0.404
NR3C1	8.756	0.463	3.853	0.797	1.244	0.418	4.419	0.636	2.202	0.757	-1.111	0.385
ERBB3	7.562	0.518	2.276	0.796	1.462	0.641	3.970	0.328	1.655	0.743	-1.135	0.361
PIK3C3	15.263	0.395	1.991	0.361	1.669	0.300	15.626	0.371	1.445	0.330	1.193	0.532
ALDH3A2	10.575	0.769	2.050	0.814	1.127	0.378	8.125	0.294	1.689	0.695	-1.386	0.366
CDKN1B	5.351	0.754	2.172	0.748	-1.311	0.273	4.783	0.322	1.999	0.663	-1.794	0.059
CCND1	-2.591	0.607	-7.175	0.620	1.415	0.404	2.167	1.000	-1.362	0.628	1.897	0.300

	12 Hour Dasatinib (Figure 4.7)						12 Hour Gefitinib Treatment (Figure 4.8)					
	BT474		SKBR3		MDAMB 453		BT474		SKBR3		MDAMB 453	
	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD	RQ	SD
RB1CC1	5.288	1.136	2.004	0.744	-1.783	0.346	1.250	0.968	2.350	1.169	-2.992	0.311
FOXO3a	1.940	0.952	-1.412	0.752	-4.184	0.577	2.331	0.841	-2.402	0.786	-11.118	0.424
NR3C1	6.206	0.843	2.599	0.655	-1.003	0.326	-2.216	0.700	-1.418	0.806	-4.514	0.304
ERBB3	1.616	0.366	-1.497	0.803	-3.810	0.557	-3.723	1.090	-1.320	0.591	-6.019	0.227
PIK3C3	1.676	0.460	-1.109	0.363	-2.230	0.256	-2.670	0.369	-1.703	0.395	-3.741	0.437
ALDH3A2	1.680	0.362	-1.102	0.763	-5.904	0.189	-1.080	0.232	1.074	0.657	-5.986	0.408
CDKN1B	2.718	0.521	1.495	0.777	-2.434	0.299	-2.328	0.536	-1.305	0.630	-5.503	0.308
CCND1	1.360	1.096	1.093	0.457	1.079	0.274	-13.438	1.221	-3.787	0.515	-2.801	0.319

12 Hour Epirubicin Treatment (Figure 4.9)

	BT474		SKBR3		MDAMB 453	
	RQ	SD	RQ	SD	RQ	SD
RB1CC1	-1.358	1.232	1.278	1.229	-4.842	0.050
FOXO3a	1.102	1.122	-4.068	0.794	-13.298	0.349
NR3C1	-4.982	0.885	-2.224	0.859	-6.321	0.323
ERBB3	-4.757	0.614	-2.084	0.614	-14.388	1.320
PIK3C3	-4.172	0.660	-2.195	0.391	-5.285	0.588
ALDH3A2	-2.857	0.493	-1.569	0.743	-8.458	0.480
CDKN1B	-3.161	0.822	-1.679	0.751	-8.046	0.296
CCND1	-6.443	1.399	-3.583	0.629	-3.212	0.328

Appendix 3:

C_t values for each of the genes in the vehicle, 1 day lapatinib and 5 day lapatinib treatment in each animal.											
	Vehicle			1 day lapatinib treatment				5 Day lapatinib treatment			
	Ct m1	Ct m4	Ct m5	Ct m1	Ct m2	Ct m3	Ct m4	Ct m2	Ct m3	Ct m4	Ct m5
RB1CC1 (H/M)	*	32.998	28.215	*	34.625	33.771	32.855	32.075	35.455	31.298	*
FOXO3a (H/M)	30.658	30.134	29.307	30.569	31.204	29.922	31.461	32.312	30.135	29.675	31.968
NR3C1 (H/M)	*	34.563	30.030	*	*	*	*	32.961	*	*	*
ERBB3 (H)	*	32.114	27.573	*	33.444	32.461	31.467	30.999	34.730	30.742	*
CCND1 (H/M)	31.068	29.467	28.485	29.458	31.405	29.007	30.808	31.857	30.007	29.540	31.880
RB1CC1 (H)	*	33.047	28.901	*	33.813	33.439	32.331	32.025	*	31.351	*
FOXO3a (H)	*	30.932	26.789	*	*	34.199	30.157	30.061	33.753	29.511	33.803
NR3C1 (H)	*	33.753	28.597	33.286	29.970	29.489	32.502	31.079	*	33.344	*
FOXO3a (M)	30.653	27.876	29.459	28.004	28.549	27.877	28.462	30.407	28.420	28.105	28.849
GAPDH (M)	21.913	21.509	25.816	22.622	23.498	23.621	23.531	26.930	22.752	22.452	25.417
GAPDH	*	26.488	21.843	31.096	28.556	28.153	26.577	26.651	28.845	24.570	31.921

*indicates that the C_t values was above 35 and as such was not considered expressed and was excluded from the calculations.

Skin C_t values							
	Vehicle			5 Day lapatinib treatment			
	C _t m1	C _t m4	C _t m5	C _t m2	C _t m3	C _t m4	C _t m5
FOXO3a (H/M)	29.19784	30.5586	30.87789	29.6879	28.15496	28.67739	*
CCND1(H/M)	29.92199	30.90163	31.42274	30.681	29.507	29.628	*
Lung C_t values							
	Vehicle			5 Day lapatinib treatment			
	C _t m1	C _t m4	C _t m5	C _t m2	C _t m3	C _t m4	C _t m5
FOXO3a (H/M)	27.51486	28.1907	28.1907	27.51962	27.42548	27.72178	27.54139
CCND1(H/M)	27.65937	27.95	27.92429	28.22269	27.47478	27.96587	27.6164
Lymph-node C_t values							
	Vehicle			5 Day lapatinib treatment			
	C _t m1	C _t m4	C _t m5	C _t m2	C _t m3	C _t m4	C _t m5
FOXO3a (H/M)	#	#	#	29.52675	29.16434	29.41843	29.07615
CCND1(H/M)	#	#	#	28.44526	28.44598	28.3298	28.10955

*indicates that the C_t values was above 35 and as such was not considered expressed and was excluded from the calculations.

#indicates that RNA extraction was not completed on these samples