BASAL-LIKE BREAST CANCERS: CHARACTERIZATION AND THERAPEUTIC APPROACHES

Tayma Khalil

Department of Human Genetics
McGill University
Montreal, Quebec, Canada
May 2008

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies of McGill University in partial fulfillment of the requirements of the degree of Masters of Science.

Your file Votre référence
ISBN: 978-0-494-51288-3
Our file Notre référence
ISBN: 978-0-494-51288-3

ABSTRACT

Background: Both basal-like subtype and BRCA1-related breast cancers tend to have a poor overall prognosis and lack of effective treatments. Given that the lung cancer drug gefitinib and the leukemia drug dasatinib inhibit proteins also belonging to the molecular signature of this subtype, we and others hypothesized that they might be useful therapies for those two breast cancer subgroups.

Methods: Eight breast cancer cell lines were characterized by immunohistochemistry and western blotting and were treated with both drugs. Response was measured by using the sulphorhodamine B (SRB) assay.

Results: Two out of six basal-like cell lines were sensitive to gefitinib and five of six to dasatinib. BRCA1-related breast cancers were also responsive to dasatinib (three out of four). Moreover, EGFR and caveolin-1 act as markers for dasatinib sensitivity, but do not appear to be the primary targets of this drug. The presence of SRC but not ABL is necessary to achieve a response to dasatinib.

Conclusion: Dasatinib is more effective in the treatment of basal-like breast cancers than gefitinib and acts by inhibiting SRC and other molecules that are yet to be determined.

RESUME

Fond de recherche: Les cancers du sein du sous-type basal ainsi que les cancers du sein héréditaires porteurs de mutations sur le gène BRCA1, sont connus pour leur mauvais pronostic et leur manque de traitements efficaces. Le gefitinib et le dasatinib, deux drogues conçues respectivement pour le traitement des cancers du poumon et de la leucémie, inhibent des protéines appartenant à la signature moléculaire de ces deux sous-types de cancer du sein. Notre groupe, ainsi que d'autres, présumons que ces deux drogues peuvent servir de thérapies efficaces contre ces sous-types de cancers du sein.

Méthodes: Huit lignées de cancer de sein humain ont été caractérisées par immunohistochimie et par western-blot, puis traitées avec le gefitinib et le dasatinib. La sensibilité de chacune de ces lignées aux deux drogues a été déterminée par titrage à base de sulphorhodamine B (SRB).

Résultats: Deux des six lignées appartenant au sous-type basal sont sensibles au gefitinib et cinq au dasatinib. Trois des quatre lignées cellulaires porteuses de mutations sur le gène BRCA1 sont aussi sensibles au dasatinib.
Caveolin-1 et EGFR, deux marqueurs de sensibilité pour le dasatinib, ne sont pas inhibés par cette drogue. De plus, pour être actif, le dasatinib nécessite la présence de SRC alors que celle de la protéine ABL n'est pas indispensable.

Conclusion: Le dasatinib est plus efficace que le gefitinib dans le traitement de cancers du sein appartenant au sous-type basal. De plus, le dasatinib agit en inhibant SRC ainsi que d'autres molécules restant à être déterminées.

ACKKNOWLEDGEMENTS

There are many people to whom I owe a debt of thanks for their unconditional support over the last two years. Without you, this accomplishment would have never been possible. First I would like to express my deepest thanks and gratitude to my supervisor, Dr. William Foulkes for his endless guidance and support, his confidence and trust in me and his inspiration. He was always there to answer any question I had even though, in my opinion, he has one of the busiest schedules as a physician/researcher in two different institutions. I would also like to thank Dr. Raquel Aloyz for co-supervising me and for guiding me and Dr. Marc Tischkowitz for his support and useful advices in the lab meetings.

To my parents, thank you for your infinite support and love. You were always there, making sure I had everything I needed to get this far. Mom, dad, I love you and I'm forever grateful. Tamara and Nadim, the best sister and brother one could have, thank you for sharing those amazing midnight laughs with me and for supporting me for more than 18 years. I love both.

To my colleagues, you were my family for two years. Not only did you help me with all the new lab techniques I had to learn, but you were also the reason I enjoyed spending so much time in the lab. Thank you for all the laughs and the good times we spent together. You all had a tremendous impact on my life. Many thanks to my colleagues at the Montréal General Hospital: Nancy, Dave, Paulina, Ashley, Neil, Luca, Michael and Suzanna. Thank you all for the good times we had and for all those soups we ate at the Thai place. A special thanks to Nancy who took care of me all throughout my masters.
To my colleagues in the Jewish General Hospital: Mike, Aaron, Anne, Lilian, Lili, Nelly, Rachel and Ahmet; thank you for all your help and for sharing many unforgettable moments.

To my friends on the fourth floor: I love you all. The friendship and bonds we developed are priceless. Mike, Riccardo, Abdel, Moutih, Aaron, Carolyn, Terry, Orlando and Sophie, I will not forget all those coffee breaks, lunches, hallway talks and overnights we had. Good luck to all of you in your studies and your job searches.

To my best friends Tina, Martine, Rola, Ramzi and all my friends: words cannot express my gratitude. Thank you for being there. I love you

Many thanks to the members of my supervisory committee for their advice, to the staff at the human genetics department for all their help, and to all the members of the human genetics student society and the Lady Davis institute student association for allowing me to be part of the association and improve as a person.

Finally, to my grandmother who I lost this year to breast cancer, I dedicate this work to you. I thank you for the inspiration and the drive you instilled in me. You were and always still are my angel; teaching me about life and making me a better person.

Table of contents

Chapter 1-Introduction:

Chapter 2-Materials and methods:

Chapter 3-Results:

Chapter 4-Discussion and future directions:

Appendix:

LIST OF FIGURES AND TABLES

LIST OF ABREVIATIONS

ABL (c-ABL): v-abl Abelson murine leukemia viral oncogene homolog 1

AKT: human version of the viral oncogene, v-akt

ALL: acute lymphoblastic leukemia

ANXA1: annexin 1

ATM: ataxia-telangiectasia mutated

BPE: bovine pituitary extract

BRCA1: breast cancer gene 1

BRCA2: breast cancer gene 2

BRIP1: BRCA1-interacting helicase

BSA: bovine serum albumin

CAV1: caveolin-1 (previously known as VIP21)

CAV2: caveolin-2

CAV3: caveolin-3

CDK: cyclin-dependent kinase

CDK2: dependent kinase 2

CDKI: CDK inhibitor

CHEK2: CHK2 checkpoint homolog

CK17: cytokeratin 17

CK18: cytokeratin 18

CK5/6: cytokeratins 5/6

CK8: cytokeratin 8

CS: Cowden's syndrome

DMSO: dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

EDTA: ethylene diamine tetraacetic acid

EGF: epidermal growth factor

EGFR (also known as HER1): epidermal growth factor receptor

EPHA2: EPH receptor A2

ER: estrogen receptor

ERK (same as MAPK1): mitogen-activated protein kinase 1

FACS: Fluorescent Activated Cell Sorter

FAK: focal adhesion kinase

FBS: fetal bovine serum

FYN: FYN oncogene related to SRC, FGR, YES

GATA3: GATA binding protein 3

GRB2: growth factor receptor-bound protein 2

GRB7: growth factor receptor-bound protein 7

H&E: hematoxylin and eosin

hCDC4 (FBW7): F-box and WD repeat domain containing 7

HER2: epidermal growth factor receptor 2

IκBα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IC_{50}: inhibitory concentration of 50%

IGFBP2: insulin-like growth factor binding protein 2

IHC: immunohistochemistry

KIP1 (P27, CDKN1B): cyclin-dependent kinase inhibitor 1B

KIT (c-KIT): v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog

LCK: lymphocyte-specific protein tyrosine kinase

LFS: Li-Fraumeni syndrome

LYN: v-yes-1 Yamaguchi sarcoma viral related oncogene homolog

MAPK: mitogen activated protein kinase

MET: met proto-oncogene (hepatocyte growth factor receptor)

NBS1: nibrin

NFκB: nuclear factor of kappa light polypeptide gene enhancer in B-cells

NSCLC: non-small cell lung cancer

PALB2: partner and localizer of BRCA2

PBS: phosphate buffered saline solution

PCR: polymerase chain reaction

PDGFR: platelet derived growth factor receptor

PI3K: phosphatidylinositol-3–kinase

PKA: protein kinase A

PR: progesterone receptor

PTEN: phosphatase and tensin homolog

PTRF: polymerase I and transcript release factor

RAD50: RAD50 (S. cerevisiae) homolog

RAS (P21): Harvey rat sarcoma virus oncogene 1

RNA: Ribonucleic Acid

SCF ubiquitin ligase complex: SKP1–cullin–F-box

SEER: Surveillance, Epidemiology, and End Results

SFK: SRC-family kinases

SHC: SHC (Src homology 2 domain containing) transforming protein

siRNA: short interference RNA

SNP: single nucleotide polymorphisms

SRB: sulphorhodamine B

SRC: v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)

TBST: Tris-buffered solution supplemented with 0.05% Tween 20

TK: tyrosine kinase

TKI: tyrosine kinase inhibitor

TMA: tissue microarray

TNP: triple negative phenotype

TP53: tumor protein p53

TSG: tumor suppressor gene

WD40: reapeats composed of 40 residues with tryptophan (W) and aspartic acid (D) at defined positions.

YES: v-yes-1 Yamaguchi sarcoma viral oncogene homolog

Chapter 1 - Introduction:

1-1 Breast cancer:

Breast cancer is one of the most common cancers affecting women worldwide. According to the Surveillance, Epidemiology, and End Results (SEER) database (www.seer.cancer.gov), women in the United States have a 12.2% lifetime risk of developing breast cancer. To date, two genes with a high penetrance and a moderate frequency have been linked to this diagnosis. In 1994, BRCA1, a breast cancer related gene located on chromosome 17 was discovered by Miki et al. BRCA1 (breast cancer gene 1) is a tumor suppressor gene (TSG) that maintains genomic integrity and prevents uncontrolled proliferation. The protein product is involved in DNA damage repair, ubiquitination, transcriptional regulation as well as other functions. One year later, in 1995, Wooster el al discovered BRCA2, the second breast cancer related gene, located on chromosome 13. BRCA2 (breast cancer gene 2), like BRCA1, is a TSG that regulates the cycle of cell division by keeping cells from growing and dividing too rapidly or in an uncontrolled manner (Miki et al., 1994;Wooster et al., 1995). The lifetime risk of developing breast cancer increases to 80% in women who have a germline mutation in either BRCA1 or BRCA2 (Ford et al., 1994;Ford et al., 1998). Other genes with either a lower penetrance or a very rare occurrence have also been described. Mutations in TP53, a gene originally implicated with the Li-Fraumeni syndrome (LFS), account for less than 1% of all breast cancer cases (Sidransky et al., 1992). Mutations in the PTEN gene are

very rare and cause Cowden's syndrome (CS); Women with CS have a 25% lifetime risk of developing breast cancer (Liaw et al., 1997). Furthermore, mutations in ATM, CHEK2, RAD50, NBS1, BRIP1 and PALB2 have been associated with a two-fold increase in breast cancer risk (Meijers-Heijboer et al., 2002;Renwick et al., 2006;Heikkinen et al., 2006;Seal et al., 2006;Rahman et al., 2007). Taken together, all the genes described above are responsible for 25% of hereditary breast cancers, and less than 10% of all breast cancers.

The diversity observed at the genetic level in hereditary breast cancers provides an example of how heterogeneous breast cancer is as a disease. Gene expression and morphological studies lead to the division of breast cancers into the distinct and clinically relevant subtypes used nowadays. However, one of the earliest classifications consisted of grouping breast cancers into estrogen receptor (ER) positive and estrogen receptor negative cancers. George Beatson was, indirectly, a pioneer in promoting this classification. More than a century ago, he theorized that all breast cancers depend on estrogen (Beatson, 1896). This theory resulted from the regression of a breast cancer tumor following an oophorectomy. As a consequence, in the years that followed, and prior to the discovery of the estrogen antagonist tamoxifen in the early 1960s, oophorectomy gained popularity as the treatment for breast cancer. This technique was later on largely replaced by tamoxifen. However, not all the patients displayed the same response to the drug. This was then explained by the fact that more than a third of all breast cancers do not express the estrogen receptor. Patients that belonged to this ER-

negative group are among those that do not respond to the treatment (Allegra and Lippman, 1980;Fisher et al., 1998;Chen and Colditz, 2007).

The classification used nowadays to classify breast cancers was devised in the past decade. Perou et al. used modern techniques such as microarray analysis to classify breast cancers into five different "intrinsic subtypes": Luminal A, luminal B, epidermal growth factor receptor 2 (HER2) positive, basal-like and normal-like (Perou et al., 2000).

1-2 The luminal A and luminal B subtypes:

Breast cancer cells that belong to the luminal subtype are known for being ER-positive (Perou et al., 2000). In the human breast anatomy, mature breast ducts usually contain two types of epithelial cells: luminal cells that do not have any contact with the basement membrane and basal (myoepithelial) cells that are usually present at the basement membrane (Purkis et al., 1990). The nomenclature of the luminal subtype results from the presence of the cytokeratins 8 and 18 (CK8 and CK18) at the cells' surface. These cytokeratins are usually specific to the luminal epithelial cells of the mammary gland (Taylor-Papadimitriou et al., 1989). Other genes that characterize this subtype include the expression of GATA3 and the progesterone receptor (PR) (Usary et al., 2004). Luminal breast cancers are further broken down into luminal A and luminal B. Luminal A tumors have high levels of ER and GATA3 and usually have a better overall prognosis when compared to the other subtypes. Luminal B tumors are distinguished by the expression of HER2 alongside ER (Sorlie et al., 2001). The luminal A subtype is the most prevalent

subtype in breast cancers, and it is present in 51% of non African-American women with breast cancer and in 36% of African-American women. The Luminal B subtype has a frequency of 18% and 9% respectively (Carey et al., 2006).

1-3 HER2 Positive subtype:

Breast cancer cells that belong to the HER2+ subtype generally have an amplification of the HER2 gene and overexpress the protein. They are associated with a poor overall prognosis. GRB7 is a protein that is commonly overexpressed in this subtype as well because the gene that encodes it is located next to the HER2 locus on chromosome 17. In general, the estrogen and progesterone receptors are not expressed in this subtype. However, as previously mentioned, when both ER and HER2 are expressed in the same breast cancer cells, they are classified within the luminal B subtype (Perou et al., 2000;Sorlie et al., 2001). HER2+ breast cancers account for 6-9% of all breast cancers and their frequency appears to be independent of race or menopausal status (Carey et al., 2006).

1-4 The basal-like subtype:

Breast cancer cells that belong to the basal-like subtype do not express ER, PR or HER2. Therefore, because they lack those three markers, they are also referred to as the triple negative phenotype (TNP). Other markers with high specificity to the basal-like subtype

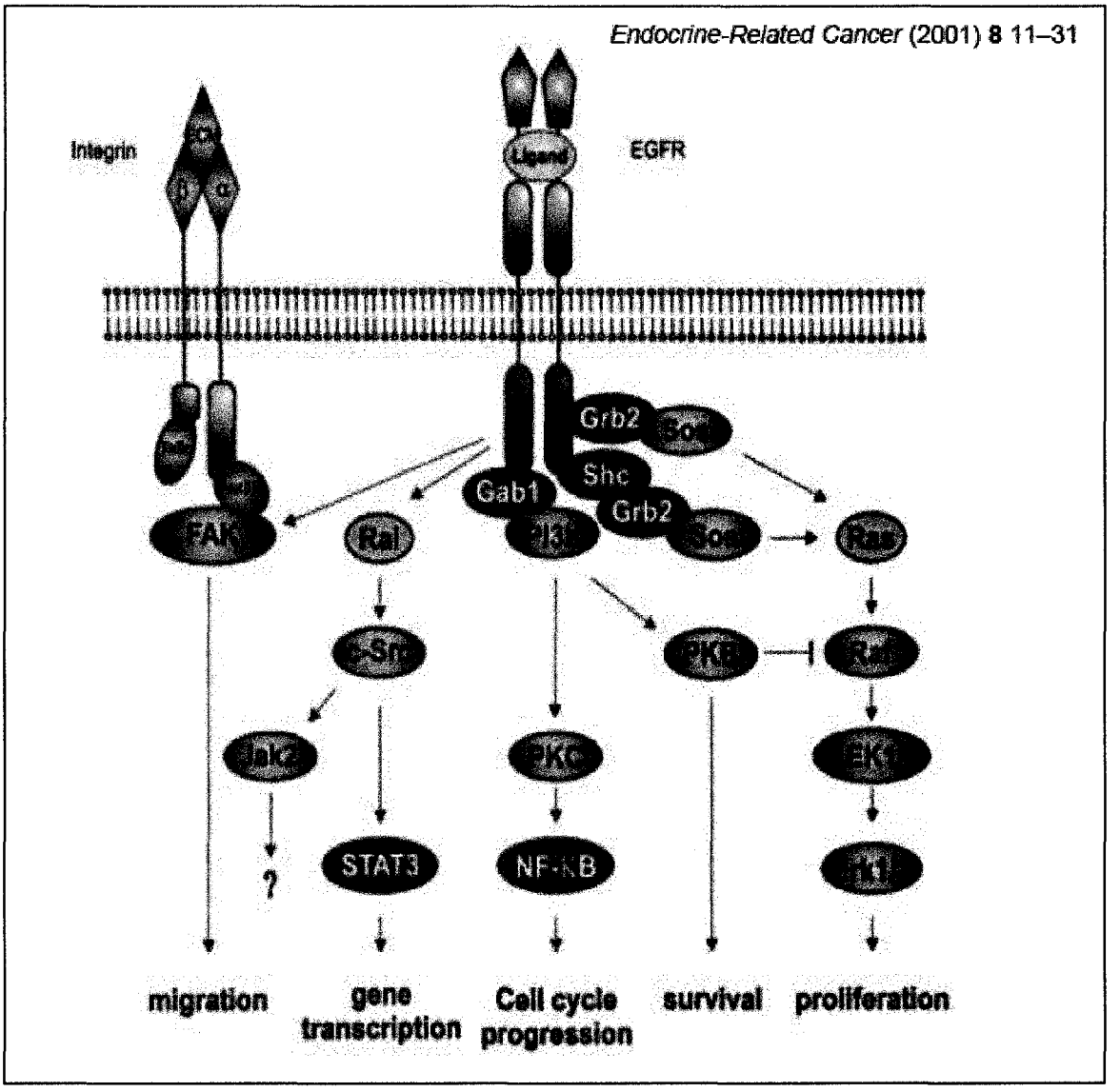
include the cytokeratins 5/6 and 17 (CK5/6 and CK17) (Nielsen et al., 2004a). Those keratins are expressed in the basal epithelial cells of the mammary gland thus; the name of the subtype. However, there is no evidence to prove that cancer cells that belong to the basal like subtype arise from this cell type (Perou et al., 2000;Sorlie et al., 2001). In contrast to the luminal subtype, the basal-like subtype is more common in African-American women with breast cancer (39%) than in white American women (16%). This subtype usually confers a poor overall prognosis (van de Rijn2002;Carey et al., 2006). Since its emergence, the list of predictive markers for the basal-like subtype is constantly growing. The epidermal growth factor receptor (EGFR), P-cadherin, caveolin-1 (CAV1), MET, c-KIT, moesin, cyclin E and other proteins make up the basal-like signature (van der Groep et al., 2004;Nielsen et al., 2004a;Pinilla et al., 2006;Charafe-Jauffret et al., 2006). These markers will be discussed in details in the following sections.

1-5 EGFR and the basal-like subtype:

The 53 amino acid epidermal growth factor (EGF) polypeptide is involved in the proliferation pathway of various cancers (Normanno et al., 1994). This protein binds to and activates the 170kDa epidermal growth factor receptor (EGFR) located at the cells' surface (Cohen et al., 1982). EGFR, also known as HER1, belongs to the "HER" family of four transmembrane proteins (HER1-4), also known as Erb family of proteins. All four proteins share a similar molecular structure: a ligand binding domain at the cell's surface, a single hydrophobic transmembrane domain and an internal cytoplasmic domain. The receptors bind to each other through their transmembrane domains to form homodimers

or heterodimers, while their tyrosine phosphorylation activity on the other hand is located at the intracellular domain and is also known as the tyrosine kinase (TK) domain. The TK domain is the most conserved region of all the HER family members (Normanno et al., 2003). Once EGFR binds to a ligand, it induces the formation of homo or hetero-dimers (mainly with HER2). Dimer formation is then followed by an auto-phosphorylation of some tyrosine residues in the TK domain (Ullrich and Schlessinger, 1990;Heldin, 1995;Alroy and Yarden, 1997). The active form of EGFR then modulates various downstream transduction pathways including the GRB2, RAS, SHC, phosphatidylinositol-3–kinase (PI3K), mitogen activated protein kinase (MAPK) pathways and many more (Prigent and Gullick, 1994;Olayioye et al., 2000;Vivanco and Sawyers, 2002). Therefore, because EGFR is involved in all those pathways, it is implicated in cell migration, gene transcription, cell cycle progression, cell survival and proliferation (figure 1). A loss in the regulation of EGFR activity or expression is often observed in various cancers and it is thought to confer a survival and growth advantage to the cells in question (Klijn et al., 1992;Franklin et al., 2002). Ligand binding is not the only means by which EGFR becomes activated. Activation or EGFR overexpression can be sometimes induced by genetic amplifications, truncations or mutations in the exons that code for the tyrosine kinase domain of the protein. For example, activating mutations in exons 19 and 21 of EGFR are seen in a subset of lung cancers (non-small cell lung cancer or NSCLC) and confer sensitivity to the EGFR tyrosine kinase inhibitor, gefitinib (ZD1839, Iressa®) (Lynch et al., 2004). In breast cancer, two separate studies conducted by Lynch et al. and Bhargava et al. did not find an activating mutation in exons 19 and 21. However, a subsequent study by Weber et al. showed that BRCA1 and BRCA2-

Figure 1: EGFR signaling



EGFR forms a dimer structure once it binds to a ligand. Specific tyrosines are then autophosphorylated and create special docking sites for effector proteins. The result is an initiation of several pathways that are involved in cell migration, gene transcription, cell cycle progression, survival and proliferation (Prenzel et al., 2001).

-related breast cancers have a higher rate of EGFR missense mutations than sporadic breast tumors but they did not look at their effect on tyrosine kinase activation. Overall, it appears that the presence of activating mutations is very rare in breast cancers (Lynch et al., 2004;Bhargava et al., 2005;Weber et al., 2005). All Receptor tyrosine kinases (RTKs) including HER1-4 and others such as MET act similarly to form illicit heterodimers when aberrantly overexpressed and confer a survival and growth advantage to the cells in question. As previously mentioned, EGFR is involved in various cancer types. The influence of EGFR expression on prognosis following a cancer diagnosis varies considerably by cancer types. In the cancers of the head and neck, cervix, ovaries, bladder and esophagus it is a marker of poor prognosis. However, it acts as a moderate to weak prognostic marker in gastric, endometrial, colorecteral, NSCLCs and breast cancer (Nicholson et al., 2001). In breast cancer, the tumors that express EGFR are usually confined to one specific subtype (Kreike et al., 2007).

A study using immunohistochemistry (IHC) to identify a specific basal-like signature showed that EGFR is present in 44.1% of breast cancers that stain positive for the basal cytokeratins, and only in 7.9% of the breast cancers that do not express those cytokeratins. By contrast, EGFR is expressed in less than 2% of ER-positive breast cancers (Nielsen et al., 2004b). In addition, Nielsen et al. demonstrated that breast cancers that are classified by microarray analysis can also be separated into their respective subtypes by IHC using markers that are specific to a distinct subtype. In that study, the defining molecular signature consisted of ER expression in the luminal subtype, HER2 expression in the HER2-positive subtype, and finally, the lack of ER and

HER2 combined with expression of CK5/6 and/or EGFR in the basal-like subtype. It was also suggested that the c-KIT protein can also act as a specific IHC marker for the basal-like subtype.

1-6 Caveolin-1 and the basal-like subtype:

Caveolae are pit-shaped invaginations of the plasma membrane. They were discovered more than half a century ago by electron microscopy (Palade, 1953). However, unlike the clathrin-coated pits, they show no coating and they are rich in cholesterol and glycosphingolipids (Stan, 2002;Stan, 2005). For a long time, very little was known about caveolae except for their overabundance in various cell types, and especially in the differentiated cells. The highest number of caveolae is present in adipocytes. They are also present in endothelial and epithelial cells, pneumocytes, fibroblasts, smooth and striated muscles cells and others (Razani et al., 2002b). The principal structural components of caveolae were discovered in the early 1990s. The proteins that belong to the caveolin gene family include VIP21, later on known as Caveolin-1 (CAV1), caveolin-2 (CAV2) and caveolin-3 (CAV3) (Rothberg et al., 1992;Scherer et al., 1996;Tang et al., 1996). The genes that code for those three proteins were subsequently localized on chromosome 7 for CAV1 and CAV2, and on chromosome 3 for CAV3 (Engelman et al., 1998a;Engelman et al., 1998b). The discovery of the protein components of caveolae instigated a cascade of discoveries as to the function of caveolae and to the pathways that they are involved in. The first observation revolved around the assembly of the caveolae. In the caveolae of muscle cells, CAV3 is the main component, while in the other cell

types; CAV1 and CAV2 make up the pit-shaped structure. Only CAV1 is needed for caveolae formation. CAV2 alone cannot induce the formation of caveolae (Galbiati et al., 2001;Razani et al., 2002a). In addition, because of their pit-like structure, caveolae contain many receptors, proteins and intracellular signaling molecule such as: growth factor receptors, SRC-family kinases (SFK), G-proteins, G-protein coupled receptors, integrins, Ras-related GTPases, protein kinase A (PKA), nitric oxide synthases (NOS) and many others (Parton, 1996;Okamoto et al., 1998;Oh and Schnitzer, 1999). Some of the functions associated with caveolae include the uptake of small molecules into the cells (by potocytosis, trancytosis and clathrin-independent endocytosis), cholesterol transport and regulation of signal transduction (Liu et al., 2002). The latter is an intriguing function of caveolae because they contain many proteins and receptors and thus were hypothesized to be involved in oncogenesis (Koleske et al., 1995).

When breast cancers were classified into their intrinsic subtypes by microarray analysis, the expression of the CAV1 gene was found to be positively correlated with the basal-like subtype (Sorlie et al., 2001;Charafe-Jauffret et al., 2006). Pinilla et al. used the IHC-based molecular signature set by Nielsen et al. to classify a panel of breast cancer samples. Then they confirmed that in those samples, CAV1 is indeed a predictive marker for both basal-like and BRCA1-mutated breast cancers (Pinilla et al., 2006). In fact, this study showed that CAV1 was present in 4.2% of all the breast cancer cases studied but in 20.7% of those that belong to the basal-like subtype ($p<0.001$). Similarly, CAV1 was found to be present in 22.2% (6/27 cases) of BRCA1-mutated breast cancers but in 0% (0/20 cases) of BRCA2-related breast cancers ($p=0.024$). In addition, as for basal-like and

EGFR positive breast cancers, CAV1 positive breast cancers correlated with a poor overall prognosis.

Those findings raised a controversy as to the role of CAV1 in cancer initiation and progression. Until recently, CAV1 was considered to be a tumor suppressor gene (TSG). Various factors leading to this assumption included: the location of the CAV1 gene on chromosome 7q31.1 and the loss of the CAV1 protein expression in various cancers such as ovarian, lung, breast and mesenchymal sarcomas (Pflug et al., 1999). Even before CAV1 was mapped to the human genome, the loss of the 7q31.1 fragile site was found to be a very common feature of various cancers including those of the breast, colon, prostate, ovary, and head and neck. This indicated that a TSG must be located within this region. The mapping of CAV1 to the 7q31.1 fragile site combined with its loss in many cancer types was the main factor as to the TSG designation of CAV1 (Williams and Lisanti, 2005). Yet, the controversy surrounding the role of CAV1 in cancer has been accentuated over the years because of the new discoveries regulating the function of this protein. In fact, many scientists now consider CAV1 to be an oncogene rather than a tumor suppressor gene (Savage et al., 2007). Advocates of this belief base their hypothesis on the following facts: first of all, CAV1 may be downregulated in the cancer types previously described, but, it is highly expressed in other cancer types. The latter group includes bladder, thyroid, prostate, esophagus and some breast cancers. Second, the presence of a specific invasion-activating CAV1 mutation (P132L) in 16% of breast cancers suggests that it has an oncogenic role (Hayashi et al., 2001). And finally, in lung cancer, just as in breast cancer, CAV1 appears to have a TSG role in one subtype, and an

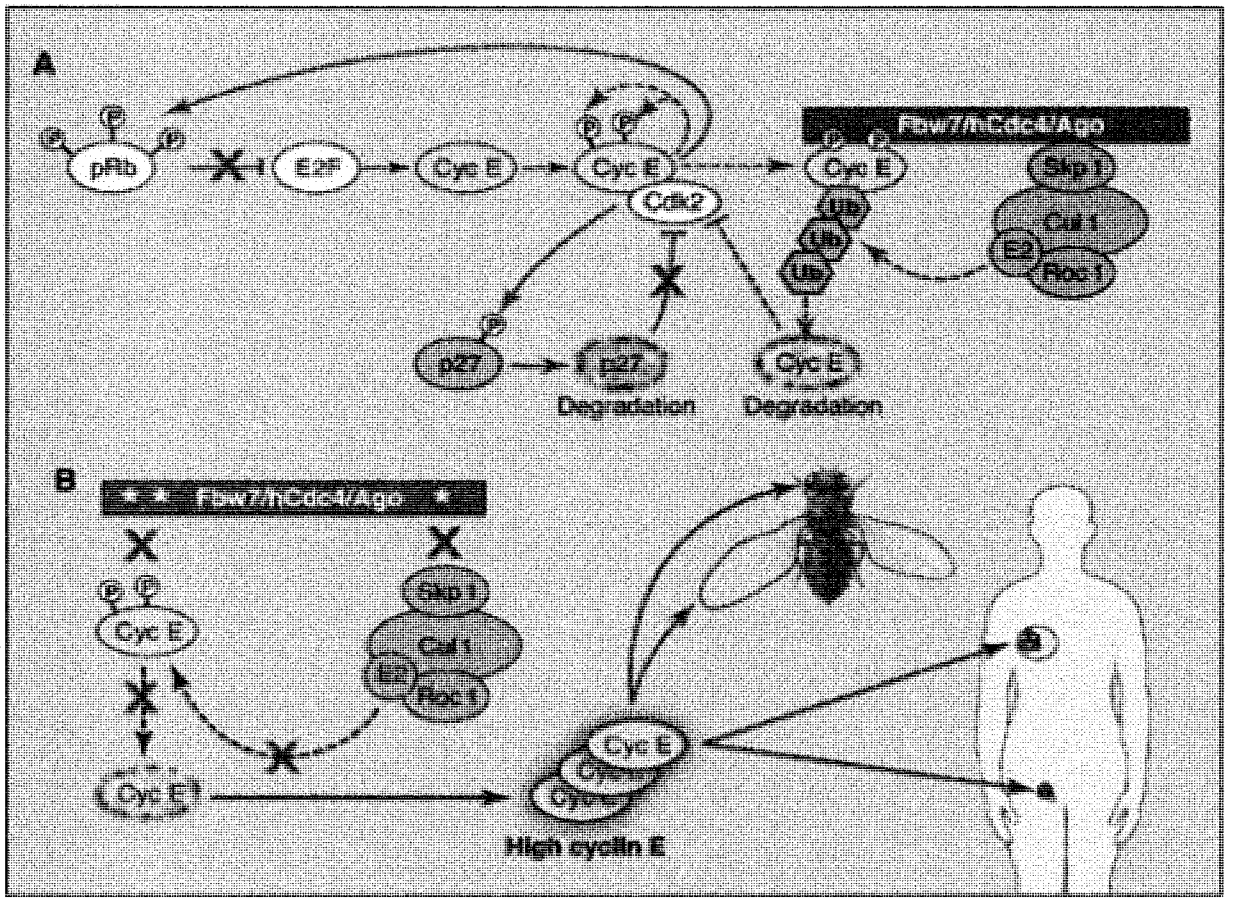
oncogenic role in another subtype. In lung cancer, 76% of non-small cell lung cancers (NSCLCs) express CAV1 (oncogenic role) while 95% of small cell lung cancers (SCLCs) lose CAV1 expression (TSG role) (Sunaga et al., 2004). The same rationale applies in breast cancer where CAV1 can be considered as a TSG in the luminal subtype, where it is absent, while it has an oncogenic role in the basal-like subtype (Savage et al., 2007).

1-7 Cyclin E and the basal-like subtype:

The cell cycle regulators mainly consist of cyclins, cyclin-dependent kinases (CDKs), the CDK inhibitors (CDKIs) and the ubiquitin family of proteins that are involved in cyclin degradation. A loss of control of the cell cycle usually results in apoptosis, cell cycle arrest or genomic instability. Cyclin E is a cyclin that associates with the cyclin dependent kinase 2 (CDK2) and is involved in the transition of the cells from the G1 phase to the S phase of the cell cycle. High levels of cyclin E accumulate during this transition and then decrease after the cells enter the S phase. Low levels of cyclin E induce cell cycle arrest while high levels can induce early entry into the S phase and can sometimes induce genomic instability. Usually, the end result of genomic instability is aneuploidy. Aneuploidy resulting from high levels of cyclin E has been observed in various cell types including epithelial breast cells (Spruck et al., 1999;Bartek and Lukas, 2001). Whether genomic instability and aneuploidy are needed for cancer formation or if they are the end result of cancer is an ongoing debate (Sieber et al., 2003;Rajagopalan et al., 2003).

The successful increase then decrease of distinct cyclins during each cell cycle is accomplished by a well-timed alternation of transcription then proteasome-dependent degradation. For example, near the end of the G1 phase, cyclin E forms a complex with CDK2 (cyclin E-CDK2) and this complex deactivates the repressor of the cyclin E transcription factor. It also phosphorylates KIP1 (also known as p27). KIP1 is a cyclin E-CDK2 inhibitor. The ubiquitin ligase SKP2 then binds to the phosphorylated KIP1 and ubiquitinates it. The ubiqutinated inhibitor is then sent to degradation allowing for an increase in cyclin E levels and for the entry of the cells into the S phase (figure 2) (Montagnoli et al., 1999). Once the cell enters the S phase, cyclin E levels must decrease. For cyclin E to be degraded by the 26S proteasome, ubiquitin residues must be first added to the cyclin. This is achieved through the SCF ubiquitin ligase complex composed of Skp1, Cul1 and Roc1. However, cyclin E and the SCF complex cannot directly interact. A bridge protein is required to link the two protein complexes together. The newly discovered F-box protein Fbw7, also known as hCDC4, assumes this bridge protein task. Mutations in the 3' end and in the region coding for the WD40 repeats of hCDC4 can prevent the binding of the two complexes and thus affect substrate binding. The repeats are called WD40 because they are usually composed of 40 residues with tryptophan (W) and aspartic acid (D) at defined positions. Therefore, by promoting high cyclin E levels and genomic instability, the presence of hCDC4 mutations in primary human tumors suggests that this protein may function as a tumor suppressor gene. The SUM149PT cell line provides an example of elevated levels of cyclin E resulting from a terminating

Figure 2: Cyclin E regulation pathway



(A) The Cyclin E-CDK2 complex induces the phosphorylation of Rb to promote cyclin E synthesis. The deactivation of Rb (E2F repressor) results in a higher cyclin E expression. This same complex phosphorylates and tags the CDK2 repressor (p27) for subsequent degradation. At this point, cyclin E levels reach a peak to induce the entry of the cell into the S phase of the cycle.
The cells then regulate the cyclin E levels through a negative feedback loop. Cyclin E becomes autophosphorylated and creates a binding site for the hCDC4 protein. HCDC4 allows the ubiquitiniation of cyclin E by the SCF complex (Skp1/Cul1/Roc1) and the ubiquitin conjuguase E2. The ubiqutinated cyclin E is then degraded by the 26S.
(B) Mutations in hCDC4 prevent the binding of this bridge protein to its substrates and thus prevent the ubiquitiniation of cyclin E and its degradation. Those mutations therefore promote high levels of the cyclin throughout the cycle and lead to premature entry into the S phase and can cause genomic instability. Those mutations can also predispose to some cancers such as breast and ovarian cancers (picture taken from Bartek and Lukas, 2001, reprinted with permission from AAAS)

mutation in hCDC4 (Koepp et al., 2001;Strohmaier et al., 2001;Moberg et al., 2001;Spruck et al., 2002;Rajagopalan et al., 2003). In breast cancer, cyclin E is overexpressed in the basal-like subtype and especially in the BRCA1-mutated cancers. Its inhibitor, KIP1, is usually underexpressed in those cases (Chappuis et al., 2000;Korsching et al., 2002;Chappuis et al., 2005). The decrease of KIP1 levels in those cases can be explained by the elevated levels of SKP2 usually found in those tumors (Signoretti et al., 2002). Rodriguez-Pinilla et al. also showed that gene amplification of cyclin E is not correlated with the protein level in breast cancers (Rodriguez-Pinilla et al., 2007) and breast cancers with high levels of cyclin E, just like BRCA1-mutated and basal-like cancers, are associated with a poor prognosis (Porter et al., 1997;Foulkes et al., 2004).

1-8 The basal-like subtype and BRCA1-mutated breast cancers:

In the previous sections, the heterogeneity of breast cancer as a disease was discussed in detail. The two most common hereditary forms of breast cancer, BRCA1 and BRCA2-related cancers, are no exception to this heterogeneity. In fact, IHC-based studies showed that, among all sub-types of breast cancers, BRCA1-mutated breast cancers have the highest probability of belonging to the triple negative subtype. The loss of ER expression is five times more likely to occur in BRCA1-mutated breast cancers than in breast cancers occurring in non-carriers (Johannsson et al., 1997). In contrast, BRCA2-related cancers are usually ER positive (Karp et al., 1997;Lakhani et al., 2002). Subsequent to the establishment of the five intrinsic breast cancer subtypes, and given that the TNP is

dominant in basal breast cancers, Foulkes et al. suggested that the BRCA1-mutated tumors must fall into the basal-like subtype. Indeed, in a study of 72 breast cancer cases occurring in Ashkenazi Jewish women, BRCA1-releted breast cancers were nine times more likely to express the basal markers CK5/6 than their non-hereditary counterparts (Foulkes et al., 2004). This observation was further confirmed by a microarray analysis study of 115 additional breast cancer cases. In this study, BRCA1-mutated breast cancers were five times more likely to belong to the basal-like subtype (Sorlie et al., 2003). It is recognized nowadays that approximately half of the BRCA1-mutated breast cancer cases are phenotypically "basal-like". In fact, ER negative and CK5/6 positive breast cancers are 36 times more likely to harbor a BRCA1 mutation than control cases belonging to the other subtypes (Lakhani et al., 2005). So far, no distinct phenotype has been assigned to BRCA2-related breast cancers (Lakhani et al., 1998;Lakhani et al., 2002).

1-9 Breast cancer treatment:

As previously discussed, the various breast cancer subtypes are different as to their prognosis and to their final outcome. A poor outcome is associated with the basal-like and HER2+ subtypes (ER negative) when compared to the luminal subtype (ER positive) (Sorlie et al., 2001;Sorlie et al., 2003). Overall, BRCA1-mutated breast cancers have a poor prognosis as well, particularly in the first few years after diagnosis. (Foulkes et al., 1997;Ansquer et al., 1998). This poor survival is not sustained however, and in one large recent study, there was no difference in breast cancer-specific death between carriers and non carriers at 10 years following the first diagnosis of the cancer (Rennert et al., 2007).

In addition, the response of the tumor cells to a variety of treatments is different among the subtypes. For example, breast cancers that belong to the ER negative subtypes (basal-like and HER2+) have a higher complete pathological response to neoadjuvant chemotherapy (chemotherapy administered prior to surgery) than cancers belonging to the luminal subtype (Rouzier et al., 2005;Berry et al., 2006). In addition, BRCA1 and BRCA2-related breast cancers have been associated with a better clinical response rate to neoadjuvant chemotherapy when compared to non-hereditary breast cancer cases (Chappuis et al., 2002). Although HER2 positive and basal-like breast cancers respond better to neoadjuvant chemotherapy, they still have a poor overall prognosis, probably due to the high rate of early relapse in patients who achieve a complete pathological response following neoadjuvant chemotherapy.

The different approaches that have been used in the cure of breast cancer are chemotherapy, radiotherapy, surgery and biological treatments. Chemotherapy is heavily used because it works by targeting rapidly dividing (cancerous) cells and by inducing high levels of DNA damage. This leads to cell death and apoptosis. A major drawback of chemotherapy is the lack of selectivity and its adverse toxic side effects.

The popularity of the targeted biological therapy approach in breast cancer treatment increased in the last decade with the introduction of the monoclonal anti-HER2 antibody, trastuzumab (Herceptin®). Now that the molecular signature of breast cancers has been revealed, tailored therapies that work by targeting specific markers in cancer cells are

being developed. To date, two types of molecular therapies are used in breast cancer treatment: tamoxifen in ER-positive breast cancers (luminal subtype) and trastazumab in HER2-positive breast cancers (Fossati et al., 1998;Baselga et al., 1998). Those therapies are not effective on basal-like breast cancers because cancers belonging to this subtype typically express neither ER nor HER2. The same lack of response is expected in BRCA1-mutated breast cancers since the majority belongs to the basal-like subtype and does not express those markers.

Therefore, the poor prognosis associated with the basal-like and BRCA1-mutated breast cancers, coupled with the lack of effective treatments, has generated an intense interest in the hunt for an efficient biological treatment that would target this subtype. The distinct signature of this subtype permits such an approach.

It is important to note that the use of biological treatments have not only gain popularity in breast cancer. Leukemia, lung, prostate cancers and many others are currently being treated via this approach as well. In addition, many examples of biological treatments that are initially designed to treat one type of cancer can be used against another type if the targeted pathway or molecule is common in both cancer types. (This concept is discussed in further detail in the following sections)

1-10 Gefitinib:

Gefitinib (also known as Iressa® or ZD1839) is an orally active tyrosine kinase inhibitor that targets EGFR and stops the proliferation of cancer cells by blocking the downstream signal transduction pathways of EGFR (Baselga and Averbuch, 2000). Gefitinib was initially designed to treat non-small-cell lung cancers (NSCLCs) and is most efficient when a gain of function mutation in the exons that code for the tyrosine kinase domain of EGFR, also known as p19 oncogene addiction, is present (Lynch et al., 2004;Paez et al., 2004). Because high levels of EGFR are present in one fifth of all breast cancers, gefitinib was used in clinical trials to assess the antitumor activity of the lung cancer drug in breast cancers that express the receptor. In most cases, inhibition of EGFR phosphorylation was present, but no sensitivity to the drug was observed, suggesting that breast tumors may not be dependant on EGFR activity alone (Baselga et al., 2005).

1-11 Dasatinib

Recent studies suggest that dasatinib (also known as Sprycel® or BMS 354825), an orally active *SRC/ABL* inhibitor, is efficient against cells that belong to the basal-like subtype of breast cancer (Finn et al., 2007;Huang et al., 2007).

Dasatinib was initially designed to treat imatinib-resistant chronic myelogenous leukemia (CML) and *BCR-ABL* positive acute lymphoblastic leukemia (ALL) (Sawyers et al.,

2002;Talpaz et al., 2006). It is a multi-targeted kinase inhibitor that mainly inhibits BCR-ABL and SRC as well as other oncogenic pathways and molecules such as LCK, YES, c-KIT, PDGFR, EGFR and others but at much higher concentrations of the drug (Lombardo et al., 2004). In their study, Finn et al. used the baseline gene expression level of breast cancer cells and generated a three-gene "predictive" set. This set identifies breast cancers that would most likely to respond to dasatinib. It consists of caveolin-1, moesin and *yes*-associated protein (Finn et al., 2007). Simultaneously, Huang et al. used a similar approach, based on gene expression levels as well, to generate a six gene model that predicts sensitivity to dasatinib. EPHA2, CAV1, CAV2, ANXA1, PTRF and IGFBP2 compose the six selected genes of this model (Huang et al., 2007).

1-12 The SRC oncogene in breast cancer:

SRC, one of the targets of dasatinib, belongs to a family of nine non-receptor tyrosine kinases. It was first recognized as the cellular form of v-SRC, the transforming agent of the oncogenic Rous sarcoma retrovirus (Thomas and Brugge, 1997). Although three members of this family: SRC, FYN and YES are all ubiquitously present in the cells, only SRC has been linked to cancer. Nonetheless, it is weakly oncogenic on its own. As a result, it has been hypothesized to promote tumor progression by assisting other oncogenic signaling molecules such as integrins, cell-cell adhesion receptors, growth factor receptors (EGFR), caveolins and steroid hormone receptors among others (Biscardi et al., 2000;Irby and Yeatman, 2000;Moro et al., 2002;Brunton et al., 2004;Shupnik, 2004;Ishizawar and Parsons, 2004). Because it is associated with all those proteins, SRC

is subsequently associated in the regulation of several pathways such as survival, differentiation, migration, proliferation and invasion in normal and cancerous cells (Frame, 2002). Its oncogenic role arises when the levels of SRC, and its activated form, p-SRC (i.e. following a phosphorylation at tyrosine 416) increase in the cells. This increase is seen in many cancer types including breast cancers (Aligayer et al., 2002;Masaki et al., 2003;Wiener et al., 2003).

As previously mentioned, EGFR is involved in many regulatory pathways and is highly expressed in breast cancers. In addition, 70% of all breast cancers overexpress either SRC or EGFR, suggesting that EGFR and CAV1 belong to important pathways of tumorigenic potential. This has been demonstrated by Biscardi et al. in breast cancer tissue and cells (Biscardi et al., 2000). SRC associates to the activated form of EGFR and becomes activated itself by phosphorylation. P-SRC then induces the activation of other downstream pathways and targets including the EGFR itself (Osherov and Levitzki, 1994;Maa et al., 1995;Biscardi et al., 2000).

SRC also promotes tumorigenesis by associating with the focal adhesion kinase (FAK). FAK is a component of the focal adhesion complex and is involved in cancer metastasis by regulating the turnover of focal adhesions. SRC binds to the activated form of FAK on tyrosine 397 (autophosphorylation site) to become activated itself. It then activates FAK further at other tyrosines mainly at tyrosine 861 to create a docking site for downstream pathways activation including the ERK pathway amongst others (Parsons, 2003).

1-13 Rationale of the study:

This study aims at determining the sensitivity of basal-like breast cancers, and in particular that of BRCA1-mutated breast cancers, to already existing biological treatments.

The main objectives of the study include:

1- Determining the sensitivity of basal-like and BRCA1-mutated breast cancer cell lines to dasatinib and gefitinib.
2- Examining the pathways targeted by dasatinib in the sensitive breast cancer cell lines.
3- Determining whether the response to gefitinib is correlated with the presence of mutations in the regions coding for the tyrosine kinase domains of the HER1-4 family members.
4- Exploring the frequency of hCDC4 mutations and the levels of cyclin E in BRCA1-mutated breast cancers.

To achieve those objectives, we will use a panel of eight cell lines, four of which (MDA-MB-436, SUM149PT, HCC1937 and SUM1315M02) have been previously described as BRCA1-mutated breast cancer cell lines (Tomlinson et al., 1998;Elstrodt et al., 2006). The remaining four cell lines include a luminal cell line (MCF7) a basal-like cell line (BT20), a HER2-positive cell line (SUM190PT) and a normal immortalized cell line (MCF10) First, the cells will be characterized by IHC and by western blotting and then

subsequently assigned to one of the five previously described subtypes. Then, to characterize the cells genetically, first the BRCA1 mutations will be confirmed in the cell lines, and the mutation analysis for the WD domains of hCDC4 and the tyrosine kinase domain of HER1-4 will be performed.

After the characterization of the eight cell lines we will treat them with the lung cancer drug, gefitinib that specifically targets one of the basal-like markers EGFR, and the leukemia drug, dasatinib, a tyrosine kinase inhibitor whose targets are also present in the molecular signature of the basal-like subtype. The sensitivity of the cells to each drug will be determined. Our goal is to create a predictive molecular signature for each drug based on the protein expression levels rather than the gene expression level of specific markers. Western blotting will be carried out to determine the baseline protein expression level of known dasatinib and gefitinib targets. We will also attempt to determine which pathway is targeted by dasatinib. This will be achieved by treating a sensitive cell line at different time points then looking for changes in protein or activation levels of selected markers.

Chapter 2-Materials and methods:

2-1 Cell lines:

MCF7, MCF10A, HCC1937, MDA-MB-436 and BT20 were purchased from the American Type Culture Collection (Rockville, MD). SUM149PT, SUM190PT and SUM1315M02 were purchased from Asterand (Anne Arbor, MI.) The cell lines were cultured in media prepared according to the manufacturer's conditions and they were maintained at 37°C in 5% carbon dioxide.

The media used to maintain MCF7, BT20 and MDA-MB-436 is composed of RPMI 1460 supplemented with L-glutamine (Invitrogen, Gibco, NY), 10% fetal bovine serum (FBS) (Invitrogen, Gibco, NY) and gentamycin.

MCF10A is maintained in complete mammary epithelial cell media (supplemented by the manufacturer with EGF, insulin, hydrocortisone, BPE and gentamycin) (LONZA, Walkersville MD) and complemented with 10% FBS.

HCC1937 is maintained in RPMI 1460 supplemented with L-glutamine and HEPES (Invitrogen, Gibco, NY), 10% FBS, 1mM sodium pyruvate (Invitrogen, CA) and gentamycin.

SUM149PT and SUM190PT are cultured in Ham's F12 media (Invitrogen, Gibco, NY) supplemented with 1mg/ml insulin (Sigma, St. Louis, MO, USA), 0.05mM Hydrocortisone (Sigma, St. Louis, MO, USA), 1M HEPES (Invitrogen, CA), 5% FBS and gentamycin.

SUM1315M02 is maintained in Ham's F12 media (Invitrogen, Gibco, NY) supplemented with 1mg/ml insulin (Sigma, St. Louis, MO, USA), 10μg/ml EGF (Sigma, St. Louis, MO, USA), 1M HEPES (Invitrogen, CA), 5% FBS and gentamycin.

To split the cells as they reached 80% to 90% confluence, the media was discarded and the cells were washed with phosphate buffered saline solution (PBS) (Invitrogen, Gibco, NY) to completely remove FBS as it blocks the activity of trypsin. To detach the cells from the membrane, 4ml of trypsin (Invitrogen, Gibco, NY) were added for 5 minutes at 37°C. The cells were then collected after 5 minutes of spinning at 1500 revolutions per minute (rpm). The trypsin-containing media was removed and the cells were resuspended in complete media and replated at the required confluence in new flasks.

2-2 Sulphorhodamine B proliferation assay:

Dasatinib (Sprycel®, Bristol Myers Squib, Princeton, NJ) was prepared as a 10 mM stock in DMSO and then serially diluted for treatment. The cells were treated at decreasing concentrations ranging from 10μM to 0.0078μM.

Gefitinib (Iressa®, AstraZeneca, UK) was obtained as a gift from Dr. Gerald Batist's lab. It was similarly diluted at a starting concentration of 30μM down to 0.078μM.

The quantitative sulphorhodamine B (SRB) colorimetric assay was used to determine the growth inhibitory effect of the drugs on the eight cell lines (Skehan et al., 1990). The cells were seeded in 96-well plates in duplicates at 1000 cells per well for MCF7, BT20 and MDA-MB-436 and at 2000 cells per well for MCF10, SUM149PT, HCC1937, SUM1315M02 and SUM190PT. They were then treated 24 hours later (to ensure the

proper adherence of the cells to the wells) with increasing concentrations of the drug. The cells were fixed 96 hours later with 50µl of 40% trichloracetic acid (TCA) (Anachemia, Montreal QC) for two hours at 4°C, washed four times with water and stained for 30 minutes at room temperature with 50µl of a 0.4% SRB solution. 0.4% SRB was prepared by dissolving powder SRB (Sigma, St. Louis, MO, USA) in an appropriate volume of 1% acetic acid. Unbound SRB was then removed by rinsing the cells four times with 1% acetic acid. The plates were air-dried and the pink protein-bound dye was dissolved in 200µl of 10 mM Tris base (pH 10) then quantified by measuring the optical density (OD) at 570 nm with a FLUOstar OPTIMA plate reader (BMG labtech, Durham NC.)

The response to the drug was expressed as an IC_{50} value, which corresponds to the drug concentration needed to inhibit the proliferation of 50% of the cells relative to untreated control cells. The IC_{50} value for each cell line was calculated from four independent experiments for dasatinib and for gefitinib. Cells that had an IC_{50} value below the mean IC_{50} of all cells combined for a specific drug were considered sensitive.

The correlations between dasatinib sensitivity and the selected markers were performed by Dr. Aloyz using a Pearson's correlation analysis (SigmaStat, Jandel Scientific, San Rafael, CA)

2-3 Western blot analysis:

The selected cells were harvested by scraping and the total protein content was extracted by lysing the cells at 4°C with a cell lysis buffer composed of 20mM Tris (pH 8), 135mM NaCl, 1% NP40, 10% glycerol containing a protease inhibitors mixture (Roche), and

1mM sodium vanadate. The protein content of the lysate was determined using a bovine serum albumin (BSA) assay (Pierce, Rockford IL.) Equal concentrations of ready-to-load lysate (2μg/ul) were prepared by adding appropriate volumes of cell lysate, 1x XT loading buffer supplemented with a reducing agent (Bio-Rad Laboratories, Hercules, CA) and lysis buffer. The prepared lysate was then incubated at 65°C for 15 minutes to reduce and stabilize the proteins. Equal amounts of ready-to-load cell lysates were loaded on a 4-12% gradient SDS–polyacrylamide gel and separated by electrophoresis. The gel was then electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was subsequently blocked for 2 hours at room temperature in a Tris-buffered solution supplemented with 0.05% Tween 20 (TBST) and 5% BSA then washed three times with TBST. Primary antibodies against the desired proteins were added to the membrane and left at 4°C overnight. Appendix A represents all the primary antibodies used in the study. Following the overnight incubation, the membrane was washed three times with TBST and incubated for 1 hour at room temperature in a 1:5000 dilution of specific anti-mouse (NA 931; Amersham Pharmacia Biotech) or anti-rabbit (SC-2004; Santa Cruz Biotechnology) horseradish peroxidase-conjugated secondary antibody and then washed three times with TBST. The selected protein was then visualized using the enhanced chemiluminescence system (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ) to see the bands on a film. The band intensity, which reflects the presence of the desired protein, was quantified by densitometry using the Scion Image program (National Institutes of Health, Bethesda, MD). All proteins were normalized against actin levels.

2-4 Tissue microarray (TMA):

A tissue microarray (TMA) for all the cell lines was assembled by Dr. Tarek Bismar's lab using the manual tissue arrayer (Beecher Instruments, Silver Spring, MD) (Rubin et al., 2002). Tissue cores from circled areas were targeted for transfer to the recipient array blocks. Three to five replicate tissue cores were sampled from each cell line sample. The 1.0 mm diameter TMA cores were each spaced at 1.5 mm from core-center to core-center. After construction, 4 μm sections were cut and stained with hematoxylin and eosin (H&E) on the initial slides to verify the histological diagnosis.

2-5 Immunohistochemistry:

Immunohistochemistry was performed by Dr. Bismar's lab using the NexES immunostainer (Ventana, Tuscon, AZ). Slides from the formalin fixed paraffin embedded tissue of the tissue microarray were stained by H&E to confirm diagnosis. Primary antibodies for ERα, PR, CK 14, CK5/6, Vimentin, HER-2 (c-erbB2) and EGFR were used (Appendix B). Immunohistochemistry for CAV1, CAV2 and MET was performed by Dr. Jorge Reis-Filho, ICR, London, UK. Immunostaining was performed on 4 μm silane-coated slides (Sigma, St. Louis, MO, USA), dried overnight at 37 °C and then dewaxed, rehydrated, and boiled (microwave) in EDTA (pH 7.0) for antigen retrieval. Slides were incubated for 32 min at 37 °C using the primary antibodies listed above. Diaminobenzidine (DAB) was used as a chromogen, and slides were counterstained with

hematoxylin before mounting. Negative controls were obtained by omitting the specific primary antibodies. Protein expression was assessed using a 4 tiered system (-, negative; +, weak; ++, moderate; and +++, high expression).

2-6 Caveolin1 siRNA:

Subconfluent cultures of SUM149PT were transfected with four different specific caveolin-1 siRNA sequences. The 3'-GCAUCAACUUGCAGAAAGAUU-5' CAV1-siRNA sequence was the most efficient in decreasing CAV1 levels and was chosen for subsequent experiments (Dharmacon J-003467-09 CAV1). An ON-TARGETplus siCONTROL non-targeting siRNA #2 was used as a control. Cells were transfected according to the supplier's instructions (Dharmacon, Lafayette CO). The Dharmafect reagent #2 was used for transfection. The cells were replated at lower confluency 24 hours following the transfection. For each transfection, the siRNA efficiency was measured 72 hours post transfection by western analysis against the CAV1 protein.

2-7 DNA extraction:

Genomic DNA was extracted from each one of the eight cell lines for DNA amplification and sequencing purposes. A pellet containing approximately 10^6 cells was collected from cultured cells, and genomic DNA was extracted using the DNeasy mini kit (Qiagen, Valencia CA). The final elution step was carried out using distilled water instead of the Tris-EDTA buffer that the kit suggested to use. This modification was carried out to

avoid any interference between EDTA and the PCR reaction. The collected genomic DNA was diluted to a concentration of 50μg/μl.

2-8 Polymerase chain reaction (PCR):

Polymerase chain reaction (PCR) was performed to amplify specific regions of interest in the genome. An Eppendorf thermocycler (model 5341) was used (Eppendorf, Hamburg Germany.) The PCR was carried out in a total volume of 25μl containing: 100ng of genomic DNA, 1x Qiagen PCR buffer (Qiagen Valencia CA), 0.5mM $MgCl_2$ (Qiagen), 200μM of an equimolar dNTP solution (Qiagen), 0.2μM forward primer, 0.2μM reverse primer and 2U (units) of HotStartTaq polymerase (Qiagen).

All thermocycling processes consisted of a 15 minutes incubation at 95°C to activate the Taq polymerase, followed by 35 cycles of a three step process composed of DNA denaturation at 95°C for 30 seconds, primer annealing at temperatures varying between 56°C and 64°C (depending on the primer used) for 30 seconds and finally an extension step at 72°C for 45 seconds. A final extension step at 72°C for 5 minutes was carried out. The products were stored at 4°C.

The PCR products were separated by migration in a 1% agarose gel (in 1X TBE buffer) containing ethidium bromide. The bands were visualized under UV light. A PCR mix containing no DNA was used as a control for all the PCR reactions performed to confirm the absence of DNA contamination.

2-9 Primer design:

The primers used to sequence the tyrosine kinase domains of EGFR (ERBB1), HER2 (ERBB2), ERBB3 and ERBB4 as previously described (Bardelli et al., 2003). Primers for exons 17 to 24 of EGFR, exons 18 to 25 of HER2, exons 18 to 25 of ERBB3 and exons 21 to 26 of ERBB4 were used.

The primers for exons 6 to 11 of CDC4 (FBXW7) and exons 2, 11 and 20 of BRCA1 were designed using the primer3 software (http://frodo.wi.mit.edu). (Appendix C and D)

All primers were designed to amplify segments that are not bigger than 500base pairs (bps). In some cases, where the exon was lager than 500bps, two overlapping primers were designed.

All primers were purchased from Invitrogen, and resuspended in water at a final concentration of 20μM.

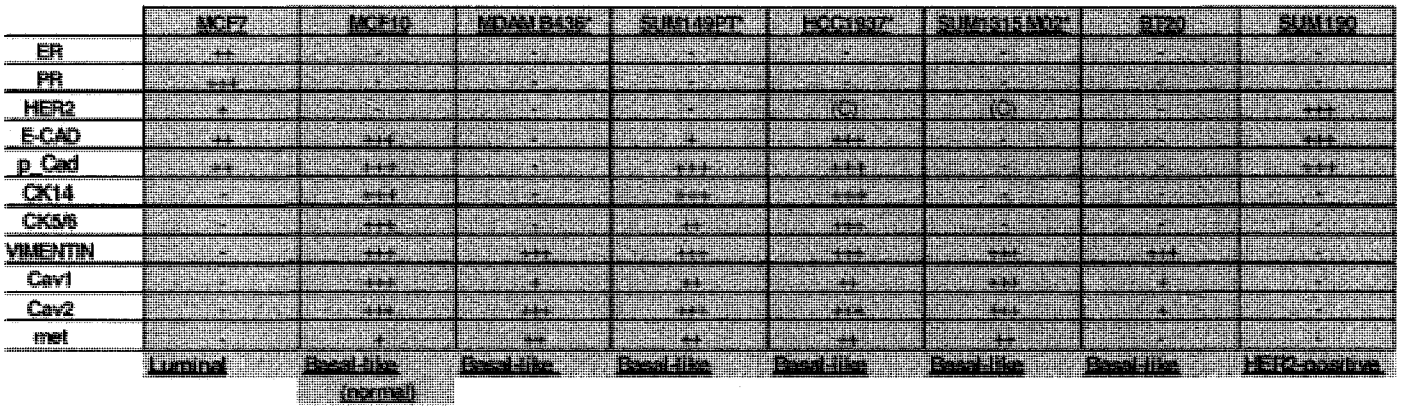
Mutation analysis was performed using the SeqMan software (DNASTAR, Madison WI). All sequences were confirmed manually. The reference sequences of the genes were obtained from the Genbank public database: EGFR (ERBB1) NM_005228, HER2 (ERBB2) NM_004448, HER3 (ERBB3) NM_001982, HER4 (ERBB4) NM_005235, hCDC4 NM_033632 and BRCA1 NM_007295.

Chapter 3-Results:

3-1 Characterization of the cell lines:

Previous microarray studies used gene expression levels to classify breast cancers, including our cell lines, into one of the five subtypes described earlier (Charafe-Jauffret et al., 2006;Neve et al., 2006). We confirmed the classification of the eight cell lines used in this study by IHC, rather than microarray analysis, for selected basal and luminal marker proteins. The markers that we checked include: ER, PR, HER2, E-cadherin, P-cadherin, CK14, CK5/6, vimentin, CAV1, CAV2 and MET (Tables 1 and 2). The cell lines that stained positive for ER, PR and HER2 altogether were classified as luminal. Within the luminal group, those that stained positive for HER2 but negative for ER were classified as HER2+. Finally, the cell lines that stained negative for ER, PR and HER2, but stained positive for CK 5, CK 14, EGFR, Vimentin or CAV1 were classified as basal-like (Carey et al., 2006). Our IHC analysis confirmed the microarray-based classification. MCF7 belongs to the luminal subtype. SUM190PT expresses HER2 but not ER and PR and is thus considered HER2+. The remaining six lines: MCF10, MDA-MB-436, SUM149PT, HCC1937, SUM1315M02 and BT20 belong to the basal-like subtype. Our results confirm that CK5/6, EGFR, CAV1 and MET are indeed markers of the basal phenotype of breast cancer (Nielsen et al., 2004a;Pinilla et al., 2006;Charafe-Jauffret et al., 2006)

Table 1: Characterization of the eight cell lines by immunohistochemistry:



	MCF7	MCF10	MDAMB436*	SUM149PT*	HCC1937*	SUM1315MO2*	BT20	SUM190
ER	++	-	-	-	-	-	-	-
PR	+++	-	-	-	-	-	-	-
HER2	+	-	-	-	(C)	(C)	-	+++
E-CAD	++	+++	-	+	+++	-	-	+++
p_Cad	++	+++	-	+++	+++	-	-	+++
CK14	-	+++	-	+++	+++	-	-	-
CK5/6	-	+++	-	++	+++	-	-	-
VIMENTIN	-	+++	+++	+++	+++	+++	+++	-
Cav1	-	+++	+	++	++	+++	+	-
Cav2	-	+++	+++	++	+++	+++	+	-
met	-	+	++	++	++	++	-	-
	Luminal	Basal-like (normal)	Basal-like	Basal-like	Basal-like	Basal-like	Basal-like	HER2-positive

EGATIVE, (+) WEAK, (++) MODRATE, (+++) STRONG. (C) Cytoplastmic
marked with an * have a BRCA1 mutation

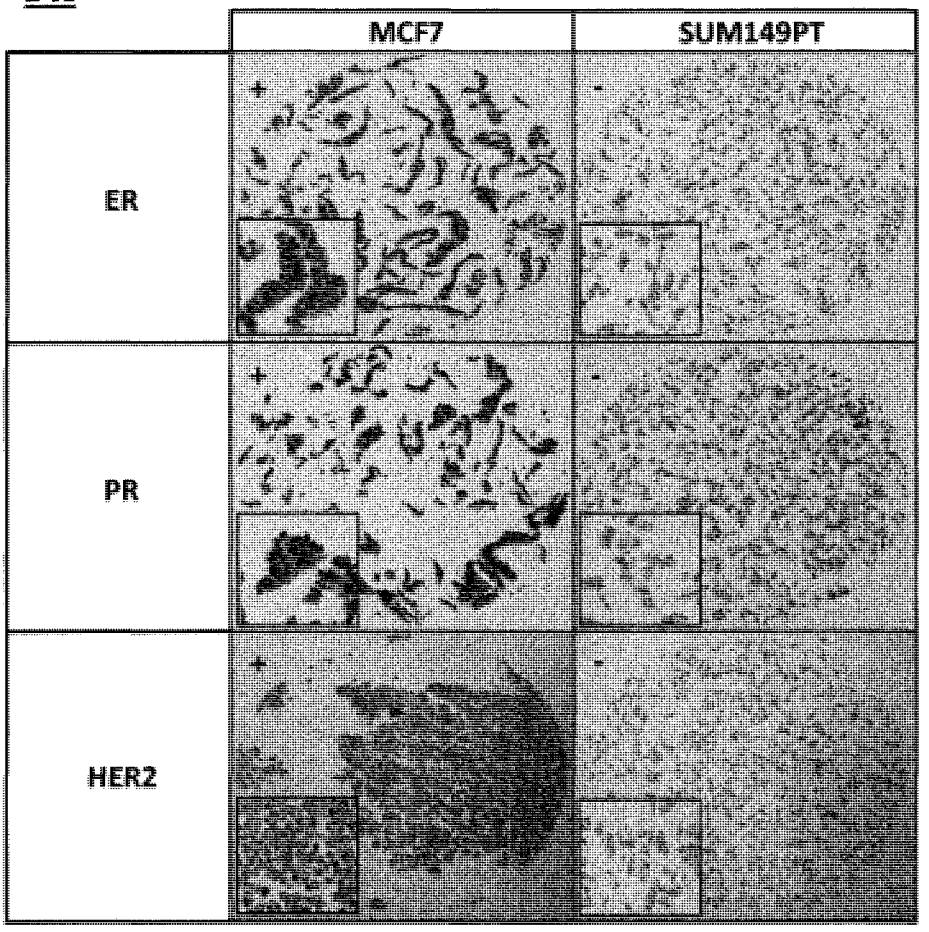
IHC for specific markers of the basal-like, the luminal and the HER2+ subtypes was performed on the eight cell lines to confirm earlier classifications done by expression microarray analysis. The cells highlighted in blue belong to the basal-like subtype. While MCF7 and SUM190 belong to the luminal and the HER2+ subtypes respectively. (Cells that exclusively express cytoplasmic HER2 are not considered HER2+. HER2 needs to be present on the cell's membrane)

Only MCF7 is positive for ER and PR. SUM190 has none of the basal-like markers, does not express ER or PR but does express HER2.

The cells that belong to the basal-like subtype express at least one of the following markers: CK14, CK5/6, vimentin, CAV1 or MET.

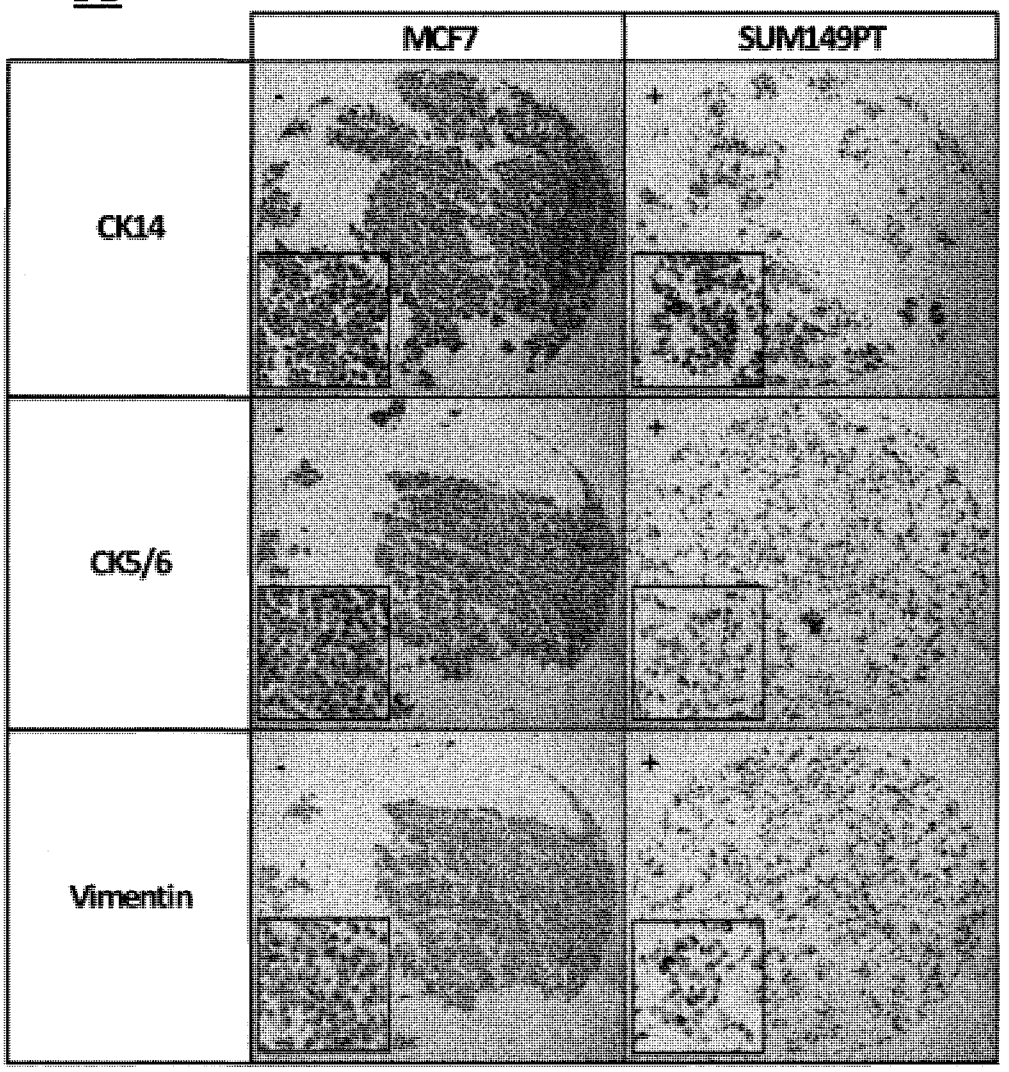
Table 2: Comparison between a luminal and a basal-like cell line for selected markers

2-A



In table 2A and 2B (following page) we compared two cell lines from the TMA we constructed: MCF7 which belongs to the luminal subtype and SUM149PT which belongs to the basal-like subtype. A few markers were selected to illustrate the difference between both subtypes.
MCF7 expresses ER, PR and HER2 (represented by a + sign next to the image) while SUM149PT expresses none of those markers (represented by a – sign next to the image)
A brown stain indicates that the cells express the markers.

2-B



In table 2B, SUM149PT expresses CK14, CK5/6 and vimentin (represented by a + sign next to the image) while MCF7 expresses none of those markers (represented by a – sign next to the image).
A brown stain indicates that the cells express the markers.

3-2 Confirmation of the BRCA1 mutations in the cell lines:

MDA-MB-436, SUM149PT, HCC1937 and SUM1315 have been previously described as BRCA1-mutated breast cancer cell lines. Each cell line harbors a BRCA1 mutation in one allele and the all have a loss of heterozygocity (LOH) for BRCA1. MCF7, MCF10, BT20 and SUM190 are wild type for BRCA1 (Tomlinson et al., 1998;Elstrodt et al., 2006). The first cell line to be characterized with a mutation was HCC1937 (5382insC). The mutations in the other three cell lines, MDA-MB-436 (5396 + 1G>A), SUM149PT (2288delT) and SUM1315M02 (185delAG) are summarized in table 3.

We confirmed the presence of the mutations in our cell lines by sequencing exons 2, 11 and 20 of BRCA1 since all four mutations are in those three exons (figure 3).

3-3 Mutation analysis of hCDC4 and cyclin E levels:

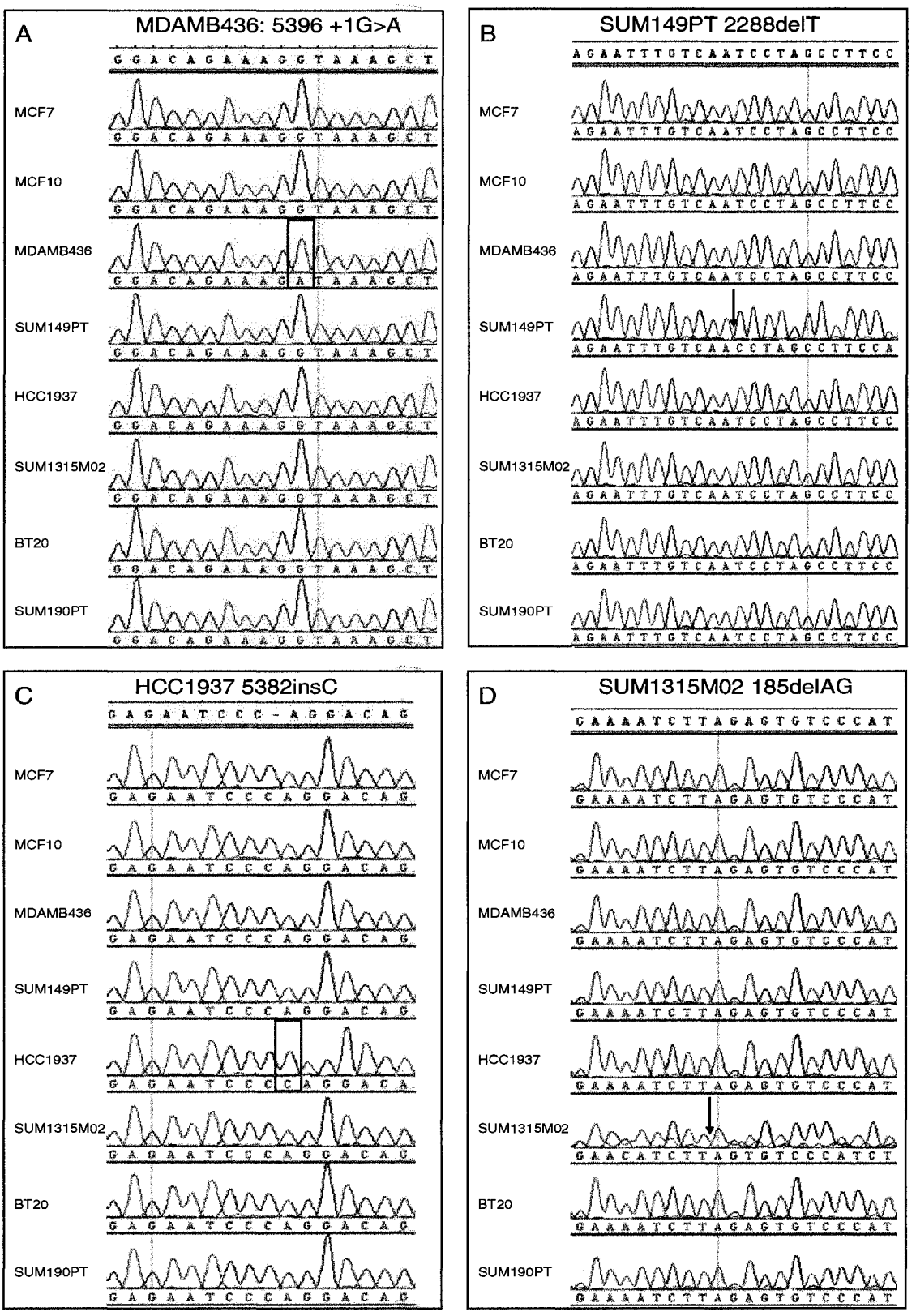
Most hCDC4 mutations previously found in a variety cancer subtypes are located at the substrate-binding domains (WD repeats) or at the amino terminal of the protein (Spruck et al., 2002). As a result, we sequenced exons six to eleven of hCDC4 in the four BRCA1-mutated cell lines. No mutations were found. SUM149PT was previously described to have a terminating mutation in this bridge protein, but we did not see it by sequencing genomic DNA. This is due to the fact that the mutation consists of a duplication of exons 8 and 9 separated by 11 base pairs of intronic sequence. It therefore appears normal by sequencing genomic DNA for those two exons. This alteration is

Table 3: BRCA1 mutations in the four cell lines

	Mutation	Exon	Predicted protein effect	No. in BIC database
MDAMB436	5396 + 1G>A	splice site of exon 20	E1731del28 and I1790insX8	46
SUM149PT	2288delT	exon 11	N723fsX13	0*
HCC1937	5382insC	exon 20	Q1756fsX74	1676
SUM1315M02	185delAG	exon 2	E23fsX17	1642

Table 3 represents the mutations present in the four cell lines. The numbering of the nucleotide changes was made according to the BRCA1 Genbank sequence (U14680) while the nomenclature of the mutations, and the number of citation of each one of them was done according to the BIC mutation database (up to July 2005, http://research.nhgri.nih.gov/bic/). All variants have a clinical pathogenic effect, and although the variant seen in SUM149PT has not been encountered in clinical cases yet (*), it is also predicted to have a pathogenic effect. The mutation seen in SUM149PT generates an incomplete protein due to a premature termination codon.
The frameshift and insertion mutations are represented by the first changed codon and the number of newly encoded codons, including premature termination codon X. All four cell lines lost one of the BRCA1 allele. (Elstrodt et al., 2006)
Figure 3 (following page): the mutations were confirmed in the four cell lines that were used in this study. The red box and the arrow indicate the position of the mutation for each of MDA-MB-436 (A), SUM149PT (B), HCC1937 (C) and SUM1315M02 (D).
The sequences clearly depict the point mutations in the four cases because only one BRCA1 allele is present in each mutated cell line (LOH).

Figure 3: Confirmation of the BRCA1 mutations in the four cell lines:



however predicted to result in chain termination by eliminating the last four (of seven) WD40 repeats and presumably rendering the polypeptide nonfunctional (Strohmaier et al., 2001). We also compared the relative levels of cyclin E in the cells and confirmed that SUM149PT has the highest levels of cyclin E (Figure 4).

3-4 Response of the cell lines to gefitinib:

Four independent SRB proliferation assays were carried out to determine the response of each cell line to gefitinib. The IC_{50} value for each cell line is represented by the average value obtained by the four assays. MCF10 and SUM149PT are sensitive to gefitinib with IC_{50}values of 0.533μM and 0.7725 μM respectively. The other six cell lines had an IC_{50} that is much higher than the attainable level of gefitinib in the serum and were thus classified as resistant (table 4).

Western blotting was then performed for EGFR and two of its activated forms (phosphor-tyrosine 845 and phosphor-tyrosine 1068). No correlation was found between the response to the tyrosine kinase inhibitor and both the activation level of EGFR and its baseline levels in the cells (Figure 5).

3-5 Mutation analysis of the HER1-4 tyrosine kinase domain:

Mutations in EGFR and especially in exons 19 and 21 have been associated with a response of lung cancers to gefitinib and there did not seem to be a clear correlation

Figure 4: hCDC4 mutation in SUM149PT:

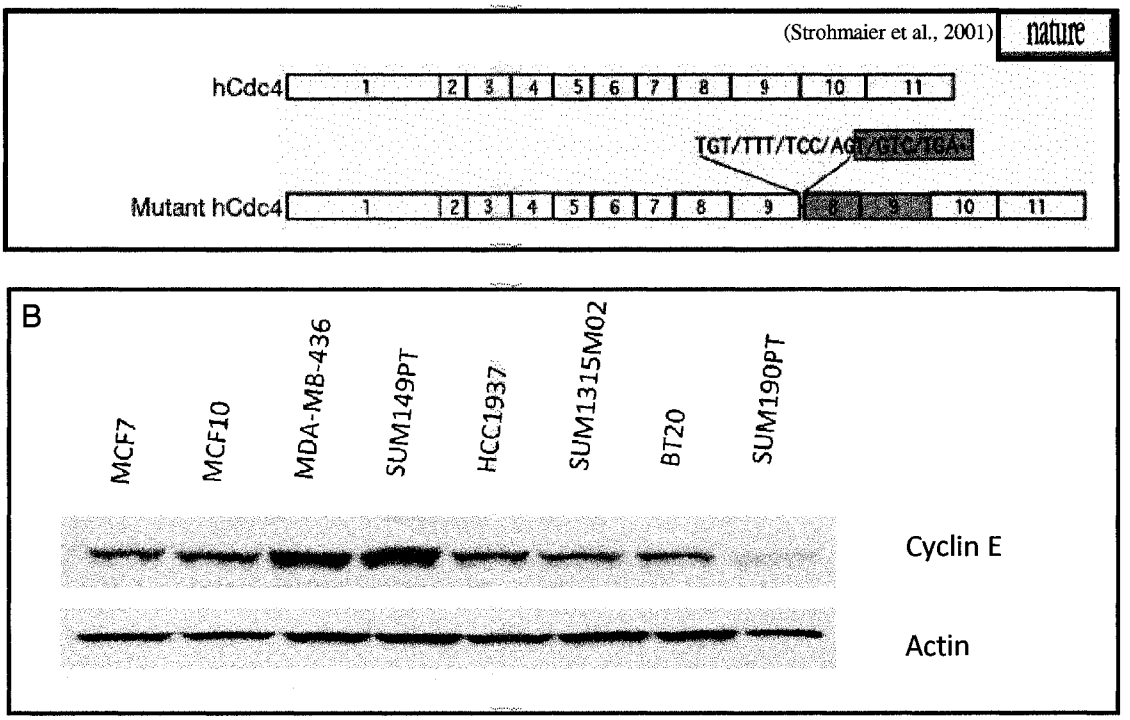


Figure 4A represents the structure of a normal hCDC4 cDNA sequence compared to that of SUM149PT. The exons are numbered and the shaded area represents a duplication of exons 8 and 9 preceded by 11 intronic base pairs. This mutation results in a chain termination by eliminating the last four WD repeats of the molecule and rendering the protein non-functional (Strohmaier et al., 2001).

Figure 4B represents the relative levels of cyclin E in the eight cell lines. SUM149PT has the highest levels of cyclin E followed by MDAMB436, another BRCA1-mutated breast cancer cell line. The protein levels were normalized compared to actin levels.

Table 4: The response of the eight cell lines to gefitnib:

A

	Gefitinib IC_{50} (μM)	Response
MCF7	20.125	resistant
MCF10	**0.533**	**sensitive**
MDAMB436	>30	resistant
SUM149PT	**0.7725**	**sensitive**
HCC1937	17.275	resistant
SUM1315M02	7.0525	resistant
BT20	12.2175	resistant
SUM190PT	9.64	resistant

B

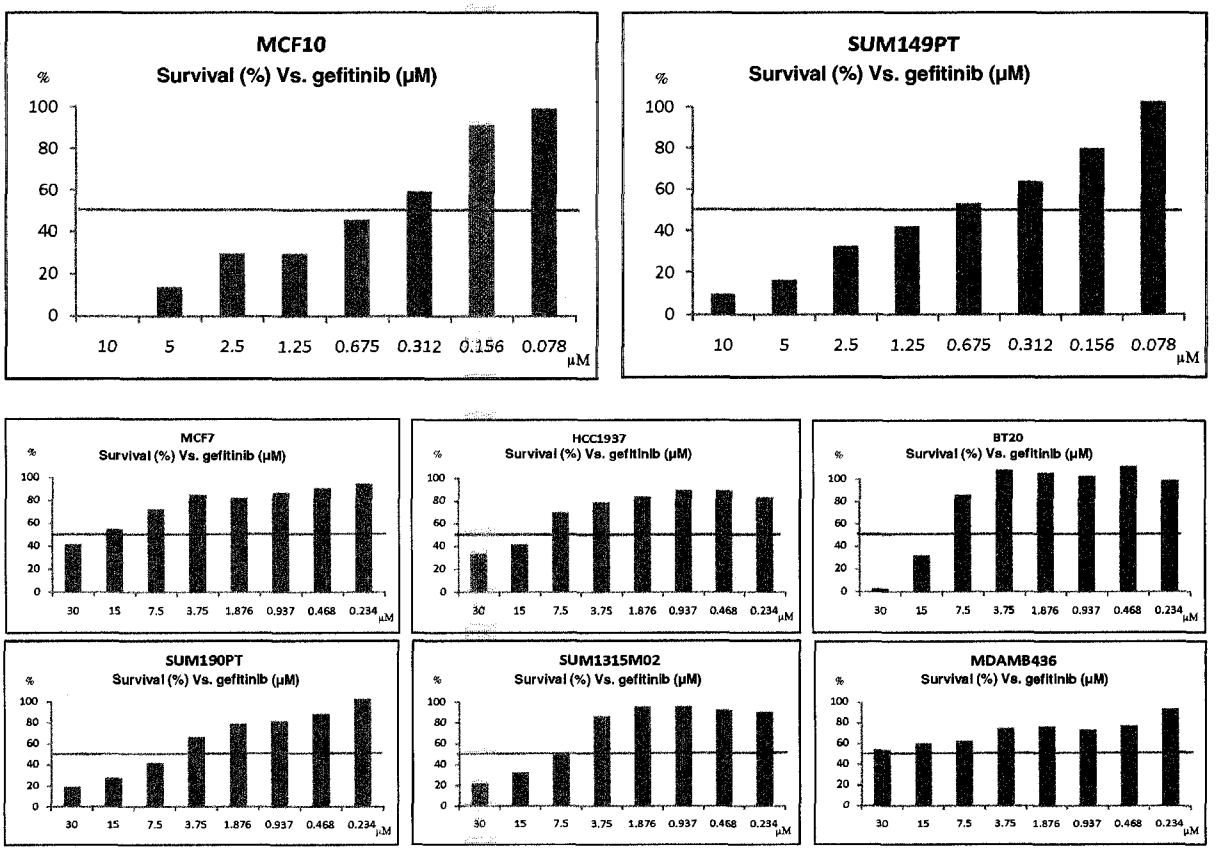


Table 4A lists the average IC_{50} of each cell line to gefitinib after four treatments. Only MCF10 and SUM149PT are sensitive to the EGFR tyrosine kinase inhibitor.

The survival of each cell line after treatment with decreasing concentrations of gefitinib in one of the four treatments is represented in table 4B. The graphs illustrate the relative response of each cell line to the drug. The red line represents 50% survival.

Figure 5: EGFR and sensitivity to gefitinib:

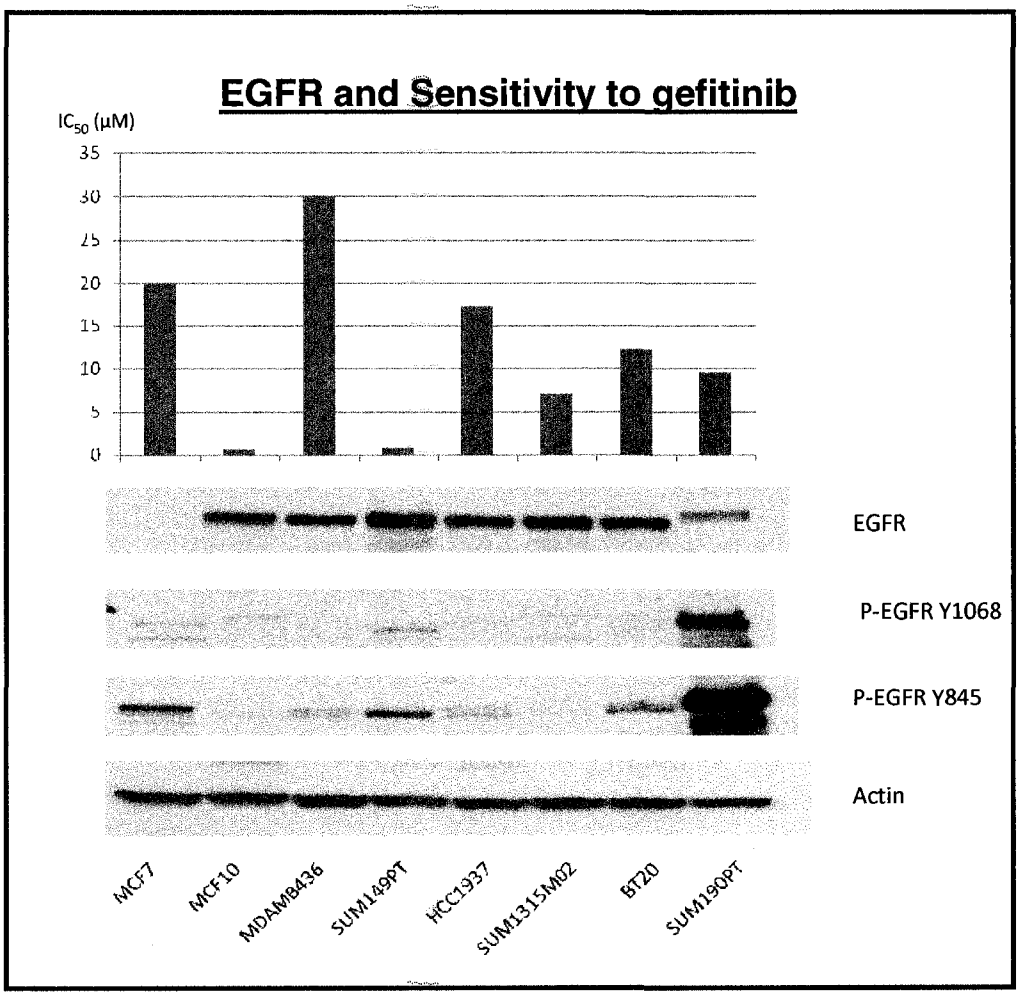


Figure 5: the sensitivity (IC_{50}) of each cell line to gefitnib is compared to EGFR and its phosphorylated levels at tyrosine 1068 and tyrosine 845. No correlation can be seen with either the basal EGFR levels or with its active form suggesting that the response to gefitinib in breast cancer cells does not depend on the levels of the receptor.

between the mutations and the EGFR levels seen by western blotting. Since the response of our cell lines did was not dependent on the protein expression levels of EGFR, we sequenced the tyrosine kinase domain of the receptor as well as the tyrosine kinase domain of HER2, HER3 and HER4 to see if the same principle applies in breast cancer. No mutation was found in any of the eight cell lines for the four genes. All the single nucleotide polymorphisms (SNPs) that we found in the exons of the four genes, and their predicted amino acid changes were previously described and are listed in table 5.

3-6 Response of the cell lines to dasatinib:

Four independent SRB proliferation assays were carried out to determine the response of each one of the eight cell lines to dasatinib. The average IC_{50} value was then calculated for each line. The four cell lines MCF7, MDA-MB-436, HCC1937 and BT20 were previously treated with dasatinib using a similar proliferation assay (Huang et al., 2007). Our results were concordant with theirs for the cell lines in common.

Finn et al. did not determine the IC_{50} value in their study and thus we could not compare our values to theirs. Five out the eight cell lines showed sensitivity to the drug and they all belonged to the basal phenotype (MCF10, SUM149PT, HCC1937, SUM1315M02 and BT20). The other three lines that did not respond were from the luminal subtype for MCF7, the HER2+ subtype for SUM190PT and the basal-like subtype for MDA-MB-436 (figure 6.) Likewise, if we take the BRCA1-mutated breast cancer cell lines alone, three out of the four lines showed a positive response to treatment with dasatinib (SUM149PT, HCC1937 and SUM1315M02).

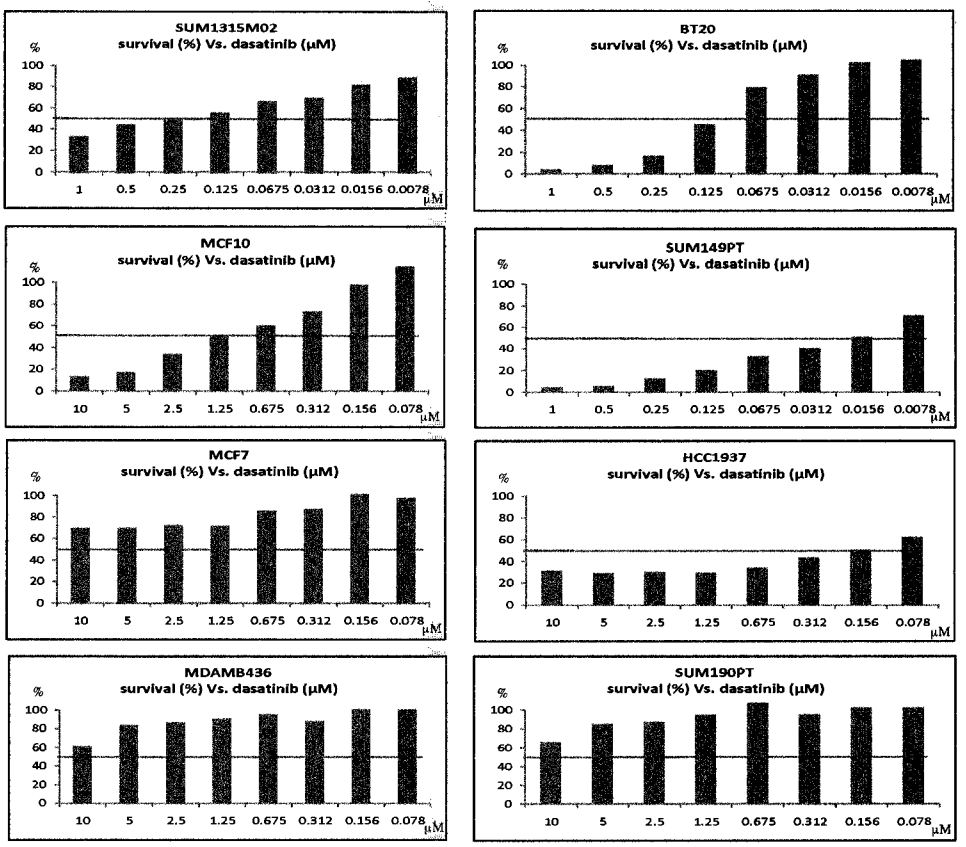
Table5: Mutation analysis of the tyrosine kinase domain of EGFR, HER2, HER3 and HER4

Gene Name		MCF7	MCF10	MDAMB436	SUM149PT	HCC1937	SUM1315M02	BT20	SUM190PT
EGFR	Exon 17	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
EGFR	exon 18	intronic SNP 2430 +19G>A (rs17337107)	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
EGFR	exon 19	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
EGFR	exon 20	SNP 2607 G>A (Q787Q) (rs1050171)	SNP 2607 G>A (Q787Q) (rs1050171)	no mutation	SNP 2607 G>A (Q787Q) (rs1050171)	SNP 2607 G>A (Q787Q) (rs1050171)	SNP 2607 G>A (Q787Q) (rs1050171)	SNP 2607 G>A (Q787Q) (rs1050171)	SNP 2607 G>A (Q787Q) (rs1050171)
EGFR	exon 21	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
EGFR	exon 22	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
EGFR	exon 23	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
EGFR	exon 24	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER2	exon 18	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER2	exon 19	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER2	exon 20	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER2	exon 21	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER2	exon 22	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER2	exon 23	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER2	exon 24	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER2	exon 25	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER3	exon 18	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER3	exon 19	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER3	exon 20	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER3	exon 21	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER3	exon 22	did not work	did not work	did not work	did not work	did not work	did not work	did not work	did not work
HER3	exon 23	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER3	exon 24	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	did not work
HER3	exon 25	intronic SNP 3322 +9 A>C	no mutation	no mutation	no mutation	no mutation	no mutation	intronic SNP 3322 +9 A>C	no mutation
HER4	exon 21	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER4	exon 22	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER4	exon 23	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER4	exon 24	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation
HER4	exon 25	no mutation	no mutation	no mutation	intronic deletion 3063 -9delTCTT	no mutation	no mutation	no mutation	no mutation
HER4	exon 26	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation	no mutation

Table 5: The exons coding for the tyrosine kinase domains of EGFR (HER1), HER2, HER3 and HER4 were sequenced in the eight cell lines. No deleterious mutations were found, and the nucleic acid changes that we saw in the exons are known SNPs. The variants seen in HER3 and HER4 are in the non-coding (intronic) region and have not been previously described.

Figure 6: The response of the eight cell lines to dasatinib

A



B

	Dasatinib IC_{50} (μM)	Response	Subtype
MCF7	>10	resistant	luminal
MCF10	**1.8275**	**sensitive**	**basal**
MDAMB436*	>10	resistant	basal
SUM149PT*	**0.065**	**sensitive**	**basal**
HCC1937*	**0.201**	**sensitive**	**basal**
SUM1315M02*	**0.22**	**sensitive**	**basal**
BT20	**0.13525**	**sensitive**	**basal**
SUM190PT	>10	resistant	HER2+

The survival of each cell line after treatment with decreasing concentrations of dasatinib in one of the four treatments is represented in figure 6A. The graphs illustrate the relative response of each cell line to the drug and the red line represents 50% survival.

Figure 6B lists the average IC_{50} of each cell line to dasatinib after four treatments. Five cells lines are sensitive to the drug and they all belong to the basal-like subtype. In addition, three out of the four BRCA1-mutated breast cancer cell lines (marked with an asterix *) are sensitive to dasatinib.

Therefore, in addition to what has previously been published on dasatinib and basal-like breast cancers, these results show that BRCA1-mutated breast cancers belong to a class of breast cancers that may be treated with this c-ABL/SRC inhibitor that was initially designed to treat leukemia (Finn et al., 2007;Huang et al., 2007). This finding opens the way to further pre-clinical studies of dasatinib in BRCA1-mutated and basal-like breast cancer.

3-7 Protein signature for dasatinib sensitivity:

In the two previous studies that associated dasatinib sensitivity to basal-like breast cancers, a panel of genes was selected to act as a predictive signature for drug response. Both studies used gene expression data collected by microarray analysis to create this panel. Although thousands of genes can be analyzed using this method, the actual levels of the proteins and the activation status are best captured by protein assays such as western blotting, FACS analysis, and IHC.

We measured the expression level of selected proteins by western blotting in our eight cell lines to confirm what has previously been found by microarray analysis and to uncover potential new markers for dasatinib sensitivity. Western blotting also allowed us to detect the activity level of certain markers and thus presented clues as to which pathways are targeted by the drug. First, the baseline protein levels of both SRC and ABL (and their active forms) were measured since dasatinib is a SRC/ABL inhibitor. The expression level of neither protein correlated with the response of the cell lines. The

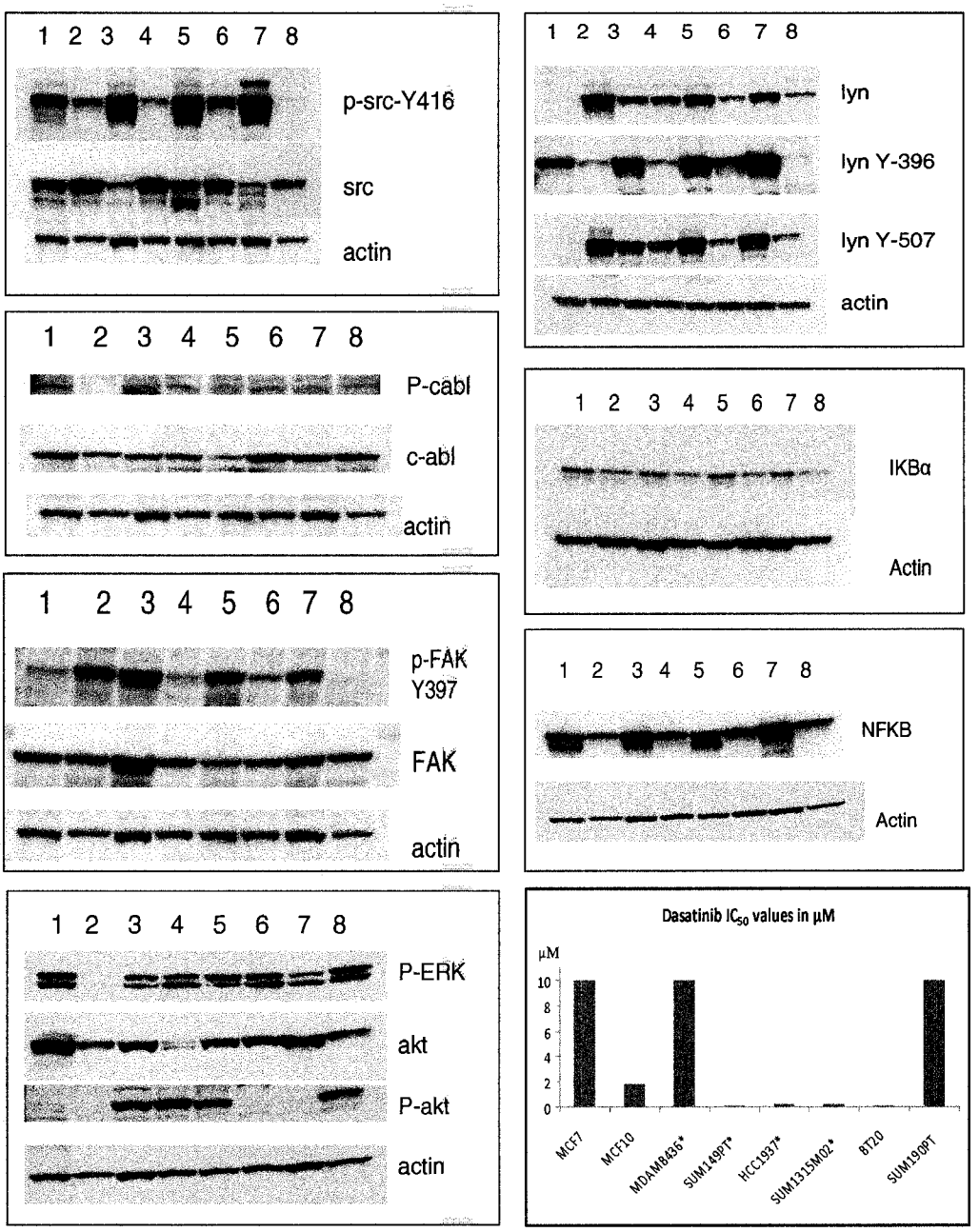
levels of LYN (and phospho-LYN), another member of the SRC family, and those of NFκB, IκBα, ERK, (and phospho-ERK) and AKT (and phospho-AKT) were measured and did not show any correlation either (figure 7).

However, as predicted by gene expression analysis, a positive correlation between drug sensitivity and CAV1 protein expression level was seen in our panel ($p=0.002$). We also demonstrated that only the sensitive cells expressed the active form of the protein (Figure 8A). EGFR levels were also associated with drug sensitivity, as five of the six EGFR positive cells lines are sensitive to dasatinib ($p=0.013$). The phosphorylated forms of EGFR at tyrosine 845 and tyrosine 1068 did not correlate with a response to dasatinib (Figure 8B).

3-8 Pathways affected by dasatinib:

In addition to developing a panel of predictive markers, we sought to delineate the pathways that dasatinib is affecting while inhibiting cell proliferation. We selected the BRCA1-mutated/basal-like/drug sensitive cell line SUM149PT and treated the cells at four different time points with 0.1 μM dasatinib. No changes in the level of c-ABL and its active form were seen, confirming that this dasatinib-target protein is not involved in the response of breast cancer cells to the drug (figure 9). We also treated the cells with the c-ABL inhibitor imatinib and the cells did not show the same response pattern as with dasatinib (Appendix E). This confirms that the inhibition of cell proliferation does not occur through c-ABL inhibition. On the other hand, the levels of the active state of SRC

Figure 7: Proteins that do not act as markers for dasatinib sensitivity



1 = MCF7
2 = MCF10
3 = MDAMB436
4 = SUM149PT
5 = HCC1937
6 = SUM1315M02
7 = BT20
8 = SUM190PT

Figure 7 represents the basal levels (and the active forms) of various dasatinib targets and of some of the proteins that act downstream to those molecules.
The sensitivity of each cell line to dasatinib (IC_{50} in µM) is represented in the graph. None of those markers act as a predictor for dasatinib sensitivity.
All proteins were normalized against actin levels.

Figure 8: Proteins that correlate with dasatinib sensitivity

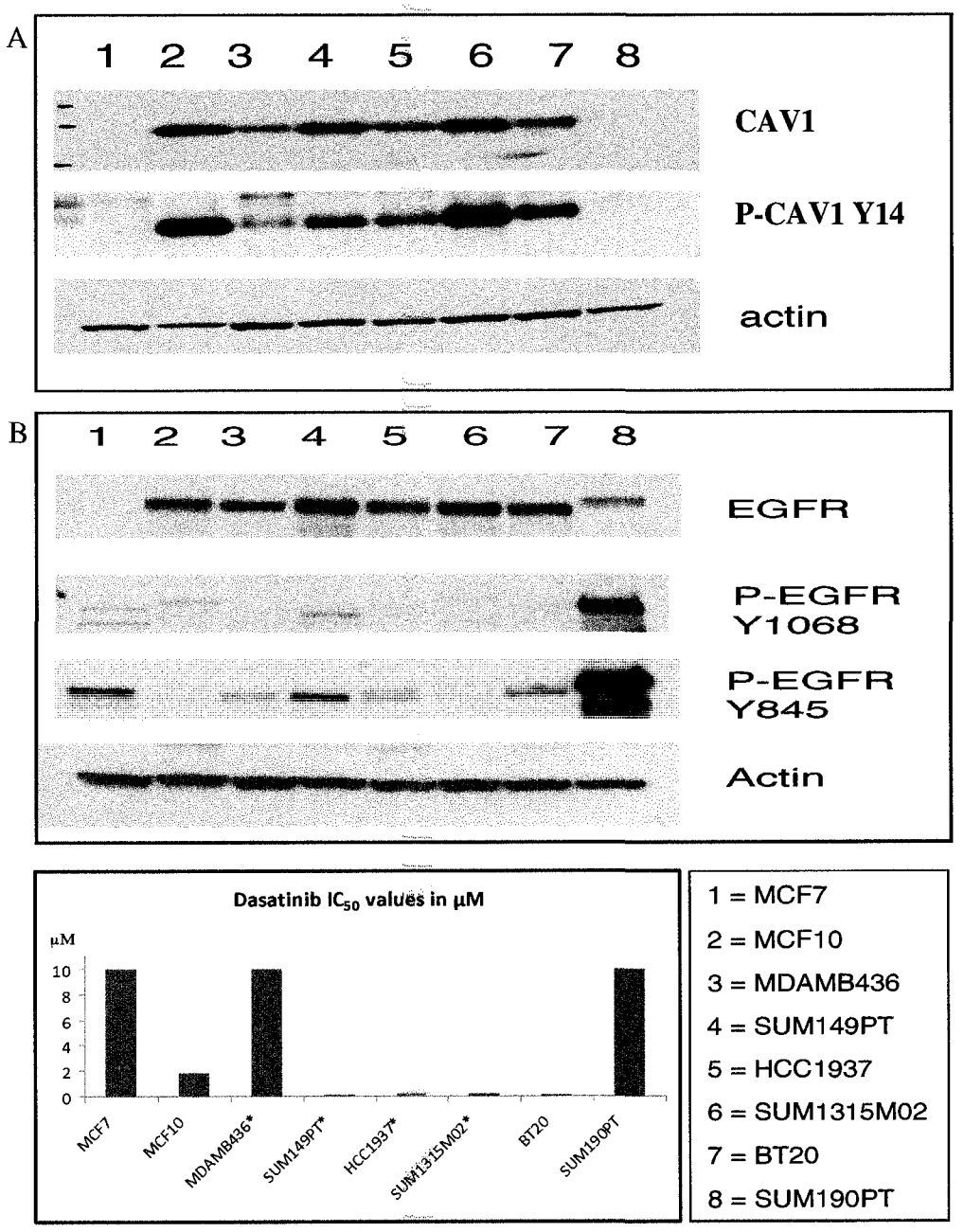
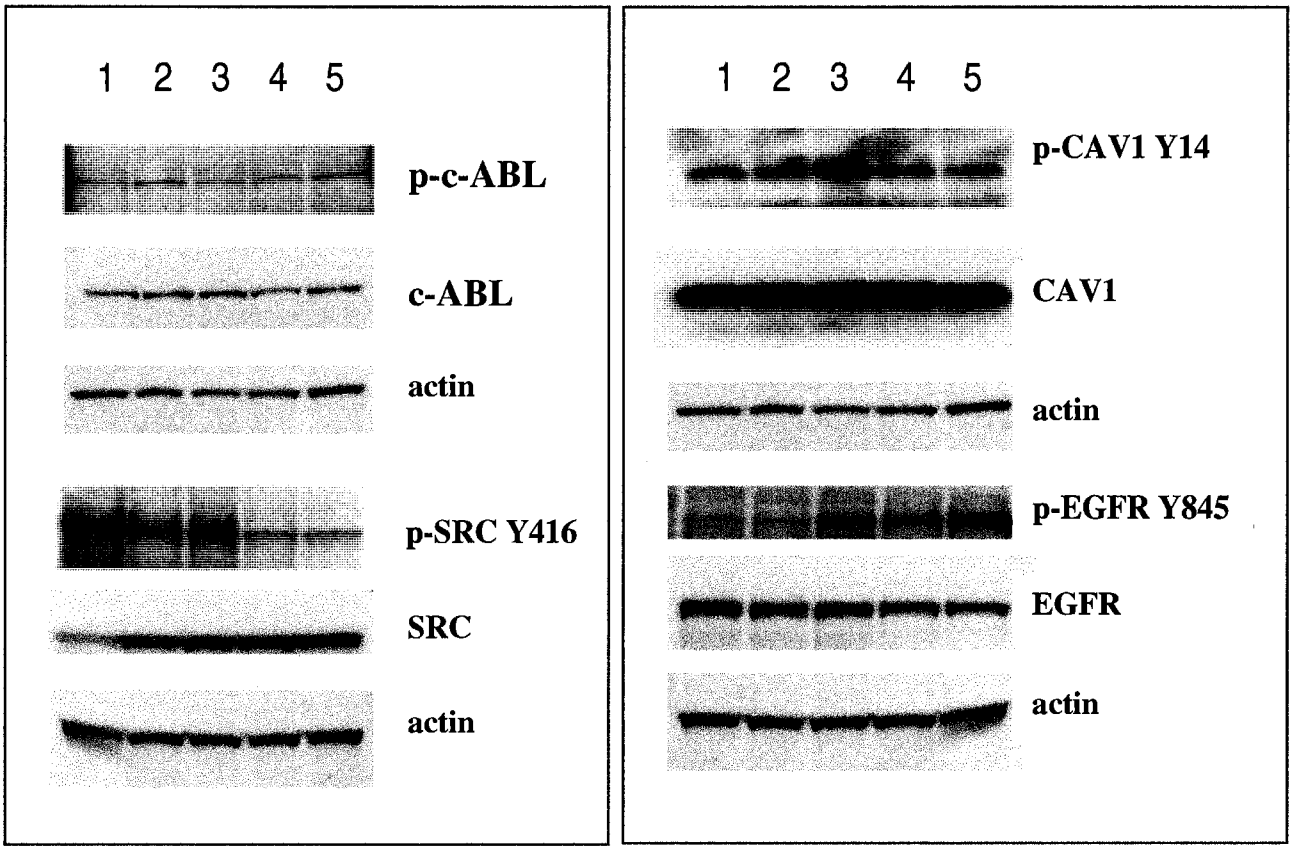


Figure 8A represents the levels of CAV1 and its active form in the eight cell lines. Those that are sensitive to dasatinib (MCF10, SUM149PT, HCC1937, SUM1315M02 and BT20) express higher levels compared to the resistant cell lines (MCF7, MDA-MB-436 and SUM190PT). Figure 8B on the other hand confirms the importance of EGFR as a marker of dasatinib response, but its active forms do not correlate with a response of the cells to the drug.

Figure 9: changes in c-ABL, SRC, CAV1 and EGFR levels after treatment with dasatinib



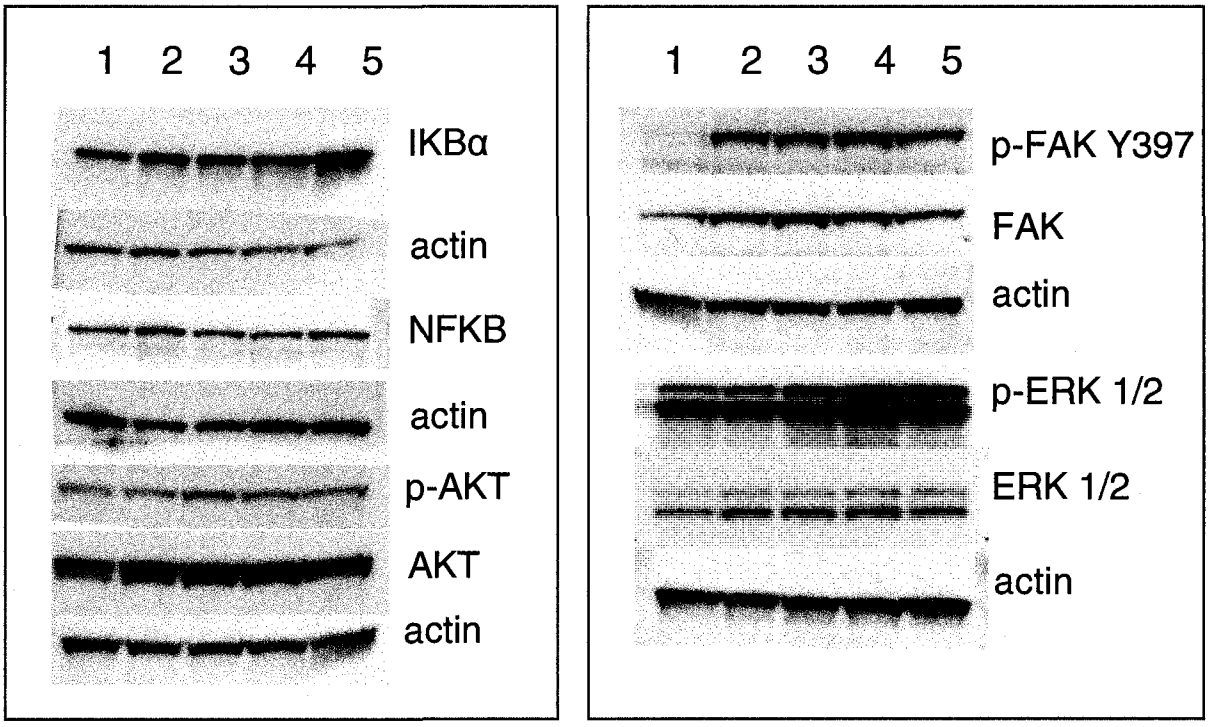
1 = SUM149PT t-0 0.1μM dasatinib
2 = SUM149PT t-1 0.1μM dasatinib
3 = SUM149PT t-6 0.1μM dasatinib
4 = SUM149PT t-24 0.1μM dasatinib
5 = SUM149PT t-48 0.1μM dasatinib

SUM149PT was treated with 0.1μM of dasatinib at five different time points. P-SRC levels decreased to reach their lowest levels at t-24 and t-48 hours post treatment. The levels of c-ABL, p-c-ABL, CAV1, p-CAV1, EGFR and SRC were not affected. P-EGFR Y845 levels increased throughout the treatment.

(p-SRC Y416), the other known target of dasatinib, gradually decreased after treatment at the different time points. This observation implies that even though the SRC and p-SRC protein levels are not predictive markers for dasatinib response, their presence is associated with a positive response to the drug (figure 9). To confirm that SRC is not the only protein that is involved in the dasatinib-response, we treated the cells in a separate experiment with the SRC/ABL inhibitor SKI-606. SKI-606 inhibited the proliferation of three out of the five dasatinib-sensitive cell lines. This confirmed that SRC is essential for dasatinib response, but suggests that it is not acting solely through SRC inhibition (Appendix E). It seems likely that another molecule or pathway is being affected by dasatinib in conjunction with SRC.

Once the two known targets of dasatinib were explored, we looked deeper at other downstream proteins that could be affected by a change in SRC activity. We mainly looked at the EGFR and CAV1 proteins since both predict sensitivity to the treatment and both their activity are regulated by SRC. However, CAV1, p-CAV1 and EGFR levels were not affected by dasatinib treatment. This suggests that although they help in determining whether the cells will respond to dasatinib or not, changes in the levels of the proteins do not mediate a response to the drug (figure 9). On the other hand, levels of p-EGFR Y845, p-ERK1 thr202 and p-ERK2 tyr204 (p-ERK 1/2), and p-FAK Y397 increased with time after addition of dasatinib. This increase of activity is usually associated with proliferation and survival but could be a side effect of SRC inhibition. The cells may be trying to "make up" for the loss of p-SRC by activating those pathways. AKT, NFκB and IκBα levels were not affected suggesting that they are not being targeted

Figure 10: The effect of dasatinib on other downstream markers

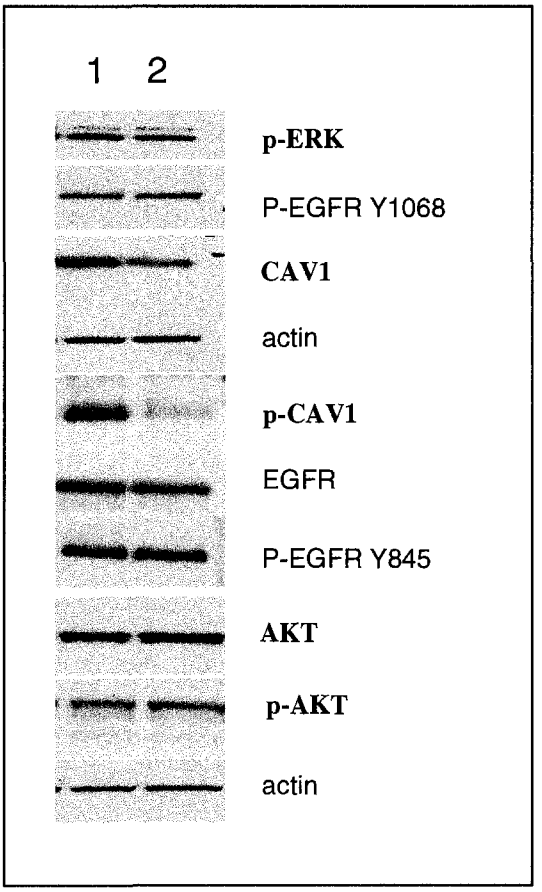


1 = SUM149PT t-0 0.1μM dasatinib
2 = SUM149PT t-1 0.1μM dasatinib
3 = SUM149PT t-6 0.1μM dasatinib
4 = SUM149PT t-24 0.1μM dasatinib
5 = SUM149PT t-48 0.1μM dasatinib

SUM149PT was treated with 0.1μM of dasatinib at five different time points. The levels of IκBα, NFκB, p-AKT, AKT FAK and ERK1/2 were not affected by the drug. P-FAK Y397 and p-ERK levels increased throughout the treatment.
The protein levels were normalized against actin.

by dasatinib, nor are their upstream regulatory pathways (figure 10). To confirm that CAV1 was not driving this inhibition of cell proliferation although it acts as a predictive marker, we downregulated its protein expression levels by siRNA transfection. The levels of CAV1 and p-CAV1 dropped, but it did not affect EGFR (and p-EGFR), p-ERK 1/2 and p-AKT levels the same way the treatment with dasatinib did (Figure 11.)

Figure 11: CAV1 downregulation and the proteins it affects:



1 = SUM149PT + non-target siRNA
2 = SUM149PT + cav1 siRNA

SUM149PT was treated with CAV1 siRNA for 72 hours. Caveolin 1 and its active form were downregulated compared to the control cells that were transfected with an on-target siRNA. Levels of p-ERK, EGFR, p-EGFR, AKT and p-AKT were not affected.
The protein expression levels were normalized against actin.

Chapter 4- discussion and future directions:

4-1 Discussion:

Despite the tremendous progress that cancer research has witnessed in the last two decades in terms of treatment, prevention and tumor characterization, the battle against cancer is yet to be won. The trend in cancer treatment is currently leaning towards tailored biological therapies because each case is unique in terms of genetic alterations, morphology and molecular signature. In breast cancer for example, the luminal and HER2-positive subtypes are being effectively treated using such tailored therapies. However, the basal-like subtype, which also includes the majority of BRCA1-mutated breast cancers, still lacks an effective and specific treatment. A successful approach that has been used in such situations revolves around using an existing drug that is used to treat one cancer type and apply it to another cancer type that exhibits similar characteristics.

The aim of this study was to evaluate the effectiveness of a lung cancer drug (gefitinib) and a leukemia drug (dasatinib) on the basal-like subtype of breast cancer. To achieve this, we selected eight breast cancer cell lines (four of which have been previously described to harbor a BRCA1 mutation), we characterized them, and then we treated them with the two drugs mentioned above. Once the response of the cell lines to each

treatment was obtained, we looked at the mechanisms by which the drug inhibited the proliferation of the cells.

The subtype of the eight breast cancer cell lines used in the study was confirmed by IHC for markers specific to each subtype. Our results were concordant with what was previously found by microarray analysis. Six cell lines were classified as basal-like (four of which are BRCA1-mutated) and the other two were classified as luminal and HER2-positive respectively. We also confirmed the presence of the BRCA1 mutations in the four cell lines that were described as BRCA1 mutants. The fact that the four BRCA1-mutated cell lines belong to the basal-like subtype validates the hypothesis set by Foulkes et al. as to the higher probability of detecting BRCA1 mutations in the cancers that are basal-like in subtype relative to the other subtypes.

To characterize the cells even further, we looked at the levels of cyclin E in the cell lines. High levels of this protein have been seen BRCA1-mutated cancers. The two lines that had the highest levels of cyclin E were in fact BRCA1-mutated (SUM149PT and MDA-MB-436.) Several proteins, including the hCDC4 bridge protein, are involved in the regulation of the cyclin levels in the cells. When mutations in the binding domains of hCDC4 are present, they can prevent the degradation of cyclin E. This imbalance in the levels of the cell cycle regulator is often linked to aneuploidy and genetic instability. We sequenced hCDC4 in our four BRCA1-mutated cell lines to see whether mutations in this gene can act as a predictive marker for BRCA1-mutated cancers. No mutations were found in exons 6 to 11 in the eight cell lines. Previous studied detected a mutation in

SUM149PT characterized by a repeat of exons eight and nine preceded by 11 base pairs. This duplication causes a frameshift and produces a non-functional protein. We did not see it in our analysis because we sequenced the genomic DNA of the cell lines and not the mRNA. For that reason, sequencing the mRNA instead of the genomic DNA in future studies should be performed to avoid missing out on such mutations.

Once the cell lines were assigned to their respective subtypes, we treated them with the EGFR tyrosine kinase inhibitor, gefitinib. This drug was chosen because most basal-like breast cancer cells express the epidermal growth factor receptor on their surface. Two out of eight lines responded to the drug (SUM149PT and MCF10). They both belong to the basal-like subtype and express the receptor on their surface. To assess whether the presence of EGFR or its active form acts as a predictive marker for gefitinib sensitivity, we measured the levels of EGFR, p-EGFR (Y845) and p-EGFR (Y1068) by western blotting. None of the three variants correlated with a sensitivity to the drug. We then looked for activating mutations in the tyrosine kinase domain of the HER family of receptors. Lung cancers with such mutations show a positive response to the drug. In our panel of eight breast cancer cell lines, no activating mutations were observed. Subsequently, the treatment of the eight cell lines with the lung cancer drug showed that gefitinib is not likely to be very effective in the treatment of the basal-like subtype of breast cancer (two out of six basal-like cell lines responded compared to five out of six for dasatinib). In addition, the level of EGFR, its activation status, and its mutational status do not act as predictive markers for the response of the breast cancer cells to the treatment.

The same cells were then treated with the leukemia drug, dasatinib. This SRC/ABL inhibitor was chosen because two recent studies showed that breast cancers that belong to the basal-like subtype are sensitive to this drug. Our results confirm this observation: five out of the six basal-like cell lines we have showed sensitivity to dasatinib while the luminal and HER2+ cell lines were resistant. We also showed that BRCA1-mutated breast cancers can be also targeted by the same drug because they are usually basal-like in phenotype. In fact, three out of the four BRCA1-mutated breast cancer cell lines showed high sensitivity to dasatinib.

The two studies by Finn et al. and Huang et al. also generated, using microarray analysis, a list of predictive markers for dasatinib sensitivity. We took this process one step further by analyzing the protein level of some of their predictive markers as well as the activation level of the proteins when possible. The presence of SRC or c-ABL did not act as predictor for dasatinib response in our eight cell lines. Other proteins that belong to the SRC family of kinases (LYN and p-LYN) or that act downstream of SRC (FAK, p-FAK, ERK, p-ERK, AKT, p-AKT, NFκB and IκBα) were also measured but they did not correlate with a response to the treatment. The only two proteins that predicted a sensitivity to the drug, when present, were CAV1 (and its active form p-CAV1-Y14) and EGFR. CAV1 was mentioned in earlier studies, however, its phosphorylated form was not. We decided to look at the active form of the protein because it is regulated by one of the dasatinib targets, SRC.

Once we established which proteins predicted a response to dasatinib, we took a sensitive cell line (SUM149PT) and treated it with the drug at five different time points. We then looked at changes in the protein levels and in the activation levels to elucidate a mechanism by which dasatinib inhibits cell proliferation. C-ABL, p-c-ABL and SRC levels did not change, but the levels of p-SRC dropped suggesting that, in breast cancers, dasatinib affects the cells by inhibiting the activity of SRC and not c-ABL. This was confirmed by treating the cells with the c-ABL inhibitor, imatinib. This drug inhibited the growth of only two of the eight cell lines. We then treated the cells with the SRC/ABL inhibitor SKI-606 to see whether dasatinib solely acts on the cells by inhibiting SRC, or if it targets other proteins as well. The results obtained with SKI-606 revealed that the presence of SRC is necessary for the inhibition of the cells with dasatinib, but it is not sufficient. In order to find the other factor that is being affected by dasatinib alongside SRC, we looked for changes in the levels of proteins in pathways that are known to be directly or indirectly affected by SRC activity in our treated cells. We also looked for changes in the levels of CAV1 and EGFR since they are both predictive markers for dasatinib sensitivity. The levels of p-AKT, AKT, ERK, NFκB, IκBα, FAK, EGFR, CAV1 and p-CAV1 did not change throughout the time course. However, the levels of p-EGFR (Y845), p-ERK and p-AKT increased with time. We expected to see a drop in the levels of those proteins. This increase may be the cells' response to the decrease seen in p-SRC levels. In this situation, the cells may be trying to make up for the decrease in p-SRC activity by increasing the activity of the three proteins. This effect has to be studied further to determine the importance of this change.

Since CAV1 was assigned as a predictive marker for the response of the cells to dasatinib, we decided to look at its role in the mechanism by which the drug affects the cells. We downregulated the levels of CAV1 in SUM149PT with a specific siRNA and then looked for changes in the levels of the proteins studied earlier. No change was seen (p-ERK and p-EGFR levels did not go up as they did with dasatinib), suggesting that a decrease in CAV1 does not mimic the effects of dasatinib in the cell. We also sought to determine whether the cells in which we knocked-down CAV1 became more resistant to the drug compared to the cells that still had the original levels of the marker. This experiment was hard to accomplish because the downregulation of CAV1 in the transiently transfected cells was not equal between the cells. This problem could be overcome in subsequent studies by performing a stable transfection using retroviruses or plasmids that contain the siRNA sequence.

In conclusion, this study was successful in determining the sensitivity and the means by which two non-breast cancer drugs affect a panel of well characterized breast cancer cell lines. We established that gefitinib is not as efficient as dasatinib in the treatment of both BRCA1 and basal-like breast cancers. We also showed that p-SRC was inhibited by dasatinib, but its inhibition is, on its own, insufficient to inhibit cell proliferation. Consequently, we looked at changes in the levels of various proteins but we have not yet pinpointed the additional agent in this process. We also showed that although CAV1 is a marker for drug sensitivity, its levels are not affected by the drug.

4-2 Future directions:

1) To determine which protein(s) is/are acting alongside SRC to inhibit the proliferation of the dasatinib-treated cells, we will look for changes in the levels of additional proteins that are involved in cell cycle progression, gene transcription, cell survival, cell proliferation and other pathways.

2) We will downregulate the dasatinib-sensitivity marker, EGFR, in a sensitive cell line then study the effects of a decrease in the EGFR levels on cell growth. We will also investigate whether the absence of this receptor affects the sensitivity of the cells to dasatinib.

3) Once we determine the protein or the proteins that associate with SRC to inhibit cell proliferation in the dasatinib treated cells, the efficiency of the drug will be further confirmed in additional *in-vivo* experiments. MCF7, SUM149PT, SUM1315M02 and BT20 can grow in immunosupressed nude mice (American Type Cell Culture; Asterand), and can thus be used to study the efficiency of dasatinib in live organisms.

Reference List

Aligayer,H., Boyd,D.D., Heiss,M.M., Abdalla,E.K., Curley,S.A., and Gallick,G.E. (2002). Activation of Src kinase in primary colorectal carcinoma: an indicator of poor clinical prognosis. Cancer *94*, 344-351.

Allegra,J.C., and Lippman,M.E. (1980). Estrogen receptor determination predicts response to tamoxifen therapy. Recent Results Cancer Res. *71*, 16-19.

Alroy,I., and Yarden,Y. (1997). The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. FEBS Lett. *410*, 83-86.

Ansquer,Y., Gautier,C., Fourquet,A., Asselain,B., and Stoppa-Lyonnet,D. (1998). Survival in early-onset BRCA1 breast-cancer patients. Institut Curie Breast Cancer Group. Lancet *352*, 541.

Bardelli,A., Parsons,D.W., Silliman,N., Ptak,J., Szabo,S., Saha,S., Markowitz,S., Willson,J.K., Parmigiani,G., Kinzler,K.W., Vogelstein,B., and Velculescu,V.E. (2003). Mutational analysis of the tyrosine kinome in colorectal cancers. Science *300*, 949.

Bartek,J., and Lukas,J. (2001). Cell cycle. Order from destruction. Science *294*, 66-67.

Baselga,J., Albanell,J., Ruiz,A., Lluch,A., Gascon,P., Guillem,V., Gonzalez,S., Sauleda,S., Marimon,I., Tabernero,J.M., Koehler,M.T., and Rojo,F. (2005). Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. J. Clin. Oncol. *23*, 5323-5333.

Baselga,J., and Averbuch,S.D. (2000). ZD1839 ('Iressa') as an anticancer agent. Drugs *60 Suppl 1*, 33-40.

Baselga,J., Norton,L., Albanell,J., Kim,Y.M., and Mendelsohn,J. (1998). Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res. *58*, 2825-2831.

Beatson,G. (1896). On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases.1. The Lancet *148*, 104-107.

Berry,D.A., Cirrincione,C., Henderson,I.C., Citron,M.L., Budman,D.R., Goldstein,L.J., Martino,S., Perez,E.A., Muss,H.B., Norton,L., Hudis,C., and Winer,E.P. (2006). Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer. JAMA *295*, 1658-1667.

Bhargava,R., Gerald,W.L., Li,A.R., Pan,Q., Lal,P., Ladanyi,M., and Chen,B. (2005). EGFR gene amplification in breast cancer: correlation with epidermal growth factor

receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations. Mod. Pathol. *18*, 1027-1033.

Biscardi,J.S., Ishizawar,R.C., Silva,C.M., and Parsons,S.J. (2000). Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. Breast Cancer Res. *2*, 203-210.

Brunton,V.G., MacPherson,I.R., and Frame,M.C. (2004). Cell adhesion receptors, tyrosine kinases and actin modulators: a complex three-way circuitry. Biochim. Biophys. Acta *1692*, 121-144.

Carey,L.A., Perou,C.M., Livasy,C.A., Dressler,L.G., Cowan,D., Conway,K., Karaca,G., Troester,M.A., Tse,C.K., Edmiston,S., Deming,S.L., Geradts,J., Cheang,M.C., Nielsen,T.O., Moorman,P.G., Earp,H.S., and Millikan,R.C. (2006). Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. JAMA *295*, 2492-2502.

Chappuis,P.O., Donato,E., Goffin,J.R., Wong,N., Begin,L.R., Kapusta,L.R., Brunet,J.S., Porter,P., and Foulkes,W.D. (2005). Cyclin E expression in breast cancer: predicting germline BRCA1 mutations, prognosis and response to treatment. Ann. Oncol. *16*, 735-742.

Chappuis,P.O., Goffin,J., Wong,N., Perret,C., Ghadirian,P., Tonin,P.N., and Foulkes,W.D. (2002). A significant response to neoadjuvant chemotherapy in BRCA1/2 related breast cancer. J. Med. Genet. *39*, 608-610.

Chappuis,P.O., Kapusta,L., Begin,L.R., Wong,N., Brunet,J.S., Narod,S.A., Slingerland,J., and Foulkes,W.D. (2000). Germline BRCA1/2 mutations and p27(Kip1) protein levels independently predict outcome after breast cancer. J. Clin. Oncol. *18*, 4045-4052.

Charafe-Jauffret,E., Ginestier,C., Monville,F., Finetti,P., Adelaide,J., Cervera,N., Fekairi,S., Xerri,L., Jacquemier,J., Birnbaum,D., and Bertucci,F. (2006). Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene *25*, 2273-2284.

Chen,W.Y., and Colditz,G.A. (2007). Risk factors and hormone-receptor status: epidemiology, risk-prediction models and treatment implications for breast cancer. Nat. Clin. Pract. Oncol. *4*, 415-423.

Cohen,S., Ushiro,H., Stoscheck,C., and Chinkers,M. (1982). A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. J. Biol. Chem. *257*, 1523-1531.

Elstrodt,F., Hollestelle,A., Nagel,J.H., Gorin,M., Wasielewski,M., van den,O.A., Merajver,S.D., Ethier,S.P., and Schutte,M. (2006). BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. Cancer Res. *66*, 41-45.

Engelman,J.A., Zhang,X.L., Galbiati,F., and Lisanti,M.P. (1998a). Chromosomal localization, genomic organization, and developmental expression of the murine caveolin gene family (Cav-1, -2, and -3). Cav-1 and Cav-2 genes map to a known tumor suppressor locus (6-A2/7q31). FEBS Lett. *429*, 330-336.

Engelman,J.A., Zhang,X.L., and Lisanti,M.P. (1998b). Genes encoding human caveolin-1 and -2 are co-localized to the D7S522 locus (7q31.1), a known fragile site (FRA7G) that is frequently deleted in human cancers. FEBS Lett. *436*, 403-410.

Finn,R.S., Dering,J., Ginther,C., Wilson,C.A., Glaspy,P., Tchekmedyian,N., and Slamon,D.J. (2007). Dasatinib, an orally active small molecule inhibitor of both the src and abl kinases, selectively inhibits growth of basal-type/"triple-negative" breast cancer cell lines growing in vitro. Breast Cancer Res. Treat.*105, 319-26*

Fisher,B., Costantino,J.P., Wickerham,D.L., Redmond,C.K., Kavanah,M., Cronin,W.M., Vogel,V., Robidoux,A., Dimitrov,N., Atkins,J., Daly,M., Wieand,S., Tan-Chiu,E., Ford,L., and Wolmark,N. (1998). Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J. Natl. Cancer Inst. *90*, 1371-1388.

Ford,D., Easton,D.F., Bishop,D.T., Narod,S.A., and Goldgar,D.E. (1994). Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. Lancet *343*, 692-695.

Ford,D., Easton,D.F., Stratton,M., Narod,S., Goldgar,D., Devilee,P., Bishop,D.T., Weber,B., Lenoir,G., Chang-Claude,J., Sobol,H., Teare,M.D., Struewing,J., Arason,A., Scherneck,S., Peto,J., Rebbeck,T.R., Tonin,P., Neuhausen,S., Barkardottir,R., Eyfjord,J., Lynch,H., Ponder,B.A., Gayther,S.A., Zelada-Hedman,M., and . (1998). Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. Am. J. Hum. Genet. *62*, 676-689.

Fossati,R., Confalonieri,C., Torri,V., Ghislandi,E., Penna,A., Pistotti,V., Tinazzi,A., and Liberati,A. (1998). Cytotoxic and hormonal treatment for metastatic breast cancer: a systematic review of published randomized trials involving 31,510 women. J. Clin. Oncol. *16*, 3439-3460.

Foulkes,W.D., Brunet,J.S., Stefansson,I.M., Straume,O., Chappuis,P.O., Begin,L.R., Hamel,N., Goffin,J.R., Wong,N., Trudel,M., Kapusta,L., Porter,P., and Akslen,L.A. (2004). The prognostic implication of the basal-like (cyclin E high/p27 low/p53+/glomeruloid-microvascular-proliferation+) phenotype of BRCA1-related breast cancer. Cancer Res. *64*, 830-835.

Foulkes,W.D., Wong,N., Brunet,J.S., Begin,L.R., Zhang,J.C., Martinez,J.J., Rozen,F., Tonin,P.N., Narod,S.A., Karp,S.E., and Pollak,M.N. (1997). Germ-line BRCA1 mutation is an adverse prognostic factor in Ashkenazi Jewish women with breast cancer. Clin. Cancer Res. *3*, 2465-2469.

Frame,M.C. (2002). Src in cancer: deregulation and consequences for cell behaviour. Biochim. Biophys. Acta *1602*, 114-130.

Franklin,W.A., Veve,R., Hirsch,F.R., Helfrich,B.A., and Bunn,P.A., Jr. (2002). Epidermal growth factor receptor family in lung cancer and premalignancy. Semin. Oncol. *29*, 3-14.

Galbiati,F., Engelman,J.A., Volonte,D., Zhang,X.L., Minetti,C., Li,M., Hou,H., Jr., Kneitz,B., Edelmann,W., and Lisanti,M.P. (2001). Caveolin-3 null mice show a loss of caveolae, changes in the microdomain distribution of the dystrophin-glycoprotein complex, and t-tubule abnormalities. J. Biol. Chem. *276*, 21425-21433.

Hayashi,K., Matsuda,S., Machida,K., Yamamoto,T., Fukuda,Y., Nimura,Y., Hayakawa,T., and Hamaguchi,M. (2001). Invasion activating caveolin-1 mutation in human scirrhous breast cancers. Cancer Res. *61*, 2361-2364.

Heikkinen,K., Rapakko,K., Karppinen,S.M., Erkko,H., Knuutila,S., Lundan,T., Mannermaa,A., Borresen-Dale,A.L., Borg,A., Barkardottir,R.B., Petrini,J., and Winqvist,R. (2006). RAD50 and NBS1 are breast cancer susceptibility genes associated with genomic instability. Carcinogenesis *27*, 1593-1599.

Heldin,C.H. (1995). Dimerization of cell surface receptors in signal transduction. Cell *80*, 213-223.

Huang,F., Reeves,K., Han,X., Fairchild,C., Platero,S., Wong,T.W., Lee,F., Shaw,P., and Clark,E. (2007). Identification of candidate molecular markers predicting sensitivity in solid tumors to dasatinib: rationale for patient selection. Cancer Res. *67*, 2226-2238.

Irby,R.B., and Yeatman,T.J. (2000). Role of Src expression and activation in human cancer. Oncogene *19*, 5636-5642.

Ishizawar,R., and Parsons,S.J. (2004). c-Src and cooperating partners in human cancer. Cancer Cell *6*, 209-214.

Johannsson,O.T., Idvall,I., Anderson,C., Borg,A., Barkardottir,R.B., Egilsson,V., and Olsson,H. (1997). Tumour biological features of BRCA1-induced breast and ovarian cancer. Eur. J. Cancer *33*, 362-371.

Karp,S.E., Tonin,P.N., Begin,L.R., Martinez,J.J., Zhang,J.C., Pollak,M.N., and Foulkes,W.D. (1997). Influence of BRCA1 mutations on nuclear grade and estrogen receptor status of breast carcinoma in Ashkenazi Jewish women. Cancer *80*, 435-441.

Klijn,J.G., Berns,P.M., Schmitz,P.I., and Foekens,J.A. (1992). The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endocr. Rev. *13*, 3-17.

Koepp,D.M., Schaefer,L.K., Ye,X., Keyomarsi,K., Chu,C., Harper,J.W., and Elledge,S.J. (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. Science *294*, 173-177.

Koleske,A.J., Baltimore,D., and Lisanti,M.P. (1995). Reduction of caveolin and caveolae in oncogenically transformed cells. Proc. Natl. Acad. Sci. U. S. A *92*, 1381-1385.

Korsching,E., Packeisen,J., Agelopoulos,K., Eisenacher,M., Voss,R., Isola,J., van Diest,P.J., Brandt,B., Boecker,W., and Buerger,H. (2002). Cytogenetic alterations and cytokeratin expression patterns in breast cancer: integrating a new model of breast differentiation into cytogenetic pathways of breast carcinogenesis. Lab Invest *82*, 1525-1533.

Kreike,B., van,K.M., Horlings,H., Weigelt,B., Bartelink,H., and van,d., V (2007). Gene expression profiling and histopathological characterization of triple negative/basal-like breast carcinomas. Breast Cancer Res. *9*, R65.

Lakhani,S.R., Jacquemier,J., Sloane,J.P., Gusterson,B.A., Anderson,T.J., van,d., V, Farid,L.M., Venter,D., Antoniou,A., Storfer-Isser,A., Smyth,E., Steel,C.M., Haites,N., Scott,R.J., Goldgar,D., Neuhausen,S., Daly,P.A., Ormiston,W., McManus,R., Scherneck,S., Ponder,B.A., Ford,D., Peto,J., Stoppa-Lyonnet,D., Bignon,Y.J., Struewing,J.P., Spurr,N.K., Bishop,D.T., Klijn,J.G., Devilee,P., Cornelisse,C.J., Lasset,C., Lenoir,G., Barkardottir,R.B., Egilsson,V., Hamann,U., Chang-Claude,J., Sobol,H., Weber,B., Stratton,M.R., and Easton,D.F. (1998). Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations. J. Natl. Cancer Inst. *90*, 1138-1145.

Lakhani,S.R., Reis-Filho,J.S., Fulford,L., Penault-Llorca,F., van,d., V, Parry,S., Bishop,T., Benitez,J., Rivas,C., Bignon,Y.J., Chang-Claude,J., Hamann,U., Cornelisse,C.J., Devilee,P., Beckmann,M.W., Nestle-Kramling,C., Daly,P.A., Haites,N., Varley,J., Lalloo,F., Evans,G., Maugard,C., Meijers-Heijboer,H., Klijn,J.G., Olah,E., Gusterson,B.A., Pilotti,S., Radice,P., Scherneck,S., Sobol,H., Jacquemier,J., Wagner,T., Peto,J., Stratton,M.R., McGuffog,L., and Easton,D.F. (2005). Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin. Cancer Res. *11*, 5175-5180.

Lakhani,S.R., van,d., V, Jacquemier,J., Anderson,T.J., Osin,P.P., McGuffog,L., and Easton,D.F. (2002). The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. J. Clin. Oncol. *20*, 2310-2318.

Liaw,D., Marsh,D.J., Li,J., Dahia,P.L., Wang,S.I., Zheng,Z., Bose,S., Call,K.M., Tsou,H.C., Peacocke,M., Eng,C., and Parsons,R. (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat. Genet. *16*, 64-67.

Liu,P., Rudick,M., and Anderson,R.G. (2002). Multiple functions of caveolin-1. J. Biol. Chem. *277*, 41295-41298.

Lombardo,L.J., Lee,F.Y., Chen,P., Norris,D., Barrish,J.C., Behnia,K., Castaneda,S., Cornelius,L.A., Das,J., Doweyko,A.M., Fairchild,C., Hunt,J.T., Inigo,I., Johnston,K., Kamath,A., Kan,D., Klei,H., Marathe,P., Pang,S., Peterson,R., Pitt,S., Schieven,G.L.,

Schmidt,R.J., Tokarski,J., Wen,M.L., Wityak,J., and Borzilleri,R.M. (2004). Discovery of N-(2-chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4- ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. J. Med. Chem. *47*, 6658-6661.

Lynch,T.J., Bell,D.W., Sordella,R., Gurubhagavatula,S., Okimoto,R.A., Brannigan,B.W., Harris,P.L., Haserlat,S.M., Supko,J.G., Haluska,F.G., Louis,D.N., Christiani,D.C., Settleman,J., and Haber,D.A. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. *350*, 2129-2139.

Maa,M.C., Leu,T.H., McCarley,D.J., Schatzman,R.C., and Parsons,S.J. (1995). Potentiation of epidermal growth factor receptor-mediated oncogenesis by c-Src: implications for the etiology of multiple human cancers. Proc. Natl. Acad. Sci. U. S. A *92*, 6981-6985.

Masaki,T., Igarashi,K., Tokuda,M., Yukimasa,S., Han,F., Jin,Y.J., Li,J.Q., Yoneyama,H., Uchida,N., Fujita,J., Yoshiji,H., Watanabe,S., Kurokohchi,K., and Kuriyama,S. (2003). pp60c-src activation in lung adenocarcinoma. Eur. J. Cancer *39*, 1447-1455.

Meijers-Heijboer,H., van den,O.A., Klijn,J., Wasielewski,M., de,S.A., Oldenburg,R., Hollestelle,A., Houben,M., Crepin,E., van Veghel-Plandsoen,M., Elstrodt,F., van,D.C., Bartels,C., Meijers,C., Schutte,M., McGuffog,L., Thompson,D., Easton,D., Sodha,N., Seal,S., Barfoot,R., Mangion,J., Chang-Claude,J., Eccles,D., Eeles,R., Evans,D.G., Houlston,R., Murday,V., Narod,S., Peretz,T., Peto,J., Phelan,C., Zhang,H.X., Szabo,C., Devilee,P., Goldgar,D., Futreal,P.A., Nathanson,K.L., Weber,B., Rahman,N., and Stratton,M.R. (2002). Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. Nat. Genet. *31*, 55-59.

Miki,Y., Swensen,J., Shattuck-Eidens,D., Futreal,P.A., Harshman,K., Tavtigian,S., Liu,Q., Cochran,C., Bennett,L.M., Ding,W., and . (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science *266*, 66-71.

Moberg,K.H., Bell,D.W., Wahrer,D.C., Haber,D.A., and Hariharan,I.K. (2001). Archipelago regulates Cyclin E levels in Drosophila and is mutated in human cancer cell lines. Nature *413*, 311-316.

Montagnoli,A., Fiore,F., Eytan,E., Carrano,A.C., Draetta,G.F., Hershko,A., and Pagano,M. (1999). Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev. *13*, 1181-1189.

Moro,L., Dolce,L., Cabodi,S., Bergatto,E., Boeri,E.E., Smeriglio,M., Turco,E., Retta,S.F., Giuffrida,M.G., Venturino,M., Godovac-Zimmermann,J., Conti,A., Schaefer,E., Beguinot,L., Tacchetti,C., Gaggini,P., Silengo,L., Tarone,G., and Defilippi,P. (2002). Integrin-induced epidermal growth factor (EGF) receptor activation

requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. J. Biol. Chem. *277*, 9405-9414.

Neve,R.M., Chin,K., Fridlyand,J., Yeh,J., Baehner,F.L., Fevr,T., Clark,L., Bayani,N., Coppe,J.P., Tong,F., Speed,T., Spellman,P.T., DeVries,S., Lapuk,A., Wang,N.J., Kuo,W.L., Stilwell,J.L., Pinkel,D., Albertson,D.G., Waldman,F.M., McCormick,F., Dickson,R.B., Johnson,M.D., Lippman,M., Ethier,S., Gazdar,A., and Gray,J.W. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell *10*, 515-527.

Nicholson,R.I., Gee,J.M., and Harper,M.E. (2001). EGFR and cancer prognosis. Eur. J. Cancer *37 Suppl 4*, S9-15.

Nielsen,T.O., Hsu,F.D., Jensen,K., Cheang,M., Karaca,G., Hu,Z., Hernandez-Boussard,T., Livasy,C., Cowan,D., Dressler,L., Akslen,L.A., Ragaz,J., Gown,A.M., Gilks,C.B., van de,R.M., and Perou,C.M. (2004a). Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin. Cancer Res. *10*, 5367-5374.

Nielsen,T.O., Hsu,F.D., Jensen,K., Cheang,M., Karaca,G., Hu,Z., Hernandez-Boussard,T., Livasy,C., Cowan,D., Dressler,L., Akslen,L.A., Ragaz,J., Gown,A.M., Gilks,C.B., van de,R.M., and Perou,C.M. (2004b). Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin. Cancer Res. *10*, 5367-5374.

Normanno,N., Bianco,C., De,L.A., Maiello,M.R., and Salomon,D.S. (2003). Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. Endocr. Relat Cancer *10*, 1-21.

Normanno,N., Ciardiello,F., Brandt,R., and Salomon,D.S. (1994). Epidermal growth factor-related peptides in the pathogenesis of human breast cancer. Breast Cancer Res. Treat. *29*, 11-27.

Oh,P., and Schnitzer,J.E. (1999). Immunoisolation of caveolae with high affinity antibody binding to the oligomeric caveolin cage. Toward understanding the basis of purification. J. Biol. Chem. *274*, 23144-23154.

Okamoto,T., Schlegel,A., Scherer,P.E., and Lisanti,M.P. (1998). Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J. Biol. Chem. *273*, 5419-5422.

Olayioye,M.A., Neve,R.M., Lane,H.A., and Hynes,N.E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J. *19*, 3159-3167.

Osherov,N., and Levitzki,A. (1994). Epidermal-growth-factor-dependent activation of the src-family kinases. Eur. J. Biochem. *225*, 1047-1053.

Paez,J.G., Janne,P.A., Lee,J.C., Tracy,S., Greulich,H., Gabriel,S., Herman,P., Kaye,F.J., Lindeman,N., Boggon,T.J., Naoki,K., Sasaki,H., Fujii,Y., Eck,M.J., Sellers,W.R., Johnson,B.E., and Meyerson,M. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304*, 1497-1500.

Palade,G.E. (1953). An electron microscope study of the mitochondrial structure. J. Histochem. Cytochem. *1*, 188-211.

Parsons,J.T. (2003). Focal adhesion kinase: the first ten years. J. Cell Sci. *116*, 1409-1416.

Parton,R.G. (1996). Caveolae and caveolins. Curr. Opin. Cell Biol. *8*, 542-548.

Perou,C.M., Sorlie,T., Eisen,M.B., van de,R.M., Jeffrey,S.S., Rees,C.A., Pollack,J.R., Ross,D.T., Johnsen,H., Akslen,L.A., Fluge,O., Pergamenschikov,A., Williams,C., Zhu,S.X., Lonning,P.E., Borresen-Dale,A.L., Brown,P.O., and Botstein,D. (2000). Molecular portraits of human breast tumours. Nature *406*, 747-752.

Pflug,B.R., Reiter,R.E., and Nelson,J.B. (1999). Caveolin expression is decreased following androgen deprivation in human prostate cancer cell lines. Prostate *40*, 269-273.

Pinilla,S.M., Honrado,E., Hardisson,D., Benitez,J., and Palacios,J. (2006). Caveolin-1 expression is associated with a basal-like phenotype in sporadic and hereditary breast cancer. Breast Cancer Res. Treat. *99*, 85-90.

Porter,P.L., Malone,K.E., Heagerty,P.J., Alexander,G.M., Gatti,L.A., Firpo,E.J., Daling,J.R., and Roberts,J.M. (1997). Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nat. Med. *3*, 222-225.

Prenzel,N., Fischer,O.M., Streit,S., Hart,S., and Ullrich,A. (2001). The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr. Relat Cancer *8*, 11-31.

Prigent,S.A., and Gullick,W.J. (1994). Identification of c-erbB-3 binding sites for phosphatidylinositol 3'-kinase and SHC using an EGF receptor/c-erbB-3 chimera. EMBO J. *13*, 2831-2841.

Purkis,P.E., Steel,J.B., Mackenzie,I.C., Nathrath,W.B., Leigh,I.M., and Lane,E.B. (1990). Antibody markers of basal cells in complex epithelia. J. Cell Sci. *97 (Pt 1)*, 39-50.

Rahman,N., Seal,S., Thompson,D., Kelly,P., Renwick,A., Elliott,A., Reid,S., Spanova,K., Barfoot,R., Chagtai,T., Jayatilake,H., McGuffog,L., Hanks,S., Evans,D.G., Eccles,D., Easton,D.F., and Stratton,M.R. (2007). PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat. Genet. *39*, 165-167.

Rajagopalan,H., Nowak,M.A., Vogelstein,B., and Lengauer,C. (2003). The significance of unstable chromosomes in colorectal cancer. Nat. Rev. Cancer *3*, 695-701.

Razani,B., Combs,T.P., Wang,X.B., Frank,P.G., Park,D.S., Russell,R.G., Li,M., Tang,B., Jelicks,L.A., Scherer,P.E., and Lisanti,M.P. (2002a). Caveolin-1-deficient mice are lean, resistant to diet-induced obesity, and show hypertriglyceridemia with adipocyte abnormalities. J. Biol. Chem. *277*, 8635-8647.

Razani,B., Woodman,S.E., and Lisanti,M.P. (2002b). Caveolae: from cell biology to animal physiology. Pharmacol. Rev. *54*, 431-467.

Rennert,G., Bisland-Naggan,S., Barnett-Griness,O., Bar-Joseph,N., Zhang,S., Rennert,H.S., and Narod,S.A. (2007). Clinical outcomes of breast cancer in carriers of BRCA1 and BRCA2 mutations. N. Engl. J. Med. *357*, 115-123.

Renwick,A., Thompson,D., Seal,S., Kelly,P., Chagtai,T., Ahmed,M., North,B., Jayatilake,H., Barfoot,R., Spanova,K., McGuffog,L., Evans,D.G., Eccles,D., Easton,D.F., Stratton,M.R., and Rahman,N. (2006). ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. Nat. Genet. *38*, 873-875.

Rodriguez-Pinilla,S.M., Rodriguez-Gil,Y., Moreno-Bueno,G., Sarrio,D., Martin-Guijarro,M.C., Hernandez,L., and Palacios,J. (2007). Sporadic invasive breast carcinomas with medullary features display a basal-like phenotype: an immunohistochemical and gene amplification study. Am. J. Surg. Pathol. *31*, 501-508.

Rothberg,K.G., Heuser,J.E., Donzell,W.C., Ying,Y.S., Glenney,J.R., and Anderson,R.G. (1992). Caveolin, a protein component of caveolae membrane coats. Cell *68*, 673-682.

Rouzier,R., Perou,C.M., Symmans,W.F., Ibrahim,N., Cristofanilli,M., Anderson,K., Hess,K.R., Stec,J., Ayers,M., Wagner,P., Morandi,P., Fan,C., Rabiul,I., Ross,J.S., Hortobagyi,G.N., and Pusztai,L. (2005). Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin. Cancer Res. *11*, 5678-5685.

Rubin,M.A., Zhou,M., Dhanasekaran,S.M., Varambally,S., Barrette,T.R., Sanda,M.G., Pienta,K.J., Ghosh,D., and Chinnaiyan,A.M. (2002). alpha-Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. JAMA *287*, 1662-1670.

Savage,K., Lambros,M.B., Robertson,D., Jones,R.L., Jones,C., Mackay,A., James,M., Hornick,J.L., Pereira,E.M., Milanezi,F., Fletcher,C.D., Schmitt,F.C., Ashworth,A., and Reis-Filho,J.S. (2007). Caveolin 1 is overexpressed and amplified in a subset of basal-like and metaplastic breast carcinomas: a morphologic, ultrastructural, immunohistochemical, and in situ hybridization analysis. Clin. Cancer Res. *13*, 90-101.

Sawyers,C.L., Hochhaus,A., Feldman,E., Goldman,J.M., Miller,C.B., Ottmann,O.G., Schiffer,C.A., Talpaz,M., Guilhot,F., Deininger,M.W., Fischer,T., O'Brien,S.G., Stone,R.M., Gambacorti-Passerini,C.B., Russell,N.H., Reiffers,J.J., Shea,T.C., Chapuis,B., Coutre,S., Tura,S., Morra,E., Larson,R.A., Saven,A., Peschel,C., Gratwohl,A., Mandelli,F., Ben-Am,M., Gathmann,I., Capdeville,R., Paquette,R.L., and Druker,B.J. (2002). Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. Blood *99*, 3530-3539.

Scherer,P.E., Okamoto,T., Chun,M., Nishimoto,I., Lodish,H.F., and Lisanti,M.P. (1996). Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. Proc. Natl. Acad. Sci. U. S. A *93*, 131-135.

Seal,S., Thompson,D., Renwick,A., Elliott,A., Kelly,P., Barfoot,R., Chagtai,T., Jayatilake,H., Ahmed,M., Spanova,K., North,B., McGuffog,L., Evans,D.G., Eccles,D., Easton,D.F., Stratton,M.R., and Rahman,N. (2006). Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. Nat. Genet. *38*, 1239-1241.

Shupnik,M.A. (2004). Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene *23*, 7979-7989.

Sidransky,D., Tokino,T., Helzlsouer,K., Zehnbauer,B., Rausch,G., Shelton,B., Prestigiacomo,L., Vogelstein,B., and Davidson,N. (1992). Inherited p53 gene mutations in breast cancer. Cancer Res. *52*, 2984-2986.

Sieber,O.M., Heinimann,K., and Tomlinson,I.P. (2003). Genomic instability--the engine of tumorigenesis? Nat. Rev. Cancer *3*, 701-708.

Signoretti,S., Di,M.L., Richardson,A., Ramaswamy,S., Isaac,B., Rue,M., Monti,F., Loda,M., and Pagano,M. (2002). Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer. J. Clin. Invest *110*, 633-641.

Skehan,P., Storeng,R., Scudiero,D., Monks,A., McMahon,J., Vistica,D., Warren,J.T., Bokesch,H., Kenney,S., and Boyd,M.R. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst. *82*, 1107-1112.

Sorlie,T., Perou,C.M., Tibshirani,R., Aas,T., Geisler,S., Johnsen,H., Hastie,T., Eisen,M.B., van de,R.M., Jeffrey,S.S., Thorsen,T., Quist,H., Matese,J.C., Brown,P.O., Botstein,D., Eystein,L.P., and Borresen-Dale,A.L. (2001). Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. U. S. A *98*, 10869-10874.

Sorlie,T., Tibshirani,R., Parker,J., Hastie,T., Marron,J.S., Nobel,A., Deng,S., Johnsen,H., Pesich,R., Geisler,S., Demeter,J., Perou,C.M., Lonning,P.E., Brown,P.O., Borresen-Dale,A.L., and Botstein,D. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc. Natl. Acad. Sci. U. S. A *100*, 8418-8423.

Spruck,C.H., Strohmaier,H., Sangfelt,O., Muller,H.M., Hubalek,M., Muller-Holzner,E., Marth,C., Widschwendter,M., and Reed,S.I. (2002). hCDC4 gene mutations in endometrial cancer. Cancer Res. *62*, 4535-4539.

Spruck,C.H., Won,K.A., and Reed,S.I. (1999). Deregulated cyclin E induces chromosome instability. Nature *401*, 297-300.

Stan,R.V. (2002). Structure and function of endothelial caveolae. Microsc. Res. Tech. *57*, 350-364.

Stan,R.V. (2005). Structure of caveolae. Biochim. Biophys. Acta *1746*, 334-348.

Strohmaier,H., Spruck,C.H., Kaiser,P., Won,K.A., Sangfelt,O., and Reed,S.I. (2001). Human F-box protein hCdc4 targets cyclin E for proteolysis and is mutated in a breast cancer cell line. Nature *413*, 316-322.

Sunaga,N., Miyajima,K., Suzuki,M., Sato,M., White,M.A., Ramirez,R.D., Shay,J.W., Gazdar,A.F., and Minna,J.D. (2004). Different roles for caveolin-1 in the development of non-small cell lung cancer versus small cell lung cancer. Cancer Res. *64*, 4277-4285.

Talpaz,M., Shah,N.P., Kantarjian,H., Donato,N., Nicoll,J., Paquette,R., Cortes,J., O'Brien,S., Nicaise,C., Bleickardt,E., Blackwood-Chirchir,M.A., Iyer,V., Chen,T.T., Huang,F., Decillis,A.P., and Sawyers,C.L. (2006). Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N. Engl. J. Med. *354*, 2531-2541.

Tang,Z., Scherer,P.E., Okamoto,T., Song,K., Chu,C., Kohtz,D.S., Nishimoto,I., Lodish,H.F., and Lisanti,M.P. (1996). Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. J. Biol. Chem. *271*, 2255-2261.

Taylor-Papadimitriou,J., Stampfer,M., Bartek,J., Lewis,A., Boshell,M., Lane,E.B., and Leigh,I.M. (1989). Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to in vivo phenotypes and influence of medium. J. Cell Sci. *94 (Pt 3)*, 403-413.

Thomas,S.M., and Brugge,J.S. (1997). Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. Biol. *13*, 513-609.

Tomlinson,G.E., Chen,T.T., Stastny,V.A., Virmani,A.K., Spillman,M.A., Tonk,V., Blum,J.L., Schneider,N.R., Wistuba,I.I., Shay,J.W., Minna,J.D., and Gazdar,A.F. (1998). Characterization of a breast cancer cell line derived from a germ-line BRCA1 mutation carrier. Cancer Res. *58*, 3237-3242.

Ullrich,A., and Schlessinger,J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203-212.

Usary,J., Llaca,V., Karaca,G., Presswala,S., Karaca,M., He,X., Langerod,A., Karesen,R., Oh,D.S., Dressler,L.G., Lonning,P.E., Strausberg,R.L., Chanock,S., Borresen-Dale,A.L., and Perou,C.M. (2004). Mutation of GATA3 in human breast tumors. Oncogene *23*, 7669-7678.

van de Rijn,M., Perou,C.M., Tibshirani,R., Haas,P., Kallioniemi,O., Kononen,J., Torhorst,J., Sauter,G., Zuber,M., Kochli,O.R., Mross,F., Dieterich,H., Seitz,R., Ross,D., Botstein,D., and Brown,P. (2002). Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. Am. J. Pathol. *161*, 1991-1996.

van der Groep,P., Bouter,A., van der,Z.R., Menko,F.H., Buerger,H., Verheijen,R.H., van der,W.E., and van Diest,P.J. (2004). Re: Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J. Natl. Cancer Inst. *96*, 712-713.

Vivanco,I., and Sawyers,C.L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat. Rev. Cancer *2*, 489-501.

Weber,F., Fukino,K., Sawada,T., Williams,N., Sweet,K., Brena,R.M., Plass,C., Caldes,T., Mutter,G.L., Villalona-Calero,M.A., and Eng,C. (2005). Variability in organ-specific EGFR mutational spectra in tumour epithelium and stroma may be the biological basis for differential responses to tyrosine kinase inhibitors. Br. J. Cancer *92*, 1922-1926.

Wiener,J.R., Windham,T.C., Estrella,V.C., Parikh,N.U., Thall,P.F., Deavers,M.T., Bast,R.C., Mills,G.B., and Gallick,G.E. (2003). Activated SRC protein tyrosine kinase is overexpressed in late-stage human ovarian cancers. Gynecol. Oncol. *88*, 73-79.

Williams,T.M., and Lisanti,M.P. (2005). Caveolin-1 in oncogenic transformation, cancer, and metastasis. Am. J. Physiol Cell Physiol *288*, C494-C506.

Wooster,R., Bignell,G., Lancaster,J., Swift,S., Seal,S., Mangion,J., Collins,N., Gregory,S., Gumbs,C., and Micklem,G. (1995). Identification of the breast cancer susceptibility gene BRCA2. Nature *378*, 789-792.

Appendix A - Antibodies used for the western blots:

Name	Source	MW (kDa)	Reference number	Brand	Dilution
Actin	Goat	44	sc-1616	Santa Cruz	$^1/_{2000}$
Akt	Rabbit	60	9272	Cell Signaling	$^1/_{1000}$
Akt (Phospho Ser ^{473})	Mouse	60	4051	Cell Signaling	$^1/_{1000}$
c-abl	Mouse	145	sc-23	Santa Cruz	$^1/_{2000}$
c-abl (phospho Tyr ^{245})	Rabbit	145	07-787	Upstate	$^1/_{1000}$
c-abl (phospho Tyr ^{412})	Rabbit	145	07-788	Upstate	$^1/_{1000}$
Caveolin	Rabbit	22	sc-894	Santa Cruz	$^1/_{1000}$
Caveolin (phospho Tyr^{14})	Mouse	22	611338	BD Pharmingen	$^1/_{1000}$
Cylcin E	Rabbit	50	sc-481	Santa Cruz	$^1/_{1000}$
EGFR	Rabbit	170	2232	Cell Signaling	$^1/_{1000}$
EGFR (phospho Tyr^{1068})	Rabbit	170	9922(kit)	Cell Signaling	$^1/_{1000}$
EGFR (phospho Tyr^{845})	Rabbit	170	9922(kit)	Cell Signaling	$^1/_{1000}$
Erk p44/42 MAPK (Phospho Thr202/Tyr204)	Rabbit	42/44	9101	Cell Signaling	$^1/_{500}$
Erk p44/42 MAPK	Rabbit	42/44	9102	Cell Signaling	$^1/_{500}$
FAK	Rabbit	125	sc-557	Santa Cruz	$^1/_{1000}$
NFKB (p65)	Rabbit	65		Santa Cruz	$^1/_{1000}$
P-FAK (phospho Tyr 397)	Rabbit	125		Biosource	$^1/_{1000}$
P-FAK (phospho Tyr^{861})	Rabbit	125		Biosource	$^1/_{1000}$
IKBα	Rabbit	37	sc-371	Santa Cruz	$^1/_{1000}$
Lyn	Rabbit	53/56	2732	Cell Signaling	$^1/_{1000}$
Lyn (phospho Tyr^{507})	Rabbit	53/56	2731	Cell Signaling	$^1/_{1000}$
Lyn (phospho Y^{396})	Rabbit	53/56	1645-1	Epitomics	$^1/_{1000}$
Src	Rabbit	60	2109	Cell Signaling	$^1/_{1000}$
Src family (phospho Tyr^{416})	Rabbit	60	2101	Cell Signaling	$^1/_{1000}$

Appendix B - Antibodies used for Immunohistochemistry:

The staining for the TMA was performed by Andrew Darnel, PhD. In Dr. Bismar's Lab.

Antibody	Clone	Type	Origin	Reference Id	Dilution	Antigen Retrieval
ERα	SP1	*rabbit monoclonal*	Ventana	517-908	Pre-diluted	EDTA, pH 7.0
PR	16	*rabbit monoclonal*	Ventana	760-2816	Pre-diluted	EDTA, pH 7.0
Cytokeratin 14	LL02	*mouse monoclonal*	Ventana	760-4251	Pre-diluted	EDTA, pH 7.0
Cytokeratin 5 & 6	D5 & 16B4	*mouse monoclonal*	Ventana	760-2253	Pre-diluted	EDTA, pH 7.0
Vimentin	3B4	*mouse monoclonal*	Ventana	760-2512	Pre-diluted	EDTA, pH 7.0
HER-2 (c-erbB-2)	CB11	mouse monoclonal	Ventana	760-2694	Pre-diluted	EDTA, pH 7.0
EGFR	n/a	rabbit polyclonal	Cell Signaling	2232	1:20	EDTA, pH 7.0

Appendix C - Primer sequences for cyclin E:

Exon	Direction	Primer	Temperature (°C)
6a	(Fwd)	5'-TGGTTTTGAGCAGAGAGATGG-3'	60.38
	(Rev)	5'-AACGGTTTCTGTTACATTGTGC-3'	59.07
6b	(Fwd)	5'-AGGGATTGATGAACCATTGC-3'	59.76
	(Rev)	5'-TCTTTCTACAGAAGAGGAGTGTCA-3'	57.43
7	(Fwd)	5'-TGCCTTCATTTTTCTCTTCACC-3'	60.59
	(Rev)	5'-CCAGTTGCTACTTGCAATGAT-3'	57.92
8a	(Fwd)	5'-CGGATGTAATATTTTCTGAAGAGC-3'	58.43
	(Rev)	5'-GAAGATGCATACAACGCACA-3'	58.29
8b	(Fwd)	5'-TGTCTGAGAACATTAGTGGGACA-3'	59.65
	(Rev)	5'-AAGAGCACACTGTCACTATTTCAG-3'	57.77
9a	(Fwd)	5'-TTGAAAATGGTTGTTGCTGTG-3'	59.62
	(Rev)	5'-ACCCTCCTGCCATCATATTG-3'	59.77
9b	(Fwd)	5'-AAAAATTCTAAACGTGGGTTTTT-3'	57.41
	(Rev)	5'-ATTAGTATGCCCCTGCAACG-3'	59.98
9c	(Fwd)	5'-TCGAGATGCCACTCTTAGGG-3'	60.35
	(Rev)	5'-TGGATCAGCAATTTGACAGTG-3'	59.71
10a	(Fwd)	5'-TCCTCTTCCCCCTTTCCTAC-3'	59.51
	(Rev)	5'-TGTCCTGTTTTGATATCCCAGA-3'	59.43
10b	(Fwd)	5'-CCATGTGGTGAGTGGATCTCT-3'	59.98
	(Rev)	5'-TGAACAAAACGAAAGGTGAGT-3'	57.38
10c	(Fwd)	5'-GTTTGGGATGTGGAGACAGG-3'	60.36
	(Rev)	5'-TTTGTGATGCTAAGGCTCCA-3'	59.42
11a	(Fwd)	5'-AAAATACTGAGGACATGGGTTTC-3'	58.44
	(Rev)	5'-CCCCACTCTCCAATGTGACT-3'	59.96
11b	(Fwd)	5'-TGGTTTTTGTCTAGGTCCCAAC-3'	60.25
	(Rev)	5'-GGTTTCTTCAGTCCCATTCC-3'	58.42
11c	(Fwd)	5'-CCAGCTCAGATGATGGAACT-3'	57.81
	(Rev)	5'-TTCTTTTTGCAGGGGGAAG-3'	60.17

Appendix D - Primer sequences for BRCA1:

185delAG (exon2) in SUM1315M02
Melting temperature: 60°C

BRCA1-2F- 5'- GGACGTTGTCATTAGTTCTTTGG -3'
BRCA1-2R- 5'- TTGCATAGGAGATAATCATAGGAA -3'

5382insC and 5396 + 1G>A (exon20) in HCC1937 and MDA-MB-436
Melting temperature: 60°C

BRCA1-20F -5'- AAATATGACGTGTCTGCTCCA -3'
BRCA1-20R-5' - CTGCAAAGGGGAGTGGAATA -3'

2288delT (exon11) in SUM149PT
Melting temperature: 60°C

BRCA1-11F- 5'- TGGAAGGTAAAGAACCTGCAA -3'
BRCA1-11R- 5'- TTTTGCCTTCCCTAGAGTGC -3'

Appendix E – Treatment of the eight cell lines with Gleevec and SKI-606:

The treatments of the cell lines with SKI-606 and gleevec was performed in collaboration with Hafedh Dekhil, PhD, in Dr. Gambacorti's Lab.

	SKI-606	STI-571 (Gleevec)
MCF7	IC_{50}: 1.46 R^2:0.84	IC_{50}: 0.75 R^2: 0.80
MCF10	IC_{50}: 2.56 R^2: 0.78	IC_{50}: 0.98 R^2: 0.67
MDAMB436	IC_{50}: 0.25 R^2: 0.705	IC_{50}: 0.79 R^2: 0.27
SUM149PT	IC_{50}: 0.39 R^2: 0.90	IC_{50}: 2.61 R^2: 0.131
HCC1937	Does not converge.	IC_{50}: 0.45 R^2: 0.514
SUM1315M02	IC_{50}: 0.67 R^2: 0.92	IC_{50}: 0.77 R^2: 0.59
BT20	IC_{50}: 0.66 R^2: 0.90	IC_{50}: 0.96 R^2: 0.3373
SUM190PT	IC_{50}: 0.108 R^2: 0.75	IC_{50}: 0.43 R^2: 0.544

The cell lines were treated separately with a SRC/ABL inhibitor (SKI-606) and a c-ABL inhibitor (gleevec) to determine the means by which dasatinib acts on the cells. The shaded areas represent the cells that are resistant to the drugs (an R^2 lower than 0.7 is an indication that the drug is non-specific to the cells) and the white areas represent the cell lines that are sensitive to the drug.

Science
AAAS

Permissions Letter

Ref # 07-23025

DATE: Friday, August 10, 2007

TO: tayma khalil
mcgill university (montreal jewish general hospital)
3755 cote st-catherine E-425
montreal, QC H3T1E2
Canada

FROM: Elizabeth Sandler, Rights and Permissions

RE: Your request for permission dated 08/07/07

Regarding your request, we are pleased to grant you non-exclusive, non-transferable permission to use the AAAS material identified below in your dissertation or thesis identified below, but limited to the formats identified below, and provided that you meet the conditions / requirements below. Such permission is for one time use and therefore does not include permission for future editions, revisions, additional printings, updates, ancillaries, other formats, translations, or promotional pieces, unless otherwise permitted below. This permission does not apply to figures / artwork that are credited to non-AAAS sources. This permission does not include the right to modify AAAS material.

The following credit line must be printed along with the AAAS material: "From [Insert Full Reference Citation]. Reprinted with permission from AAAS."

This permission covers the use of the AAAS material identified herein in the following format versions of your dissertation/thesis:
[x] print
[x] microform
[x] Digitized / electronic versions , provided the reprinted AAAS material remains in situ and is not made digitally available separated from your dissertation/thesis

AAAS agrees that ProQuest/UMI may supply copies of the dissertation/thesis on demand.

If the requested material is sourced to or references non-AAAS sources, you must obtain authorization from that source as well.

Permission fees are waived in this instance. AAAS reserves the right to charge for reproduction of AAAS controlled material in the future.

AAAS must publish the full paper prior to use of any text.

AAAS does not supply photos or artwork. Use of the AAAS material must not imply any endorsement by the American Association for the Advancement of Science. This permission is not valid for the use of the AAAS and/or SCIENCE logos.

Permission is valid for use of the following AAAS material only:
Fig w/caption"Keeping the balance" from Bartek and Lukas, SCIENCE 294:66 (10/5/2001)
In the following work only:
BASAL-LIKE BREAST CANCER: THERAPEUTIC APPROACHES published by mcgill

Thank you for writing. If you have any questions please call me at (202) 326-6765 or write to me via fax at (202) 682-0816. For international calls, +1 is the country code for the United States.