Y-BOX BINDING PROTEIN-1 (YB-1) IS A BIO-MARKER OF AGGRESSIVENESS IN BREAST CANCER AND IS A POTENTIAL TARGET FOR THERAPEUTIC INTERVENTION

by

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ABSTRACT

Early detection is one of the most important factors for successful treatment of cancer. Currently, scientists are searching for molecular markers that can help identify and predict outcome and chance of recurrence in patients. In this study, we demonstrate the potential impact of Y-Box binding protein-1 (YB-1) as a marker of aggressiveness and cancer recurrence in breast malignancies by screening one of the largest tissue microarrays in North America.

YB-1 is an oncogenic transcription/translation factor, which is over-expressed in the majority of malignancies, including breast cancer. In the cohort of 4049 primary breast tumours, we show that YB-1 is a strong marker of aggressiveness, poor survival and cancer recurrence in all subtypes of human breast cancer with a particularly high frequency of expression in the ER negative basal-like and HER-2 breast cancer subtypes. This suggests that targeting YB-1 may provide a new avenue for therapeutic intervention in these breast cancers that are currently challenging to treat. Cox regression multivariate analysis indicates that YB-1 is second only to nodal status as a strong independent prognostic marker for poor outcome and relapse compared to established clinico-pathological biomarkers, including tumour size, age, grade, ER and HER-2 status. This finding suggests that YB-1 has great potential to be in a priority list of biomarkers for identifying the patients with a higher risk of relapse and poor outcome. Subsequently, we find an association between YB-1 and urokinase Plasminogen Activator (uPA) expression in the basal-like subtype. We then show that YB-1 is involved in the regulation of uPA expression. More importantly, silencing YB-1 or uPA results in a significant reduction in cancer cell invasion. As there are no commercially available YB-1inibitors we examin the efficacy of BMS-536924, a small molecule inhibitor for activated IGF-1R/IR on SUM149 cells. We demonstrate that activated IGF-1R is associated with poor survival in primary breast tumours and, that BMS-536924 reduces uPA expression through inhibition YB-1 in SUM149 cells.

We therefore conclude that YB-1 is a bio-marker for poor survival and relapse. We also indicate that YB-1 has potential use as a molecular marker in a clinical setting. Inhibiting YB-1 may provide an ideal opportunity for targeted therapy in breast cancer.

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BLBC	Basal-like breast cancer
CRS	Cytoplasmic retention site
CSD	Cold shock domain
CTD	C-terminal domain
DbpB	DNA binding protein B
DMSO	Dimethyl Sulphonite
ECM	Extracellular matrix
4E-BP1	4E-bindining protein 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELB	Egg lysis buffer
ER	Estrogen receptor
ERK1/2	Extracellular-signal-regulated kinase 1
EMSA	Electrophoretic mobility shift assay
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GSK3	Glycogen synthase kinase 3
HIV-I	Human T-cell lymphotropic virus type I
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase

MDR-1	Multidrug resistance 1
MHC	Major histocompatibility complex
MMP-2	Matrix metalloproteinase-2
MMP-12	Matrix metalloproteinase-12
mRNP	Messenger ribonucleoprotein particles
MRP 1	Multidrug resistance-related protein 1
MVP	Major vault protein
NLS	Nuclear localization signal
NSCLC	Non-small-cell lung cancer
NSEP-1	Nuclease-sensitive element protein-1
pAKT	Phosphorylated AKT
PCNA	Proliferating cell nuclear antigen
PDK1	3-phosphoinositide-dependent protein kinase-1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homologue
RTK	Receptor tyrosine kinase
SERM	Selective estrogen receptor modulator
SH2	SRC-homology-2
TMA	Tissue microarray

TNP	Triple negative phenotype
YB-1	Y-box binding protein-1

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TO MY DEAR PARENTS AND MY LOVELY SISTER
All I have is because of you

CO-AUTHORSHIP STATEMENT

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CHAPTER 1

INTRODUCTION

Development of cancer is a multi-step process, which represents genetic alterations that drive uncontrolled growth and transformation of normal human cells into malignant derivatives that can metastasize to other sites in the body (Hanahan et al. 2000). Worldwide, this disease affects millions of people every year; in fact, a study reported by Jemal et al showed that currently one in four deaths is due to cancer in developed countries (Jemal et al. 2006). Taking these statistics into consideration, it is not surprising that cancer has outweighed heart disease as the leading cause of death for people under the age of 85 in the United States since 1999 (Jemal et al. 2006).

1.1 Breast cancer

1.1.1 Frequency and origin

Worldwide, breast cancer is the most common female malignancy and it accounts for 22% of all new cancer diagnoses in women. Every year, there are 22,000 new cases of breast cancer diagnoses in Canada and approximately 5000 of Canadian women will die of this disease. Unfortunately, one in nine women will be diagnosed with breast cancer and 1 in 27 will die of it (www. cancer.ca).

The vast majority of breast cancers arise from epithelial tissues of either the ducts (this is call ductal carcimona) or the lobules (lobular carcinoma). Ductal carcinoma is making up to 90 percent of all human breast cancers (www. cancer.ca). Based on histopathological studies, ductal breast carcinoma is believed to evolve through a sequential progress from normal epithelium to usual ductal hyperplasia (UDH), to atypical ductal hyperplasia (ADH), to ductal carcinoma in situ (DCIS) and finally to invasive ductal breast carcimona (Gong et al. 2001). Several reports from American Cancer Society indicate that there is a 98% chance of survival five years post-diagnosis for the patients that have localized breast tumours. Nevertheless when the cancer has metastasized to distant sites the five-year survival rate decreases to approximately 16%. Breast cancer has the potential to spread to almost any region of the body. The most common regions that breast cancer spreads to are the bone, followed by the lung and liver (www.cancer.org).

Both genetic and non-genetic or environmental factors, such as age, obesity and increased hormone exposure play a part in the development of breast cancer. All these mentioned factors are involved in the accumulation of mutations in essential genes that mainly have important roles in cell survival and growth, cell cycle check points, and DNA repair (Baselga et al. 2002). The most frequent genetic mutations in breast cancer result in losses of heterozygosity (LOH) in tumour suppressor genes (Baselga et al. 2002). Loss of heterozygosity in *brca1* (Hall et al. 1990; Miki et al. 1994), *brca2* (Wooster et al. 1994); (Wooster et al. 1995), *p53* (Malkin et al. 1990), and *pten* (Li et al. 1997) are known to be associate with hereditary breast cancers. Amplification or activating mutations in oncogenes including *erbb2* (Slamon et al. 1987), *c-myc* (Kniazev et al. 1986), and *cyclin D1* (Buckley et al. 1993), play an important role development and progression of breast cancer (Baselga et al. 2002). Moreover, the activation of some receptors such as the estrogen receptor (ER) and the epidermal growth factor receptor (EGFR) and the insulin like growth factor receptor (IGF-1R) also contribute to the process of tumourgenesis in breast (section 1. 2). Although 5-10 percent of all breast cancers in females are hereditary, most breast tumours are categorized as sporadic (Mincey 2003).

1.1.2 Classification of breast cancer

Recent advances in gene expression profiling combined with advanced bioinformatics radically changed our ability to study and treat breast cancer (Sorlie 2007). Perou and Sorlie et al. in the year 2000 categorized sporadic breast tumours into distinct subtypes by using DNA microarrays and their "intrinsic" gene list. They also discussed the statistical relationships of the subgroups with clinical features of the disease (Perou et al. 2000).

Breast cancers are broadly divided into 2 distinct groups based on their estrogen receptor (ER) status. ER-positive tumours are also called the luminal subtype because patterns of gene expression in these tumours are similar to the luminal epithelial cells of the breast. These tumours, which represent greater than 70% of all breast cancer cases, tend to be more differentiated, less aggressive and express lower levels of growth factor receptors compared to ER-negative breast tumours (reviewed in (Tannok et al. 2005)).

ER-positive breast cancers are further sub-divided into luminal A and B, which usually express cytokeratin-8 or cytokeratin-18. GATA-3, hepatocyte nuclear factor 3α and X-box binding protein expression are also associated with luminal phenotype (Sorlie et al. 2003). ER-negative breast cancers also classified into 3 distinct subgroups: the ERBB2+, basal-like and the "normal breast-like". Normal breast-like tumours are distinguished by having similar gene expression pattern to the adipose tissue and other non-epithelial cell types. In comparison with the "normal breast-like", the ERBB2+ and basal-like breast cancers seem to be more aggressive and patients with these types on breast cancer normally have have shorter relapse free survival time (Sorlie et al. 2003). ERBB2+ subtype over-expresses ERBB2 protein, or the human epidermal growth factor receptor-2 (HER-2), however the basal-like tumours express genes such as the basal cytokeratin-5/6 and 17. These tumours have often been referred by the "triple negative" phenotype (TNP); ER-negative, progesterone receptor (PR)-negative and human epidermal growth factor receptor-2 (HER-2)-negative (Finnegan et al. 2007). The basal-like breast cancers (BLBC) are currently the mostly challenging subtype of breast cancer in regards to treatment.

1.1.3 Standard treatment of breast cancer

The primary therapy of non-metastatic breast cancer is either surgery and radiation or mastectomy with or without reconstruction. In most cases, patients also receive systemic adjuvant therapies in order to completely eradicate microscopic deposits cancer cells that may have spread from the primary tumour. This process results in decreased recurrences and improved overall survival (Baselga et al. 2002).

1.1.3.1 Chemotherapy

Chemotherapy is given for 4-6 months and normally combination of different agents is used to for treatment of patients. Chemotherapeutic agents such as alkylating agents (eg. cyclophosphamide), antimetabolites (eg. 5-fluorouracil), topoisomerase inhibitors (eg. etoposide) and anti-microtubular agents (eg. paclitaxel) all interfere with DNA synthesis and/or cell replication despite of having different mechanism of action. For instance, etoposide interferes with the activity of topoisomerase II, and paclitaxel

inhibits microtubule formation (Baselga et al. 2002). Although these processes are critical for cancer cells, they can also control the growth of normal cells including the cells in gastro-intestinal lining and hair follicles. In addtion, these traditional chemotherapeutic agents are often not very selective and have unfavorable side effects such as hair loss, vomiting and nausea. Currently, more targeted therapies are being sought.

1.1.3.2 Targeted therapy

Hormonal treatment is the first example for targeted therapy. The female hormones, estrogen and progesterone, are not only involved in normal development of mammary gland but also have essential role in breast tumourigenesis. Hirohashi et al. shown that the growth of breast cancer cells was arrested by ovariectomy in female mice. This study provided experimental evidence for hormone dependency of the human breast cancer *in vivo* and strongly suggested the important role of estrogen in the growth regulation of ER-positive human breast cancer cells (Hirohashi et al. 1977).

Currently, there are 3 classes of anti-estrogen drugs that are approved for the treatment of breast cancer patients that are estrogen-receptor-positive: 1) Aromatase inhibitors 2) Selective estrogen receptor modulators (SERMs) 3) Estrogen receptor antagonists. These three types of drugs block the ability of estrogen to turn on and stimulate the growth of breast cancer cells; however they do so through different mechanisms. 1) Aromatase inhibitors: these agents inhibit the conversion of peripheral androgens to estrogen, and reduce the amount of estrogen produced outside the ovaries in postmenopausal women. Currently, Femara® (letrozole), Arimidex® (anastrozole), and Aromasin® (exemestane) are three anti-aromatase drugs that are approved by Food and Drug Administration (FDA) for the treatment of postmenopausal women with breast cancer. Exemestane is a type one steroidal inhibitor, which binds to the aromatase permanently and stops the activity of this enzyme forever. Letrozole and anastrozole have a similar chemical structure and are considered to be type two nonsteroidal aromatase inhibitors that bind reversibly to the aromatase enzyme (Lake et al. 2002). 2) Selective Estrogen Receptor Modulators (SERM): these agents block estrogen receptors and result in reduction of estrogen-stimulated growth. Currently, tamoxifen is the most common SERM used for the hormonal treatment of breast cancer. Raloxifene is another example

of a SERM that unlike tamoxifen, have both estrogenic and anti-estrogen properties, depending on the specific tissues or cell types examined (McKenna et al. 2000). 3) Estrogen Receptor Antagonist: these drugs block and down-regulate estrogen receptors. Fulvestrant, is the first estrogen receptor antagonist, which binds to estrogen receptors, inactivates the ER complexes, and degrades them (O'Regan et al. 2001). The FDA has approved Fulvestrant for hormone treatment in postmenopausal women with ER-positive breast cancer that didn't respond to previous hormone therapies (O'Regan et al. 2001).

Although hormonal therapy has a significant impact on improving the treatment of breast cancer, there are still few limiting factors in using hormonal drugs for anti-cancer therapies. While anti-estrgen drugs such as tamoxifen were shown to be beneficial to ER-positive breast cancer patients, about 30-40% of the breast tumours do not express this receptor (Shao et al. 2004). Trastuzumab, a humanized monoclonal antibody against HER-2, was approved for treatment of patients with ER-negative HER-2-overexpressing metastatic breast cancers (section 1.2.2.3). However, treatment of other ER-negative breast tumours, such as BLBC, is still challenging. In addition, most of the patients with advanced breast cancer who originally were sentisitive to tamoxifen therapy became resistant to this type of drug. The common factors that contribute to tamoxifen resistance are the infrequent loss and mutation in ER, altered levels of ER co-regulators, and importantly, abnormal activation of additional growth factor signaling pathways (Shao et al. 2004). Thus, there is an urgent need to obtain more profound perception of other signaling pathways and receptor tyrosine kinases that are deregulated in breast cancer.

1.2 Receptor tyrosine kinases (RTK)

RTKs are key regulators of cellar responses to the environmental factors. These receptors can link the environmental signals through ligand binding to downstream signaling cascades and gene expression. Over the past 25 years scientists have discovered that RTKs are not only involved in mediating the normal processes in the cell but also have very important roles in the development, transformation and progression of human malignancies. Thus, receptor tyrosine kinases and the signaling pathways that are being controlled by them, provide special opportunities for therapeutic intervention (Zwick et al. 2001).

Based on their structural characteristics, RTKs can be categorized into 20 subfamilies, which consist of a common homologous domain that defines the catalytic tyrosine kinase function (Ullrich et al. 1990). All RTKs have a transmembrane domain that separates the extracellular part from the intracellular tyrosine kinase portion (Ullrich et al. 1990). The tyrosine kinase domain consist of the ATP-binding site that catalyses receptor autophosphorylation and tyrosine phosphorylation of RTK downstream substrate (Yarden et al. 1988). Upon ligand binding, receptors undergo conformational changes that result in stabilization and dimerization of RTKs. This process leads to increased kinase activity and autophosphorylation of tyrosine residues (Ullrich et al. 1990). Tyrosine phosphorylation of specific amino acid residues of the receptor creates an optimal binding site for Src homology 2 (SH2)- and phosphotyrosine binding (PTB) domain-containing proteins (Zwick et al. 2001). In addition, Pawson et al in 1995 reported that adaptor molecules or enzymes that are tyrosine phosphorylated such as Src and phospholipase C, are being recruited to the plasma membrane via these binding motifs (Pawson 1995; Zwick et al. 2001).

1.2.1. Epidermal growth factor receptor (EGFR)

EGFR, a 170-kDa protein, was the first RTK to be molecularly cloned (Ullrich et al. 1984). It is a member of the ErB family of receptors, a subfamily of four closely related receptor tyrosine kinases: the EGFR (ErbB-1), HER-2/c-neu (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4) (Zwick et al. 2001).

EGFR, like other receptors of the family has an extracellular ligand-binding domain, a single transmembrane lipophilic segment, and an intracellular cytoplasmic tyrosine kinase domain with a regulatory carboxyl terminal segment (Hynes et al. 2005; Zwick et al. 2001). This cell surface receptor gets activated upon binding of its specific ligands, including epidermal growth factor (EGF), heparin-binding EGF (HB-EGF), transforming growth factor-α (TGF-α), amphiregulin, betacellulin and epiregulin (Yarden 2001). Upon activation by its growth factor ligand, EGFR undergoes conformational change, and this leads to the formation of homo- or heterodimers with other members of the EGF receptor family (Graus-Porta et al. 1997). Dimerization of the receptors stimulates its intrinsic intracellular protein-tyrosine kinase activity. As a result,

autophosphrylation of several specific tyrosine residues in the C-terminal domain of EGFR occurs. This autophosphorylation elicits downstream activation and signaling by several other proteins that associate with the phosphorylated tyrosines through their own phosphotyrosine-binding SH2 domains. These downstream signaling proteins initiate several signal transduction cascades, principally the mitogen-activated protein kinase (MAPK), and the phosphatidylinositol-3 kinase (PI3K) pathways, leading to several cellular responses such as proliferation, survival, differentiation, and migration (Prenzel et al. 2001; Slichenmyer et al. 2001; Yarden 2001; Zwick et al. 2001). Following ligand binding and tyrosine kinase activation, the receptor-ligand complex can be recycled or degradated by the proteosomal complex (Yarden 2001).

1.2.1.1 The oncogenic property of EGFR

Although activation of EGFR signaling cascade mediates cell proliferation, it can also regulate many essential tumourgenic processes such as cell survival, motility, adhesion, angiogenesis, and metastasis (Mendelsohn et al. 2000). The oncogenic property of EGFR was illustrated originally in a study where human EGFR was over-expressed by a retroviral vector and induced a transforming phenotype in NIH 3T3 cells. In this study, Velu et al. showed that cells expressing 4x 10^5 EGF receptors formed tumours in nude mice, while control cells did not (Velu et al. 1987). In another study, over-expression of EGFR in transgenic mice resulted in development of tumours in the bladder and the breast (Brandt et al. 2000; Cheng et al. 2002). Induced or constitutively active EGFR signaling has been reported in many malignancies, such as lung, colon, ovarian, and breast cancers (Laskin et al. 2004). In addition, increased expression of the receptor and/or the ligands is believed to be the causes for elevated EGFR signaling.

1.2.1.2 Role of EGFR in development of breast cancer

Over-expression of EGFR has been detected almost very frequently in breast cancer (Bucci et al. 1997; Klijn et al. 1992; Osaki et al. 1992; Salomon et al. 1995). Several teams also reported that the expression of EGFR is inversely correlated with ER (Lebeau et al. 2003; Tsutsui et al. 2002; Wu et al. 2006). Subsequently, a study by Nielsen et al in 2004 showed that EGFR is highly expressed in the ER-negative basal-like

breast cancer (Nielsen et al. 2004). These reports further supported the fact that over-expression of EGFR is associated with poor outcome, tumour aggressiveness, and metastasis (Klijn et al. 1992; Nielsen et al. 2004; Tsutsui et al. 2002; Wu et al. 2006). In contrast with other cancers such as glioblastoma that overexpression of *egfr* gene is amplified, in breast cancer over-expression of EGFR is mainly due to transcriptional activation (Kersting et al. 2004).

1.2.1.3 EGFR as a target for anti-cancer therapy

Considering aberrant EGFR signaling has been reported in many human malignancies and EGFR over-expression is correlated with poor prognosis, it is believed to be an attractive target for therapeutic intervention (Hong et al. 2000; Huang et al. 1999). In the past few years, cetuximab (C225), a monoclonal antibody targeting EGFR, has been developed. Cetuximab blocks the ligand binding to the extracellular domain of EGFR, inhibits receptor activation and induces receptor internalization. Several studies have reported the growth inhibiting and anti-tumour effect of cetuximab in many human cancer cells such as pancreatic, renal and breast cancers (Overholser et al. 2000; Prewett et al. 1998). Therefore, it is currently undergoing a number of clinical trials either alone or in combination with other chemotherapeutic drugs (Baselga et al. 2000; Bruns et al. 2000; Butts et al. 2007; Inoue et al. 2000; Zwick et al. 2001).

Since EGFR is a receptor tyrosine kinase, inhibiting it with tyrosine kinase inhibitors (TKIs) is an excellent approach towards anti-cancer therapy. In the past few years, the compounds that are ATP analogues of the quinazoline and pyridopyrimidine family have been developed. These TKIs bind to the ATP-binding site in the kinase domain of the receptor and thereby lead to inhibition of EGFR activation (Levitzki 1992; Noonberg et al. 2000). Gefitinib (Iressa) was the first EGFR kinase inhibitor approved by U.S. Food and Drug Administration (FDA) for cancer therapy. It is an EGFR small molecule kinase inhibitor that blocks ligand-stimulated EGFR phosphorylation and suppress EGF dependent tumour growth of *in vitro* and *invivo* (Wakeling et al. 2002). In May 2003, FDA in united states approved gefitinib for treatment of non-small cell lung cancer (NSCLC) that progressed after or failed to response docetaxel and platinum-based therapy (http://www.fda.gov/CDER/Drug/infopage/gefitinib/default.htm). However, Kris

et al. reported that in a phase II randomized trial approximately 10 percent of the patients respond to gefinitib (Kris et al. 2003). Considering the fact that EGFR is over-expressed in some subtypes of breast cancer, a phase II clinical trial has been organized to evaluate the efficacy of combining gefitinib and docetaxel as a first-line treatment for metastatic breast cancer patients (Takabatake et al. 2006). Although, the response to gefitinib was promising in the preclinical testing, this drug as a single agent is largely ineffective in several clinical trials (Smith et al. 2007). For instance, in breast cancer, addition of gefitinib to neoadjuvant anastrozole had no additional clinical or biologic effect on the patients (Smith et al. 2007). Moreover, the results from a phase II clinical trials in prostate cancer patient were disappointing. In these trials patients where treated with 250mg or 500mg of Iressa daily and unfortunately there was no evidence from any tumour type that a higher dose than what was used in this study has a greater benefit (Canil et al. 2005). These findings indicates that infectiveness of gefitinib as an anti-cancer drug could be due to compensatory signaling by other members of the ERBB family, such as ERBB-2.

1.2.2 Human epithelial growth factor receptor-2 (HER-2)

Originally, HER-2 was discovered as both the transforming gene in a rat neuroblastoma cell line (Schechter et al. 1984) and as a cDNA clone which was related to EGFR (Coussens et al. 1985). HER-2 has also been commonly implicated in variety human malignancies (Hynes et al. 1994; King et al. 1985; Slamon et al. 1987). Although, no ligand has been identified to bind to HER-2 with high affinity, this RTK dimerizes with the other Erb family members. Specifically, either the neuregulins or the EGFR ligands bind to HER-3 or HER-4 and induce heterodimer formation between these receptors and HER-2. The heterodimer formation is very important in activation of downstream signaling cascade, which leads to uncontrolled growth, development and transformation of malignant tumours. Activation of PI3K, MAPK and JNK signaling pathway have been detected in cells that over-express HER-2 (Zwick et al. 2000; Zwick et al. 2001).

1.2.2.1 The oncogenic property of HER-2

Originally, Di Fiore et al. (1987) showed that the high levels of *erbB-2* product associated with malignant transformation of NIH3T3 cells were observed in human mammary tumour cells that over-expressed the gene (Di Fiore et al. 1987). Subsequently, another study by the same group, the normal *erbB-2* gene was introduced into immortalized human mammary epithelial cells (184B5) by transfection conferred a growth advantage to these cells both *in vitro* and *in vivo* (Pierce et al. 1991). Over-expression and/or gene amplification of HER-2 was detected in many types of human malignancy and particularly in human breast cancers where *erbB-2* gene amplification has been found with a frequency of approximately up to 30% (Slamon et al. 1987; Slamon et al. 1989). Paik et al in 1990 also reported that abnormal elevated levels of HER-2, either with or without gene amplification, is associated with poor survival and aggressiveness in patients with breast cancer (Paik et al. 1990; Zwick et al. 2001).

1.2.2.2 HER-2 as a prognostic and predictive indicator

In normal epithelial cells, the expression level of HER-2 is relatively low however over-expression or amplification of HER-2 was detected in variety of human malignancies such as breast, bladder, gastric, lung, kidney and ovarian carcinomas (Hynes et al. 1994). Slamon et al in 1987 reported that particularly in patients with breast cancers that over-express HER-2 have much shorter survival time compared to the patients that express HER-2 at the normal level (Slamon et al. 1987). Furthermore, a study by Yu et al shows that over-expression of HER-2 is associated with the number of lymph node metastases in node-positive breast cancer patients (Yu et al. 2000). Moreover, in advanced breast cancers high levels of circulating extracellular domain of HER-2 is associated with reduced the efficacy of certain chemotherapy combinations (Colomer et al. 2000). Mostly importantly, HER-2 is an independent predictive marker for response to anti-estrogen drugs. Houston et al in 1999 showed that HER-2 over-expression is associated with lack of response to endocrine therapy in patients with advanced ER-positive breast cancer (Houston et al. 1999; Zwick et al. 2001).

1.2.2.3 HER-2 as a target for anti-cancer therapy

There are many approaches towards inhibiting HER-2 mediated signaling (Bange et al. 2001). Studies by Hudziak et al (1989) and Shepard et al 1991 showed that MAb 4D5, a monoclonal antibody targeting the extracellular domain of HER-2, reduced the growth of HER-2-over-expressing cell lines and inhibited tumour growth *in vivo* (Hudziak et al. 1989; Shepard et al. 1991). Herceptin (trastuzumab), a'humanized' version of murine MAb 4D5 developed by Genentech, was approved by the US Food and Drug Administration (FDA) in 1998 as a first line of treatment for women with metastatic breast cancer (Pegram et al. 1998). Several studies reported that up to 15 % of HER-2-overexpressing breast cancer patients who received trastuzumab as a single-agent in their treatment regime responded to this drug (Baselga et al. 1996; Cobleigh et al. 1999). However, trastuzumab combined with paclitaxel or doxorubicin with cyclophosphamide significantly increased relapse free survival time of HER-2 ever-expressing metastatic breast cancer patients (Burris 2000; Stebbing et al. 2000; Zwick et al. 2001).

1.2.2.4 Other anti-cancer drugs against HER family RTKs

Due to lack of efficiency and the possible chance of developing resistance to gefitinib or trastuzumab as a single agent, pharmaceutical companies developed drugs that target both EGFR and HER-2. Lapatinib also known as GW572016, is an anti-cancer drug developed by GlaxoSmithKline for treatment of breast and lung cancer patients. The FDA approved laptinib on March 13, 2007, for treating patients with advanced metastatic breast cancer in combination with certain chemotherapy drugs. Lapatinib is an epidermal growth factor receptor (EGFR) and HER-2/neu (ErbB-2) dual tyrosine kinase inhibitor (Xia et al. 2002). It binds to the intracellular domain to inhibit receptor autophosphorylation upon ligand binding. The exact binding site for lapatinib has currently not been confirmed. Xia et al showed that lapatinib inhibits receptor phosphorylation, blocks MAPK signaling cascade and thereby reduces the growth of EGFR or HER-2 over-expressing cell lines (Xia et al. 2002). Several studies tested the effect of lapatinib in combination with other drugs both in cell lines and in mouse xenograft. For instance, lapatinib in combination with PI3K inhibitor, LY294002 synergistically induced apoptosis. In addition, the improved anti-tumour activity *in vivo*

was observed when lapatinib was used with paclitaxel or doxorubicin (Rabindran 2005).

CI-1003 is also another type of tyrosine kinase inhibitor that targets EGFR, HER-2 and HER-4 (Allen et al. 2002). CI-1003 irreversibly inhibits ligand-stimulated phosphorylation of the receptor by binding to a conserved cysteine residue in the ATP binding site in the tyrosine kinase domain (Slichenmyer et al. 2001). This drug can induce apoptosis in HER-2 over-expressing cells by increasing p38, downstream substrate of MAPK, and blocking the Akt signaling (Nelson et al. 2001). The efficacy of this inhibitor has been examined in phase I clinical trials in combination with other chemotherapeutic drugs such as docetaxel for treatment of advanced solid tumours or with paclitaxel and carboplatin for treatment of NSCLC (Chiappori et al. 2006; Garland et al. 2006).

1.2.3 Insulin growth factor receptor (IGF-R) family

The insulin-like growth factor-1 (IGF-1) and the insulin receptor (IR) are structurally quite different from the EGFR family. These two receptors both have two extracellular α subunits, which are designated for ligand binding, and two membrane-spanning β subunits, which consist of the tyrosine kinase domain and the autophosphorylation sites (Yarden et al. 1988). Insulin, IGF-I and IGF-II are the ligands that bind to these receptors. Insulin is mainly a metabolic hormone, whereas IGF-I and IGF-II are critical for normal development and cancer progression. IGFs are bound to various different IGF-binding proteins (IGFBPs) in the circulating system (Jones et al. 1995). There are six IGFBPs that have been discovered so far which are transporters for these ligands. These binding proteins are also involved in modifying the stability and the proliferative effect of the growth factors (Jones et al. 1995; Zwick et al. 2001).

1.2.3.1 IGF-1R signaling cascade

Upon ligand binding to IGF-1R, tyrosine kinase activity of the receptor gets induced and leads to transphosphorylation of one α-subunit by the other. Subsequently the activated receptor phosphorylates adaptor proteins such as the insulin receptor substrate (IRS) family and Src homology 2 domain-containing (SHC) on multiple tyrosine residues. These adaptor proteins then recruit other downstream substrates with

src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. Phosphorylation of the extracellular signal-regulated kinase (ERK) 1/2 downstream of MAPK signaling pathway and phosphatidylinositol-3 kinase (PI3K) results in cell replication. In addition, activation of PI3K results in activation of Akt and leads to phosphorylation of mammalian target of rapamycin (mTOR). Subsequently, activated mTOR phosphorylates p70S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4EBP1), which leads to induction of cap-dependent mRNA translation. IGF1-R signaling cascade results in up-regulation of cyclin D1, leading to over-expression of growth promoting genes (Sachdev et al. 2006).

1.2.3.2 Role of IGF-1R in breast cancer progression

In the past 17 years, it has been reported that IGF-IR and its ligands are involved in development and progression of many human malignancies such breast and prostate cancer (Perks et al. 2000; Perks et al. 2000). A report by Sell et al. (1993) indicated that mouse fibroblast cells with genetic deletion of IGF-1R lost their transforming abilities unless IGF-1R was stably reintroduced into the fibroblasts (Sell et al. 1993). Subsequently DeAngelis et al in 2006 has shown that tyrosine phosphorylation of IRS-1, and activation of the PI3K pathway seems to be crucial for the ability of IGF-1R to transform cells (DeAngelis et al. 2006). In addition, inhibition of IGF-1R function by dominant negative mutants, antibodies against IGF-1R, or antisense approaches prevented tumour growth *in vitro and in vivo* (Arteaga et al. 1989; Salatino et al. 2004).

Activation of IGF-IR pathway also stimulates motility of breast cancer cells *in vitro* suggesting a role for this system in metastasis. A number of reports indicated that blocking of IGF-1R results in significant reduction in invasion and metastasis of breast carcinoma cells (Dunn et al. 1998; Sachdev et al. 2004). Dunn et al in 1998 showed that a soluble, truncated IGF-1R (called 486/stop) created by inserting a premature stop codon at amino acid 486 suppressed the invasion and metastasis of MDA-MB-435 cells (Dunn et al. 1998). Another study by Sachdev et al. (2004) illustrated that a C-terminally truncated dominant negative IGF-1R significantly inhibited invasion and metastasis of MDA-MB435/LCC6 cells to lung (Sachdev et al. 2004).

1.2.3.3 IGF-1R as a target for cancer therapy

Currently, there are two different approaches towards inhibiting IGF-1R activation: 1) Antibodies that binds to extracellular domain of IGF-1R and 2) Small molecule inhibitors that block the kinase activity of the receptor.

1.2.3.3.1 Antibodies against IGF-1R

Several monoclonal antibodies directed against IGF-IR have been created such as: CP-751, 871 from Pfizer, IMC-A12 from Imcloned Systems, 19D12 from Schering-Plough, EH164 from ImmunoGen/Aventis, and hC7C10 from Pierre Fabre/Merk (Burtrum et al. 2003; Garber 2005; Goetsch et al. 2005; Lee et al. 2000; Maloney et al. 2003; Wang et al. 2005). Currently, all antibodies seem to work in a similar manner, which is binding to extracellular ligand binding domain and result in down-regulation of IGF-IR. Although binding of IGF-1 results in receptor recycling, monoclonal antibody binding leads to endosomal degradation of the receptor (Sachdev et al. 2003; Wang et al. 2005; Yee 2006). Besides blocking ligand binding and inhibiting IGF-1R signaling, the majority of antibodies also inhibit tumour growth *in vivo* (Li et al. 2000; Sachdev et al. 2003; Yee 2006). Cohen et al. (2005) reported that CP-751, 871 is able to bind to IGF-1R with high affinity and inhibit its function by blocking IGF-1 binding. They also showed that CP-751, 871 induces IGF-1R down-regulation *in vitro* and *in vivo* (Cohen et al. 2005). This humanized monoclonal antibody also illustrated significant anti-tumour activity as a single agent and in combination with doxorubicin or tamoxifen in multiple tumour models (Cohen et al. 2005; Sachdev et al. 2006).

1.2.3.3.2 Small molecule inhibitors against IGF-1R

Given that IGF-IR is a tyrosine kinase, several small molecule inhibitors have also been developed against it (Carboni et al. 2005; Garcia-Echeverria et al. 2004; Mitsiades et al. 2004). Most inhibitors such as NVP-AEW541, NVP-ADW742 from Novartis Institutes, INSM-18 from InsMed, BMS-55417 and BMS-536924 from Bristol-Myers Squibb inhibit the kinase activity of IGF-1R by competing with the ATP binding site of the receptor (Sachdev et al. 2006). However, small molecules such as cycloligans picropodophyllin are substrate inhibitors that disrupt tyrosine in the activation loop of the

kinase domain of IGF-1R (Vasilcanu et al. 2004). In a study, Vasilcanu et al. demonstrated that the cyclolignan picropodophyllin efficiently inhibited phosphorylation of IGF-1R without interfering with insulin receptor activity. This reagent preferentially reduced phosphorylated Akt, as compared to phosphorylated ERK1/2, and caused apoptosis (Vasilcanu et al. 2004).

Another compound, INSM-18, is nordihydroguaiaretic acid (NDGA) has also been shown to disrupt the activity of both IGF-IR and HER-2. Although this might seem undesirable, recent report by Nahta et al shows that IGF-IR plays a role in development of resistance to Herceptin (Nahta et al. 2005). Thus, a drug that disrupts both pathways may be desirable in specific situations.

Two recent dual specificity inhibitor, BMS-554417 and BMS-536924, are found to target both the IGF-1R and IR (Hofmann et al. 2005). Haluska et al. (2006) reported BMS-554417 inhibits tumour growth of IGF-1R-Sal tumour xenografts (Haluska et al. 2006). In another study, BMS-536924 was also shown to inhibit tumour growth *in vivo* (Kim et al. 2007).

While these above-mentioned compounds, show some selectivity of IGF-IR over IR in cell model systems, it is uncertain as to whether this selectivity will be observed *in vivo*. Moreover, whether or not IGF-IR selectivity is even desirable is unclear. There are several reports indicate that insulin receptor (IR) mediates a substantial portion of IGF-stimulated tumour cell biology, especially via IGF-II activation, then cancer cell inhibition of IR would be necessary (Haluska et al. 2006; Sachdev et al. 2006).

All these findings lead us to believe that activation of IGF-1R signaling cascade has a crucial role in breast carcinogenesis. One of the roles IGF-1R in cancer progression and metastasis is through upregulation of urokinase plasminogen activation (uPA).

1.2 Down stream substrates of RTKs which are potential therapeutic targets

1.3.1 Urokinase plasminogen activator (uPA)

1.3.1.1 Protein structure and function

uPA is a 53-kDa serine protease. Similar to majority of mammalian proteases, uPA is initially produced by the cells as a pro-enzyme in a catalytically inactive single-chain polypeptide (Duffy 2004). Prostate specific antigen (PSA), plasmin, cathepsin B,

cathepsin L and human kallikrein type 2 have been shown to catalyze activation of uPA *in vitro* (Andreasen et al. 1997; Yoshida et al. 1995). All these proteases, convert the single-chain pro-form of urokinase-type plasminogen activator (scuPA) to an active 2-chain form. Mature uPA includes a two-chain protein, which are linked by a single disulfide bond (Andreasen et al. 1997; Duffy et al. 2004). uPA can be divided into three main domains, an N-terminal domain, which includes the binding site for uPAR, a kringle domain of unknown function, and a C-terminal domain that contains the catalytic site (Andreasen et al. 1997; Duffy et al. 2004).

uPA converts inactive plasminogen to active plasmin which can degrade majority of ECM proteins. Moreover, plasmin is also a serine protease that can activate the precursor forms of a number of matrix metalloproteinases (MMPs) such as MMP-3, MMP-9, MMP-12 and MMM-13 (Carmeliet et al. 1997). Activation of the above mentioned MMPs results in further degradation and remodeling of the ECM. Interestingly, plasmin is involved in activation or release of insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2) and transforming growth factor-beta (TGF-β) (Rifkin et al. 1997). uPA also activates IGF-1R signaling pathway by mediating the levels IGFBP in blood (Sorrell et al. 2006; Yoshida et al. 1995). Therefore, uPA plays a very important role in cancer development, invasion and metastasis (Duffy et al. 2004).

The most important inhibitors of urokinase are plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2), which inhibit the protease activity irreversibly. Both PAI-1 and PAI-2 belong to serine protease inhibitor family which are a superfamily of proteins that fold into a conserved structure and demonstrate a special suicide substrate-like inhibition mechanism (Silverman et al. 2001; Duffy et al. 2004).

1.3.1.2 uPA and breast cancer

High levels of uPA in breast tumours predicts poor survival (Janicke et al. 1989; Look et al. 2002). In experimental models of breast cancer, uPA increases cell migration (Nguyen et al. 2000) and metastasis (Yu et al. 1990). Consistent with this, inhibition of uPA decreases tumour growth and metastasis (Guo et al. 2002). Importantly, uPA is also significantly associated with a high rate of relapse for patients with breast cancer (Janicke

et al. 1989). The European Organization for Research and Treatment of Cancer regard uPA as a Level I category biomarker for cancer and therefore new agents targeting this protease would clearly be beneficial (Harbeck et al. 2004; Dos Santos et al. 2007).

1.3.1.3. uPA as a target for cancer therapy

Strategies to inhibit uPA include abrogation of uPA protease activity (Wendt et al. 2004) and disruption of uPA binding to its receptor (Guo et al. 2002). Approaches to inhibit uPA production have resulted in suppression of cancer development and metastasis suggesting that this could be an important target for therapeutic intervention. For instance, silencing uPA using small interfering RNA (siRNA) inhibits the growth of prostate cancer cell lines (Pulukuri et al. 2005). The peptide inhibitor A6, that particularly bind to the amino acid sequence crucial for uPA to bind to uPAR, has demonstrated very promising results in phase I clinical studies in ovarian cancer (Berkenblit et al. 2005). In addition, other drugs such as 17-allylamino geldanamycin (17-AAG) (Nielsen et al. 2004) and celecoxib (Andrews et al. 2005) were shown to inhibit uPA production. Nevertheless they are hindered by formulation and cytotoxicity problems therefore there are some limitations for their use in the clinic (Dos Santos et al. 2007).

Our team previously reported that the up-regulation of uPA by IGF-1R depends on a convergence of PI3K/Akt and MAPK pathways, leading to the activation of the uPA (Dunn et al. 2001). Inhibition of both PI3K/Akt and MAPK optimally inhibited uPA-reporter activity (Dunn et al. 2001). Recently, our team demonstrated that QLT0267 or integrin-linked kinase (ILK) siRNA suppresses uPA production, invasion and ultimately the growth of tumour cells representing aggressive model of cancer (Dos Santos et al. 2007). In addition to inducing uPA, our group has also shown that IGF-1R signaling engages oncogenic transcription factors such as activating protein-1 (AP-1), hypoxic-inducible factor-1 alpha (HIF-1α) (Oh et al. 2002) and Y-box binding protein-1 (YB-1) (Sutherland et al. 2005).

1.3.2 The Y-box binding protein-1 (YB-1)

Initially, the Y-box binding protein-1 (YB-1) was identified for its ability to bind to the inverted CCAAT box of major histocompatilibity complex (MHC) class II

promoter (Didier et al. 1988). In 1988 Sakura et al also discovered YB-1 as one of the DNA binding proteins that binds to the *egfr* enhancer and *HER-2* promoter (Sakura et al. 1988). Subsequently, YB-1 was referred to DNA binding protein B (DbpB), and nuclease-sensitive element protein-1 (NSEP-1), which was independently cloned based on its ability to bind to CT-rich elements in the *c-myc* promoter (Kolluri et al. 1991). Eventually, Evdokimova et al in 1995 also named YB-1 as the human homolog of p50, which was initially discovered to be one of the major proteins in messenger ribonucleoprotein particles (mRNPs) in rabbit reticulocytes (Evdokimova et al. 1995).

1.3.2.1 Gene structure and expression

The *yb-1* gene is located on chromosome 1p34 and consists of 8 exons (Toh et al. 1998). The mRNA is about 1.5 kb long and encodes a 43 kDa protein which has 324 amino acids (Didier et al. 1988). The expression of YB-1 varies among tissues and developmental stages. Spitkovsky et al. (1992) detected high level of YB-1 mRNA in fetal cerebrum, heart, muscle, liver, bone marrow, and kidney. In contrast, low amounts of YB-1 mRNA were found in thymus, kidney, bone marrow and spleen (Spitkovsky et al. 1992). On the other hand, YB-1 transcript is not detected or is expressed at very low level in many adult tissues including breast tissues (Spitkovsky et al. 1992). These findings suggest that the expression of YB-1 must be tightly regulated but the exact mechanisms that control YB-1 expression remains largely unknown. Nonetheless, Uromoto et al. (2002) showed that c-Myc binds to multiple E-boxes and GF-boxes on the *yb-1* promoter and interacts with p73 to transactivate *yb-1* promoter (Uramoto et al. 2002). In another study by Yokoyama et al. transcription factor, GATA binding protein-1 (globin transcription factor-1; GATA-1) was also shown to bind to the 5'-untranslated region and activates the *yb-1* promoter in erythoid cells(Yokoyama et al. 2003).

1.3.2.2 Protein structure and function

YB-1 is composed of three domains: a non-conserved variable N-terminal domain, a highly conserved cold-shock domain (CSD), and a C-terminal domain (CTD) (Kohno et al. 2003). The N-terminal domain is alanin and proline rich and is believed to be involved in transactivation (Kohno et al. 2003). The cold-shock domain is the most

evolutionary conserved nucleic acid-binding protein domain that so far has been discovered in prokaryotes and eukaryotes (Wolffe 1994; Wolffe et al. 1992). It is also known as the nucleic-acid binding domain of the vertebrate proteins, shows about 40% homology with the 70 amino acid cold-shock proteins found in bacteria where the proteins act as RNA chaperones (Yamanaka et al. 1998). Finally, the C-terminal domain mediates protein-protein interactions and consists of regions of about 30 alternating basic and acidic amino acids (Bouvet et al. 1995). This structure is termed as the B/A repeats or charged zipper and is unique to vertebrate organisms (Kohno et al. 2003). These repeats are also common in proteins that bind to ribonucleoprotein complexes involved in shuttling between cytoplasm and nucleus (Ranjan et al. 1993). Bader et al. (2005) identified nuclear localization signal (NLS) and a cytoplasmic retention site (CRS) in the CTD to monitor YB-1 localization in the cell (Bader et al. 2005).

1.3.2.3 Functions of YB-1

YB-1 is a multi-functional protein involved in several biological processes including gene regulation working as transcription or translation factor, cell proliferation, DNA repair, drug resistance, and stress responses to extracellular signals (Kohno et al. 2003).

1.3.2.3.1 Role of YB-1 as a transcription factor

As a transcription factor, YB-1 binds to the sequence motif CTGATTGG that is known as the Y-box in the promoter regions of several eukaryotic genes such as growth-associated genes (Kohno et al. 2003). YB-1 can activate or repress gene transcription in there different ways. YB-1 interacts directly with the Y-boxes and related sequences to regulate target genes. Conversely, it can mediate gene expression in a "Y-box-independent manner" through interacting with other transcription factors such as p53 (Okamoto et al. 2000), AP-1 (Lasham et al. 2000), smad3 (Higashi et al. 2003). In addition, YB-1 could also bind to single-stranded region of the promoters, called S1-sensitive sites, to inhibit or enhance binding of other transcription factors to DNA (Kohno et al. 2003; Wilusz et al. 2001).

As mentioned previously, YB-1 is involved in cell proliferation and growth. It can up-regulate transcription of *cyclin A* (Jurchott et al. 2003), *cyclin B1* (Jurchott et al.

2003), *topoisomerase II α* (Shibao et al. 1999) and *DNA polymerase α* (En-Nia et al. 2005). These findings suggest that YB-1 can accelerate cell growth by promoting both cell cycle progression and DNA replication. Swamynathan et al. (2002) reported that targeted disruption of one allele of the Y-box protein gene in DT40 cells results in defects in cell cycle and reduces cell growth (Swamynathan et al. 2002). Another studies also demonstrated that YB-1 interacts with proliferating cell nuclear antigen (PCNA) (Ise et al. 1999) and DNA polymerase δ (Gaudreault et al. 2004). However, the exact role of YB-1 in DNA replication is not very clear and needs further investigation.

Since YB-1 is involved in the cell proliferation, scientists were interested to investigate its role in tumourgenesis. It has been reported that YB-1 up-regulates the transcription of protein tyrosine phosphatase 1B (PTP1B) (Fukada et al. 2003), matrix metalloproteinase-2 (MMP-2) (Mertens et al. 2002), matrix metalloproteinase-12 (MMP-12) (Samuel et al. 2005). YB-1 can also repress the expression of collagen α1 (Norman et al. 2001) and collagen α2 (Higashi et al. 2003). All the above mentioned genes are involved in cell adhesion, motility, invasion and thus metastasis. Stein et al. in 2001 reported high level of YB-1 expression is associated poor clinical response to chemotherapies. This is due to the ability of YB-1 in promoting the expression of multi-drug resistance gene (*mdr1*) (Bargou et al. 1997), multi-drug resistance-related protein-1 (*mrp1*) and major vault protein (*mvp*) (Stein et al. 2005).

On the other hand, YB-1 can also down-regulate the transcription of genes such as the class II major histocompatibility complex (Class II MHC) (Didier et al. 1988) and FAS (Apo-1/CD95) (Lasham et al. 2000). Since YB-1 is involved in repressing the immune system by regulation of the genes mentions above, it would a wise choice for pathogenic viruses to use YB-1 to facilitate their own gene expression and evade from immunosurveillance. YB-1 up-regulates the gene expression of many viruses, such as the Rous sarcoma virus d (RSV-d) (Kandala et al. 1994), adenovirus (Holm et al. 2002), the human immunodeficiency virus type I (HIV-1) (Sawaya et al. 1998).

1.3.2.3.2 Role of YB-1 as a translation factor

Despite of the fact that YB-1 acts as a transcription factor in the nucleus, this protein has been mainly detected in the cytoplasm suggesting that YB-1 can also be involved in translational regulation (Evdokimova et al. 2006).

YB-1 was found to be a major component of messenger ribonucleoprotein particles (mRNPs) (Evdokimova et al. 1998; Evdokimova et al. 1995). Matsumoto et al. (1996) also reported that the CSD and the CTD of YB-1 is required for incorporating mRNP into mRNA (Matsumoto et al. 1998). As a translation factor, YB-1 can act as an activator or repressor of this process based on its concentration in the cell (Kohno et al. 2003). However, the recent reports are emphasizing more on the role of YB-1 in repressing the translation. Low levels of YB-1 facilitate the cap binding to mRNA whereas high level of YB-1 inhibits interaction of cap-binding protein and enhances the stability of RNA (Evdokimova et al. 1998). Basically, YB-1 competes with translation initiation factor 4E (elF4E)- driven translation initiation complex for binding to the certain capped mRNA terminus (Evdokimova et al. 2001). Moreover, Evdokimova et al. (2006) discovered that the majority of these YB-1 bound transcripts encode for proteins essential for cell growth or viability (Evdokimova et al. 2006). Recently, another study by Evdokimova et al. also demonstrated Akt-mediated phosphorylation of YB-1 at serine 102 negatively regulates the cap-dependent repressor activity of YB-1 and activates translation of silent mRNAs (Evdokimova et al. 2006). It is very important that YB-1 can control translational silencing and RNA stabilization under conditions of stress, nutrient deprivation or meiosis, when translation is restricted (Evdokimova et al. 2006).

In another study, YB-1 was found to be involved in translation control of ferritin protein. Ashizuka et al. (2002) demonstrated that under low iron conditions, the iron-responsive element-binding protein (IRE-BP/IRP2) binds to iron-responsive element (IRE) at the 5'UTR of ferritin mRNA lead to translational repression. However, in high iron conditions, IRP2 binds to YB-1. This interaction results in the release of IRP2 from IRE, activating the translation of ferritin protein (Ashizuka et al. 2002).

1.3.2.3.3 Role of YB-1 in drug resistance

As mentioned previously, YB-1 can induce expression of *mdr-1* and mrp-*1* that encode for the drug efflux pumps, ABC transporter P-glycoprotein and MRP1, respectively (Stein et al. 2005). In addition, YB-1 mediates the expression of MVP, which also plays an important in drug resistance (Stein et al. 2001). Bargou et al. (1997) demonstrated that over-expression of YB-1 cause cancer cells to become resistant to doxorubicin and etoposide (Bargou et al. 1997). In another study, silencing YB-1 using antisense, exhibited increased sensitivity to cisplatin and mitomycin C (Ohga et al. 1996; Ohga et al. 1998). Consistently, Shibahara et al. illustrated that the targeted disruption of one allele of *yb-1* (YB-$1^{+/-}$) sensitized mouse embryonic stem cells to cisplatin and mitomycin C (Shibahara et al. 2004). A few reports illustrated the nuclear localization of YB-1 and up-regulation of the drug resistance genes immediately after exposure to UV radiation and chemothrapeutic drugs such as doxorubicin and 5-fluorouracil (Bargou et al. 1997; Stein et al. 2005). The association of nuclear YB-1 and p-glycoprotein expression has been reported in many malignancies such as; breast cancer (Bargou et al. 1997; Saji et al. 2003), prostate cancer (Gimenez-Bonafe et al. 2004), osteosarcoma (Oda et al. 1998), ovarian cancer (Kamura et al. 1999) and synovial sarcoma (Oda et al. 2003). All these findings suggest that YB-1 may provide a potential predictive marker for response to chemotherapy.

1.3.2.4 YB-1 and breast cancer

YB-1 has been found to be associated with tumour aggressiveness, relapse and poor patient survival in breast cancer (Bargou et al. 1997; Wu et al. 2006). Janz et al. (2002) reported that cancer relapsed in 66% of the patients with high level of YB-1 expression in their breast tumours after post-operative chemotherapy treatment while patients with low YB-1 expression had no evidence of cancer recurrence (Janz et al. 2002). In an interesting study, Bergmann et al. (2005) showed that YB-1 was able to induce mammary tumour formation through induction of genetic instability in a transgenic mouse model (Bergmann et al. 2005). Our team also demonstrated that activated Akt can phosphorylate YB-1 at serine102, resulting to nuclear localization of this protein (Sutherland et al. 2005). In the nucleus, activated YB-1 can directly bind to

egfr and *HER-2* promoter to up-regulate the expression of these receptors, which lead to tumour growth (Wu et al. 2006).

These findings motivated us to investigate more deeply into the expression of YB-1 in different subtypes of breast cancer and further understand the prognostic value of this protein.

1.4 Thesis hypothesis

YB-1 is marker of aggressiveness and recurrence in breast cancer. Therefore, it may provide a novel potential target for therapeutic intervention.

1.4.1 Thesis objectives

Objective 1: To determine if YB-1 is a marker of aggressiveness in breast cancer subtypes using Tissue Microarray

Aim 1: To find out if YB-1 expression is correlated with biological subtypes of breast cancer.

Aim 2: To find out if YB-1 expression is correlated with poor survival in subtypes of breast cancer.

Aim 3: To determine if YB-1 is a poor prognostic marker compared to other established markers used in the clinic.

Aim 4: To investigate if YB-1 expression is correlated with earlier time of relapse in subtypes of breast cancer.

Significance

We have previously shown that YB-1 expression is associated with poor outcome in 438 cases of primary breast tumours. However, we have not investigated the correlation of YB-1 expression with individual subtypes of breast cancer, poor survival and relapse in a larger independent cohort (4042 cases of breast cancer). In this study we compare YB-1 with other established makers, which will allow us to assess the effect of YB-1 expression in breast cancer progression and lead us to better individualized therapy.

Objective 2: To understand the molecular pathways connecting YB-1 with poor survival in the aggressive basal-like breast cancers.

Aim 1: To understand new downstream targets of YB-1 which are involved in the aggressive nature of basal-like breast cancers using chromatin immunoprecipitation on chip (ChIP on chip) in SUM149 cells (basal-like breast cancer model).

Aim 2: To validate uPA as one of down stream targets of YB-1 and determine if YB-1 is involved in regulation of uPA production using YB-1 siRNA.

Aim 3: To examine the effect of silencing YB-1 on invasion of breast cancer cells.

Aim 4: To examine the effect of the IGF-1R small molecule inhibitor (BMS- 536924) on uPA and YB-1 expression and investigate the signaling cascade that might be involved.

Significance

We have previously shown that high level of uPA is correlated with metastasis and cancer recurrence. This study will allow us to understand the mechanism that is involved in uPA production, resulting in metastatic spread. Ultimately it may provide evidence for YB-1 as a potential therapeutic target.

Objective 3: To identify tumours that can be benefited from BMS-536924

Aim 1: To determine if IGF-1R is activated in primary tissues using TMA.

Aim 2: To determine if down stream components of phospho-IGF-1R signaling are elevated in primary tissue.

Aim 3: To investigate the expression level of phospho-IGF-1R in the subtypes of breast cancer.

Aim 4: To examine the effect of BMS-536924 on the growth of aggressive basal-like breast cancer cells.

Significance

We have previously reported that IGF-1R expression in primary tumours is a common event however until now we have not had the opportunity to characterize whether or not the receptor is indeed activated in primary tumours. This study may lead us to a predictive tool for prioritizing patients that might benefit from inhibitors of this pathway.

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CHAPTER 2

Epidermal growth factor receptor (EGFR) is transcriptionally induced by the Y-box binding protein-1 (YB-1) and can be inhibited with Iressa in basal-like breast cancer providing a potential target for therapy

INTRODUCTION

Identifying molecular targets for aggressive types of breast cancer is a milestone in the pursuit of individualized therapies. Gene expression profiling of primary tumours has led to the following subcategories: 1) luminal A, 2) luminal B, 3) the human epidermal growth factor receptor-2 (HER-2) and 4) the basal-like subtypes (Sorlie et al. 2003). Our attention was drawn to the basal-like subtype because these tumours do not respond to available targeted therapies and patients often die within two years of diagnosis (Sorlie et al. 2003; Van 't Veer et al. 2002). Approximately 16% of all breast cancers are basal-like (Sorlie et al. 2001); this corresponds to 46,400 women among the ~290,000 women in North America who will be diagnosed with breast cancer each year. What sets these tumours apart is that unlike many breast cancers, basal-like tumours do not express the estrogen receptor (ER) or progesterone receptor (PR), nor do they have amplified HER-2. In the clinic, these tumours are therefore often referred to as "triple negative". Women with "triple negative" tumours are not eligible for effective treatments targeting ER (tamoxifen, aromatase inhibitors) or HER-2 (trastuzumab). Instead they are treated with conventional chemotherapies, which have limited efficacy and numerous side effects. Therefore, it is critically important to identify alternative therapeutic strategies for basal-like breast cancer (BLBC).

We recently found that the transcription factor, Y-box binding protein-1 (YB-1), protein is commonly expressed in ER negative breast cancers (Wu et al. 2006) and loss of this receptor is one of the hallmarks of BLBC (Perou et al. 2000; Sorlie et al. 2001). More recently, YB-1 was pulled out of a screen from the BLBC cell line SUM149 in an attempt to identify genes that promote malignant transformation and tumour cell growth (Berquin et al. 2005). It has also been recently demonstrated that epidermal growth factor receptor

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(EGFR) is highly expressed in approximately 50% of BLBC (Nielsen et al. 2004).

Interestingly YB-1 was originally isolated as a transcription factor that bound to enhancer sites on the EGFR gene, a finding which could explain, at least in part, why it promotes the growth of breast tumour cells (Sakura et al. 1988). In keeping with this possibility, Berquin et al expressed YB-1 in mammary epithelial cells and observed a concomitant induction of EGFR (Berquin et al. 2005). We demonstrated in MCF-7 (ER positive breast cancer cells) that over-expression of YB-1 leads to an elevation in the levels of EGFR mRNA and protein (Wu et al. 2006). This depends upon phosphorylation of YB-1 at S102 (Wu et al. 2006). The YB-1 S102 site is located in the DNA binding domain suggesting to us that the effect on EGFR expression was likely through transcriptional regulation. We demonstrated that Akt binds directly to YB-1 and phosphorylates the S102 site, an observation that was subsequently confirmed in NIH3T3 cells (Evdokimova et al. 2006). We now believe that Akt is one of several kinases capable of phosphorylating the S102 site of YB-1. In support of this idea, inhibition of the kinase mTOR with rapamycin also inhibits YB-1 phosphorylation (Evdokimova et al. 2006). To understand this further, we demonstrated that YB-1 binds directly to the EGFR promoter within the -1kb of the transcription start site and this occurs in a phosphorylation-dependent manner (Wu et al. 2006). Consistent with these preclinical developments, we find that YB-1 is strongly correlated with EGFR in primary breast tumours by screening a tissue microarray of ~490 cases (Wu et al. 2006). More recently, we have confirmed this observation in a cohort of ~2222 primary breast tumours. In this study, YB-1 and EGFR are once again tightly correlated ($p=1.414 \times 10^{-24}$) (data not shown).

Since both YB-1 and EGFR are expressed in BLBC we questioned whether there was a relationship between these two genes in this particular subtype of breast cancer. Firstly, we determined whether the over-expression was due to gene amplification and then further dissected the regulatory relationship between the two. Finally, we addressed whether inhibiting EGFR with Iressa (also referred to as ZD1839 or gefitinib) would slow the growth of BLBC.

MATERIALS AND METHODS

Tumour tissue microarrays and Cluster Analysis

Patients in this cohort and their tumours were previously described by us (Sutherland et al. 2005). The staining conditions for YB-1, HER-2, ER and PR have been previously described (Sutherland et al. 2005). EGFR and CK5/6 staining was performed according to Nielsen et al (Nielsen et al. 2004). In total, we had interpretable data on these proteins from 285/438 total breast cancer cases. For our analysis, YB-1 scored as 0 or 1 was considered negative, and as 2 or 3 was considered positive. Data was filtered to exclude patients who were missing diagnostic or survival information. Results were considered statistically significant with $P<0.05$. The data was analyzed using SPSS software (Chicago, IL).

Comparative Genomic Hybridization

Ten formalin fixed and paraffin embedded archival BLBC cases from the Vancouver General Hospital archival TMA438 series were identified based on a distinct immunohistochemical (IHC) staining pattern (ER-, HER-2-, PR-, CK5/6+). Tissue cores (1.5 mm) extracted from the source blocks were treated with xylene and ethanol as described by Garnis et al (Garnis et al. 2003). Samples were placed into DNA lysis buffer comprised of 10 mM Tris, 50 mM NaCl, 1 mM EDTA, 0.5% SDS placed at 55°C, and digested with Proteinase K (Invitrogen) over a period of 48-72 h. DNA was extracted as previously described, RNase-treated, and quantified by ND-1000 Full Spectrum UV/Vis Spectrophotometer (Nanodrop, Wilmington DE) (Garnis et al. 2003). The ten BLBC specimens were assayed for genetic alterations using a whole genome tiling path bacterial artificial chromosome (BAC) array in comparative genomic hybridization (CGH) experiments as previously described (Shadeo et al. 2006). The submegabase resolution tiling set (SMRT) array contained 32,433 overlapping BACs derived DNA segments that provide tiling coverage over the human physical genome map. All clones were spotted in triplicate resulting in 97,299 elements over two sides. Hybridizations were scanned using a CCD based imaging system (Arrayworx eAuto,

Applied Precision) and analyzed using SoftWoRx Tracker Spot Analysis software as previously described (Chi et al. 2004; Lockwood et al. 2006). Data was filtered and breakpoints were identified as previously described by Baldwin et al. (Baldwin et al. 2005). Clones with standard deviations between replicate spots of >0.075 and with signal-to-noise ratios of <3 were filtered from raw data. Genomic breakpoint boundaries were defined by a CGH-Smooth software and visual inspection. Log 2 signal intensity ratio thresholds were used to determine regions of gain and loss, with >0.5 representing a gain and <-0.5 representing a loss.

Characterization of YB-1 and EGFR in basal-like breast cancer cells in vitro

184htert cells were obtained from Dr. J. Carl Barrett at the National Institute of Health and were cultured as previously described by us (Oh et al. 2002). SUM149 cells, selected because they express markers of BLBC (Bertucci et al. 2005; Charafe-Jauffret et al. 2006), were purchased from Astrand (Ann Arbor, MI) and were grown according to the manufacturer's recommendation. The cells were cultured in F-12 (Ham's) media (Gibco/Invitrogen, Burlington, ON) supplemented with 5μg/ml insulin (Sigma, Oakville, ON) 1μg/ml hydrocortisone (Sigma), 10 mM HEPES (Sigma), 5% fetal bovine serum (Gibco/Invitrogen), and 100 units/ml of penicillin/streptomycin (Gibco/Invitrogen). MDA-MB-468 cells were obtained from the ATCC and cultured in Dulbecco's Modified Eagle's Medium, 10% FBS and 100 units/ml penicillin/streptomycin. HCC1937 breast cancer cells, also triple negative (Jönsson et al. 2007), were cultured in RPMI-1640 media supplemented with 5% FBS, 10 mM HEPES, 4.5g/L glucose (Sigma), 1 mM sodium pyruvate (Sigma) and 100 units/ml penicillin/streptomycin. Cells were maintained at 37°C in 5% CO_2 and passaged every two days.

Proteins were isolated from log growing 184htert, SUM149 and HCC1937 cells using an ELB buffer (Wu et al. 2006). YB-1, EGFR and actin were detected by immunoblotting. The YB-1 polyclonal antibody (generous gift from Dr. Colleen Nelson, University of British Columbia) was used at a dilution of 1:10,000. The EGFR monoclonal (clone 6F1, StressGen, San Diego CA) and actin (Sigma, St Louis MO) antibodies were diluted 1:1000.

Chromatin Immunoprecipitation

SUM149 cells were plated at a density of 1 x 10^7 in a 150mm dish and YB-1 promoter complexes were isolated by chromatin immunoprecipitation (ChIP) as previously described by us (Wu et al. 2006). The primers to each of the potential YB-1 binding sites were previously described (Wu et al. 2006). The EGFR promoter was amplified (40 cycles) using primers that span regions within the first 2kb upstream of the start site. The input DNA was diluted four fold prior to amplification.

Serial ChIP to determine YB-1 phosphorylation status

To determine whether YB-1 is serine phosphorylated at the EGFR promoter, complexes were isolated as described above with the chicken YB-1 antibody and then eluted by incubation in 10 mmol/L DTT at 37°C for 30 min with agitation. The eluate was diluted 1:50 with buffer (20 mmol/L Tris (pH 8.1), 150 mmol/L NaCl, 2 mmol/L EDTA, and 1% Triton X-100) and re-immunoprecipitated with 5μg of anti-phosphoserine antibody (StressGen) overnight at 4°C. Secondary immuno-complexes were incubated with salmon sperm DNA/protein A agarose for 2 h at 4°C. Subsequent steps followed the ChIP protocol described previously by us (Wu et al. 2006) and PCR was performed with primers to the EGFR 2a site as described above. To test for non-specific binding species matched IgY and IgG were incubated with an equal amount of SUM149 cross-linked DNA. The sample was then processed as described above and amplified with primers to EGFR 2a. The input DNA was also introduced as a positive control.

ChIP was also performed using a phospho-YB-1(S102) antibody (in collaboration with Dr Peter Mertens, Germany). The peptide sequence and supportive data demonstrating the specificity of the antibody was recently described by us (To et al. 2007). The immunoprecipitation was carried out as described above for YB-1 with protein G-agarose used in place of PreciPhen beads and rabbit IgG instead of IgY.

Electrophoretic mobility shift assay (EMSA)

Nuclear and cytoplasmic protein was extracted from log-growing SUM149 cells, MDA-MB-468 or HCC1937 cells using the NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford IL) following the manufacturer's protocol.

Briefly, cells were centrifuged to obtain a packed cell volume and lysed in ice cold CER I with protease inhibitors. Following 5 min on ice, ice-cold CER II was added and samples centrifuged at 13000g for 10 min. Cytoplasmic protein was retained and the pellet resuspended in ice-cold NER with protease inhibitors. The sample was incubated on ice for 40 min with frequent mixes and then centrifuged at 13,000g for 10 min. The supernatant containing nuclear protein was stored. Proteins were quantified using the Bradford Assay. EMSA's were carried out using the Lightshift® Chemiluminescent EMSA kit (Pierce Biotechnology), following the manufacturer's protocol. 5' Biotin-labeled complementary oligonucleotides with the following sequences,
Wild-type (-979 to -937)
TTCACACATTGGCTTCAAAGTACCCATGGCTGGTTGCAATAAACAT,
-968 mutant
5'-TTCACAC*CCCCC*GCTTCAAAGTACCCATGGCTGGTTGCAATAAACAT,
-940 mutant
5'- TTCACACATTGGCTTCAAAGTACCCATGGCTGGTTG*CCCCC*AAACAT and
double mutant 5'-
TTCACAC*CCCCC*GCTTCAAAGTACCCATGGCTGGTTG*CCCCC*AAACAT
were annealed to form double stranded DNA. Binding reactions consisted of 1 x binding buffer, 50ng/μl poly dIdC, 20 fmol Biotin-labeled DNA and 5μg nuclear protein in a 20μl reaction. Competition reactions included 16 pmol unlabelled oligonucleotide (800 fold excess) and to determine YB-1 involvement 1μg chicken anti-YB-1 antibody was included. An antibody to Creb (1μg) was introduced as a negative control. The protein was incubated with the unlabelled oligonucleotide or the antibody for 20 min prior to the addition of the biotin-labeled oligonucleotide. The samples were incubated for 20 min at room temperature. The reaction mixture was run on a 6% non-denaturing polyacrylamide gel and transferred to a positively charged nylon membrane (Amersham Biosciences, Little Chalfont, UK). DNA was crosslinked to the membrane at 120mJ/cm^2 using a UV-light crosslinker (Stratalinker, Stratagene) and detected using chemiluminescence (Pierce Biotechnology).

Nuclear extraction of primary BLBC tumours

Tissue slices from 6 BLBC tumour specimens were obtained from the BC Cancer Agency. Nuclear fractions were extracted using the NE-PER nuclear and cytoplasmic extraction reagents as described above. Since tissue was limited the samples were pooled prior to the nuclear extraction step. Electrophoretic mobility shift assays were carried out as described above with 10μg protein.

EGFR Luciferase assay

To determine whether YB-1 has a direct effect on EGFR promoter activity the normal breast cell line, 184htert, was transfected with a 1kb EGFR promoter construct (Nishi et al. 2002) (kind donation from Dr Johnson, NCI, MA), a renilla expression vector, pRL-TK (Promega), and a YB-1 expression plasmid, a YB-1 serine 102 mutant (A102) or empty vector. The cells were plated in 6 well plates (4×10^5 cells/well) and transfected with a total of 1.5μg DNA using lipofectamine 2000 (Invitrogen). Cells were harvested 24 h post-transfection in 1 x PLB buffer (Promega) and luciferase activity measured. All luciferase measurements were normalized to the renilla reading from the same sample. To carry out the inverse experiment the Fast-Forward Protocol provided with the HiPerFect Transfection Reagent (Qiagen, Mississauga ON) was used to achieve knock-down of YB-1 in SUM149 and HCC1937 cells using siRNA (Control and YB-1 siRNA sequences see (Wu et al. 2006)). Briefly, cells were seeded at 4×10^5 cells per well of a 6-well plate in 2ml media shortly before transfection. siRNA was diluted to 100μl in serum free media to achieve a final concentration of 5nM (SUM149) or 20nM (HCC1937), and 3μl HiPerFect was added. Samples were vortexed, incubated at room temperature for 10 min, and then added drop-wise to the cells. At 48 h the cells were re-plated in 6 well plates (4×10^5 cells/well and transfected with the pER1, pRL-TK and empty vector and harvested at 24 h post-transfection as described above. Cell lysates were also collected at the time of re-plating to ensure successful knock-down of YB-1. The experiments were performed in triplicate on two separate occasions. The results are reported as the average of two experiments.

Cell viability following treatment with Iressa

SUM149 breast cancer cells were plated in 96 well plates (5 x 10^3 cells/well) and incubated for 24 h at 37°C in the growth media described above. Cells were treated with Iressa (isolated from tablets purchased from Astra Zeneca and kindly provided by Dr. Ching-Shih Chen, Ohio State University) at the following concentrations; 0, 0.25, 0.5, 1 and 2μM with dimethyl sulphoxide (DMSO) as vehicle control. Cell number was ascertained after 72 h treatment. Cells were washed in PBS and then incubated with Hoechst dye (1μg/ml) for 15 mins. Nuclei counts/well were determined using the ArrayScan VTI high throughput analyser. Statistical analyses were carried out using the Student t test with significance accepted when $p<0.05$.

Growth in soft agar

SUM149 cells were plated at a density of 2.5 x 10^4 in a 24 well plate in 0.6% agar as previously described (Sutherland et al. 2005) and supplemented with Iressa in the cell layer (concentrations as above). HCC1937 cells were treated with CTRL and YB-1 siRNA for 48 hours and then plated at a density of 10 x 10^3 in 0.6% agar. At the time of seeding the agar was supplemented with Iressa (0.25-2μM) as described earlier. Colonies developed over 30 days and were then counted. Each experiment was performed in replicates of four and repeated twice.

EGFR sequencing from SUM149 cells

Genomic DNA was isolated from 2x10^7 SUM149 cells using phenol chloroform extraction followed by alcohol precipitation (modified from (Sambrook et al. 1989)). DNA was quantified in a UV spectrophotometer. EGFR exons 1-28 were amplified by PCR and sequenced using standard techniques employed by the BC Cancer Agency Michael Smith Genome Sciences Centre. PCR primers were designed using human genome reference sequence acquired from the UCSC Genome Browser (Kent et al. 2002) ((http://genome.ucsc.edu/cgi-bin/hgGateway; range=chr7:54860434-55049739)) and the Primer3 program (Rozen et al. 2000). The PCR primer sequences are listed in Table 1 supplemental. Each PCR reaction was performed on 10ng of SUM149 DNA and the products were visualized on a 2% agarose gel. PCR products were cleaned-up using

Ampure magnetic beads (Agencourt) and sequenced using a standard BigDye Terminator v3.1 cycle sequencing chemistry and Applied Biosystems 3730xl DNA Analyzer.

RESULTS

YB-1 and EGFR amplification is not common in BLBC indicating changes in transcriptional control

Breast tumour tissue microarrays were profiled to evaluate the frequency to which EGFR and YB-1 are expressed in triple negative breast cancers. Such tumours express YB-1 and EGFR in 73% and 57.1% of the BLBC cases, respectively (Table 1). Representative immunohistochemical images for both EGFR and YB-1 are shown in Figure 1. As indicated by the arrow-heads YB-1 was expressed in the cytoplasm as well as the nucleus. While we have established that YB-1 and EGFR are frequently expressed in triple negative breast cancers, it is not clear why this occurs. One possibility is that these genes are both amplified during the development of BLBC. To study this, we isolated DNA from ten primary BLBC and evaluated them for copy number changes by array CGH using a genome spanning tiling path array (SMRT) (Ishkanian et al. 2004). Copy number changes were not observed at the YB-1 locus (1p34.2) or the EGFR locus (7p13-11.2) in 10/10 and 9/10 cases, respectively (Figure 2). A borderline 10Mb segmental gain was present in one (referred to as BLC9) of the ten cases at 7p13-11.2 encompassing many gene loci including EGFR (Figure 2). The lung cancer adenocarcinoma cell line (HCC827), known to have amplified EGFR, was evaluated as a positive control (Figure 2). Overall neither YB-1 nor EGFR were commonly amplified suggesting expression is increased due to promoter activation.

YB-1 regulates the expression of EGFR in BLBC

To perform functional investigations into the role of YB-1 and EGFR in BLBC, we tested the SUM149 and HCC1937 cell lines, which have a basal phenotype (Bertucci et al. 2005; Charafe-Jauffret et al. 2006; Jönsson et al. 2007; Tomlinson et al. 1998). Initially the levels of YB-1 and EGFR were compared between 184htrt (immortalized breast epithelial cells) and the cancer cells. SUM149 and HCC1937 cells had high levels

of YB-1 and EGFR compared to the 184htert cells (Figure 3A). Building on the observation that YB-1 binds to the EGFR promoter within -1 kB of the start site (Wu et al. 2006) we then investigated whether there was a causal link between YB-1 and the expression of EGFR in the SUM149 and HCC1937 cells. Firstly, we have determined that YB-1 was able to stimulate EGFR promoter activity using a luciferase reporter construct containing the first 1kB of the EGFR promoter. Immortalized breast cells (184hterts) confirmed to not express YB-1 (Figure 3A) transfected with a hYB-1 plasmid increased EGFR luciferase activity 1.5 fold compared to the control cells ($P = 0.04$, $N = 6$) (Figure 3B). Interestingly, when cells were transfected with the YB-1 mutant (A102) that could no longer be phosphorylated at serine 102 there was a significant attenuation in reporter activity compared to control cells ($P = 0.013$, $N = 6$) (Figure 3B). We then addressed whether silencing the high levels of YB-1 in the SUM149 and HCC1937 cells would attenuate EGFR reporter activity. YB-1 was knocked down with siRNA for 48 h and then transfected with the EGFR reporter. Under these conditions, we observed a 78% and 77% loss in EGFR reporter activity, in SUM149 and HCC1937 cells respectively ($P = 4.53 \times 10^{-5}$ and $P = 5.98 \times 10^{-7}$, $N = 6$) (Figure 3C and 3D). Thus, through gain-of-function and loss-of-function studies we demonstrated that YB-1 transactivates the EGFR promoter and that this occurs in a manner that is dependent on S102 DNA binding site.

Having demonstrated YB-1 can transactivate EGFR we next determined whether YB-1 interacted with the EGFR promoter in the basal-like breast cells to further confirm binding observed in breast cancer cell lines that were not basal-like (Wu et al. 2006), and to address whether this occurs in a manner that is dependent upon S102 phoshorylation using a newly developed antibody directed at YB-1(S102) (To et al. 2007). Using the primer sets previously described by us (Wu et al. 2006) we show that in SUM149 cells YB-1 binds to the EGFR promoter within the first 1kb and most strongly at the 2a site (Figure 4A, lane 2). This interaction is also observed in the basal-like MDA-MB-468 cells which we have previously reported (To et al. 2007). In the SUM149 cells binding did not occur in the regions designated 2b and 3 (Figure 4A, lanes 3-4). We confirmed that binding was specific and did not bind to the IgY alone (Figure 4A, lanes 5-8) and that the primers indeed could amplify genomic input DNA (Figure 4A, lanes 9-13)

compared to the negative controls where no DNA was added to the amplification reaction (Figure 4A, lanes 13-16). This binding pattern is in keeping with our previous work showing that YB-1 binds to the EGFR promoter within the 1kB in a manner that was dependent on phosphorylation at S102 (Wu et al. 2006). Since the phosphorylation status of YB-1 affected its ability to transactivate EGFR we assessed whether this was also the case in the interaction between YB-1 and 2a site of the promoter. We therefore questioned whether YB-1 is serine phosphorylated when it binds to the 2a site. To address this, we initially developed serial ChIP protocol, where YB-1 was initially used to pull-down protein: DNA complexes and then the resulting samples were subsequently immunoprecipitated with an antibody to phospho-serine. Using this method, we were able to show that YB-1 is serine phosphorylated when it binds to the 2a site (Figure 4B). More recently, we have had the opportunity to test a new polyclonal antibody raised against YB-1(S102) specifically (To et al. 2007). In this case, binding to the 2a site is observed as well (Figure 4C) further supporting the idea that YB-1 is serine phosphorylated at S102 when it binds to the EGFR promoter.

The ability of YB-1 to bind to the EGFR promoter specifically at the 2a region was further confirmed using gel shift assays. Nuclear extracts from SUM149, MDA-MB-468 and HCC1937 cells were incubated with a biotin labelled oligonucleotide probe spanning -979 to -934 of the EGFR promoter (Figure 5A). MDA-MB-468 and HCC1937 cells were used as an additional basal-like cancer cell lines as they are "triple negative" and they over-express EGFR. Compared to the unbound probe (Figure 5B, lanes 1, 5 and 10), the introduction of the nuclear extract from both cell lines produced intense binding to the EGFR promoter (Figure 5B, lanes 2, 6 and 11) that could be competitively inhibited with unlabelled probe (Figure 5B, lanes 3, 7 and 12). Co-incubation of the nuclear extract with an YB-1 antibody caused a supershift (Figure 5B, lanes 4, 8 and 13), an effect not observed when an unrelated Creb antibody was used in the same reaction, (Figure 5B, lanes 9 and 14), thus, we validated our ChIP results by demonstrating that YB-1 binds directly to the EGFR promoter. We have also been able to show that YB-1 binds to the 2a region of the EGFR promoter in primary BLBC cancer samples (Figure 5C, lane 2). This interaction could be competed off with unlabelled oligo (Figure 5C, lane 3) and supershifted using the YB-1 antibody (Figure 5C lane 4). To further dissect YB-1

binding within the 2a region we designed biotin-labelled oligonucleotides in which the YB-1 responsive elements (YRE's) were mutated at -968, -940, or both sites (Figure 5A). Losing either of the YRE's resulted in less YB-1 binding compared to the wild-type EGFR promoter sequence (Figure 5D). These data verify that the -968 and -940 binding sites are bona fide YRE's. Together these data show that YB-1 is able to bind to the first 1 kB of the EGFR promoter and this leads to transactivation in a phosphorylation dependent manner.

Inhibiting EGFR suppresses the growth of BLBC cells

Since there are several commercially available EGFR inhibitors available (such as Iressa and erlotinib) we questioned whether targeting this receptor tyrosine kinase would be effective in cells in which it is highly expressed. Monolayer cell growth could be inhibited by up to 40% when SUM149 cells were treated with Iressa (0-2μM) for 72 h (Figure 6A), however, more interestingly, if we grew SUM149 cells in anchorage-independent conditions then formation of colonies, and therefore ability of the cells to transform, was completely abolished when in the presence of as little as 0.25μM Iressa compared to vehicle treated cells (control 1867±363, 0.25-2μM Iressa 0±0) (Figure 6B). These concentrations are achievable in patients (Herbst et al. 2002) and have previously been shown to inhibit MAP kinase signalling (Noro et al. 2006). To confirm this observation, we also found that low doses of Iressa inhibited signalling through the MAP kinase pathway (data not shown). To ascertain whether this sensitivity was inherent to other BLBC cell lines we repeated the same experiment in HCC1937 cells and somewhat surprisingly these cells were still able to form colonies in anchorage-independent conditions in the presence of up to 2μM Iressa. Similarly, the MDA-MB-468 basal-like breast cancer cells are insensitive to Iressa initially but can be sensitized by targeting PI3 kinase with LY294002 (She et al. 2003); an observation that we independently confirmed (data not shown). In a separate study, LY294002 has been shown to inhibit phosphorylation of YB-1 (Evdokimova et al. 2006). This is in keeping with our previous studies demonstrating that YB-1 is phosphorylated by Akt in response to PI3 kinase activation (Sutherland et al. 2005). We therefore questioned whether knocking down YB-1 in HCC1937 cells prior to treating with Iressa would be effective at reducing the

ability of these cells to grow in soft agar. The suppression of YB-1 alone caused a 42% reduction in the number of colonies compared to control ($P = 0.0008$) but there was further significant decreases in colony number with the addition of as little as 0.25μM Iressa ($P<0.001$ for all concentratons)(Figure 6C). Thus, our studies indicate that although some BLBC cells may be sensitive to Iressa, for others the inhibition of YB-1 may be necessary to sensitize the cells to drug.

We were rather surprised that the SUM149 cells were so sensitive to the drug. An obvious explanation would be that these cells express activating mutations in EGFR that would make them sensitive to Iressa as has been described in lung cancer (Lynch et al. 2004). We therefore sequenced EGFR but unexpectedly did not find such mutations. All 28 exons coding for this gene were amplified by PCR and sequenced. Activating mutations such as L858R or delL747-P753insS previously reported to be associated with Iressa sensitivity (Lynch et al. 2004) were not found. However, we did identify 5 single nucleotide polymorphisms (SNPs) in exons 12, 13, 15 and 20 (Table 2, supplemental). There was a homozygous non-translated SNP (rs712830), three heterozygous synonymous SNPs (rs17290005, rs17290162, rs17337198), and 1 heterozygous non-synonymous SNP (rs11543848). These dbSNP's have been previously identified for EGFR ((http://www.ensembl.org)) although as of yet their functional significance is not known. The SNP of most interest is R521K located on exon 13 because it results in an amino acid change located in the extracellular domain of the receptor (Ogiso et al. 2002).

We concluded that irrespective of "activating mutations" in EGFR, Iressa inhibits the growth of basal-like breast cancer cells. In some cases, co-targeting EGFR and YB-1 may be necessary to optimally inhibit the growth of these aggressive breast cancer cells. Given these data, we concluded that inhibiting EGFR and YB-1 significantly slows the growth of BLBC cells.

DISCUSSION

It has previously been reported that both YB-1 and EGFR are highly expressed in aggressive forms of breast cancer (Nielsen et al. 2004; Wu et al. 2006). In this study we demonstrate that while these proteins are a feature of BLBC neither gene is over-

expressed due to amplification. In further studying YB-1 as a transcription factor, we show that it transcriptionally induces EGFR in basal-like cell lines, which could lead to the elevated expression that is observed. Importantly, we have been able to pinpoint that YB-1 binds specifically to YRE's located at -968 and -940. Upon precisely identifying the bona fide YRE's on the EGFR promoter, we demonstrate for the first time that binding to this region occurs when YB-1 is phosphorylated at S102. The high levels of both EGFR and YB-1 in BLBC opens the question of either of them being potential therapeutic targets. Based on the poor survival rates previously reported (Sorlie et al. 2003; Van 't Veer et al. 2002) it is clear that the BLBC subtype represents a very aggressive form of the disease and EGFR is a rational target for treatment of BLBC. In fact, since it was reportedly associated with this subtype of breast cancer in 2004 (Nielsen et al. 2004) the use of EGFR in classifying basal-like tumours by immunohistochemistry has become widely accepted (Carey et al. 2006; Rodriguez-Pinilla et al. 2006).

We demonstrate for the first time that the EGFR inhibitor Iressa suppresses the growth of SUM149 cells, a model for BLBC, *in vitro* at concentrations achievable in patients (Herbst et al. 2002). This is not the case for other BLBC models as no inhibition of anchorage-independent growth was evident in the HCC1937 cells when they were treated with Iressa alone. This insensitivity is also reported in MDA-MB-468s (She et al. 2003) and MDA-MB-231 cells, another triple negative cell line with high levels of EGFR expression (Campiglio et al. 2004; Takabatake et al. 2006). Why the SUM149 cells alone are sensitive to the drug is not clear. Several studies suggest that activating mutations in EGFR are predictive of whether inhibitors, such as Iressa, would be effective in lung cancer patients (Lynch et al. 2004; Sordella et al. 2004). The same could be true for breast cancer yet it is not known whether BLBC harbour such mutations. We did however sequence the entire EGFR gene from SUM149 cells and did not find activating mutations previously described for lung cancer. Whether the SNP at R521K influences sensitivity to Iressa is not known, warranting further investigation. Another factor that may influence the sensitivity to EGFR inhibitors is the level of expression of the target itself and also presence of alterations in downstream signalling independent of receptor activation. For example, both the HCC1937 (Jönsson et al. 2007) and MDA-MB-468 cells (Li et al. 1997) are PTEN null resulting in enhanced propogation of the PI3-

kinase pathway. She et al have previously shown that by inhibiting the PI3-kinase pathway with LY294002 they can sensitize cells to Iressa (She et al. 2003) and similarly in this study we find that by suppressing the expression of YB-1, which is downstream of phospho-Akt (Sutherland et al. 2005), using siRNA in the HCC1937 cells we also enhance the effect of Iressa. The question as to why YB-1 sensitizes BLBC cells to Iressa is an interesting one. YB-1 has been shown to regulate the MDR-1 gene (Ohga et al. 1996) (Ohga et al. 1998) and thus the P-glycoprotein pump, a member of the ABC family of transporters. This pump is involved in the efflux of many drugs and has been associated with resistance to many chemotherapeutic agents (Kuwano et al. 2003). We recently performed a ChIP on chip analysis of YB-1 target genes in SUM149 cells in our lab and identified ~15 ABC transporter family members that were putatively bound by YB-1 including ABCG2, ABCA5 and ABCC3. Studies carried out by Özvegy-Laczka et al showed that multidrug transporters such as ABCG2 may be involved in the resistance to tyrosine kinase inhibitors such as Iressa by modulating the uptake and extrusion of these drugs to and from cells (Özvegy-Laczka et al. 2004). In fact, they specifically show that ABCG2, but not mutant ABCG2, protects the lung cancer cell line A431 from Iressa induced growth inhibition (Elkind et al. 2005). A more recent study (Li et al. 2007) also confirms these findings with demonstration of decreased intracellular accumulation of low concentrations of Iressa (0.1μM-1μM) and higher efflux with 1μM Iressa. Although further work would be required to ascertain the mechanism involved, the suppression of YB-1 expression could therefore indirectly increase the levels of these inhibitors in the cells allowing them to bind to their target and reduce cell growth.

Not withstanding that SUM149 cells are sensitive to Iressa suggesting that some BLBC may be also, we recognize that acquired resistance to inhibitors such as Iressa is a common problem. There are numerous studies, which implicate over-activation of alternative signalling pathways, such as the insulin-like growth factor-1 pathway (Morgillo et al. 2007) and MET receptor amplification leading to activation of ErbB3-AKT pathway (Morgillo et al. 2007). Alternatively, downstream pathways can become constitutively activated, an example being k-ras, which has been reported in lung and colon cancers (Aviel-Ronen et al. 2006; Janmaat et al. 2006; Rosell et al. 2006; Taron et al. 2005). Given this problem of acquired resistance, and the fact that many BLBC

cases will not be sensitive, using Iressa in combination with an inhibitor for a downstream component may provide more long-term benefits.

While we have established an association between YB-1 and EGFR in BLBC it is likely that this transcription factor regulates the expression of other proteins linked to BLBC. For example, YB-1 regulates proliferating cell nuclear antigen (PCNA) and topoisomerase II alpha (Kohno et al. 2003) both of which are expressed in BLBC (Perreard et al. 2006). In colorectal carcinomas, YB-1 and topoisomerase II alpha are co-ordinately expressed (Shibao et al. 1999). Likewise, similar expression patterns are reported in lung cancer (Gu et al. 2001) and synovial sarcomas (Oda et al. 2003). More direct evidence for this association is supported by Shibao et al who reported that knocking down YB-1 with antisense attenuates topoisomerase II alpha reporter activity (Shibao et al. 1999). These and other YB-1 target genes are yet to be confirmed in BLBC. If indeed PCNA and topoisomerase II alpha are YB-1 responsive genes in this type of cancer it would explain why the expression of this transcription factor is clearly associated with poor survival based on work previously done by us (Wu et al. 2006), and others (Bargou et al. 1997). There are currently no commercially available inhibitors to YB-1. However, since YB-1 transactivates many growth-promoting genes and we have shown that it can enhance sensitivity to approved agents in BLBC, the question as to whether it would also be a potent therapeutic target this aggressive type of breast cancer is being actively pursued in our laboratory.

We conclude from our data that YB-1 plays a role in EGFR gene expression in BLBC. Furthermore, we demonstrate that tumour cell growth can be attenuated by blocking YB-1's target gene EGFR, alone or in combination with YB-1 inhibition, providing new possibilities for the treatment of this highly aggressive disease.

FIGURES

Table 1. YB-1 is highly expressed in triple negative breast cancer. YB-1 is expressed in 73% of triple negative breast cancers in the TMA438. EGFR is expressed in 57.1% of these cases.

Marker	Correlation	Likelihood Ratio Value
YB-1	**$p=3.899 \times 10^{-4}$** **n=27/37 (73%)**	**12.58**
EGFR	**$P=9.206 \times 10^{-12}$** **n=20/37 (57.1%)**	**46.491**

Table 1 supplemental. PCR Primers for 28 exons of EGFR. Forward primer sequences were prefixed with a -21M13 sequencing tag, TGTAAAACGACGGCCAGT and reverse primer sequences were prefixed with an M13R sequencing tag, CAGGAAACAGCTATGAC. The primers (-21M13 and M13R) were then used in the corresponding sequencing reaction.

Table 1 supplemental.

Exon	Annealing Temperature, °C (T_{ann})	Forward Primer Sequence	Reverse Primer Sequence	Product Length (with seq tags)
1	60	tcgcattctcctcctctct	cgcagctgatctcaaggaa	622
2	57	tggaccttgagggattgttt	ccaggcctttctccacttag	329
3	59	tcgtgtgcattagggttcaa	ttctccgaggtggaattgag	428
4	59	tgcatccttcatgggaattt	cccagtgctgtagagctgtc	300
5	60	agccagccaaacaatcagag	aactgcatgcggtgagattt	412
6	60	catgaaaaagtctgcaagtgct	aagtcttctgtcctggtgtgg	322
7	60	gctttctgacgggagtcaac	aggagacagagcgggacaag	334
8	58	ccatcacccctcaagaggac	gaggaagatgtgttcctttgg	333
9	55	gcctgtggatccctagctatt	ctgaaacaaacaacagggtga	331
10	55	gtcacaggttcagttgcttgt	gggaacaggaaatatgtcgaa	285
11	58	ccctgagagtctagagtaatgtctcat	tctcctgttaagcctaatttcca	280
12	56	tcaatcaaaggtggtctgga	aaatgggaatagcccttcaa	414
13	61	caaggtcatggagcacagg	aacaacaacctggagcctta	333
14	56	ggtgatttgtgttcctgcaa	tcatcactgttcggcttctg	262
15	60	atcatttggctttccccact	acaaacctcggcaatttgtt	407
16	56	caacatccagacacatagtgatttt	gtcagaaatgcaggaaagca	300
17	60	gccaaggccatggaatct	aactgctaatggcccgttct	322
18	60	gtgtcctggcacccaagc	ccccaccagaccatgaga	340
19	60	cagcatgtggcaccatctc	cagagcagctgccagacat	273
20	60	cattcatgcgtcttcacctg	catatccccatggcaaactc	412
21	60	agccataagtcctcgacgtg	acccagaatgtctggagagc	372
22	56	agactgaaatcccctgttgc	tcagtacaatagatagacagcaa tga	435
23	60	gaagcaaattgcccaagact	atttctccagggatgcaaag	413
24	58	gcaatgccatctttatcatttc	gctggcatgtgacagaacac	281
25	59	agacccctgctcctatagcc	ccatgtgagtttcactagatggtt	379
26	57	cctgcattcaggaaaagtgg	ggaaaaacccacacaggaag	284
27	56	caaggagatctcgggtga	gagatgctggagggagcac	329
28	62	atcctgcatgggatggtg	gtggcttggtcctgggtat	517

Table 2 supplemental. Sequence analysis of EGFR from the SUM149 cells. Variants were identified in exons 1, 12, 13, 15, and 20. The variants in exons 12, 13, 15 and 20 relate to SNP's that have been previously reported for EGFR.

EGFR Variants Detected in SUM149 Cell Line

Exon #	Variants			Interpretation
	bp # within exon	bp # within transcript	Amino Acid #	
1	A56CC	A56CC	Not translated	SNP: rs712830
2	No variants			
3	No variants			
4	No variants			
5	No variants			
6	No variants			
7	No variants			
8	No variants			
9	No variants			
10	No variants			
11	No variants			
12	A19AG	A1563AG	A439A	SNP: rs17290005
13	G64AG	G1808AG	R521K	SNP: rs11543848
14	No variants			
15	G66AG	G2034AG	P596P	SNP: rs17290162
16	No variants			
17	No variants			
18	No variants			
19	No variants			
20	G78AG	G2607AG	Q787Q	SNP: rs17337198
21	No variants			
22	No variants			
23	No variants			
24	No variants			
25	No variants			
26	No variants			
27	No variants			
28	No variants			

These variants can be viewed using Ensembl:
http://www.ensembl.org/Homo_sapiens/transview?db=core;transcript=ENST00000275493

Figure 1. YB-1 and EGFR are detected in basal-like breast cancer specimens on a tumour tissue microarray. A) EGFR negative staining (40X). B) Brown cells indicate EGFR positivity (40X), a segment of the core is magnified at 200X. C) YB-1 negative staining (40X). D) Brown staining indicates YB-1 positivity (40X) which is detected in both the nucleus and cytoplasm (arrowheads 200X).

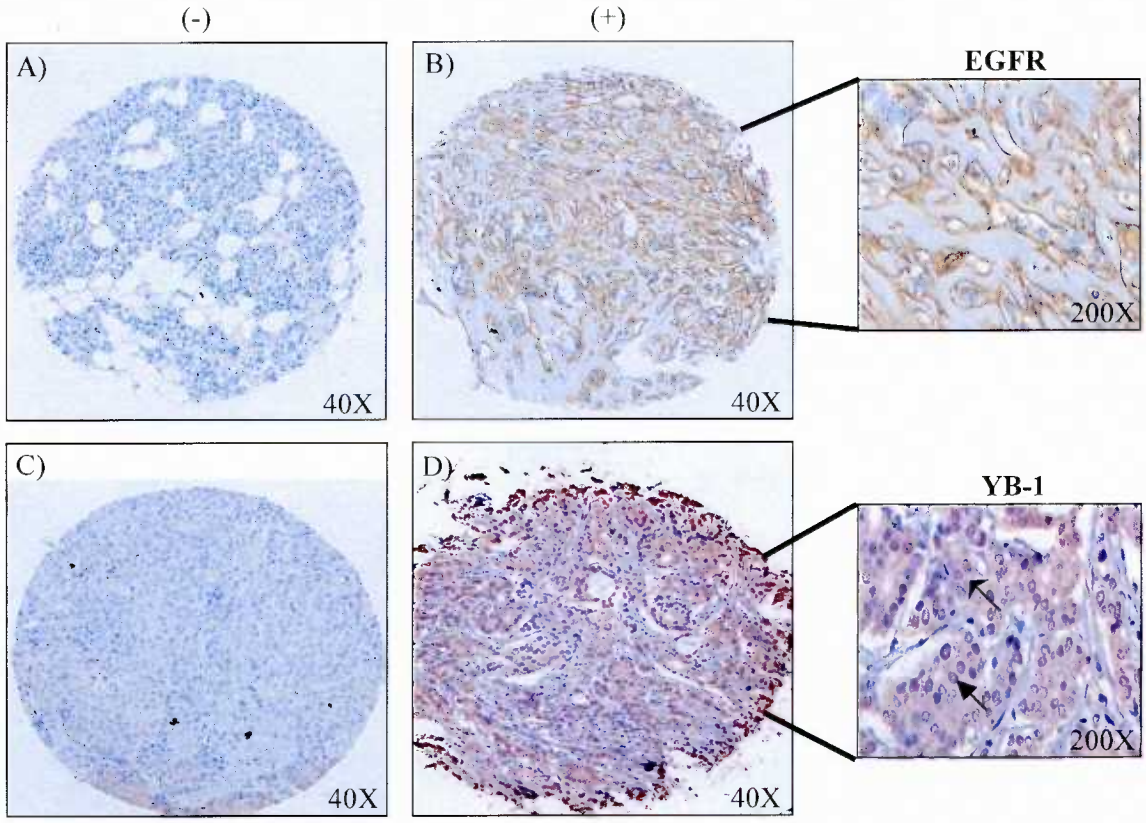


Figure 2. Basal-like breast tumours do not exhibit amplifications for YB-1 or EGFR. Primary breast tumours were evaluated for genetic amplifications using SMRT array CGH. DNA was isolated from ten primary basal-like breast tumours and genomic profiles were generated by SMRT array CGH. There was no obvious gain of copy number on chromosomes 1, or 7 representing the loci for YB-1, and EGFR respectively. The exception to this trend was BLC9 where there was a large amplicon on chromosome 7. The lung adenocarcinoma cell line HCC827 was included as a positive control of EGFR amplification.

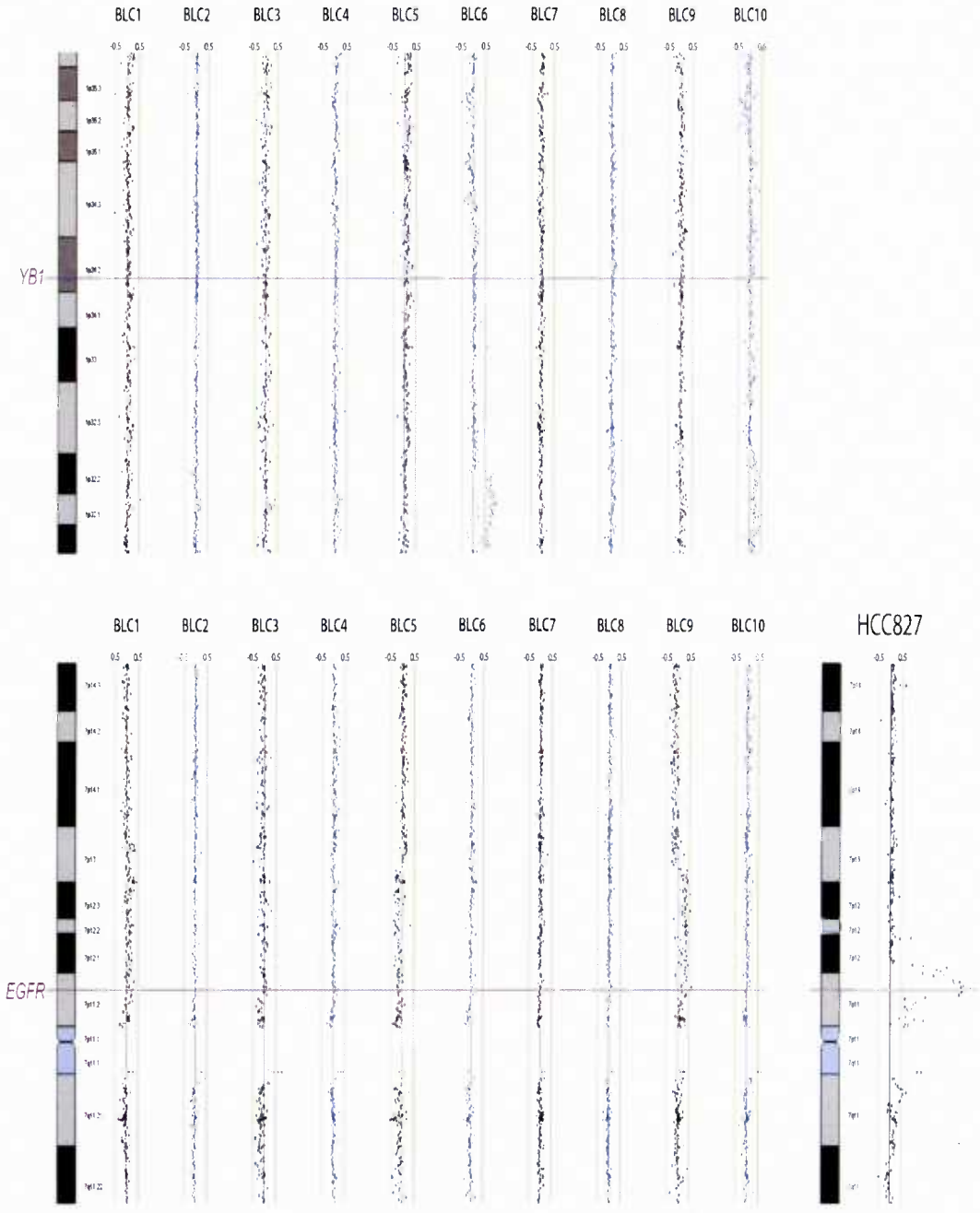
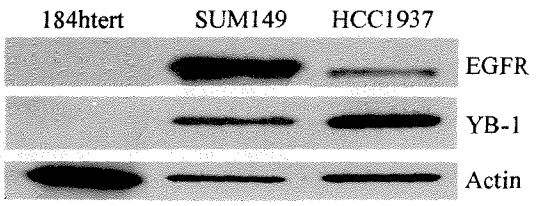
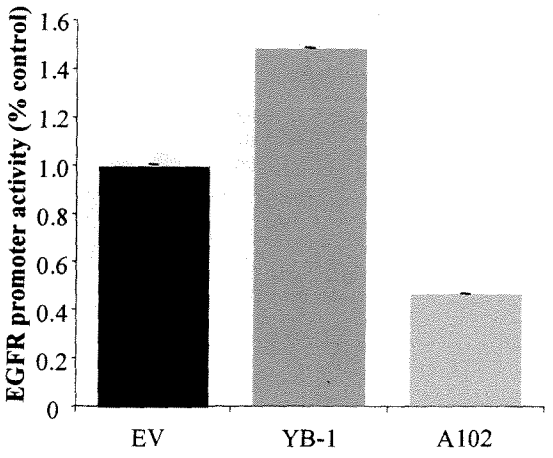


Figure 3. YB-1 regulates the expression of EGFR in basal-like breast cancer cells. **A)** The levels of YB-1 and EGFR proteins were compared between immortalized breast epithelial cells, 184htert, SUM149 and HCC1937 basal-like breast cancer cells. Actin was evaluated as a control for equal protein input. **B)** 184htert cells were transfected with an EGFR promoter (-1kb) luciferase construct (pER1), a control renilla plasmid (pRL-TK) and either flag-EV or flag-YB-1 or flag-YB-1(A102). Luciferase and renilla activity were measured after 24 hours. YB-1 induced EGFR promoter activity by 1.5 fold ($P = 0.04$, $N = 6$) whereas the A102 mutant did not. **C)** SUM149 cells were treated with YB-1 siRNA (5nM) for 48 hours. The cells were then transfected with the EGFR reporter for 24 hours and compared to the empty vector. Loss of YB-1 expression resulted in a 78% decrease in EGFR reporter activity ($P = 4.53 \times 10^{-5}$, $N = 6$). **Inset.** Evidence that siRNA targeting YB-1 cause a decrease in expression of the protein. Actin was used as a loading control. **D)** The same experiment was repeated using HCC1937 cells treated with 20nM YB-1 siRNA for 48 hours. Loss of YB-1 expression resulted in a 77% reduction in EGFR promoter activity ($P = 5.98 \times 10^{-7}$, $N = 6$)

A.



B.



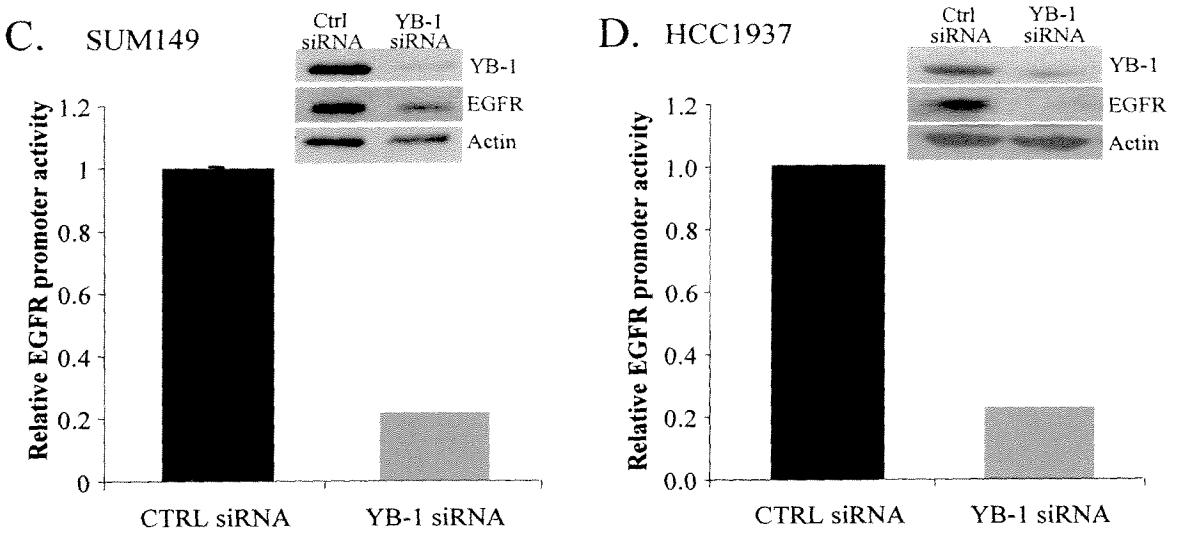
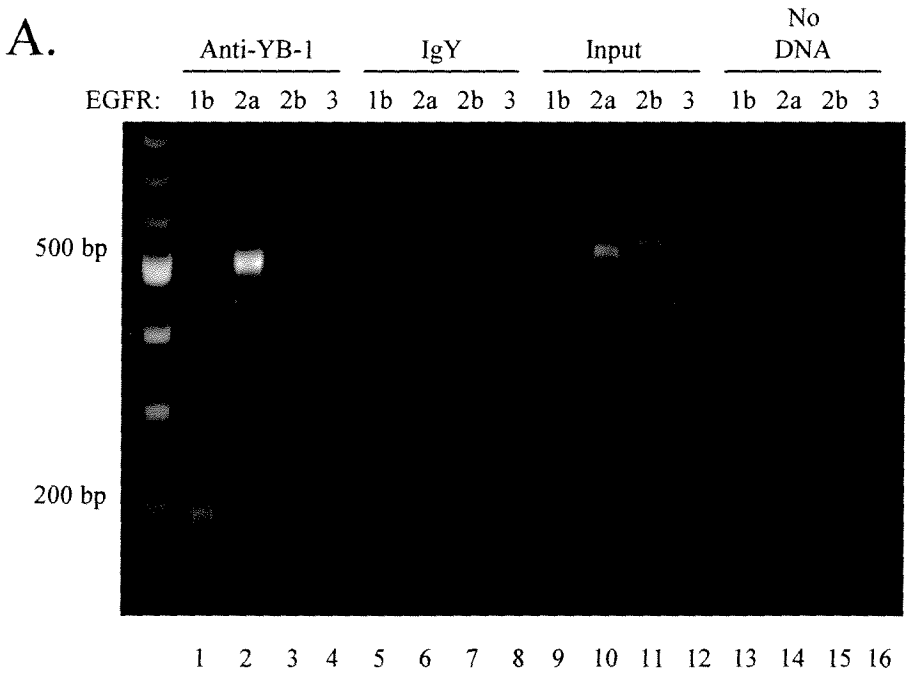
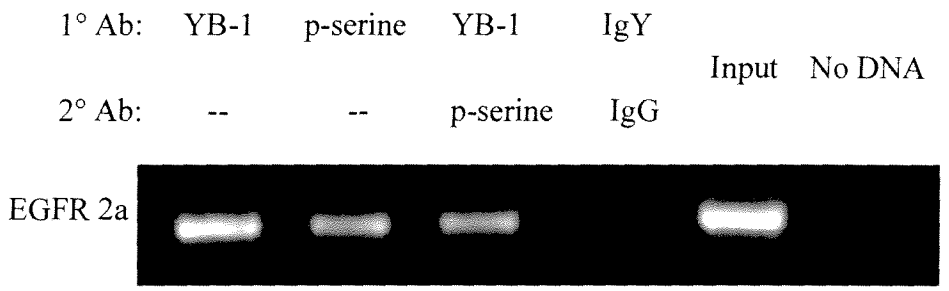

C.
SUM149
Ctrl siRNA
YB-1 siRNA
YB-1
EGFR
Actin
Relative EGFR promoter activity
1.2
1
0.8
0.6
0.4
0.2
0
CTRL siRNA
YB-1 siRNA
D.
HCC1937
Ctrl siRNA
YB-1 siRNA
YB-1
EGFR
Actin
Relative EGFR promoter activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CTRL siRNA
YB-1 siRNA

Figure 4. YB-1 binds to the EGFR promoter. A) Chromatin immunoprecipitation was performed on SUM149 cells. YB-1 binds to the EGFR promoter in the basal-like cells where the 2a loci is the preferred binding site (lane 2). Weak binding was also detected with the 1b primers (lane 1). No binding was observed in the 2b or 3 sites (lanes 3-4) nor was there any non-specific binding detected in the IgY negative controls (lanes 5-8). Input DNA was diluted 4 fold and amplified to demonstrate that the primer produced an expected product (lanes 9-12). The no input controls (lanes 13-16) are presented to show a lack of non-specific amplifications. **B)** Serial ChIP was performed by sequentially pulling down YB-1 and then immunoprecipitating with a phospho-serine antibody. This demonstrated that at least some of the YB-1 is serine phosphorylated when bound to EGFR 2a site. YB-1 binds to the 2a site (lane 1) as expected. Similarly, the p-serine antibody pulls down a complex that can be amplified with the 2a primers (lane 2). Re-ChIP with the YB-1 antibody and subsequently with the p-serine antibody also bound to EGFR at the 2a site (lane 3). A phospho-ser YB-1 complex bound to the 2a site on EGFR (lane 3). Species matched IgG and IgY controls were included to show that the binding was specific lane 4). The input DNA and no DNA controls were also included (lanes 5-6). **C)** ChIP was carried out using a phospho-YB-1 antibody (Ser 102) and binding was detected for the EGFR 2a region (lane 4). There was no binding observed when immunoprecipitation was performed using IgG as a control (lane 3). Input DNA was diluted 4 fold and amplified to demonstrate that the primer produced an expected product (lanes 5-6). Lane 1 is the DNA ladder.



B.


1° Ab: YB-1 p-serine YB-1 IgY
Input No DNA
2° Ab: -- -- p-serine IgG
EGFR 2a

C.

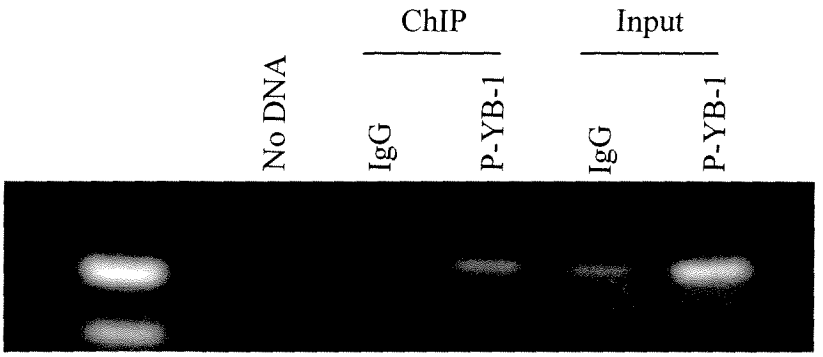
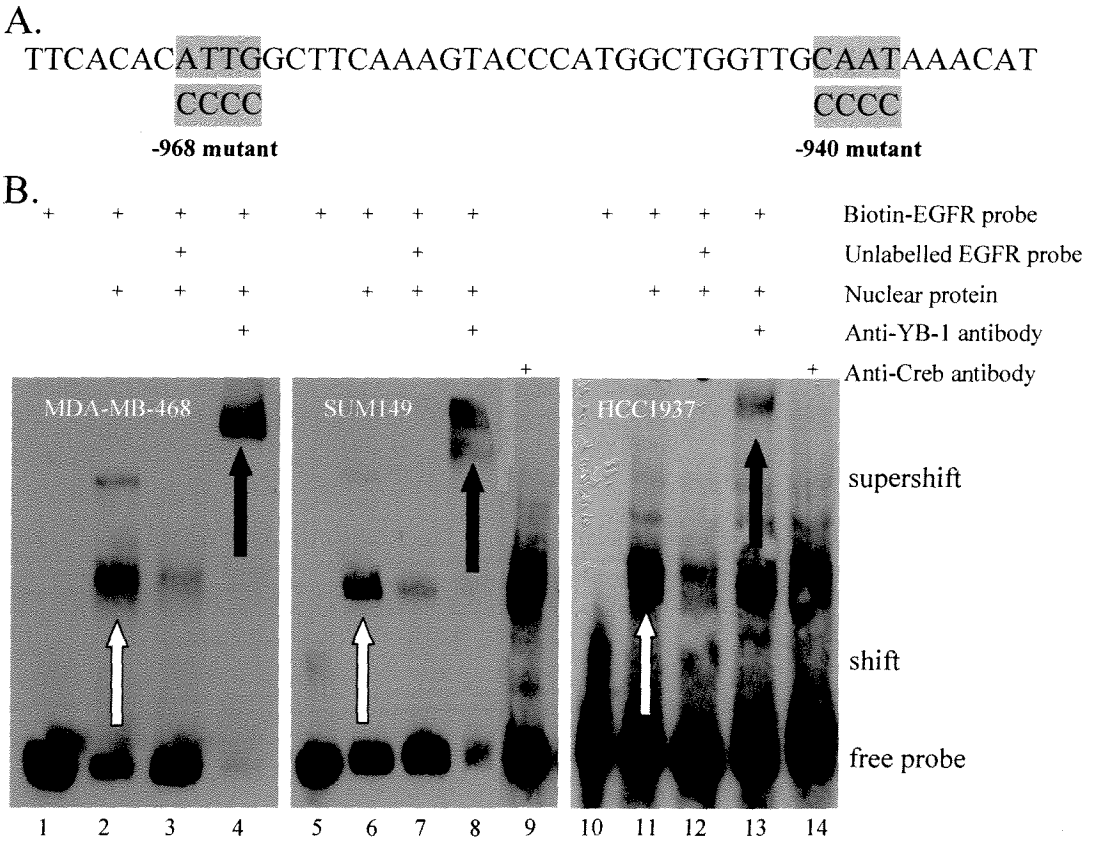
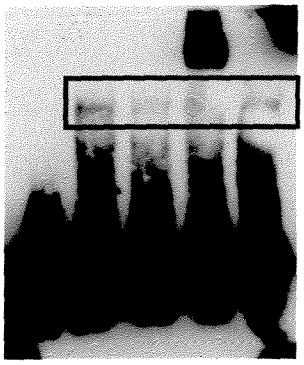

ChIP
Input
No DNA
IgG
P-YB-1
IgG
P-YB-1

Figure 5.YB-1 binds to specific sites within the EGFR promoter. A) Sequence of the EGFR2a oligonucleotide used in the gel shift assays (-979 to -934). Highlighted sequences are the potential YB-1 binding sites. The substitutions made in the two mutants are given under the wild-type sequence. **B)** Direct evidence for YB-1 binding to the EGFR promoter using gel shift assays. Nuclear extract from SUM149, MDA-MB-468 or HCC1937 cells were incubated in the presence of the EGFR oligonucleotide spanning -979 to -934. In the absence of protein, there was no binding (lanes 1, 5 and 10) while the addition of the nuclear extract (lanes 2, 6 and 11) resulted in strong binding that could be inhibited with the unlabelled oligonucleotide (lanes 3, 7 and 12). The addition of an YB-1 antibody elicited a supershift (lane 4, 8 and 13) that did not occur when the non-related Creb antibody was used instead (lanes 9 and 14). C) Nuclear extract from 6 primary BLBC samples were pooled and used in a gel shift assay for the EGFR 2a site. Lane 1 contains EGFR2a biotin-labelled oligo only. Binding to the probe is evident in lane 2, which was competed off in lane 3 and supershifted with a YB-1 antibody in lane 4. A creb antibody was used to demonstrate specificity of the supershift (lane 5). **D)** Validation of putative YRE's in on the EGFR promoter. SUM149 nuclear extracts were incubated with either wild-type (lane 1) or mutant biotin oligo nucleotides (lanes 3, 4, and 5). A competition reaction was carried out against the wild-type (lane 2). Nuclear extract bound to the wild-type sequence (lane 1) but was unable to bind the mutants (lanes 3, 4 and 5).



C.

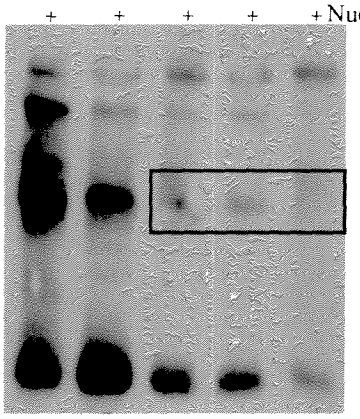
+	+	+	+	Biotin-EGFR probe
		+		Unlabelled EGFR probe
	+	+	+	Nuclear protein
			+	Anti-YB-1 antibody
				+ Anti-Creb antibody



1 2 3 4 5

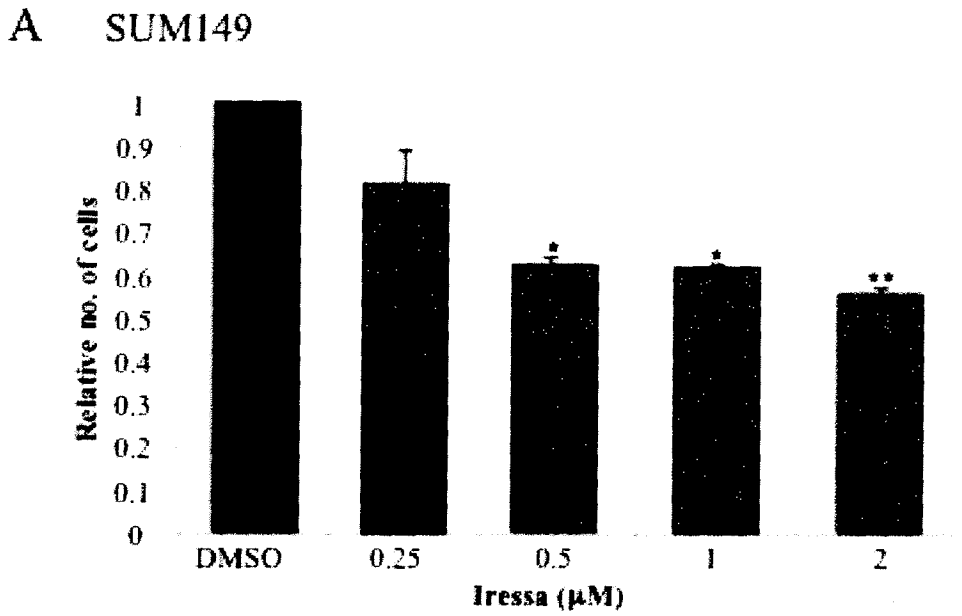
D.

+	+	+	+	+ Biotin-labelled probe
	+			Unlabelled probe
+	+	+	+	+ Nuclear protein

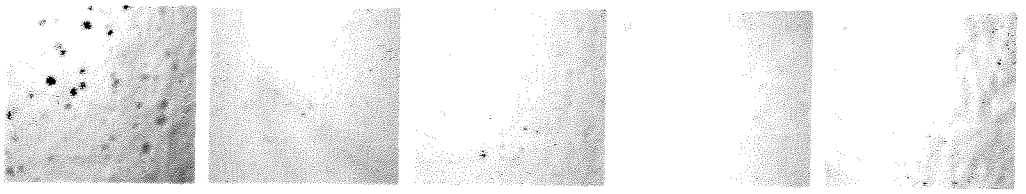


Wild-type -968 mutant -940 mutant Double mutant

Figure 6. Inhibiting EGFR suppresses the growth of basal-like breast cancer cells. **A)** Inhibition of EGFR with Iressa (0.25, 0.5, 1 and 2 μM) blocks the growth of basal-like breast cancer cells by up to 40% when the cells were treated for 72h (0.5μM P = 0.02, 1μM P = 0.02, 2μM P = 0.07). Each experiment was performed in replicates of six on two separate occasions. **B)** Anchorage-independent growth was measured by counting colonies formed after 4 weeks exposure to Iressa or vehicle control. Representative images of colonies following each treatment are shown with average colony number/well shown underneath. The ability to form colonies was completely lost in the presence of concentrations of Iressa as low as 0.25μM. **C)** The ability of HCC1937 cells to form colonies was not effected by Iressa alone, however, knockdown of YB-1 significantly reduced the number of colonies (P<0.001). The addition of Iressa further reduced the number of colonies. This was highly significant at all concentrations (P<0.001). Statistical analysis carried out using students t-test; * p<0.05, ** p<0.01.

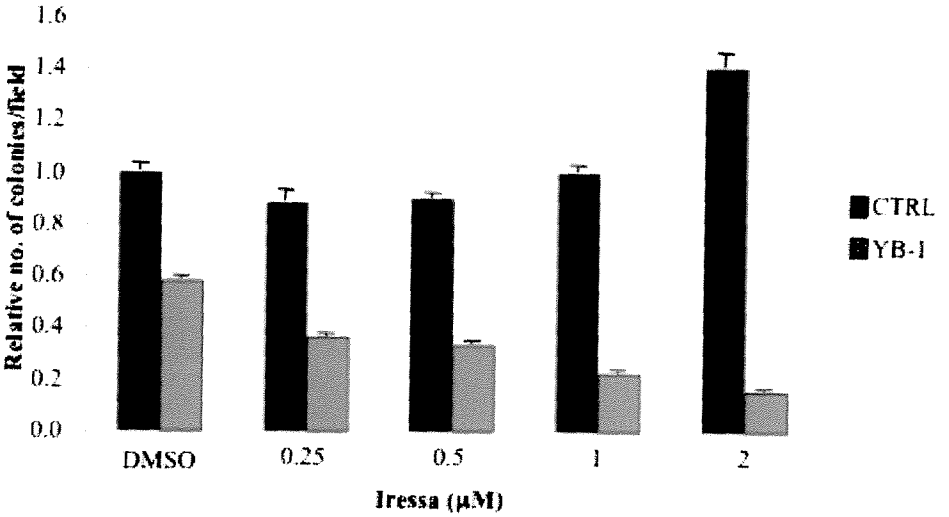


B SUM149



Iressa	DMSO	0.25μM	0.5μM	1μM	2μM
Average colonies/well	1867±363	0±0	0±0	0±0	0±0

C HCC1937



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CHAPTER 3

The Y-box binding protein-1 (YB-1) defines aggressive forms of breast cancer representing a new opportunity for targeted therapy.

INTRODUCTION

Breast cancers have recently been subdivided into four subtypes: luminal A, luminal B, basal-like and HER-2 over-expressing, based on their gene expression signatures (Sorlie et al. 2001). From these and other studies, over-expression of the human epidermal growth factor receptor-2 (HER-2) is clearly associated with a very aggressive pathogenesis (Sorlie et al. 2001). The strength of HER-2 as a marker of poor prognosis, and the fact that tumours depend upon this factor for survival has led to the development of several targeted inhibitors, such as trastuzumab, pertuzumab and more recently lapatinib. These agents have shown some promise, but have also demonstrated utility in only a limited number of patients. For example, trastuzumab used as a single agent benefited only ~30% of patients with metastatic disease (Vogel et al. 2002). In addition, initial responses are frequently accompanied by the subsequent emergence of resistant tumours. Thus, the principle of developing targeted therapies has been established for human breast cancer, but additional approaches are clearly required.

The Y-box binding protein-1 (YB-1) is a transcription and translation factor that has been reported to promote tumour growth and render cancer cells resistant to chemotherapy (Wu et al. 2007). YB-1 induces growth-promoting genes such as HER-2 (Wu et al. 2006), EGFR (Wu et al. 2006), proliferating cell nuclear antigen (Ise et al. 1999), the multi-drug resistance-1 gene (Bargou et al. 1997), and cyclin A (Jurchott et al. 2003) and B (Jurchott et al. 2003). YB-1 is activated by kinases such as Akt (Sutherland et al. 2005) that has also been linked to breast cancer (Wu et al. 2007). Finally, forced expression of YB-1 in the mammary gland in mice induces tumour formation with 100% incidence suggesting that YB-1 can act experimentally as an oncogene (Bergmann et al. 2005).

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What is not known at this time is whether YB-1 carries prognostic value for specific types of breast cancer. In a pilot study, we recently reported that YB-1 was expressed in more than 70% of triple negative breast cancers (chapter 2) however this study was limited by a lack of sufficient size to determine patient survival or recurrence. This encouraging study prompted us to examine the expression of YB-1 in a much larger cohort that had the additional benefits of including patients with defined treatment procedures (ie no chemotherapy or Tamoxifen alone). To address these questions, we screened a large tissue microarray (TMA) series consisting of 4049 invasive breast cancers with 20-years of clinical follow-up. An equally challenging question is whether breast cancers typically associated with the worst prognosis are indeed dependent upon YB-1 for continued growth and survival. In order to address this question we determined the effect of inhibiting YB-1 using small interfering RNAs on the growth *in vitro* and *in vivo* of aggressive models of human breast cancer.

MATERIALS AND METHODS

TMAs

The study cohort and construction of the TMAs used here has been described previously (Cheang et al. 2006; Rajput et al. 2007). Sections from were cut at 4 μm and immunostained with a rabbit polyclonal anti-YB-1 antibody (1:1,400) (a gift from Dr. Colleen Nelson, University of British Columbia, Vancouver, BC). The immunohistochemical staining was performed using an Automated Secondary System (Ventana, Tucson, AZ, cat # 760-4205). Slides were stained concurrently for estrogen receptor (ER), HER-2, and progesterone receptor (PR) using standard immunoperoxidase techniques as previously described (Cheang et al. 2006; Rajput et al. 2007). Breast cancer subtypes were determined using immunohistochemical markers (HER-2, ER and PR) to define each type. Subtype definitions were as follows: luminal A (ER+ and/or PR+, HER2–), luminal B (ER+ and/or PR+, HER2+), triple negative (ER– PR–, HER2–), HER2+ (ER–, PR–, and HER2+), and unassigned (missing data any of the 3 markers). TMAs were evaluated independently by two pathologists (Dr. Hamid Masoudi, Dr.

Dimithry Turbin) to quantify the percentage of tumour cells that showed positive YB-1 and ER staining as follows: negative (<1%); positive: 1+ (1-25%), positive: 2+ (25-75%), or positive: 3+ (>75%). Of the 4049 samples, 3097 could be scored for YB-1 and had breast cancer-specific survival (BCSS) outcome data. In addition, 3101 cases could be scored for YB-1 had data regarding their relapse survival outcome. Immunohistochemical data was collected on samples that were blinded as to clinical outcomes.

Statistical analysis was performed using SPSS software version 13.0 (SPSS Inc, Chicago, IL) and was described previously (Cheang et al. 2006). In brief, for univariate analyses, BCSS and relapse free survival (RFS) was estimated using Kaplan-Meier curves. Survival differences were determined by Breslow tests. For BCSS, survival time was censored at death if the cause was not breast cancer or if the patient was still alive at the end of the study. Six patients with an unknown cause of death were excluded from the BCSS analysis. In addition, relapse free survival time was also censored at death if the cause was not breast cancer and if the patients were alive without any relapse at the end the study. For multivariate analyses, Cox proportional hazards models were used to calculate adjusted hazard ratios (HRs) accounting for independent co-variates. A likelihood ratio was used to determine the extent of association of YB-1 expression with particular subtypes. Spearman's correlation was also used to determine the extent of correlation between YB-1 expression and other markers such as ER.

Cell preparation and transfection

BT474-m1 cells were grown in 50% F-12/50% Dulbecco's modified essential medium (DMEM) (Gibco/Invitrogen, Burlington, ON) supplemented with 5% fetal bovine serum (FBS, Gibco/Invitrogen). SUM149 cells were purchased from Astrand (Ann Arbor, MI) and were grown as recommended by the supplier in F-12 medium supplemented with 5 μg/ml insulin (Sigma, Oakville, ON), 1 μg/ml hydrocortisone (Sigma), 10 mM HEPES (Sigma), 5% FBS and 100 units/ml of penicillin/streptomycin (Gibco/Invitrogen). MDA-MB-468, MDA-MB-231, and MDA-MB-453 cells were obtained from the American Type Culture Collection (Rockwood, MD) and cultured in DMEM plus 10% FBS and 100 units/ml penicillin/streptomycin. The immortalized breast epithelial cells, 184htrt, were grown as previously described by us (Oh et al. 2002).

Cells were maintained at 37°C in an atmosphere of 5% CO_2 in air and subcultured every two days.

For the siRNA transfections, BT474-m1 cells were plated with $3x10^5$cells per well in a 6 well dish and the next day they were transfected with 5 nM of control or siRNA oligonucleotides (Dharmacon, IL) according to manufacturer's protocol. The sequence for siYB-1#1 was as previously reported (Wu et al. 2006). A second siRNA oligonucleotide referred to as siYB-1#2 was designed using the following sequence (CCACGCAAUUACCAGCAAAdTdT) and matched to a scrambled control oligonucleotide (UUC UCC GAA CGU GUC ACG UdT dT). In brief, control or siRNA (5 nM) were diluted in serum-free media and HiPerfect (Qiagen, MD) transfection reagent was added to the diluted siRNA solution. The mixture was gently vortexed for a few seconds and incubated at room temperature for 5-10 minutes. The complex was then added to the cells in each well and the plates incubated at 37°C, in a 5% CO_2 incubator before harvesting the cells for subsequent studies.

Western blotting

Cells were harvested by scraping and lysed in ELB buffer (5 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA (pH 8.0), 1% Triton-X100, 1% deoxycholate and 0.1% SDS) with protease and phosphatase inhibitors. The lysates were sheared by passage through a 21-gauge needle and centrifuged at 13,000 rpm for 10 minutes at 4°C. The lysates were electrophoresed (50 μg) in 12% SDS polyacrylamide gels at 45V and transferred to nitrocellulose membranes overnight at 4°C. The membranes were then probed with the following antibodies: YB-1 (1:10,000), EGFR (1:1000; StressGen, San Diego, CA), HER-2 (1:1000; AbCam, Cambridge, UK), P-ERK1/2$^{T202/Y204}$ (1:1000; Cell Signaling Technology; Danvers, MA), P-Akt^{S473}, GSTb^{S9}, P-Stat3^{S727}, P-Stat3^{Y705}, Stat3, MCL-1 (Santa Cruz), and survivin (all diluted 1:1000, CST) and anti-P-H2AX^{S139} (1:500, AbCam). Vinculin (1:2000; clone Vin 11-5, V4505 antibody from Sigma) and pan-actin (1:1000; CST) antibodies were used as loading controls.

MTS Assays

One day after transfection, 5000 BT474-m1 cells were seeded into each well of a 96-well plate. The cells were subjected to the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) at different time-points to evaluate cell viability. For a 96-well plate, 2 ml of MTS was mixed with 100 μl of PMS and this solution was added to 9.8 ml of serum-free media. 120 μl of this mixture was added to each well. The cells were incubated in the dark at 37°C, in a 5% CO_2 incubator for 2 hours before the absorbance at 490 nm of each well was determined.

High content screening assays

A high content screen was performed on 184htrt, BT474-m1, SUM149, HCC1937, MDA-MB-468, MDA-MD-453, and MDA-MB-231 to assess whether or not inhibiting YB-1 would suppress their growth in monolayer. All cell lines were plated at a density of 8000 cells/well except the SUM149 cells, which were plated at 5000 cells/well. The cells were plated in 150 μl of medium at the stated density in Collagen I coated 96 well plates (Falcon, BD) and allowed to attach overnight. The cells were then transfected with either the scrambled oligonucleotide or siYB-1#2 (5nM) according to the manufacturer's instruction (HiPerfect®, Qiagen), and 50 μl of siRNA/transfection complex was added to each well to give a final concentration of 5 nM siRNA in 200 μl medium. The plates were then incubated for 72 hr at 37°C with 5% CO_2. The degree of YB-1 knock down was assessed by immunofluorescence. In brief, the medium was aspirated at the end of siRNA treatment, and the cells were fixed in 2% paraformaldehyde in PBS for 20min. The cells were then permeablized in 0.1% Triton-100 in PBS for 15 min. After blocking in 1% BSA in PBS for 30 min, the cells were incubated with the primary rabbit anti-YB1 antibody for 1h at RT followed by the secondary mouse anti-rabbit antibody (FITC labeled, Jackson ImmunoResearch) for 1h at RT. The nuclei of the cells were stained with Hoechst (1 μg/ml) for 5 min. The cells were washed with PBS three times after each of the above steps and were finally kept in PBS (100μl/well) at 4 °C. The plates were analyzed and the images taken on the ArrayScan VTI (Cellomics, Pittsburgh). The degree of cell growth was measured by Hoechst staining (To et al. 2007). There are six replicates for each treatment and the

experiment was repeated 2-3 times. Because the HCC1937, MDA-MB-468 and MDA-MB-231 cells were more difficult to transfect with siYB-1 these cell lines were plated in a six-well dish and treated with siYB-1 (20nM) for 48 hours and then replated into a 96 well dish. Changes in cell growth were assessed after 72 hours as described above.

A high content screen was performed on BT474-m1 to assess whether or not inhibiting YB-1 would suppress their growth when combined with Taxol. Cells were plated at a density of 8000 cells/well on Collagen I coated 96 well plates (Falcon, BD) and allowed to attach overnight. The cells were then transfected with either the scrambled oligonucleotide or siYB-1#2 (5nM) according to the manufacturer's instruction (HiPerfect®, Qiagen), and 50 μl of siRNA/transfection complex was added to each well to give a final concentration of 5 nM siRNA in 200 μl medium. The plates were then incubated for 48 hours at 37°C with 5% CO_2 and then treated with Taxol (0.1, 1.0 and 10 nM) for 48 hours. After siRNA treatment the sample plates were processed for nuclear condensation using Hoechst (To et al. 2007). All cells in each well were scanned and analyzed. There are six replicates for each treatment and the experiment was repeated 2-3 times.

Apoptosis assays

BT474-m1 cells were treated with either scrambled control, siYB-1#1 or siYB-1#2 YB-1 oligonucleotides over a time course of 1-4 days. The floating and attached cells were harvested and then analyzed for P-H2AX^{S139} (1:500 dilution) by immunoblotting. Evidence for apoptosis was further investigated in the cultures of siYB-1 treated cells by staining cells with either propidium iodide (PI) or Annexin V. For PI staining, all cells were collected from each well (both unattached and attached), washed once with 1% FBS-containing phosphate buffered saline (PBS) and the cells were then stained with 30 μg/ml of PI (Sigma) in 1% FBS-containing PBS for 3-5 minutes. The cells were then centrifuged and resuspended in 500 μl of 1% FBS-containing PBS before being analyzed on a FACSCalibur (BD). For Annexin V staining, the manufacturer's protocol was followed. In brief, the harvested cells were washed twice with cold PBS and 1x binding buffer containing Annexin V-PE and 7AAD dyes were added to the cell pellets. The cell pellets were then gently vortexed for a few seconds before being

incubated in the dark at room temperature for 15 minutes. 1X Binding buffer (250 μl) was then added to each sample and within an hour the cells were analyzed by flow cytometry on a FACSCalibur (BD).

Pathway evaluation of apoptosis induction

To understand the pathway leading to the induction of apoptosis, cell extracts from siYB-1 treated cells were immunoblotted for P-Erk1/2$^{T202/Y204}$, mTOR, P-mTOR, P-Stat3^{S727}, P-Stat3^{Y705}, Stat3, and MCL-1. From these experiments, it was not clear which pathway could be responsible for changes in Stat3 phosphorylation therefore, BT474-m1 cells (50,000/6 well) were treated with Rapamycin (18 hours, 40 nM) or PD98059 (6 hours, 20 μM) to inhibit mTOR or MEK respectively. The proteins were evaluated by immunoblotting for changes in P-Stat3^{S727}, MCL-1, P-S6 (CST) or P-Erk1/2$^{T202/Y204}$.

Stat3C rescue experiments

Constitutively active Flag:Stat3C (Bromberg et al. 1999) was transiently transfected into BT-474 m1 cells (5x10^4/6 well) for 48 hours using Lipofectamine 2000 1:3 DNA:lipofectamine ratio. The Flag: Stat3C plasmid was introduced at increasing concentrations (0.5, 1.0 and 2 μg) and comparisons were made to the RcCMV empty vector at the same concentration. Recombinant Flag: Stat3C was detected using by immunoblotting 50 μg of total protein with the M2 antibody against Flag (Sigma Chemical Co.). The blot was reprobed for MCL-1 to illustrate that increasing Flag: Stat3C was functional. Following the optimization of Flag: Stat3C, the cells were transfected as above with 2 μg of the plasmids and then treated with siYB-1#2 (5 nM) or scrambled control (5nM) for 96 hours whereby the proteins were isolated for expression of P-H2AX^{S139}, MCL-1, Flag: Stat3 (M2 antibody, Sigma) and YB-1 by immunoblotting.

Soft agar assays

The bottom layer was prepared by mixing 2x DMEM with a solution of 1.2% agarose in water in a 1:1 ratio. Two ml of this mixture was then added to each well of a 6-well plate. Top layers were prepared by adding 1x10^4 BT474-m1 cells, SUM149 cells

or MDA-MB-468 cells to a 1:1 mixture of 2x DMEM and a solution of 0.6% agarose in water.

Inhibition of YB-1 in vivo

BT474-m1 cells were transfected with 5 nM of control or siRNA oligonucleotides and the cells were harvested by trypsinization (0.25% typsin-EDTA; Invitrogen, Burlington, ON). Cells were washed with Hanks Balanced Salt Solution twice and counted to prepare $1x10^6$ cells, which were subsequently mixed with matrigel (BD) in 1:1 ratio of total volume 200 µl. This mixture was injected subcutaneously into the right lower hind flank of female six-*nu/nu* mice per treatment group using a 26-gauge needle. Tumour growth was measured using calipers. Measurements of width (a), length (b) and depth (c) were taken twice weekly. Tumour volumes were calculated according to the equation $4/3\pi$ (a/2xb/2xc/2). Animal body weight measurements were also taken at that time. Harvested tumours were snap-frozen in liquid nitrogen at 4 weeks post-injection.

RESULTS

YB-1 expression is associated with earlier cancer recurrence in subtypes of human breast cancers defined by hormone receptors and HER-2 status

YB-1 was detected by immunostaining in 41% (n=1644/4049 cases) of the patient samples for which 20 years of follow-up clinical data was available, and at a higher frequency (~65%) in the triple negative breast cancer and HER-2-positive subtypes (Figure 1A and data not shown). YB-1 staining was also consistently associated with disease recurrence and shorter survival independent of breast cancer subtype as defined by ER, progesterone receptor (PR), and HER-2 expression, (Figure 1A and B), possibly because of a strong negative correlation between ER and YB-1 expression within and across subtypes (Supplementary Figure 1A and B and Supplementary Table 1). These associations were confirmed using two Cox regression models (Table 1 and Supplementary Tables 1A and B), one of which further showed that YB-1 expression posed an even greater hazard risk (HR=1.456, $p=5.6x10^{-7}$) than tumor grade (HR=1.416,

$p=8.9x10^{-6}$), patient age (HR=1.160, p=0.05), ER status (HR=0.816, p= 0.038) or HER-2 expression (HR=1.259, p=0.012) in this series (Table 1A and B).

Evidence that YB-1 expression might identify a subset of patients requiring more aggressive treatment was obtained by analysis of the 437 (34%) YB-1-positive tumours in a low-risk subset of 1292 cases with small tumours and/or no nodal involvement (T1N0). These cases were also associated with an increased risk of breast cancer-specific death (Figure 2A), as was seen in the 185 (22%) YB-1-positive cases identified in another subset of 856 ER-positive tumors treated with tamoxifen (Figure 2B). In addition, YB-1 expression was a significant adverse prognostic biomarker in patients that did not receive chemotherapy (HR=1.311, $p=1.7x10^{-6}$, Supplementary Table 3) but not in cases that were treated with tamoxifen (Cox regression analysis, data not shown). Taken together, these data indicate YB-1 expression may be a valuable independent biomarker for the most aggressive forms of breast cancer across all subtypes.

Aggressive breast cancer cells are dependent upon YB-1 for survival

Given the strength of YB-1 as a prognostic marker we addressed whether tumour cells depend upon it for sustained grow. If they are, YB-1 could become a new molecular target for breast cancer. To approach this, we inhibited YB-1 with small interfering RNAs (siRNA) in the HER-2 over-expressing BT474-m1 cells. The oligonucleotides suppressed YB-1 expression 2 days post-transfection and continued to inhibit it for up to 14 days (Figure 3A). We also noted that after 14 days of exposure siYB-1#2 still inhibited YB-1 by ~50% (Figure 3A). The loss of YB-1 expression decreased HER-2 and EGFR expression with a resultant loss of signaling through the MAP kinase pathway (Figure 3B). In contrast, there was no effect in the siRNA-transfected cells on the levels of P-Akt^{S473} or P-$GSK\beta^{S9}$ (Figure 3B). YB-1 inhibition also affected the ability of the cells to grow in monolayer cultures with siYB-1#2 having a more robust inhibitory effect (Figure 3C). To further this we developed a high content screening assay to monitor the potential growth inhibitory effect of siYB-1#2 on a panel of breast cancer cell lines. Each cell line was exposed to 5-20nM siYB-1 or the equivalent amount of scrambled control for 72 hours. To ensure that the knock down was effective, levels of YB-1 were imaged by immunofluorscence while cell number was enumerated by Hoechst staining.

To illustrate this, the siYB-1#2 decreased YB-1 by more than half in the SUM149 and MDA-MB-231 basal-like breast cancer cells (Figure 3D, top panel). The loss of YB-1 correlated with a 60% inhibition of tumour cell growth (Figure 3D, bottom panel). Similarly, the HCC1937 basal-like breast cancer cells were exquisitely sensitive to YB-1 inhibition as were the BT474-m1 cells (Figure 3D). The normal immortalized breast epithelial cells, 184htrt and the breast cancer cell lines, MDA-MB-453, and MDA-MB-468 cells were not nearly as sensitive under these conditions. It therefore appears that inhibiting YB-1 in most breast cancer cell lines inhibits growth by at least 50%. Next we questioned whether inhibitingYB-1 with Taxol would enhance cell killing. Taxol was selected because it is routinely used in the clinic for the treatment of advanced breast cancer. BT474-m1 cells were treated with siYB-1#2 for two days and then exposed to increasing concentrations of Taxol (0.1-10 nM). Taxol alone inhibited the growth of the cells by only 20% at doses under 10 nM yet the combination of siYB-1#2 brought growth inhibition up to 80% (Figure 3E). Thus, we developed HCS screens to show that the growth inhibition of YB-1 can be generalized to most breast cancer cell lines. This platform could also be used to screen combinations of agents. As such, we show that inhibiting YB-1 improved the inhibitory effect of Taxol.

During the HCS screens it was noted that the nuclei were often fragmented following long-term exposure to siYB-1 suggesting that it also triggers apoptosis in addition to having a growth suppressive effect. We therefore investigated whether knocking down YB-1 engaged programmed cell death initially by examining H2AX phosphorylation in BT474-m1 cells (Figure 4A). After two days, siYB-#2 induced high levels of P-H2AX^{S139} that increased out to day 4. Treatment with siYB-1#1 had a similar effect but caused less P-H2AX^{S139} initially. To further confirm the induction of apoptosis, we observed an 8 and 30-fold increase in propidium iodide incorporation with both siYB-1#1 and with siYB-1#2 respectively following exposure for three days (Figure 4B). The degree of annexin V staining was also increased following treatment with either of the siYB-1 oligonucleotides for the same amount of time (Figure 4C, top panel). During the conducted time course (1-4 days) the annexin V staining was up to 6-fold higher than in the siYB-1#2 compared to the cell treated with the scrambled control while it was on average 3-fold higher in cells exposed to siYB-1#1. To further confirm these

results we developed a high content screening assay to monitor chromatin condensation, propidium iodide uptake and phosphorylation of $H2AX^{S139}$. BT474-m1 cells exposed to either of the YB-1 siRNA's caused an induction of apoptosis after 72 hours (Figure 4D). Based on these studies, we find that knocking down YB-1 suppressed the tumour cell growth in several models of aggressive breast cancer. Furthermore, inhibiting it induced apoptosis, an endpoint that is highly desirable for the development of a new therapeutic target for cancer.

Evidence that YB-1 inhibitory effect is mediated by the mTOR/Stat3/MCL-1 pathway

To understand why cells undergo apoptosis, we turned to the signal transducer and activator of transcripton 3 (Stat3) pro-survival pathway because it is phosphorylated at S727 in breast cancer (Yeh et al. 2006), it lies down-stream of HER-2 (Fernandes et al. 1999) EGFR (Garcia et al. 1997) and it protects these from cells from death (Gritsko et al. 2006) in part through the induction of MCL-1 (Huang 2007). We determined that inhibiting YB-1 with siYB-1#2 correlates with a decrease in $P\text{-}mTOR^{S2448}$, total m-TOR, $P\text{-}Stat3^{S727}$ and MCL-1 while $P\text{-}Stat3^{Y705}$ was undetectable (Fig 4E). This was somewhat surprising because the link between YB-1 and the Stat3 pathway had never previously been reported. Moreover, it introduced the possibility that phosphorylation of Stat3 at S727 was sufficient for activation of this transcription factor in the absence of Y705 phosphorylation. We confirmed the absence of Y705 phosphorylation by comparing the BT-474-m1 cells to the MDA-MB-231 cells which are known to express high levels of Stat3Y705 (Berishaj et al. 2007) (data not shown).

It has previously been reported that mTOR (Yokogami et al. 2000) and Erk1/2 phosphorylate $Stat3^{S727}$ (Wen et al. 1995). In keeping with this, we found that inhibiting either mTOR or MEK with Rapamycin (40 nM) or PD98059 (20 μM) suppressed $Stat3^{S727}$. These inhibitors also caused a decrease in MCL-1 (Figure 4F). Collectively, our data indicates that inhibiting YB-1 has a major bearing on expression of the EGFR and HER-2 receptors leading to a decrease in signaling through the MEK/mTOR/Stat-3/MCL-1 pathway (Schematic, Figure 4F). We therefore reasoned that adding back Stat3 could rescue the effects of YB-1 induced cell death. To investigate this possibility a

constitutively active form of Stat3 known as Stat3C (Bromberg et al. 1999) was introduced in an attempt to rescue the YB-1 induced apoptosis. Initially we verified that Stat3C induced MCL-1 expression as expected following transient transfection into the BT474-m1 cells (Fig 4G). We then rescued the induction of apoptosis by siYB-1#2 by introducing Stat3C as indicated by a decrease in P-H2AX^{S139} (Figure 4H). The decrease in apoptosis was consistent with the induction of MCL-1 by Stat3C (Figure 4H). These data suggested that Stat3 is down-stream of the YB-1 pro-survival pathway.

Suppression of YB-1 inhibits tumourigenic properties of breast cancer cells

The growth suppressive effect of inhibiting YB-1 *in vitro* led us to question whether it could block tumour growth in mice. To begin to study this, we showed that knocking down YB-1 with siRNA inhibited the growth of BT474-m1 cells in soft agar by 70-90% (Figure 5A). This was achieved with either siRNA. Similarly, inhibition of YB-1 with siRNA suppressed the growth of SUM149 and MDA-MB-468 cells in soft agar by ~50-75% (Figure 5B). This was an exciting finding because it suggested that inhibiting YB-1 could suppress tumour growth *in vivo*. To test this idea, BT474-m1 cells were transfected with 5nM of either scrambled control, siYB-1#1, or siYB-1#2 then harvested the cells after 24 hours and $1x10^{-6}$ cells were injected into the rear hind flank of BalbC nu/nu mice (n=6 animals). Tumour development was then assessed over three weeks. Remarkably, inhibition of YB-1 expression suppressed the growth of BT474-m1 cells in mice for the first two weeks (Figure 5C-D). There was an impressive suppression in tumour development in the first week where 5 of the 6 mice injected with cells treated with the scrambled control siRNA developed tumours, whereas only 2 of the 6 mice that received siYB-1#1 developed tumours in the same time frame. Knock-down of YB-1 expression with siYB-1#2 had an even more impressive effect resulting in only 1 of the 6 mice injected with these cells developing a tumour after a week (Figure 5C). After 2 weeks all control mice developed tumours, whereas, the mice injected with siYB-1#1 or siYB-1#2-treated cells still showed a reduced tumour incidence (3 of 6 and 1 of 6, respectively). By the third week, smaller but detectable tumours were apparent in 4 of 6 and 5 of 6 mice injected with siYB-1#1 and in siYB-1#2-treated cells, respectively (Figures 5C). At the termination of the experiment, the tumours that arose from the mice

pre-treated with siRNA's against YB-1 were notably smaller (Figure 5D-E). We suspected that the mice eventually developed small tumours because YB-1 was not inhibited in 100% of the cells or that the siRNA had a limited half-life of approximately 14 days *in* vivo based on the characterization of the oligonucleotides *in vitro*. To determine whether the delayed appearance of the tumours derived from siRNA-treated cells might have been due to the eventual loss of the siRNAs, the harvested tumours were assessed for YB-1 expression. The results showed this to be the case with no difference in YB-1 expression between the tumours that eventually arose from the treated cells and the controls (data not shown). Because the siRNAs have a limited half-life we attempted to stably express a short-hairpin inhibitory RNA to YB-1 that was cloned into an expression plasmid. The shYB-1 was transfected into the BT-474-m1 cells however we were not able to establish stable clones because the loss of YB-1 killed the cells. Conversely, cells transfected with the empty vector were as viable as the untransfected controls. We concluded that YB-1 was essential for these cells further supporting our idea that it could be a molecular target for cancer. It also indicates that HER-2 over-expressing breast cancers could be addicted to YB-1.

DISCUSSION

Our data indicates that YB-1 is a strong prognostic marker for all subtypes of human breast cancer with a particularly high frequency expression in ER-negative TNP and HER-2 breast cancer subtypes. We also show that the survival and growth of aggressive models of breast cancer are YB-1-dependent suggesting a new strategy for the targeted therapy of breast cancers that currently cannot be treated effectively. Our study supports preliminary clinical data from others that also suggests that YB-1 may define a high-risk group of breast cancer patients. Notably, Janz et al (Janz et al.) showed that YB-1 was expressed in 49% (20/42) of the cases receiving chemotherapy and was suggestive of a trend towards a poor disease free survival although their data did not reach statistical significance. The same authors also examined YB-1 expression in a cohort of 42 low-risk cancers not treated with chemotherapy and found that YB-1 was expressed in 76% (32/42) of cases. However none of the patients that had low YB-1

expression relapsed; however, 30% of those with high levels did ($p<0.011$). As well, Huang et al reported in a study of 42 patients that YB-1 was associated with recurrence following adjuvant chemotherapy although long-term follow-up were not evaluated (Huang et al. 2005). We now provide definitive retrospective data indicating YB-1 expression in low-risk patients to be strongly associated with higher rates of breast cancer-related deaths. Importantly, YB-1 was a significant factor in a Cox regression model when other variables that typically define risk were taken into consideration. In this analysis, the prognostic value of YB-1 was comparable to tumour size and was superior to HER-2, ER, age or grade as a prognostic marker.

In the past few years, several new approaches have been proposed to identify patients at high risk. For example, diagnostic tests have been developed to stratify patients based upon gene expression and are currently being prospectively evaluated. The first test, Mammaprint®, stratifies node-negative breast cancer patients based on a set of 70 genes using cDNA microarrays (Glas et al. 2006; Van de Vijver et al. 2002). A quantitative PCR-based method called Oncotype Dx® is commercially available and appears to define a group of low-risk patients who may be able to avoid chemotherapy. We propose that YB-1 could be monitored in a low-cost manner by immunohistochemistry, just as HER-2 and ER proteins are currently evaluated clinically. It should be noted we have already optimized the detection of YB-1 using an automated immunostaining system thus this new biomarkers could easily be translated in routine clinical practice for diagnostic purposes.

Development of YB-1 inhibitors could also be of interest, given the demonstrated dependency of many breast cancer cells on it. Here provide the first pre-clinical evidence that YB-1 may be essential for the viability and growth of aggressive forms of breast cancer both *in vitro* and *in vivo*. This is due in part to the ability of YB-1 to protect these cells against apoptosis. Since YB-1 is highly expressed not only in breast cancer but also in a wide range of adult and pediatric malignancies (Wu et al. 2007), it may be of broad interest as a cancer pathway target. In a recent study, a short hairpin RNA against YB-1 was used to inhibit the growth of melanoma cells although the mechanism of this effect was not investigated (Schittek et al. 2007). Here we provide evidence of cell death by apoptosis caused by an inhibition of the mTOR/Stat3/MCL-1 pro-survival pathway.

Stat3 is an oncogenic transcription factor (Bromberg et al. 1999) that is phosphorylated at S727 by mTOR (Yokogami et al. 2000). Following cytokine stimulation, Stat3 is also phosphorylated at Y705 (Wen et al. 1995) leading to co-operation with S727 for maximal transcriptional activation. However, this does not seem to be consistent for all stimuli in that Notch signaling leads to P-Stat3^{S727} in the absence of P-Stat3^{Y705} (Androutsellis-Theotokis et al. 2006). Similarly, P-Stat3^{S727} is essential for the survival of macrophages in the absence of P-Stat3^{Y705} (Liu et al. 2003). Upon activation by cytokines and growth factors, Stat3 protects cells against apoptosis by inducing genes such as MCL-1, Bcl-xl, c-myc and survivin (Buettner et al. 2002). Following YB-1 knock-down, we determined that only MCL-1 was decreased following P-Stat3^{S727} loss while there was no change in survivin; and Bcl-xl was not detectable in our cells (data not shown). This is consistent with a study showing that silencing Stat3 in breast cancer cells induces apoptosis (Gritsko et al. 2006), although in this instance a loss of survivin rather than MCL-1 was noted, perhaps due to the fact that a different breast cancer cell line was used. Interestingly, we found that inhibiting YB-1 did not decrease signaling through the AKT/GSK pathway. This could have explained a decrease in MCL-1 mediated through the ubiquitination and degradation pathway (Ding et al. 2007). The finding that YB-1 promotes cell survival by engaging the mTOR/Stat3/MCL-1 pathway provides a novel insight into how this transcription factor may render cells resistant to chemotherapy and radiation (Kohno et al. 2003). It also helps to explain why YB-1 is associated with poor patient outcome and it is likely no coincidence that YB-1 (Wu et al. 2006) and Stat3^{S727} (Yeh et al. 2006) are both strongly associated with ER-negative breast cancers, which tend to be associated with poor survival.

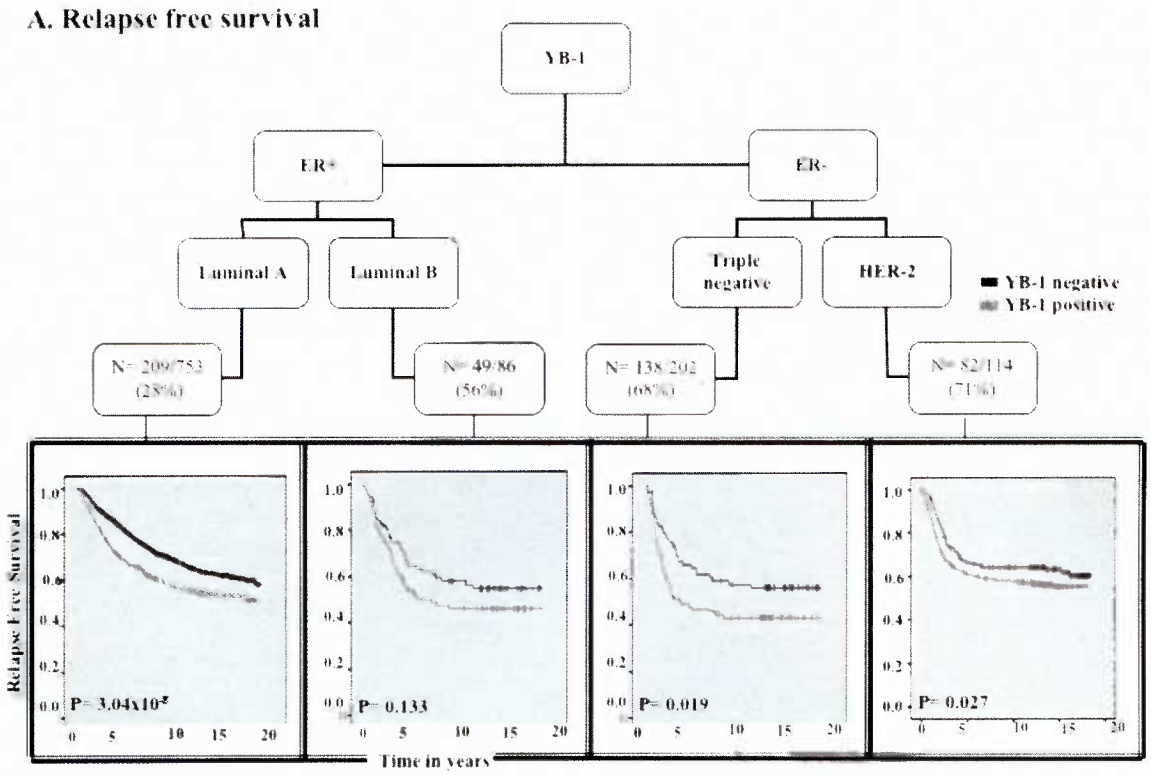
Herein we demonstrate that inhibiting YB-1 with siRNA suppresses the growth of aggressive forms of breast cancer when used as a single agent or when combined with Taxol. It is possible that the suppression of YB-1 could augment other current therapies for breast cancer. Inhibiting YB-1 with siRNA has also been found to sensitize melanoma cells to cisplatin and etoposide but not doxorubicin (Schittek et al. 2007). Thus, by combining YB-1 inhibition with agents such as cisplatin and/or etoposide could improve the treatment of certain breast cancers. Combining YB-1 inhibition with targeted agents such as trastuzumab, gefitinib or lapatanib might also be anticipated to

enhance the outcome of poor prognosis breast cancer patients. In a recent study, we demonstrated that co-targeting YB-1 and EGFR with gefitinib (Iressa) optimally inhibited the growth of BLBC cells in culture (Stratford et al. 2007). Importantly, clinically relevant doses were used indicating that combining YB-1 with gefitinib could potentially have a clinical benefit. Furthermore, we showed that inhibiting YB-1 could reverse gefitinib resistance by mechanisms that are yet to be understood (Stratford et al. 2007). Perhaps the mechanism relates to alterations in the mTOR/Stat3/MCL-1 pathway. Because EGFR targeted therapies have had limited success in clinical trials we propose that perhaps combining a YB-1 inhibitor could improve their efficacy. Similarly, it is possible that combining YB-1 with inhibitors to HER-2 could have a beneficial effect. This is particularly important for the >70% of breast cancer patients that are eligible for trastuzumab but do not respond (Meric-Bernstam et al. 2006; Nahta et al. 2006). It is therefore conceivable that inhibiting YB-1 in combination with other agents could improve the upfront treatment of breast cancer and this could also translate into reduced rates of recurrence.

While siRNA is useful for proof-of-concept studies, this strategy is unlikely to be directly translatable into the clinic. Therefore an immediate challenge is to identify novel ways of inhibiting YB-1. One approach is to inhibit YB-1 indirectly by disrupting signal transduction leading from the PDK-1/AKT pathway (To et al. 2007). Another strategy would be to develop cell permeable peptides or peptidomimetics against YB-1 as was done for Stat3 (Turkson et al. 2003; Turkson et al. 2001). Small molecules could also be designed against the DNA binding domain of YB-1 since the crystal structure of this region as been resolved (Kloks et al. 2002). The present data provide new impetus for such approaches and their future application to improving the diagnosis and /or treatment of incurable forms of breast cancer by targeting YB-1.

FIGURES

Figure 1. YB-1 is associated with relapse and poor survival in subtypes of breast cancer defined by hormone receptors and HER-2 status. A. Tumor tissue microarrays were stained for YB-1, ER, PR and Her-2 (n=2978 cases in total for all markers). The relative distribution of YB-1 was then evaluated by breast cancer subtypes. YB-1 is expressed in 23.1% (480/2078) of luminal A, 51.6% (95/183) of luminal B, and more likely to be expressed in the ER negative breast cancers where it was found in 64.6 (320/493) and 66.4% (148/223) of TNP and HER-2 subtypes, respectively. In all subtypes, the expression of YB-1 was associated with shorter relapse free survival. N represent the number of YB-1 positive patients that had breast cancer relapse (number of events) in each subtype. **B)** Similarly, YB-1 was associated with poor survival all subtypes of breast cancer. N represent of number of YB-1 positive patients that died of breast cancer (number of events) in each subtype. Examples of the immunostaining are represented in the inset (magnification 200x).



B. Breast cancer specific survival

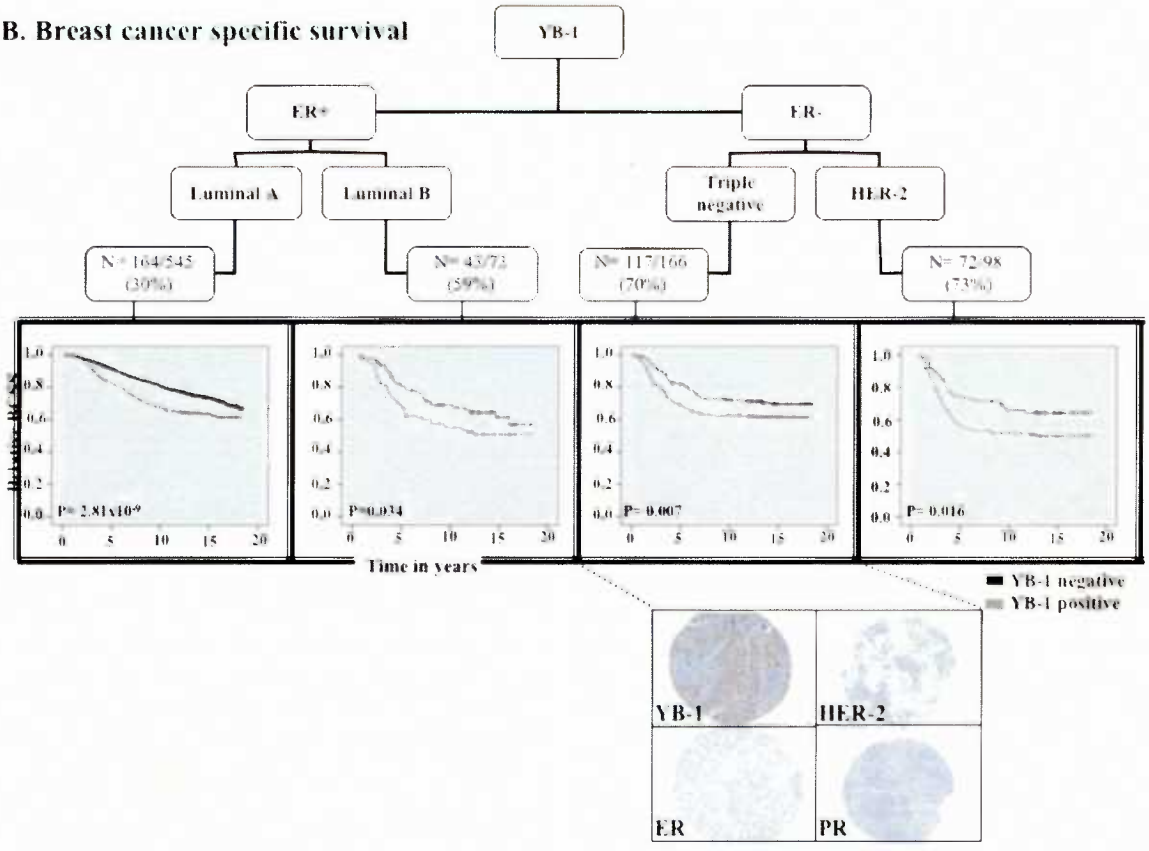


Figure 1 supplemental. YB-1 expression is associated with poor survival in the entire cohort. YB-1 is expressed in 35% (n=1081/3097) of the patients that had RFS and BCSS data. Patient survival was based on deaths specifically related to breast cancer and not other causes. **A.** YB-1 expression is associated with shorter relapse free survival time in the entire cohort. N represents the number of patients that had breast cancer recurrence (N of event). **B.** YB-1 expression is associated with poor survival in the entire cohort. N represents the number of patients that died of breast cancer (N of event).

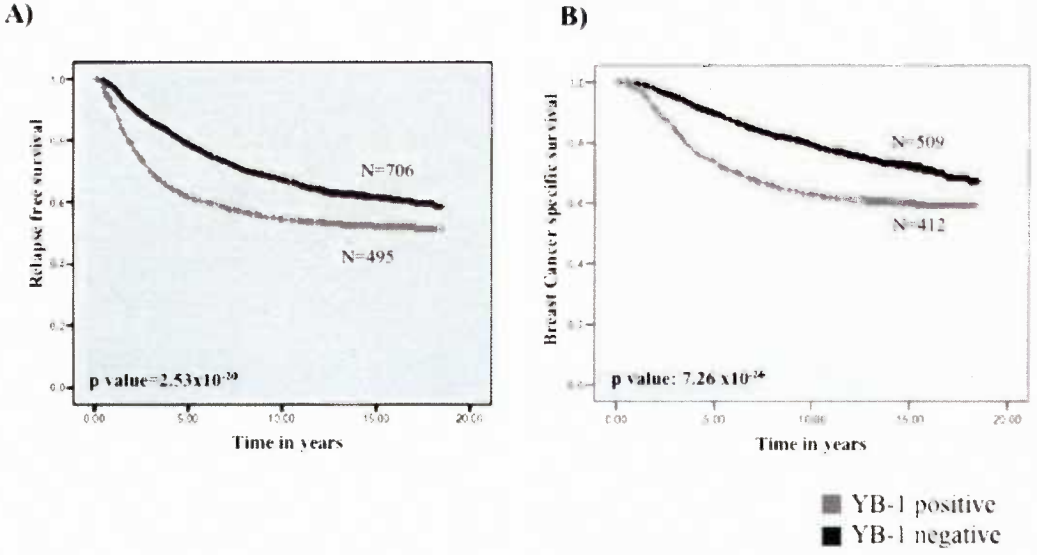


Table 1 Supplemental. A. The expression of YB-1 was associated with shorter relapse free survival (HR=1.284, p=0.008) independent of breast cancer subtype defined by hormone receptor and HER-2 status based on a Cox regression analysis. **B.** Likewise, the expression of YB-1 was associated with breast cancer specific survival (HR=1.46, p=6.74x10-7) independent of subtypes.

A.

		ALL PATIENTS		
	HAZARD RATIO	**HAZARD RATIO 95% C.I.**		**P value**
		LOWER	UPPER	
Nodal status				
Positive vs negative	1.724	1.451	2.050	6.41E-10
Tumour size (cm)				
2 < size ≤ 5 vs ≤ 2	1.338	1.120	1.600	0.001
> 5 vs ≤ 2	1.995	1.443	2.759	2.97E-005
Grade				
III vs II and I	1.262	1.046	1.524	0.015
Age				
<50 vs > = 50	0.984	0.821	1.180	0.865
YB-1				
Positive vs negative	1.284	1.068	1.544	0.008
Molecular characteristics of breast cancer				
Luminal B vs Luminal A	1.178	0.829	1.675	0.360
HER-2 vs Luminal A	1.279	0.947	1.727	0.109
Tripleneg vs Luminal A	0.982	0.765	1.262	0.889
Unassigned vs Luminal A	1.012	0.656	1.560	0.957

B.

		ALL PATIENTS		
	HAZARD RATIO	**HAZARD RATIO 95% C.I.**		**P value**
		LOWER	UPPER	
Nodal status				
Positive vs negative	2.397	2.079	2.762	1.72E-033
Tumor size (cm)				
2 < size ≤ 5 vs ≤ 2	1.577	1.364	1.822	7.01E-010
> 5 vs ≤ 2	2.241	1.730	2.904	1.01E-009
Grade				
III vs II and I	1.436	1.233	1.672	3.34E-006
Age				
<50 vs > = 50	1.159	1.001	1.342	0.048
YB-1				
Positive vs negative	1.452	1.253	1.682	6.74E-007
Molecular characteristics of breast cancer				
Luminal B vs Luminal A	1.296	1.003	1.675	0.047
HER-2 vs Luminal A	1.467	1.167	1.845	0.001
Tripleneg vs Luminal A	1.155	0.951	1.405	0.147
Unassigned vs Luminal A	1.240	0.890	1.726	0.203

Table 1. In Cox regression models, the harzard ratio for YB-1 is higher than age or grade, ER, HER-2 status and suggest that YB-1 is strong potential marker for A) cancer recurrence and B) poor outcome.

A.

		ALL PATIENTS		
	HAZARD RATIO	**HAZARD RATIO 95% C.I.**		**P value**
		LOWER	UPPER	
Nodal status				
Positive vs negative	1.951	1.725	2.206	1.74E-026
Tumour size (cm)				
2 < size ≤ 5 vs ≤ 2	1.392	1.227	1.579	2.76E-007
> 5 vs ≤ 2	1.921	1.509	2.445	1.16E-007
Grade				
III vs II and I	1.300	1.139	1.482	9.61E-005
Age				
< 50 vs > = 50	1.000	0.878	1.139	1.000
ER				
Positive vs negative	0.942	0.816	1.087	0.413
HER-2				
Positive vs negative	1.256	1.066	1.479	0.006
YB-1				
Positive vs negative	1.331	1.169	1.516	1.61E-005

B.

		ALL PATIENTS		
	HAZARD RATIO	**HAZARD RATIO 95% C.I.**		**P value**
		LOWER	UPPER	
Nodal status				
Positive vs negative	2.405	2.083	2.776	4.62E-033
Tumour size (cm)				
2 < size ≤ 5 vs ≤ 2	1.605	1.387	1.859	2.83E-010
> 5 vs ≤ 2	2.269	1.749	2.943	2.82E-010
Grade				
III vs II and I	1.416	1.215	1.651	8.94E-006
Age				
<50 vs > = 50	1.160	1.000	1.345	0.050
ER				
Positive vs negative	0.816	0.720	0.991	0.038
HER-2				
Positive vs negative	1.259	1.052	1.506	0.012
YB-1				
Positive vs negative	1.456	1.257	1.656	5.56E-007

Table 2 supplemental. Correlations between YB-1 and clinicopathological features of breast cancer. YB-1 was not associated with nodal status and weakly related to tumour size. The expression of YB-1 was however closely associated with tumour grade, patient age, estrogen receptor negative tumours and HER-2 expression.

	YB-1	
	Spearman's correlation value	**Significance (p value)**
Nodal status	0.008	0.895
Tumor size	0.089	2.853E-004
Grade	0.268	2.957E-027
Age	-0.203	6.512E-017
ER	-0.343	5.452E-047
HER-2	0.217	8.588E-019

Figure 2. Patient survival was assessed following either surgical resection and no chemotherapy (A) or treatment with tamoxifen (B). YB-1 is expressed in 34% (437/1292) of patients that didn't receive any systemic therapy and 22% (185/856) of tamoxifen-treatet patients. N represents the number of patients that died of breast cancer (N of event) in each category. Survival was stratified based on whether tumours expressed YB-1 (grey line) or not (black line).

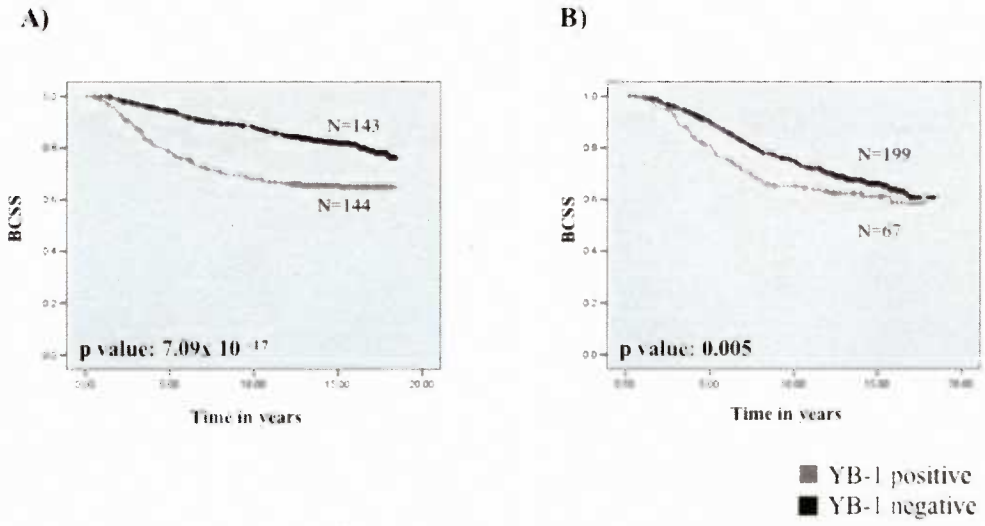
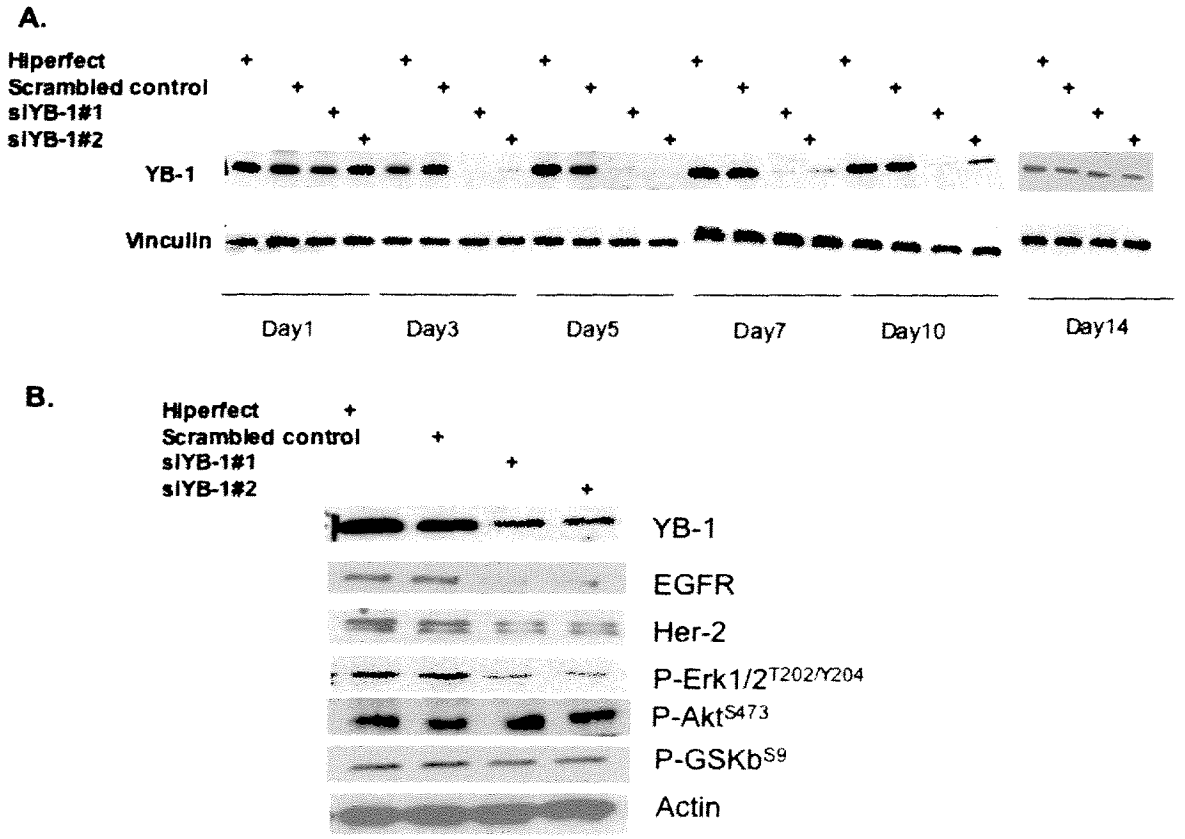


Table 3 supplemental. Cox regression model for patients that were treated with surgical resection and no chemotherapy. Nodal status, tumour size and YB-1 expression were associated with poor outcome.

		ALL PATIENTS		
	HAZARD RATIO	**HAZARD RATIO 95% C.I.**		**P value**
		LOWER	UPPER	
Nodal status				
Positive vs negative	4.097	3.006	5.583	4.31E-019
Tumor size (cm)				
2 < size ≤ 5 vs ≤ 2	1.644	1.286	2.102	7.37E-005
> 5 vs ≤ 2	1.063	.431	2.625	0.894
Grade	1.311	.996	1.725	
III vs II and I	1.126	.832	1.523	0.054
Age	1.041	.783	1.385	
<50 vs > = 50	1.352	.984	1.857	0.443
ER	1.897	1.460	2.465	
Positive vs negative	4.097	3.006	5.583	0.782
HER-2				
Positive vs negative	1.644	1.286	2.102	0.063
YB-1	1.063	.431	2.625	
Positive vs negative	1.311	.996	1.725	1.67E-006

Figure 3. Inhibition of YB-1 suppresses tumour growth by inducing apoptosis. A) Time-course study (1-14 days) examining the reduction of YB-1 protein levels by siRNA oligos. BT474-m1 cells at log-growing phase were transfected with control or siRNA oligos targeting YB-1 and whole cell extracts were prepared at the time-points stated on the graph. Proteins in the cell lysates were separated by SDS-polyacrylamide-gel electrophoresis and immunoblotted with antibody against YB-1 protein. Pan-actin antibody was used as loading control. **B)** Knocking-down YB-1 decreased expression of YB-1 target genes. Exponentially proliferating BT474-m1 cells were transfected with control or siRNA oligos targeting YB-1. Protein extracts were prepared 4 days after transfection to examine the expression of EGFR, and HER-2, which are YB-1 target genes. P-Erk1/2$^{T202/Y204}$, P-AKT^{S473} and P-GSKβ^{S9} were also evaluated. Pan-actin was used as a loading control. **C)** Decreasing YB-1 protein level inhibited cell growth in monolayer over 7 days. BT474-m1 cells were transfected with control or siRNA oligos targeting YB-1 for 2 day before being re-plated in 96-well plates. Cells were subjected to MTS assay 1, 3, 5 and 7 after re-plating. **D)** High content screen of a panel of breast cancer cell lines for the effect of siYB-1#2 on cell growth. The degree of YB-1 knock-down was imaged by immunofluorescence staining (Top panel) and the effect of siYB-1#2 on cell growth was quantified relative to the scrambled control (Bottom panel). **E)** BT-474m1 cells were treated with a combination of siYB-1#2 and Taxol (0.1-10 nM). The cells were treated with siYB-1#2 for 2 days and then exposed to Taxol for an additional 3 days. Each treatment was performed in replicates of six. Cell viability was evaluated by high content screening based on Hoechst staining.



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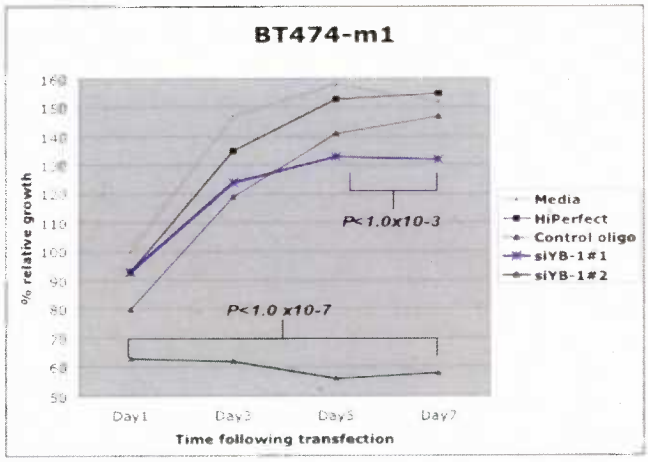
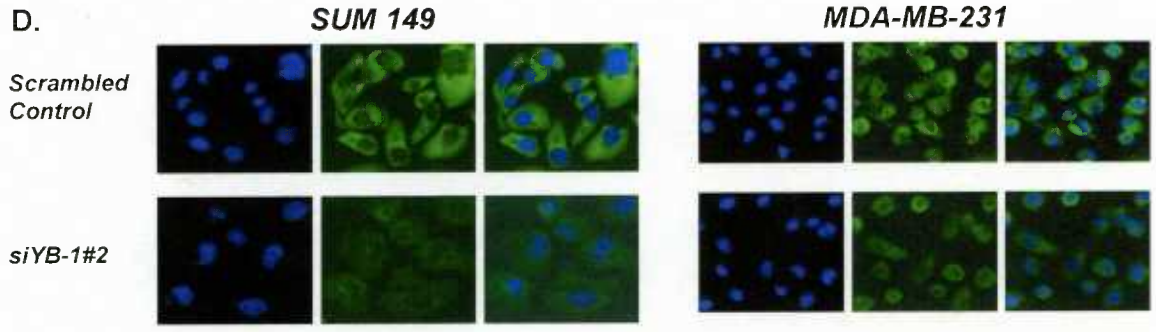
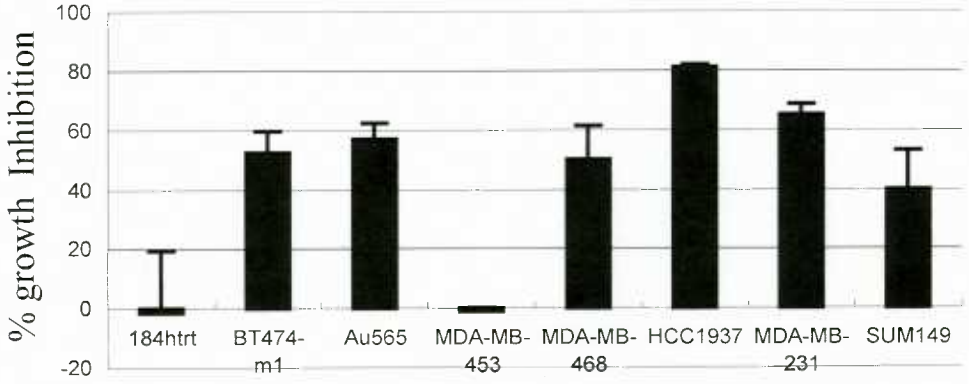

BT474-m1
% relative growth
P<1.0x10-3
P<1.0 x10-7
Media
HiPerfect
Control oligo
siYB-1#1
siYB-1#2
Day1
Day3
Day5
Day7
Time following transfection

D.


SUM 149
MDA-MB-231
Scrambled Control
siYB-1#2


% growth Inhibition
100
80
60
40
20
0
-20
184htrt
BT474-m1
Au565
MDA-MB-453
MDA-MB-468
HCC1937
MDA-MB-231
SUM149

E.

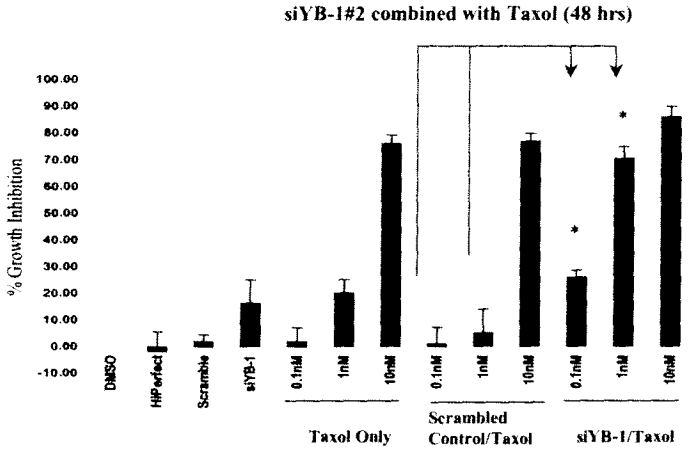
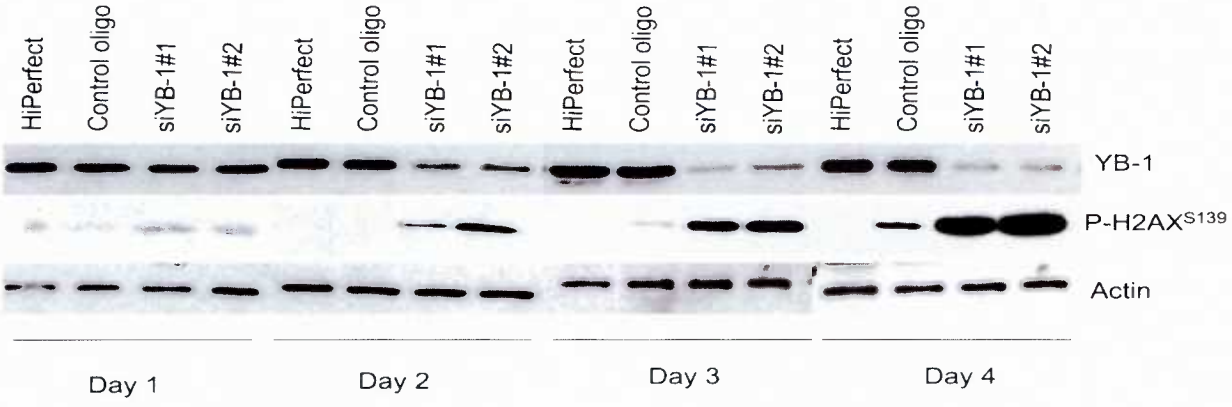

siYB-1#2 combined with Taxol (48 hrs)
% Growth Inhibition
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
DMSO
HiPerfect
Scramble
siYB-1
0.1nM
1nM
10nM
0.1nM
1nM
10nM
0.1nM
1nM
10nM
Taxol Only
Scrambled Control/Taxol
siYB-1/Taxol

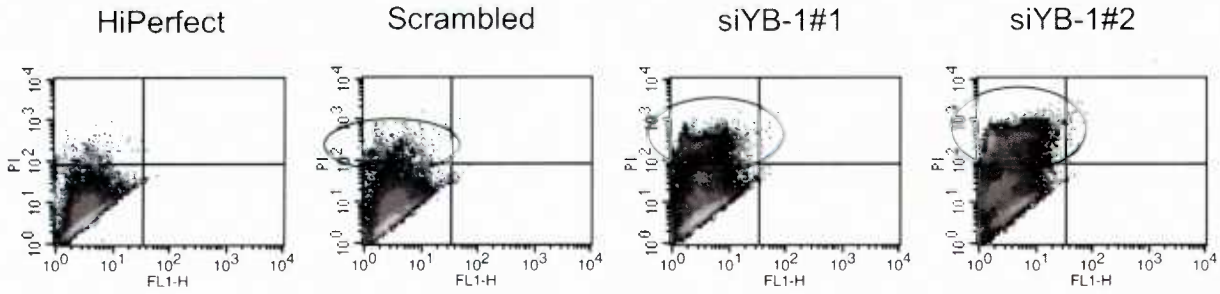
Figure 4. Inhibition of YB-1 leads to the induction of apoptosis through the mTOR/STAT3/MCL-1 pathway. A) BT474-m1 cells were transfected with control or siRNA oligos targeting YB-1 and harvested 1, 2, 3 and 4 day after transfection. Proteins were isolated from the cells and the lysates were subjected to western blotting to examine the level of P-H2AX^{S139}. Pan-actin was used as loading control. **B)** Isolation of BT474-m1 cells following YB-1 knock down for 3 days led to the induction of apoptosis based on propidium iodide incorporation. **C)** Small interfering RNAs against YB-1 induced annexin V staining after treatment for 3 days (upper panel). A time course was then conducted to profile the effect of knocking down YB-1 (1-4 days, lower panel). There was an incremental increase in annexin V staining with siYB-1#2 as a function of time. **D)** Based on a high content screening method, cells treated with siYB-1#2 for 3 days caused an increase in chromatin condensation (first panel), propidium iodide uptake (second panel), P-H2AX^{S139} (third panel). All three apoptotic markers could be imaged simultaneously (merged, fourth panel). **E)** Loss of YB-1 expression with siYB-1#2 (2 days post-transfection) was associated with a decrease in P-mTOR^{S2448}, mTOR, P-Stat3^{S727} and MCL-1 relative to the scrambled control (lane 1) or the HiPerfect control (lane 2). There was no change in total levels of Stat3 or survivin. Stat3^{Y705} was undetectable. Actin was used as a control for equal sample loading. **F)** Flag:Stat3C (0.5-2 ug) was transiently transfected into BT474-m1 cells and 2 days later the cells were harvested and evaluated for Stat3C by immunoblotting for Flag. There was a dose dependent increase in Flag:Stat3C correlative with an increase in MCL-1 expression. **G)** Flag:Stat3C (2 ug) was transiently transfected into cells for 2 days and then subsequently treated with siYB-1#2. The cells were then examined for changes in P-H2AX^{S139} and MCL-1.

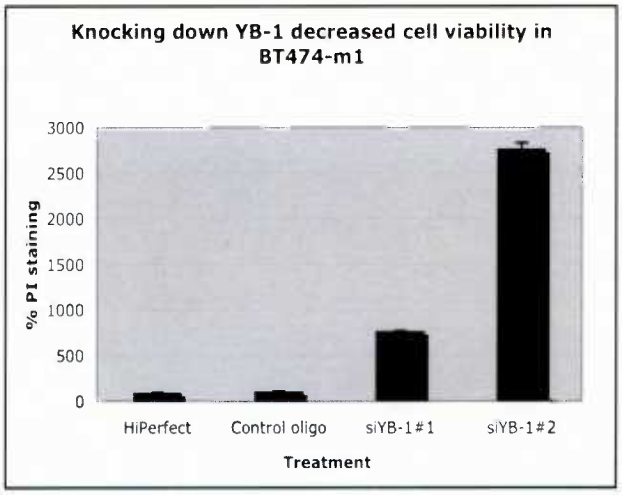
Figure 4

A.

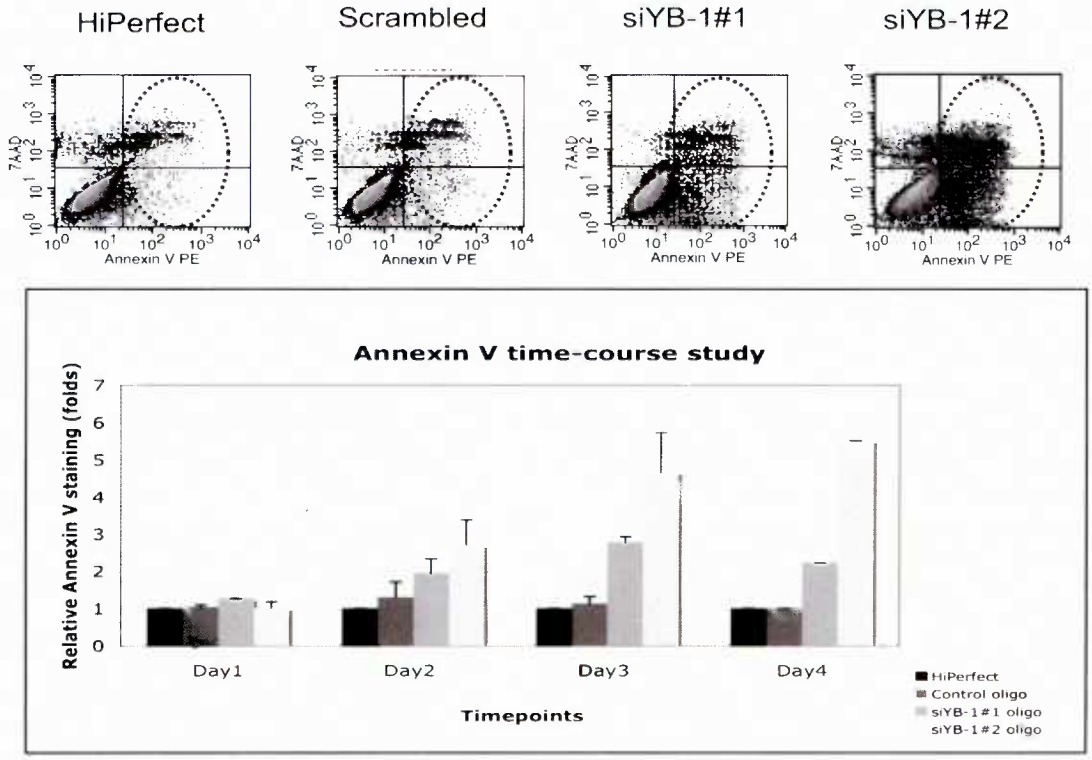


B.

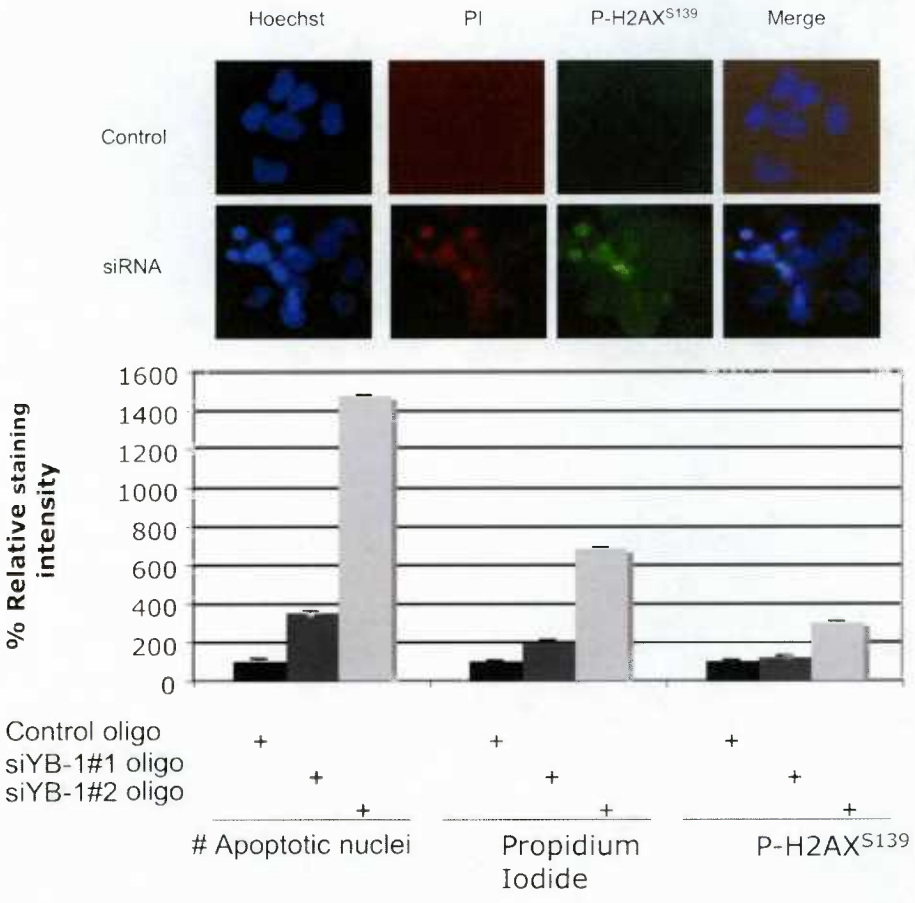

HiPerfect
Scrambled
siYB-1#1
siYB-1#2
PI
FL1-H


Knocking down YB-1 decreased cell viability in BT474-m1
% PI staining
3000
2500
2000
1500
1000
500
0
HiPerfect
Control oligo
siYB-1#1
siYB-1#2
Treatment

C.


HiPerfect
Scrambled
siYB-1#1
siYB-1#2
7AAD
Annexin V PE
Annexin V time-course study
Relative Annexin V staining (folds)
Day1
Day2
Day3
Day4
Timepoints
HiPerfect
Control oligo
siYB-1#1 oligo
siYB-1#2 oligo

D.


Hoechst
PI
P-H2AX^{S139}
Merge
Control
siRNA
% Relative staining intensity
1600
1400
1200
1000
800
600
400
200
0
Control oligo
siYB-1#1 oligo
siYB-1#2 oligo
+
+
+
Apoptotic nuclei
Propidium Iodide
P-H2AX^{S139}

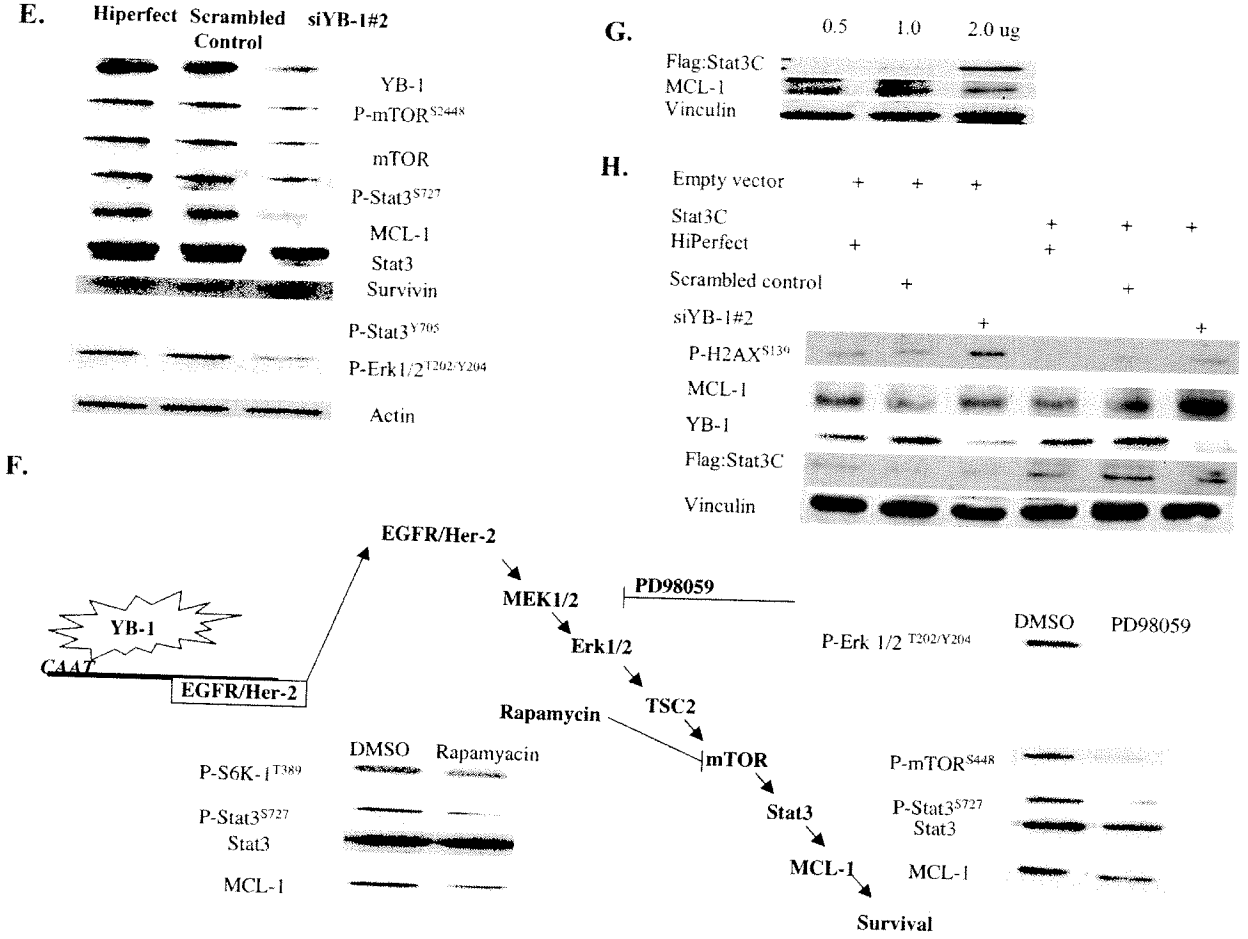
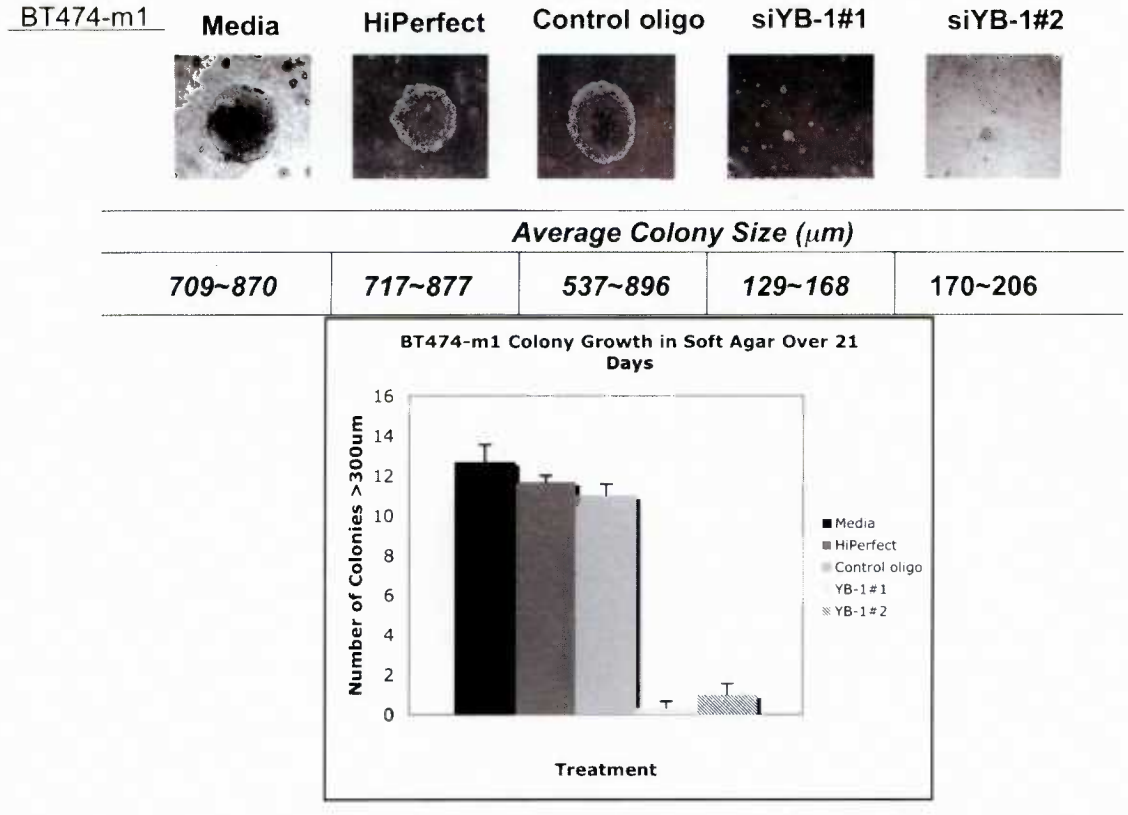
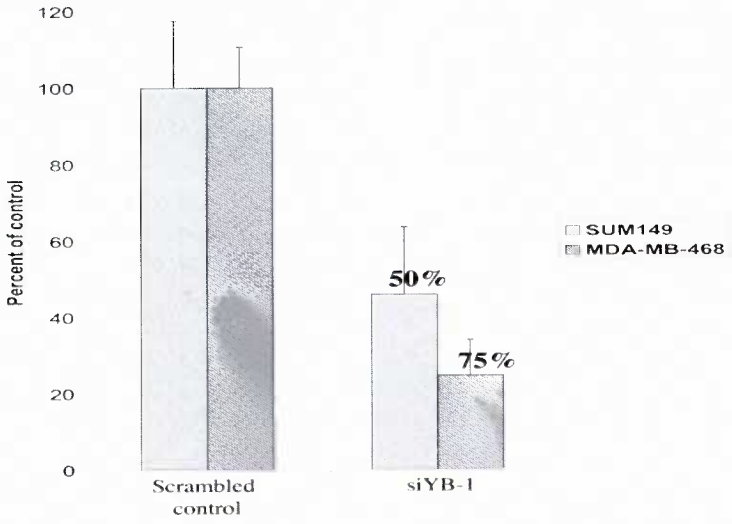

E.
Hiperfect
Scrambled Control
siYB-1#2
YB-1
P-mTOR S2448
mTOR
P-Stat3 S727
MCL-1
Stat3
Survivin
P-Stat3 Y705
P-Erk1/2 T202/Y204
Actin
G.
0.5
1.0
2.0 ug
Flag:Stat3C
MCL-1
Vinculin
H.
Empty vector
Stat3C
HiPerfect
Scrambled control
siYB-1#2
P-H2AX S139
MCL-1
YB-1
Flag:Stat3C
Vinculin
F.
EGFR/Her-2
MEK1/2
PD98059
YB-1
CAAT
EGFR/Her-2
Erk1/2
P-Erk 1/2 T202/Y204
DMSO
PD98059
Rapamycin
TSC2
mTOR
DMSO
Rapamyacin
P-S6K-1 T389
P-Stat3 S727
Stat3
MCL-1
P-mTOR S448
Stat3
P-Stat3 S727
Stat3
MCL-1
MCL-1
Survival

Figure 5. Suppression of YB-1 inhibits the growth of aggressive breast cancer cells in soft agar and in mice. A) BT474-m1 cells were transfected with siRNA directed against YB-1 and colony growth was assessed after 28 days. The loss of YB-1 markedly inhibited the growth of these aggressive models of breast cancer. **B)** SUM149 and MDA-MB-468 cells were treated as above with siYB-1. Similarly, loss of YB-1 inhibited the growth of these aggressive models of breast cancer by 50-75%. Statistical significance was determined using the student's T test. Each experiment was performed in triplicate on two separate occasions. Colony size was measured and colonies greater than 300μm were counted under the microscope after 28 days in soft agar. **C-E)** BT474-m1 cells were transfected with either scrambled control, siYB-1#1 or siYB-1#2 for 48 hours and then the cells were injected subcutaneously into nu/nu mice. The loss of YB-1 expression remarkably suppressed tumour formation within the first two weeks ($p<0.01$). By the third week, tumours developed in the mice however they were remarkably smaller in size. Compared to the control oligonucleotide, the tumours that arose in siYB-1#1 or siYB-1#2 were ~50-67% smaller ($p<0.01$) respectively. Significance was determined using a student' T test. *<0.01.

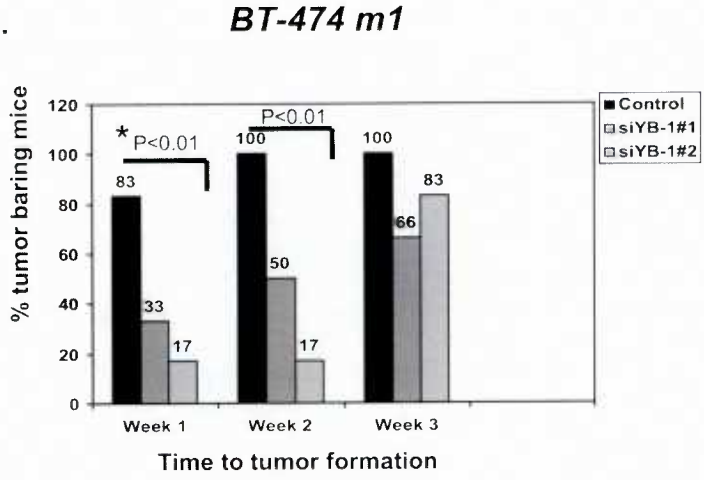
A.



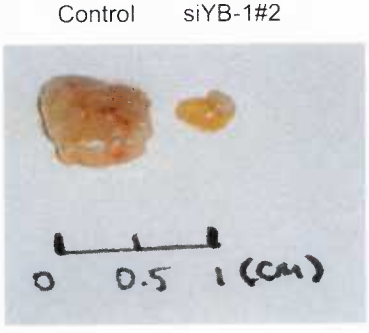
B.


120
100
80
60
40
20
0
Percent of control
50%
75%
SUM149
MDA-MB-468
Scrambled control
siYB-1

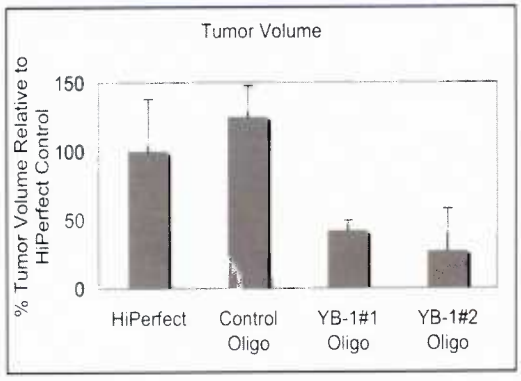
C.


BT-474 m1
% tumor baring mice
120
100
80
60
40
20
0
*P<0.01
P<0.01
83
33
17
100
50
17
100
66
83
Week 1
Week 2
Week 3
Time to tumor formation
Control
siYB-1#1
siYB-1#2

D.


Control
siYB-1#2
0
0.5
1 (cm)

E.


Tumor Volume
% Tumor Volume Relative to HiPerfect Control
150
100
50
0
HiPerfect
Control Oligo
YB-1#1 Oligo
YB-1#2 Oligo

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CHAPTER 4

Inhibition of YB-1, a marker of cancer recurrence, reduces breast cancer invasion

INTRODUCTION

One of the most important current issues in breast cancer is early identification of patients with high risk of relapse. Approximately 1.1 million women will be diagnosed with breast cancer worldwide this year. Based on several reports from the American Cancer Society, patients with localized breast tumours can expect a 98% chance of survival five years post-diagnosis. However, once the cancer has metastesized to distant sites the five-year survival rate decreases to as low as 16%. Breast cancer has the potential to spread to almost any region of the body. The most common regions that breast cancer spreads to are the bone, followed by the lung and liver (www.cancer.org).

Therefore, there is a desperate need for prognostic markers for patient outcome and relapse and potential targets for treatment of aggressive types of breast cancer. In this study we focus on Y-Box binding protein-1 (YB-1), which is an oncogenic transcription/translation factor that is overexpressed in many malignancies including breast cancer. Recent work by our team identified YB-1 as a marker of aggressiveness, poor outcome and cancer recurrence. We also demonstrated that YB-1 is a much strong indicator of cancer recurrence in comparison to estrogen receptor (ER) and HER-2 status (chapter 3). Schittek et al. (2007) demonstrated expression and nuclear translocation of YB-1 is induced in metastatic melanoma cells *in vitro* and *in vivo* (Schittek et al. 2007). They also found significant reduction in migration and invasion of melanoma cells *in vitro* with stable down regulation of YB-1 (Schittek et al. 2007).

Our attention was also drawn to urokinase plasminogen activator (uPA), which is a serine protease that is involved development, progression and metastasis of breast cancers (Duffy et al. 1988; Foekens et al. 2000). It is also significantly associated with a high rate of relapse for patients with breast cancer (Janicke et al. 1989). Importantly, the European Organization for Research and Treatment of Cancer regard uPA as a Level I category biomarker for cancer and therefore new agents targeting this protease would clearly be

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benficial (Harbeck et al. 2004).

uPA converts inactive plasminogen to active plasmin that can degrade majority of proteins present in the extracellular matrix (ECM). Moreover, plasmin is also a serine protease that can activate the precursor forms of a number of matrix metalloproteinases (MMPs) such as MMP-3, MMP-9, MMP-12 and MMM-13 (Carmeliet et al. 1997). Activation of these MMPs results in further degradation and remodeling of the ECM. Interestingly, plasmin is involved in activation or release of insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2) and transforming growth factor-beta (TGF-β) (Rifkin et al. 1997). UPA also activates IGF-1R signaling pathway by mediating the levels IGFBP in blood (Sorrell et al. 2006; Yoshida et al. 1995). Therefore, uPA plays a very important role in cancer development, invasion and metastasis (Duffy et al. 2004).

We previously showed that YB-1 is a strong maker of cancer recurrence and poor prognosis by screening 4049 primary breast tumours (chapter 3). Since uPA is also involved in relapse, we questioned whether there is a relationship between these two genes. Finally, we addressed whether inhibiting IGF-1R pathway with the small molecule BMS-536924 would have any effect on expression of these two proteins.

MATERIALS AND METHODS

Tissue microarray analysis

The study cohort and construction of the TMAs used here has been described previously (Cheang et al. 2006 ; Rajput et al. 2007). Sections were cut at 4 μm and immunostained with a rabbit polyclonal anti-YB-1 antibody (1:1,400) (a gift from Dr. Colleen Nelson, University of British Columbia, Vancouver, BC). The immunohistochemical staining was performed using an Automated Secondary System (Ventana, Tucson, AZ, cat # 760-4205). Slides were stained concurrently for estrogen receptor (ER), HER-2, and progesterone receptor (PR) using standard immunoperoxidase techniques as previously described (Cheang et al. 2006; Rajput et al. 2007). Breast cancer subtypes were determined using immunohistochemical markers (HER-2, ER and PR) to define each type. Subtype definitions were as follows: luminal A (ER+ and/or PR+, HER2–), luminal B (ER+ and/or PR+, HER2+), triple negative (ER– PR–, HER2–),

HER2+ (ER–, PR–, and HER2+), and unassigned (missing data any of the 3 markers). Scoring system for YB-1 was previously described by us (chapter 3). TMAs were evaluated independently by two pathologists (Dr. Ashish Rajput, Dr. Blake C Gilks) to quantify the percentage of tumour cells that showed positive uPA staining as follows: negative (<1%); positive: (>1%). Of the 4049 samples, 3145 cases could be scored for uPA had data regarding their relapse survival outcome. Immunohistochemical data was collected on samples that were blinded as to clinical outcomes.

Statistical analysis was performed using SPSS software version 13.0 (SPSS Inc, Chicago, IL) and was described previously (Cheang et al. 2006). In brief, for univariate analyses, BCSS and relapse free survival (RFS) was estimated using Kaplan-Meier curves. Survival differences were determined by Breslow tests. For BCSS, survival time was censored at death if the cause was not breast cancer or if the patient was still alive at the end of the study. Six patients with an unknown cause of death were excluded from the BCSS analysis. In addition, relapse free survival time was also censored at death if the cause was not breast cancer and if the patients were alive without any relapse at the end the study. For multivariate analyses, Cox proportional hazards models were used to calculate adjusted hazard ratios (HRs) accounting for independent co-variates. Spearman's correlation was also used to determine the extent of correlation between YB-1 expression and other markers such as uPA.

Cell culture

SUM149 cells were purchased from Astrand (Ann Arbor, MI) and were grown according to the manufacturer's recommendation. The cells were cultured in F-12 (Ham's) media (Gibco/Invitrogen, Burlington, ON) supplemented with 5μg/ml insulin (Sigma, Oakville, ON) 1μg/ml hydrocortisone (Sigma), 10 mM HEPES (Sigma), 5% fetal bovine serum (Gibco/Invitrogen). Cells were maintained at 37°C in 5% CO_2 and passaged every two days.

Drug and siRNA treatments

For the siRNA transfections, SUM149 cells ($4x10^5$/6 well dish) were transfected with 5 nM of control or YB-1/uPA siRNA oligonucleotides according to manufacturer's

protocol. The siRNA oligonucleotides had sequences as follows; siYB-1#2 (CCACGCAAUUACCAGCAAAdTdT) (Dharmacon, IL), control oligonucleotide (UUC UCC GAA CGU GUC ACG UdT dT) (Qiagen non-targeting siRNA) and uPA siRNA (Qiagen, S102662135). In brief, control, or YB-1 siRNA (5 nM) or uPA siRNA (20 nM) were diluted in serum-free media and HiPerfect transfection reagent (Qiagen, MD) was added to the diluted siRNA solution. The mixture was gently vortexed for a few seconds and incubated at room temperature for 10 minutes. The complex was then added to the cells in each well and the plates incubated at 37°C, in a 5% CO_2 incubator before harvesting the cells for subsequent studies. To create siYB-1 stables, SUM149cells were transfected with pSUPER which contains YB-1 short hairpin RNA (GATCCCCGGTCATCGCAACGAAGGTTTTCAAGAGAAACCTTCGTTGCGATGA CCTTTTTGGAAA) and selected with G418.

SUM149 cells ($4x10^5$/6 well dish) were also treated with 4 μM BMS-536924 or DMSO as a control for 72 hours prior to harvesting. YB-1 and uPA mRNA and protein was measured.

Immunoblot analysis

Cells were harvested by scraping and lysed in ELB buffer (5 mmol/L HEPES (pH7.4), 150 mmol/L NaCl, 1 mmol/L EDTA (pH8.0), 1% Triton-X100, 1% deoxycholate and 0.1% SDS) with protease and phosphatase inhibitors. The lysates were sheared by passage through a 21-gauge needle and centrifuged at 13,000 rpm for 10 minutes at 4°C. The lysates were electrophoresed (50 μg) in 12% SDS polyacrylamide gels at 150V and transferred to nitrocellulose membranes at 4°C for 2 hours. The membranes were then probed with the following antibodies: anti-YB-1 (1:5,000) and anti-pan-actin (1:1000; Cell Signaling Technology), which was used as the loading control.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

This method was previously described by us (Andrews et al. 2001). In brief, the conditioned media from SUM149 cells was collected after 48 and 72 hours and stored at –70 °C. The media was diluted 1:5 and uPA protein concentrations were quantified according to the manufacturer's protocol (Immunobind uPA ELISA kit; American

Diagnostica Inc., Montreal, QC).

Invasion assays

The Matrigel invasion assays were performed as previously described (Dunn 2000). In brief, SUM149 cells were treated with YB-1 siRNA (5nM), uPA siRNA (20nM) or control for 72 hours. Cells were then re-plated at $1x10^6$ cells/ well in DMEM media containing 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in a transwell chamber (Becton Dickenson Biosciences, Mississauga, Ontario) containing a filter 6.5 mm in diameter and 8 μm pores coated with matrigel (Collaborative Research Co., Bedford, MA) at concentration of 10 μg/100 μl/ well. The cells were incubated for 24 hours at 37 °C in humidified air with 5% CO2. Following incubation, cells on the upper chamber were removed with cotton swabs. The cells that invaded the lower surface of the membrane were fixed with methanol and stained with Hoechst 33258 (Sigma Chemical Co., Bedford, St. Louis, MA). Cellular invasion was measured by counting the cells within the field of view of fluorescence microscope (40X magnification).

Quantitative real time polymerase chain reaction (qRT-PCR)

SUM149 cells were treated with either DMSO or BMS-536924 (4μM) to examine the effect of P-IGF-1R inhibition. In another experiment, SUM149 cells were transfected with YB-1 siRNA and control siRNA (5nM) for 48 and 72 hours, RNA was isolated (Qiagen, Inc., Mississauga, ON) and reverse transcribed (Super- scriptTM III First-Strand Synthesis System, Invitrogen). The cDNA was amplified (ABI Prism® 7000 Sequence Detection System, Applied Biosystems, Foster City, CA) using uPA assay on demand (Applied Biosystems, Streetsville, ON) according to our previously reported method (Andrews et al. 2005). Each treatment was analyzed in triplicate on two separate occasions. 18S ribosomal protein was used as controls to assess differences in sample input.

Chromatin Immunoprecipitation (ChIP) on chip

SUM149 cells grown on 15-cm plates to 80% confluence ($8x\ 10^6$ cells) were fixed with formaldehyde for 10 minutes. Lysates were pooled from 12 plates and subjected to chromatin immunoprecipitation for endogenous YB-1 using chicken anti-

human polyclonal antibody as previously described by us (Wu et al. 2006). Immunoprecipitated DNA was amplified using the protocol provided by NimbleGen. In brief, eluates and input control were subjected to DNA end blunting using T4 DNA polymerase (NEB, #203L) and incubated for 20 minutes at 12°C. Samples were then vortexed for 1 minute after the addition of 3 mol/L NaOAc, 20 mg/ml glycogen, and phenol/chloroform. Aqueous supernatants were precipitated using ethanol and the DNA pellet was resuspended in 25μl water. Subsequently, the blunted DNA was ligated using T4 DNA ligase (NEB, #202L) and 15μmol/L of the annealed linkers oJW 102 (5'-GCGGTGACCCGGGAGATCTGAATTC) and oJW103 (5'- GAATTCAGATC). Following overnight incubation at 16°C, samples were precipitated in ethanol. LM-PCR was performed using oJW 102 primers. Conditions used for the amplification were as follows: 55°C for 2 minutes, 72°C for 5 minutes, and 95°C for 2 minutes, followed by 22 cycles of 95°C for 1 minutes, 60°C for 1 minute and 72°C for 5 minutes. Samples were purified using the QIAquick PCR purification kit (#28104) and quantified with a NanoDrop® ND-1000 Spectrophotometer. Samples were sent to Nimblegen for ChIP on chip analysis. This assay was performed using Nimblegen standard single promoter array protocol where 2.2 kb upstream and 500 bp downstream of approximately18000 promoter regions was analyzed. The resolution of this tiling array was one probe every 100 bps. The ChIP DNA and the input DNA were probed with Cy5 and Cy3 respectively. Signal intensity of the ChIP DNA was normalized to the input. Log-2 signal intensity ratios lower that 2 were filtered from the original raw data and 6691 genes remained as YB-1 potential down-stream targets. This assay was performed in duplicate and standard deviations are derived from the fold enrichment values from two replicates.

RESULTS

YB-1 is correlated with uPA expression in the "triple negative" breast cancers

In this study we focused on "triple negative" subtype of breast cancer because there is no targeted treatment available for triple negative patients and they frequently die within 2 year of diagnosis (Van 't Veer et al. 2002). Since YB-1 expression is detected in greater percent of ER-negative breast cancers in this cohort, we examined the correlation

between YB-1 and uPA in the triple negative subtype. Surprisingly, we found that there is an association between YB-1 and uPA expression in this subtype (Table 1). In order to further confirm this finding we performed chromatin immunoprecipitation on chip (ChIP on chip) in the SUM149 cell line, which was our in vitro model for triple negative breast cancer. Interestingly, we found uPA along with many other genes that are involved in the invasion and metastasis process, are in the list of potential YB-1 target genes (Table 2). We also determined that area of YB-1 binding to uPA promoter was enriched 7 fold.

TMA analysis indicated uPA expression is correlated with earlier cancer recurrence and poor outcome

In the cohort of 4049 patients we evaluated the expression of urokinase plasminogen activator in the tumour tissue microarray (TMA). We determined that 45% (n=1424/3145) of the patients in the entire cohort express uPA in their tumours. We found that uPA positive patients have a shorter relapse free survival (Breslow p value= 0.006; Figure 1A) and breast cancer specific survival time (Breslow p value= $3.18x10^{-5}$, Figure 1B) compared to the patients who do not. Representative immunohistochemistry images for uPA staining in this cohort are illustrated in Figure 2.

Inhibition of YB-1 expression results in a significant reduction in uPA transcript

In order to further confirm the association between YB-1 and uPA, we silenced YB-1 using small interference RNA (siRNA) for 48 and 72 hours. Using qRT PCR analysis, we found that treating SUM149 cells with YB-1 siRNA (48 hours) resulted in a 83% and 84% reduction in YB-1 and uPA mRNA respectively. Consistent with this, after 72hours YB-1 siRNA treatment, we found an 86% reduction in YB-1 mRNA and a concordant 76% loss in uPA transcript (Figure 3A). Similarly, we were able to confirm these findings using SUM149 cells, which had stable over-expression of a vector encoding a short hairpin RNA to YB-1 (shYB-1) (Figure 3B).

Inhibition of YB-1 expression results in a significant reduction in uPA protein

We were also able to confirm our qRTPCR findings at the protein level. We silenced YB-1 using si RNA for 48 and 72 hours and we confirmed the YB-1 knock

down with immunoblot analysis (Figure 4B). In order to assess uPA protein expression levels we used a quantitative uPA-specific enzyme-linked immunosorbent assay (ELISA) kit. We observed a 71% and 51% reduction in uPA protein after 48 and 72 hours respectively (Figure 4A). In concordance with this result we confirmed these findings when we used the stable SUM149-shYB-1 cells (Figure 4 C-D).

Silencing YB-1 and uPA results in a significant reduction in cell migration and invasion

To determine the role of YB-1 in recurrence we assessed the effect of reducing YB-1 expression by siRNA on the ability of cells to invade through a matrigel. When SUM149 cells were treated with YB-1 siRNA (72 hours) the invasion through matrigel was inhibited by 77%. To confirm that uPA was playing a part in cancer cell invasion in this system we also used uPA siRNA (72 hours) in the invasion assay and we indeed observed a 63% reduction in cell migration (Figure 5).

BMS-536924 inhibits uPA expression through reducing YB-1 expression

In 2001, Dunn et al. demonstrated that up-regulation of uPA by IGF-1 is dependent upon the AKT pathway (Dunn et al. 2001). Subsequently, our team has shown that AKT phosphorylates YB-1 at serine 102 upon IGF stimulation (Sutherland et al. 2005). Since there are no commercially available inhibitors to YB-1, we were therefore interested to evaluate the effect of BMS-536924, a small molecule inhibitor to phospho-IGF1R/IR, on YB-1 and uPA. As was expected, we found a significant reduction in uPA mRNA and protein secretion after 72-hour treatment with BMS-536924 (Figure 6A). Interestingly, we also observed a very significant reduction in YB-1 mRNA and protein after 72 hour treatment with BMS-536924 (Figure 6B).

DISCUSSION

In this study we evaluate effect of YB-1 expression on the process of invasion and metastasis in the triple negative breast cancers. Specifically, we focused on this subtype of breast cancer because patients with triple negative breast carcinoma frequently die within the first two years after diagnosis. In addition, there are no targeted treatment

currently available for triple negative patients. In this report, we showed that there is an association between YB-1 and uPA expression in the primary triple negative breast tumours. Furthermore, we showed that YB-1 can regulate uPA expression and that silencing YB-1 results in a significant reduction in cancer cell invasion. We also evaluated the effect of a phospho-IGF-1R inhibitor as a potential drug for treatment of triple negative breast cancer patients.

There are a number of studies on involvement of YB-1 in cancer recurrence and metastasis. In 2005, Huang et al reported in a study of 42 patients that YB-1 was associated with recurrence although long-term follow-up was not evaluated (Huang et al. 2005). In a small pilot study, Janz et al. also reported that cancer relapsed in 66% of the patients with high levels of YB-1 expression in their breast tumours after post-operative chemotherapy treatment while patients with low YB-1 expression had no evidence of cancer recurrence (Janz et al. 2002).

More recently, we examined a large cohort of 4049 breast tumours and found that YB-1 expression is associated with shorter relapse free survival time in both the overall cohort and in each subtype of breast cancer defined by hormone receptor and HER-2 status (chapter 3). Importantly, in a multivariate survival analysis, we found that YB-1 is a stronger indicator for earlier relapse compared to other markers that are currently being used in clinic such as tumour size and grade, ER and HER-2 status (chapter 3).

There have been several reports on the role of YB-1 in metastasis and relapse. YB-1 was shown to mediate the expression of matrix metalloproteinase-2 (MMP-2) (Mertens et al. 1997; Mertens et al. 2002), matrix metalloproteinase-12 (MMP-12) (Samuel et al. 2005), collagen α1(I) (Norman et al. 2001), and collagen α2(I) (Dooley et al. 2006; Higashi et al. 2003). All the described genes are involved in cell adhesion, motility, invasion and thus metastasis of breast cancer cells. In this study, we determine that there is an association between YB-1 and uPA in the triple negative breast cancer subtype. In addition, we found that uPA is one of the YB-1 target genes using ChIP on chip in SUM149 cells. Therefore, we proposed that one possibility for the involvement of YB-1 in relapse and metastasis is the role in regulation of uPA, an important biomarker for cancer recurrence and metastasis.

We also found that uPA expression is associated with poor outcome and earlier time to relapse in this cohort of patients. Furthermore, we were able to determine that YB-1 is involved in regulating uPA expression using both siRNA and shRNA system. However the mechanism of regulation of uPA by YB-1 requires further investigation. Since, there are no commercially available YB-1 inhibitors we sought to investigate other pathways that might be involved in regulation of uPA and YB-1. Previous studies report that activated IGF-1R results in activation of PI3K/Akt signaling cascade leading to up-regulation of uPA expression in breast cancer cells (Dunn et al. 2001). Extensive studies in our laboratory illustrated that Akt phosphorylated YB-1 at serine 102 through IGF stimulation, resulting in nuclear localization of YB-1 (Sutherland et al. 2005). In the nucleus, YB-1 acts as a transcription factor, directly or indirectly (via binding to other transcription factors), and regulates downstream substrates such as epidermal growth factor receptor (EGFR). We now show that BMS-536924, a small molecule inhibitor of activated IGF-1R/1R, could inhibit uPA expression and postulate that this is through the observed reduction of YB-1 expression. We therefore believe that uPA is a potential downstream target of YB-1; whether this is through direct transcriptional activation needs further investigation.

In conclusion, YB-1 demonstrated prognostic significance in breast cancer by identifying patients that have a higher risk of cancer recurrence and may have a predictive role if these patients can benefit from treatment with a phospho-IGF1R inhibitor as the cell line data suggests. Since YB-1 can be detected by visual analysis automated immunostaining, it is very feasible to include this marker in routine clinical practice. With this method, we will be identifying patients with high risk of relapse who may require more aggressive and specialized treatment.

FIGURES

Table 1. There is an association between YB-1 and uPA expression in the triple negative breast cancers.

		YB-1	
uPA		Negative	Positive
	Negative	85/231 (36.8%)	146/231 (63.2%)
	Positive	50/206 (24.3%)	156/206 (75.7%)

Spearman's correlation value= 0.135
P value= 0.005

Table 2. List of potential YB-1 target genes determined by ChIP on chip analysis that are involved in invasion and metastasis. Standard deviations are derived from the fold enrichment values from two replicates. Fold enrichment values represent the degree of amplification of the promoter sequence bound by YB-1.

	Potential genes that are bound by YB-1	Fold change	Standard deviation
BRMS1	Breast cancer metastasis suppressor 1	3.67	0.747
CDH1	Cadherin type 1-E cadherin	4.01	0.682
CXCR4	Chemokine (C-X-C) receptor 4	4.14	0.615
KISS1	KiSS-1 metastasis suppressor	3.67	0.65
MMP 9	Matrix metallopeptidase 9 Gelatinase B	7.32	0.499
MMP 15	Matrix metallopeptidase 15	7.90	0.415
MMP16	Matrix metallopeptidase 28	12.62	0.27
MMP19	Matrix metalloprotease 19	5.06	0.600
MTA	Metastasis associated 1 family- member 3	6.29	0.344
MTSS1	Metastasis suppressor1	5.29	0.714
uPA	Urokinase plasminogen activator	6.95	0.694
VEGFC	Vascular endothelial growth factor C	5.19	0.475

Figure 1. uPA expression is associated with poor survival in the entire cohort of 4090 primary breast tumours. In this cohort, 3145 had interpretable data on uPA. uPA is expressed in 45% (n=1424/3145) of the patients that had RFS and BCSS data. Patient survival was based on deaths specifically related to breast cancer and not other causes. **A.** uPA expression is associated with shorter relapse free survival time in the entire cohort. N represents the number of patients that had breast cancer recurrence (N of event). **B.** uPA expression is associated with poor survival in the entire cohort. N represents the number of patients that died of breast cancer (N of event).

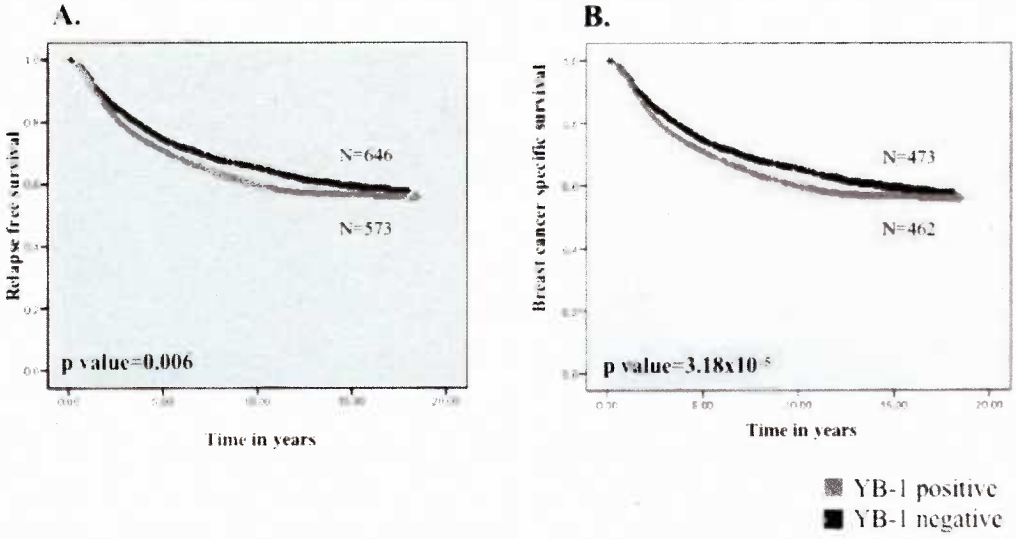
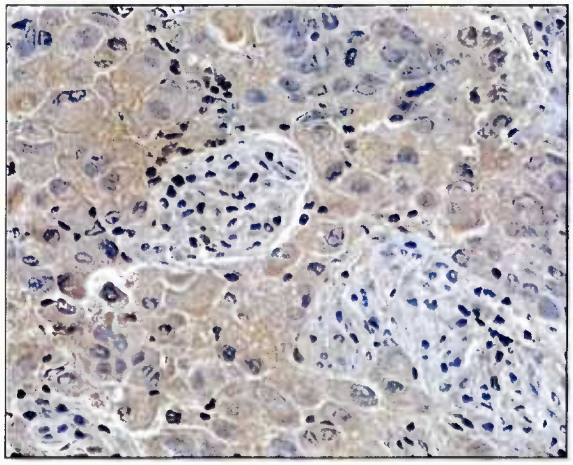


Figure 2. Representative immunohistochemistry images using uPA primary antibody in this cohort.

Positive staining



Negative staining

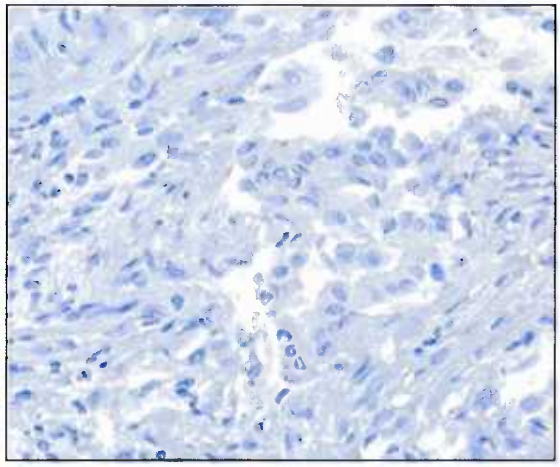


Figure 3. Silencing YB-1 results in a significant reduction in uPA mRNA expression. A) SUM149 cells were treated with 5μg of YB-1 siRNA or control siRNA for 48 and 72 hours. B) SUM149 cells were transfected with YB-1 shRNA containing vector and cells were selected with G418. The experiments were performed in triplicate in two separate occasions.

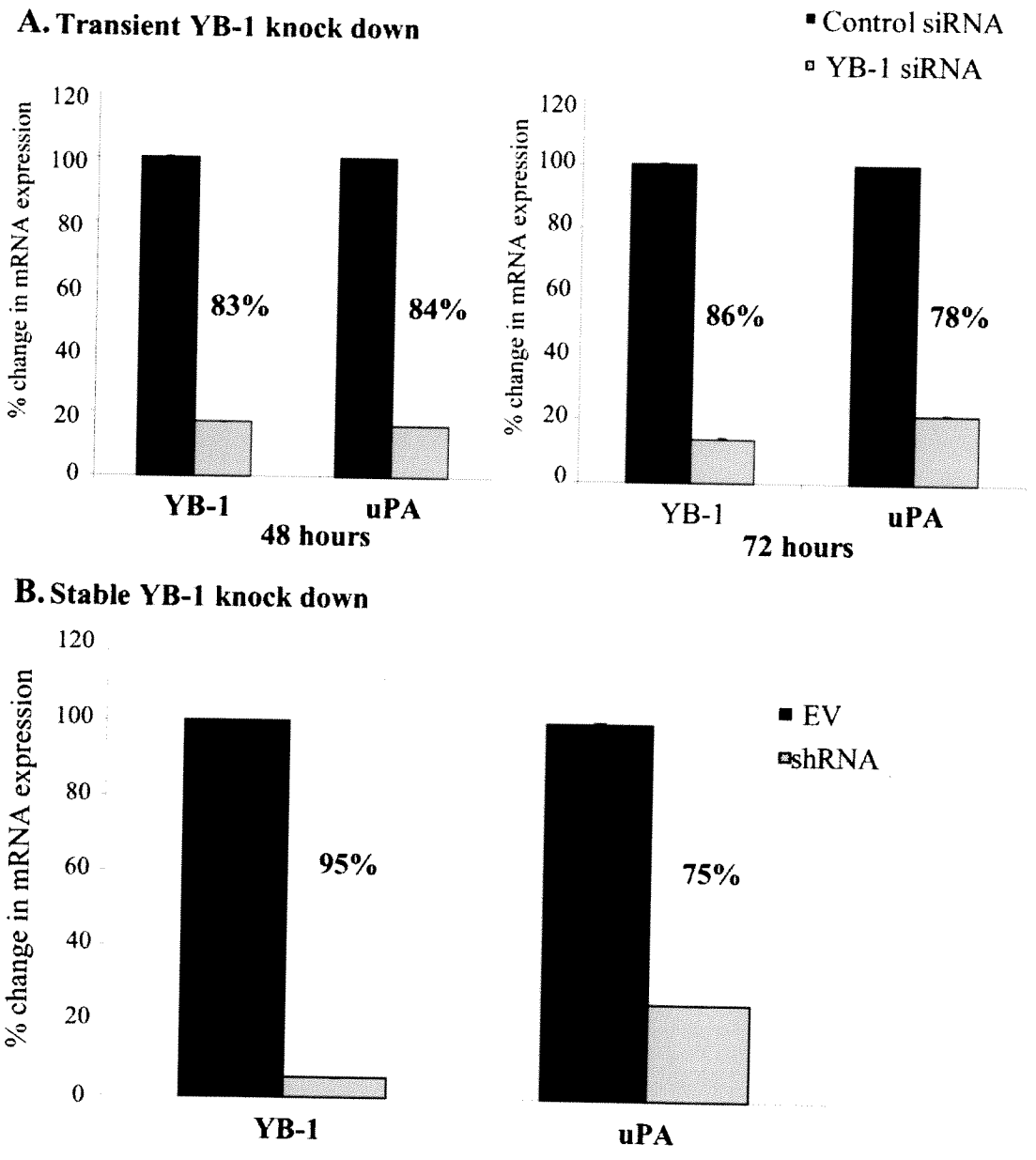


Figure 4. Silencing YB-1 with siRNA results in a significant reduction in uPA protein secretion (A), YB-1 knock down confirmed (B). Inhibiting YB-1 with shRNA results in significant reduction in uPA protein secretion (C), YB-1 knock down confirmed (D).

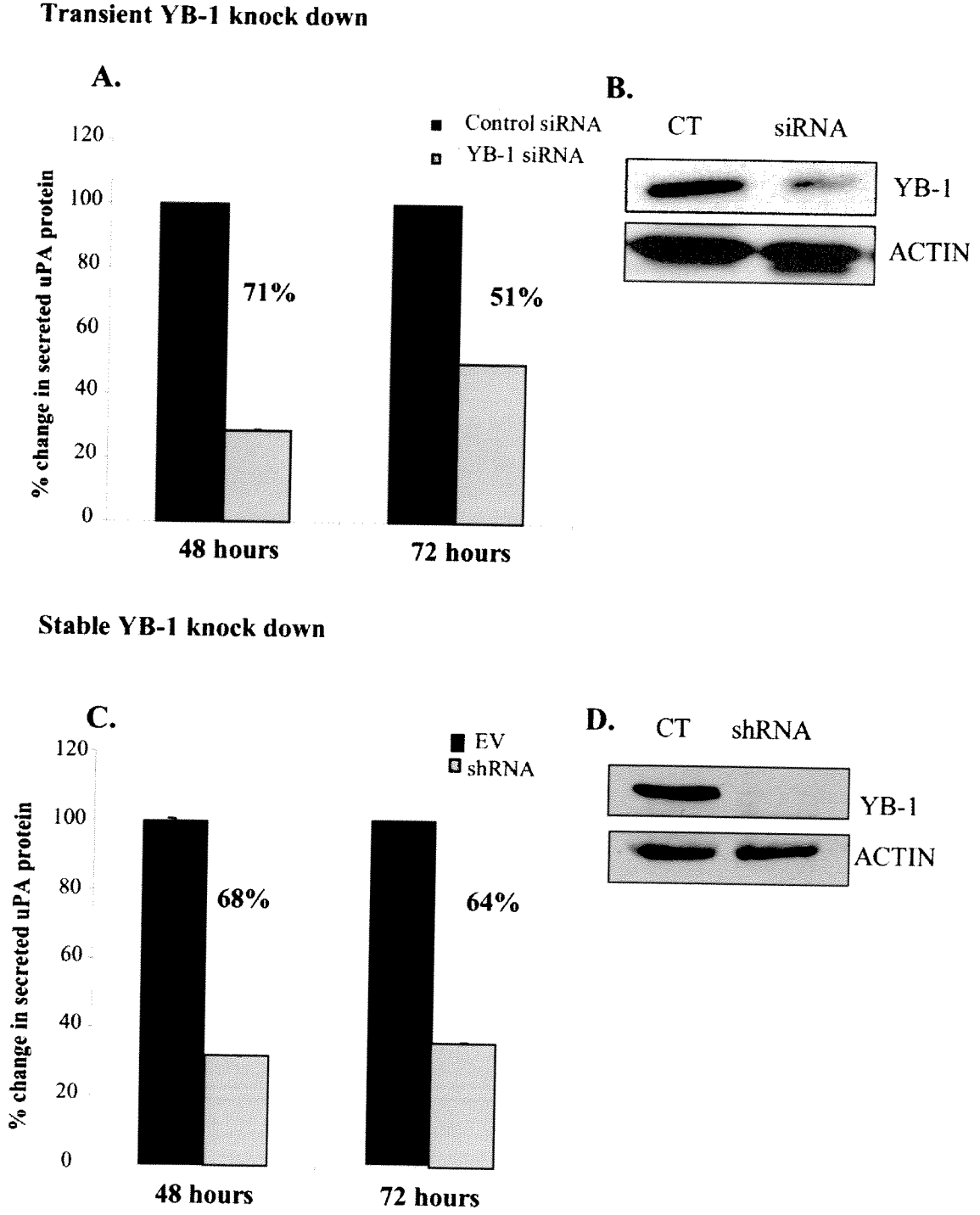


Figure 5. Suppression of YB-1 or uPA for 72 hour with siRNA results in inhibition of the SUM149 cells ability to invade through matrigel. A) uPA knocked down was confirmed by ELISA **B)** Silenced YB-1 was confirmed by Western blot analysis. **C)** Invasion assay was performed to evaluate the effect YB-1 or uPA siRNA on number of SUM149 cell that invade through matrigel.

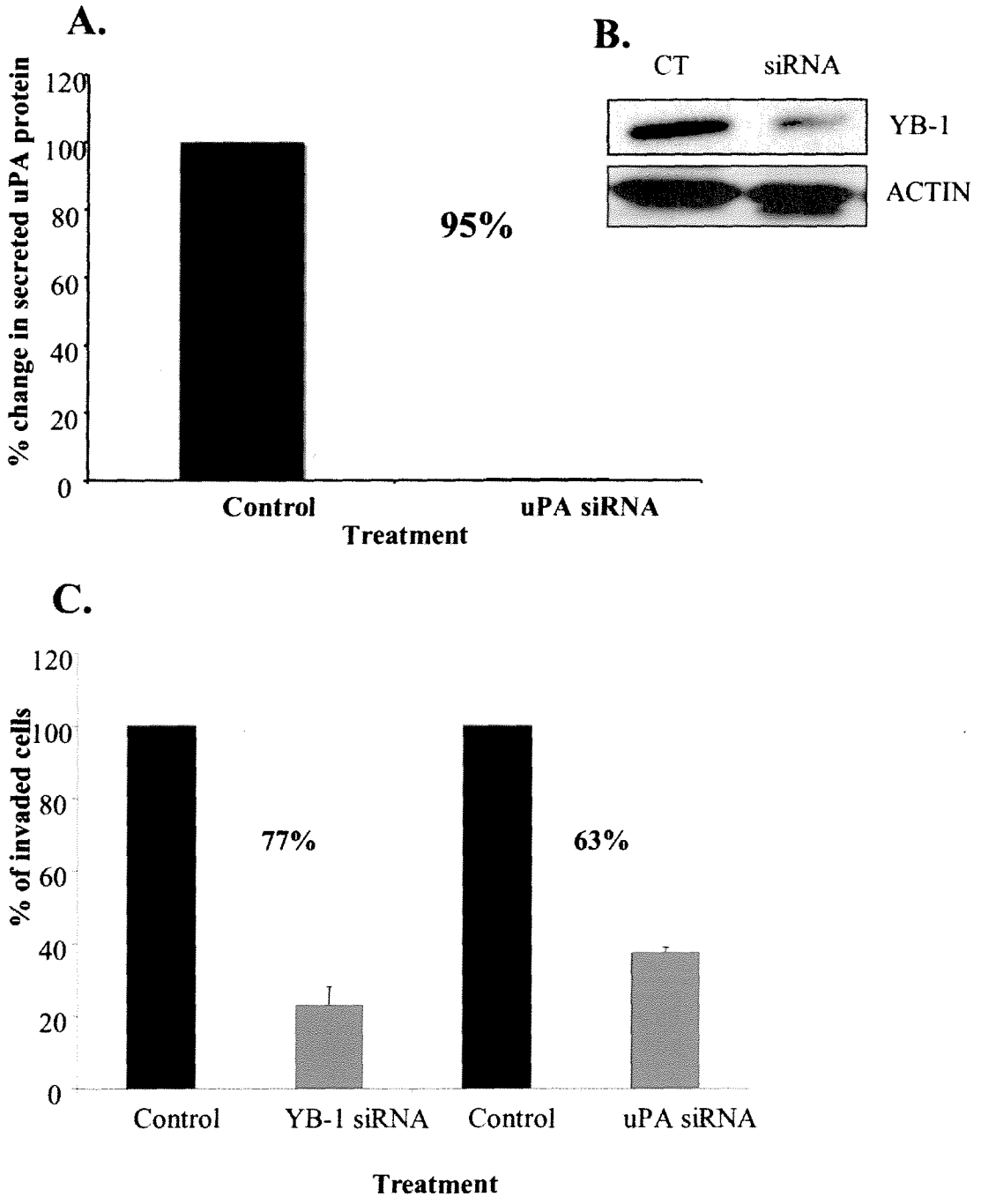
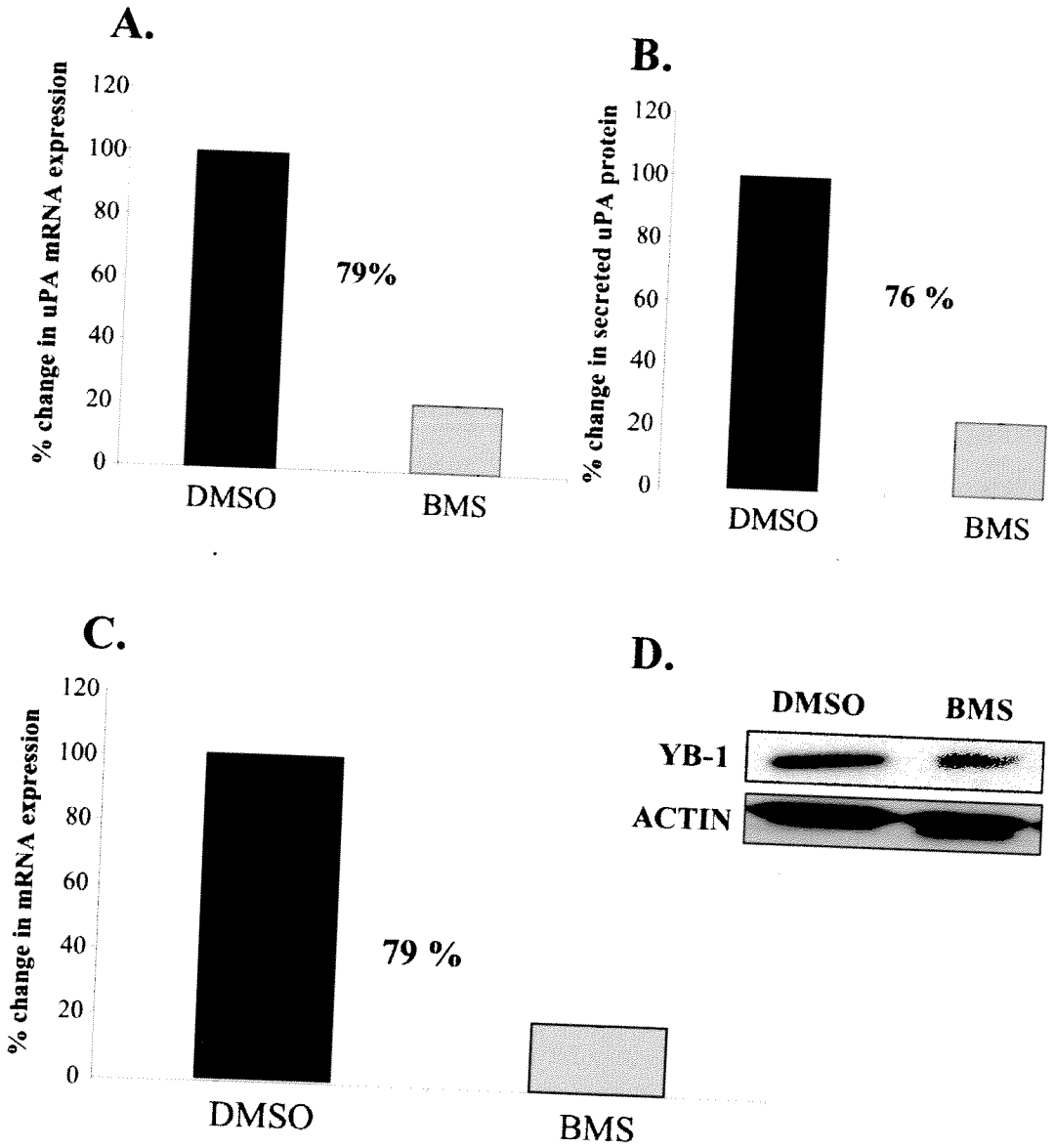


Figure 6. BMS-536924 inhibits uPA expression at the mRNA (A) and the protein (B) level. This small molecule inhibitor also suppresses YB-1 expression at mRNA (C) and protein (D) level.



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CHAPTER 5

Tumour Tissue microarray screen for assessing patients with activated insulin-like growth factor-1/insulin receptors: A predictive tool for prioritizing patients that may benefit from inhibitors to this pathway.

INTRODUCTION

The insulin-like growth factor-1 receptor (IGF-1R) has become an attractive molecular target for cancer given that it is expressed in a wide range of tumours including those that arise in the breast. Several studies indicate that IGF-1R is associated with the growth, invasion and metastasis of breast cancer (Kucab et al. 2003). Importantly, the expression of constitutively activated IGF-1R in the mammary gland leads to the development of tumours (Carboni et al. 2005). In addition, Kim et al. (2007) reported that over-expression of a constitutively activated IGF-IR (CD8-IGF-IR) was sufficient to cause transformation of immortalized human mammary epithelial cells and growth in immunocompromised mice (Kim et al. 2007). Conversely, silencing IGF-1R with small molecules (Carboni et al. 2005) inhibits the growth of mammary cells (IGF-1R-Sal) expressing constitutively activated IGF-1R in a xenograft model (Carboni et al. 2005). Further to this concept, disruption of IGF-1R with a dominant negative inhibitor suppresses breast cancer metastases (Dunn et al. 1998; Sachdev et al. 2004). This supports earlier studies demonstrating that IGF-1R neutralizing antibodies suppress the growth of breast cancer cells implanted as xenografts (Arteaga et al. 1989). These and many other studies support the goal of targeting IGF-1R for the treatment of breast cancer.

Given the growing interest in IGF-1R as a target for cancer therapy (Pollak et al. 2004), several pharmaceutical companies have developed inhibitors to it (Garber 2005). It is therefore timely that we now evaluate the role for activated IGF-1R/IR in the large series of primary breast tumours in relation to outcome. Understanding the clinical relationship for IGF-1R signaling in different subtypes of breast cancer is also very important in treatment of breast cancer patients since each subtype has clinically

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distinct outcome and requires different treatment protocols. The ER-positive or luminal breast cancers usually have better outcome compared to aggressive ER-negative breast cancers, HER-2 and basal-like (Perou et al. 2000). Tamoxifen or other aromatase inhibitors are usually suggested for patients with cancers expressing hormone receptors whereas trastuzumab is prescribed for HER-2 over-expressing breast cancer patients. Unfortunately, there is no commercially available targeted treatment for basal-like breast cancers at the moment. These patients do not express estrogen receptor (ER), progesterone receptor (PR) and do not have amplified HER-2 in their tumours. They are also often referred to as "triple negative" patients and frequently die within two years of diagnosis (Van 't Veer et al. 2002).

IGF-1R pathway has not only been implicated substantially in growth of hormone responsive breast cancer models such as MCF-7 through its synergy with the estrogen receptor (ER), but has also been reported to contribute to acquired drug resistance to clinically relevant agents such as tamoxifen (Knowlden et al. 2005). Activation of IGF-1R signaling cascade is also associated with resistant to Herceptin (Lu et al. 2004; Lu et al. 2001; Nahta et al. 2005) or anti-EGFR agents such as gefitinib (Lu et al. 2001).

We previously explored total IGF-1R expression in clinical disease, where levels were evident in 87% of primary breast tumours by screening a cohort of 930 patient samples (Nielsen et al. 2004). At that time, we were unable to determine whether or not the receptor was activated because suitable antibodies were not available at that time, although a subsequent small study has indicated that activation of IGF-1R should be detectable in clinical disease (Gee et al. 2005). If we were able to identify patients that express activated IGF-1R it would then help us to prospectively select patients that might benefits from inhibitors to it particularly as the IGF-1R pathway has not only been implicated substantially in growth of hormone responsive breast cancer models such as MCF-7 through its synergy with the estrogen receptor (ER), but has also been reported to contribute to acquired drug resistance to clinically relevant agents such as tamoxifen (Knowlden et al. 2005). Activation of IGF-1R signaling cascade is also associated with resistance to tratuzumab (Lu et al. 2004; Lu et al. 2001; Nahta et al. 2005) or anti-EGFR agents such as gefitinib (Lu et al. 2001). Due to this, we also felt it would be beneficial to develop high-content screening (HCS) assays to evaluate potential P- IGF-1R

inhibitors in this disease. Herein we describe a system to identify activated IGF-1R and the impact of the inhibitor BMS-536924 (Wittman et al. 2005) on SUM149 cell growth and the induction of apoptosis in a multi-channel single well assay performed in a 2-dimensional system where the cells were plated on collagen I. This was addressed in several models of breast cancer including MCF-7 cells (as a model ER-positive breast cancer), MCF-7 Tamoxifen resistant (Tam-R) and SUM149 (as a model for ER-negative basal-like breast cancer).

MATERIALS AND METHODS

Clinical breast cancer TMA studies

The TMA was comprised of duplicate cores from 481 primary breast tumours. Construction of the TMA and the clinical correlates were previously described by us (Kucab et al. 2005; Sutherland et al. 2005). The TMA's screened for P-IR/IGF-1R once we confirmed the specificity of the antibody. Previous studies by this group (JG, HJ, RN) demonstrated that IGF-1R is highly expressed and activated in DU145 prostate cancer cells that had acquired resistance to Gefitinib (DU145/TKIR) (Jones et al. 2004) based on immunoblotting. We therefore selected the DU145/TKI cells for the initial validation. The cells were maintained in DCCM-1 media containing 1 μM gefitinib were plated at 7.5 x 10^3 cells/ 22mm^2 coverslip and grown to 60% confluence as previously described (Jones et al. 2004). The DU145/TKI cells were treated with or without the IGF-1R-selective tyrosine kinase inhibitor AG1024 for 24hr (20uM; Jones et al. 2004). For in situ detection, the cells plated on coverslips were immunostained for phosphorylated IGF-1R as described in Jones et al., 2004, in this instance using the phosphorylated IGF-1R 1131/IR 1146 antibody (Cell Signaling Technologies #3021). Following this, the cells were treated above and then cell pellets were formalin fixed and embedded in histogel. The resulting samples were then embedded in parafin and processed the same way that clinical samples would be from the TMA. The specificity of the phosphorylated IGF-1R 1131/IR 1146 antibody was further confirmed by immunoblotting where a single band was detected and this could be inhibited with AG1024. Western blotting was performed in parallel on DU145/TKIR cells +/- 24hr 20μM AG1024 treatment using a

1/1000 dilution of the same phosphorylated IGF-1R 1131/IR 1146 antibody according to previously described methods (Jones et al. 2004).

Following this initial verification of antibody staining specificity, staining in primary breast tumours with the P-IGF-1R/IR antibody (Cell Signaling Technologies #3021) was assessed immunohistochemically in the formalin-fixed, paraffin embedded TMA's. The phosphorylated IGF-1R 1131/IR 1146 antibody (Cell Signaling Technologies #3021) staining procedure as applied to 5μm sections from the TMA involved an initial blocking of endogenous peroxidases with 3% hydrogen peroxide for 5 minutes. An antigen retrieval step was then performed by placing the slides in a pressure cooker in pH 8 EDTA followed by 10 min under gently running tap water. The slides were then blocked in 0.02% Tween/PBS blocking solution for 5 min. The p-IGF1R/IR antibody was diluted 1:20 in PBS, and the slides were incubated overnight at 23 °C. The next day, the slides were washed in PBS three times then the rabbit EnVision peroxidase labeled polymer antibody (Dako; #K4011) was applied for 2 hours at 23 °C and then developed with DAB/H_20_2 chromagen for 8 minutes and counterstained with Hematoxylin. Internal positive and negative control slides for the TMA assay comprised 5μm sections of formalin-fixed/paraffin embedded DU145/TKIR cell pellets (Jones et al., 2004) and R-(IGF-1R-null) fibroblast pellets respectively (kind gift from V. Macaulay, Weatherall Institute of Molecular Medicine, Oxford, UK & R. Baserga Kimmel Cancer Centre, Philadelphia) (Sell et al. 1994).

To assess P-S6 the TMA slides were incubated at 60 °C prior to deparaffinization and rehydration step. In the antigen retrieval step, slides were in citrate buffer and heated for 98 °C in steamer for 30 minutes. Slides were held at room temperature for 20 minutes to cool down and were then washed three times in PBS for 5 minutes. Then the slides were incubated with hydrogen peroxide for 10 minutes and they were again washed three times in PBS for 5 minutes. The slides were blocked with DAKO protein block (Dako Cytomation Protein Block Serum-Free, ref no X0909) and stained with rabbit polyclonal P-S6 antibody (Cell Signaling, Ser235/236, cat no 2211) at a dilution of 1:200 overnight. The secondary antibody was applied directly to the slides (Dako Cytomation Envision R system, HRP, anti-rabbit, cat#K4002) for 30 minutes. The arrays were washed with PBS/tween 20 (0.1%) 3 times for 5 minutes after each step and stained with Nova Red

(Vector NovaRED substrate kit from Vector laboratories, cat no, SK-4800) for 1 minute and counterstained with Hematoxylin for 30 minutes. YB-1, P-Akt, ER and HER-2 staining and scoring criteria has been described previously by us (Kucab et al. 2005; Sutherland et al. 2005). IGF-1R staining and scoring was performed according to Nielsen et al. (Nielsen et al. 2004).

Statistical analysis

Correlations between P-IGF-1R/IR, IR, IGF-1RP-S6, P-Akt, YB-1, Ki67 and ER staining in the clinical samples were determined using a Spearman's test (SPSS software, version 13, Chicago, IL). Patient survival was determined using Breslow univariate statistical analysis and Kaplan-Meier curves. Data was filtered to exclude patients who were missing diagnostic or survival information. Results were considered statistically significant with $P< 0.05$.

Breast cancer cell line studies and drug treatments

Hormone responsive parental MCF-7 cells and the tamoxifen resistant subline MCF-7 Tam-R (Knowlden et al. 2003) were obtained from R Nicholson (University of Cardiff, Wales). For experimental purposes, the parental cell line was grown in 5% phenol red-free RPMI, 1% penicillin/streptamyacin and 4mM L-glutamine. Tam-R cells were routinely grown in the same media with the addition of 100 nM 4-OH-tamoxifen (Sigma Chemical Co. Oakville, ON) to sustain resistance. Cell culture for the basal-like breast cancer models, SUM149 cells were previously described (Stratford et al. 2007). To examine the effect of BMS-536924 *in vitro* cells were plated ($2x10^5$ cells/well) in 6-well dishes and allowed to attach overnight. The next day they were changed to serum free media for 24 hours. BMS-536924 was introduced (0.5-2 μM) 1 hr prior to the addition of IGF-1 (100 ng/ml, Long-R3 IGF-1, Cederlane, ON). Proteins were then extracted and evaluated for changes in P-S6 by immunoblotting.

IGF-1R inhibitor evaluation in breast cancer in vitro

High content screening

To understand the fate of the MCF-7 cells following drug treatment we established a multi-channel high content screening protocol to simultaneously measure different aspects of apoptosis. Using the ArrayScan VTI (Cellomics, Pittsburg PA), we first established that IGF-1R/IR was activated by developing a simple immunofluorescence method to detect phosphorylated IGF-1R/IR. MCF-7 cells were seeded in 96 well plates in RPMI medium (Gibco/Invitrogen) supplemented with 5% FBS and incubated (37°C, 5% CO_2) for 48 hours. Immediately after aspirating the medium, 100 µL of 2% paraformaldehyde in PBS was added and the cells were kept at room temperature (RT) for 20 minutes. After washing with PBS three times, the cells were permeabilized in 0.1% Triton-100 in PBS for 15 minutes followed by 1% BSA in PBS for 30 minutes. The cells were then incubated with rabbit anti-phospho-IGF-1R/IR antibody diluted 100X (Cell Signaling Technologies #3021) overnight at 4 °C followed by the secondary goat anti-rabbit antibody conjugated with Fluro®488 diluted 200X (Invitrogen) for 1 hour at room temperature. The nuclei of the cells were stained in Hoechst dye (100 µL at 1 µg/ml) for 5 minutes. The cells were washed with PBS 3 times after each of the steps mentioned above. The images were taken on an ArrayScan® Reader (Cellomics, Pittsburgh, PA). The control cells were treated as above however there was no primary antibody added.

Once we established that IGF-1R was indeed activated under the culture conditions used, we established a screen for apoptosis indices following exposure to BMS-536924. MCF-7 and MCF-7 Tam-R cells were seeded in 96-well plates (Collagen I coated, BD, Franklin Lakes, NJ) at 5,000 cells/well in 100µl of Ham's F12 medium (Gibco/Invitrogen) supplemented with 5% FBS. We found that the Collagen I coated plates provides two advantages. The first advantage was this two-dimensional culture system was more representative of the epithelial-stromal environment that breast cancer cells would normally encounter. Collagen I was selected because it constitutes one of the most abundant extracellular matrix proteins in the breast (Provenzano et al. 2006). Secondly, the cells attached to the plates better when plated on collagen I which was a particular benefit because there are several wash steps in the protocol which otherwise

caused significant variability due to cell detachment. The plates were incubated at 37°C with 5% CO_2 for 24h. BMS-536924, dissolved in DMSO, was diluted in the medium containing either IGF-1 (100 ng/m) or IGF-1 plus estradiol (10 nM) and added accordingly (100 μL/well) to each well to give final concentrations of 0 (DMSO only), 0.05, 1.0 or 2.0 μM of the drug (total volume = 200 μL/well). Tamoxifen was tested at a range of concentrations (0.5-4 μM). There were three replicate wells for each treatment. Thirty minutes before each endpoint (2, 6 or 24 hours), 20 μL of Hoechst and propidium iodide (PI) solutions were added to each well to give a final concentration of 1 μg/ml of each dye in the medium. Following 30-minute incubation, the medium was aspirated and the cells were gently washed with PBS three times. The cells were then fixed in 2% paraformaldehyde in PBS for 20 minutes and permeabilized in 0.1% Triton-100 in PBS for 15 minutes. After blocking in 1% BSA in PBS for 30 minutes, the cells were incubated with primary mouse anti-phospho-H2AX antibody (AbCAM, Cambridge, MA) diluted 100X for 1 hour at RT followed by secondary donkey anti-mouse antibody (FITC) (Jackson ImmunoResearch, West Grove, PA) diluted 200X for 1 hour at RT. The cells were washed with PBS three times after each step mentioned above and were finally kept in PBS (100 μL/well) at 4 °C. The plates were analyzed and the images taken on the ArrayScan® Reader. Six hundred cells were analyzed for each replicate well and the results were presented as a mean ± SD. Repeated tests (n=3) showed identical results.

Soft agar studies

MCF-7 parental and MCF-7 Tam-R cells ($2.5x10^4$) were placed into soft agar as previously described (Sutherland et al. 2005) and allowed to grow for 28 days at which time the colonies were counted. Each treatment was performed in replicates of three on two separate occasions.

RESULTS

Initially the P-IGFR/IR 1131/1146 antibody was assessed for specificity of immunostaining using the acquired gefitinib resistant prostate cancer subline DU145/TKI. These cells have previously shown to have enhanced IGF-1R activity (as

measured using an alternative activated IGF-1R antibody) in relation to the parental DU145 cells that can be depleted by using IGF-1R tyrosine kinase inhibitors such as AG1024 (Jones et al. 2004). In keeping with these data, P-IGF-1R1131/IR 1146 staining was detected as a crisp plasma membrane signal in the DU145/TKI cells, which was depleted by treating with AG1024 (20uM) when the receptor was stained in situ (Figure 1A). Similarly, P-IR/IGF-1R was detected when the cells were formalin fixed and parafin embedded but not when the cells were treated with the inhibitor (Figure 1B). These findings corroborated by an equivalent profile by immunoblotting (Figure 1C). Having confirmed the specificity of the antibody, breast cancer TMA's were screened for levels of P-IGF-1R/IR by immunohistochemistry along with P-S6, IR and IGF-1R (representative images, Figure 2A-D). In this cohort of 481 patients, P-IGF-1R/IR was expressed in 49% of tumours (199/407 scorable cases, Table 1). In the clinical breast cancers, positive P-IGF-1R immnostaining was associated with poor survival when assessed at 15 (Breslow p value=0.046) and 20 years (Breslow p value=0.051) (Figure 3A and B). Activated receptors directly correlated with staining level of P-S6 ($p = 1.4x10^{-4}$) in the TMAs (Table 2 and 3). However, P-IGF-1R/IR did not correlate with IGF-1R, IR, ER, YB-1, P-Akt, or Ki67 (Table 3). It appeared that P-Akt was a less reliable indicator of P-IGF-1R/IR given that the association between these variable was not significant ($p = 0.836$), suggesting that P-S6 is a better sentinel marker for activated state of these receptors. There was also surprisingly no correlation with the estrogen receptor (Table 3) suggesting that P-IGF-1R/IR targeted inhibitors would benefit patients independent of their hormone receptor status. We have also evaluated insulin receptor (IR) expression in this cohort, although its expression was associated with poor survival ($p = 0.006$, Figure 4A) but there was no correlation between either activated P-IR/IGF1R or P-S6 (data not shown). However, total level of IGF-1R, were not significantly associated with poor patients outcome (Figure 4 B). Yet, it was correlated with P-S6 expression further confirming the value of P-S6 as a specific marker for activation of IGF-1 receptor. We determined that activation of P-IGF-1R/IR is frequent in breast cancer, implying it may comprise an important new therapeutic target in this disease.

Because we determined that approximately half of breast cancer in our cohort expressed activated P-IGF-1R/IR we sought to develop a high content screen to evaluate

potential inhibitors. Using the same antibody that was applied to the TMA study, we developed an immunofluorscence HCS method to detect activated levels of P-IGF-1R/IR using the Cellomics ArrayScan VTI. This antibody produced crisp membranous staining as it did on the tissues in the MCF-7 cells (Figure 5A). Following the identification of activated P-IGF-1R/IR we screen the cells for their response to the small molecule inhibitor BMS-536924. The drug inhibited the growth of MCF-7 cells as a single agent and improves the growth suppressive effects of Tamoxifen. This was demonstrated in presence of IGF-1 (Figure 5B left panel) as well as cultures containing IGF-1 plus estradiol (Figure 5B, right panel). Further to this, we demonstrate that BMS-536924 inhibits signaling through s6 (Figure 5C).

This prompted us to address whether the drug would have a similar effect on the growth of tamoxifen resistant cells given that so many patients ultimately succumb to their disease once resistance is mounted. BMS-536924 in the presence of IGF-1 alone (Figure 5D, left panel) or combined with estradial (Figure 5D, right panel) suppressed the growth MCF-7 Tam-R cells. It was noteworthy that the MCF-7 TamR cells were actually more sensitive to the growth inhibitory effects of the drug than the MCF-7 WT cells. Moreover, in the MCF-7 Tam R cells the drug induced apoptosis as shown by an increase in chromatin condensation, phosphorylation of H2AX and propidium iodide uptake based upon high content screening (Figure 6 Aand B). Similar results were obtained in a screen with the MCF-7 wild-type cells (data not shown).

Consistent with these findings, BMS-536924 also had a robust inhibitory effect on the growth of the MCF-7 and MCF-7 Tam-R cells in soft agar (Figure 7A and B). However the IC50 was approximately 2 μM for MCF-7 Tam-R cells whereas it is 0.5 μM for the MCF-7 parental cells.

We have demonstrated that estrogen receptor positive breast cancer cells, MCF-7 and MCF-7 Tam are sensitive to P-IGF-1R inhibition. However based on our tumour tissue microarray analysis the expression of P-IGF-1R is not restricted to ER-positive breast cancer, in fact we find it expressed in 45.5% (n=15/33) of the triple negative breast cancers as well (Table 4). We therefore screened a panel of breast cancer cells lines for IGF-1R expression and noticed that the basal-like breast cancer cell line SUM149 express moderate levels of the receptor (Figure 8A). Using high content screening method, we

were able to detect the activated IGF-1 receptor in these cell lines (Figure 8B). We also discovered that SUM149 cells were also very sensitive to inhibition of P-IGF-1R with BMS-536924 (Figure 8C). This inhibitor has a very robust effect on inhibiting the growth of SUM149 cells in a dose dependent manner.

DISCUSSION

In this study, we find that P-IGF-1R/IR is associated with increased rate of breast cancer-related deaths. We also find a strong correlation between P-IGF-1R/IR and P-S6 suggesting that this down-stream marker could be used to monitor the effect of inhibitors in the clinic. More importantly, we observed no correlation between total IR and P-IGF1R or P-S6 further upheld the prognostic value of P-S6 as specific marker for IGF-1 receptor activation in primary tumours. While we find that P-IGF-1R/IR is associated with poor survival this is in contrast to the Nottingham Breast unit study where P-IGF-1R was stained with a different antibody, pY1316, which detects only activated IGF-1R. In that study, P-IGF-1R was not associated with poor survival however the cohort differed from ours in that the cases (n=64) were all ER-positive patients who underwent sequential needle-core biopsies before being treated with Tamoxifen and subsequently when the disease progressed through the treatment (Gee et al. 2005).

Currently, there are two major approaches to inhibit IGF-1R signaling pathway; blocking access to the activating ligand and down-regulating receptor expression at plasma membrane mostly with antibodies versus inhibiting the receptor kinase activity with small molecules (Hofmann et al. 2005). IGF-1R neutralizing antibodies, CP-751, 871 (Cohen et al. 2005) and 19D12 (Wang et al. 2005) inhibit the growth of MCF-7 cell *in vitro* (Cohen et al. 2005). This approach to inhibiting IGF-1R also improves the efficacy of chemotherapeutic agents used to treat cancer including adriamycin, 5-fluorouracil and tamoxifen (Cohen et al. 2005). Furthermore, combining CP-751,871 enhances the tamoxifen responsiveness of MCF-7 cells in mice (Cohen et al. 2005). It is therefore highly likely that small molecule inhibitor, BMS-536924, will have a similar effect.

Two recent dual specificity inhibitor, BMS-554417 and BMS-536924, are found to target both the IGF-1R and IR (Hofmann et al. 2005). One advantage of these inhibitors is that they are orally available whereas neutralizing antibodies are administrated intravenously (Hofmann et al. 2005). Haluska et al. (2006) reported BMS-554417 inhibits tumour growth of IGF-1R-Sal tumour xenografts (Haluska et al. 2006). In another study, BMS-536924 was also shown to inhibit tumour growth *in vivo* (Kim et al. 2007)

This possibility that BMS-536924 treatment leads to enhanced response to Tamxifen would translate in to better care for women given that 290,000 of them will be diagnosed with breast cancer in North America. In this population, 60%-70% of them will have tumours that express the estrogen receptor and therefore will be candidates for anti-hormone treatment ie Tamoxifen, or Raloxifen. What still remains a serious concern is the de novo and acquired resistance to Tamoxifen and this will be a problem for 30-40% of patients. In previous studies, MCF-7 Tam-R growth was previously reported to be sensitive to IGF-1R inhibition using AG1024 (Knowlden et al. 2005). However, AG1024 is an agent that can be used only for research purposes. Taking this into consideration, we determined that MCF-7 Tam-R cells were sensitive to IGF-1R inhibition using BMS-536924. Thus it is possible that breast cancer could be more effectively treated by combining IGF-1R inhibitors with anti-estrogens such as Tamoxifen or more recently Raloxifen. Furthermore, we evaluated the P-IGF-1R in different subtypes of breast cancer. Since P-IGF-1R was also expressed in ER-negative breast cancers, we were motivated to assess the effect of BMS-536824 on aggressive basal-like breast cancer cells. Interestingly, treating SUM149 cells with this inhibitor resulted in inhibition of cancer cell growth in a dose dependent manner and suggesting that BMS-536924 might be a great candidate for treatment of aggressive BLBC which are very challenging to treat in clinic at the moment.

In closing, we now have the opportunity to a priori evaluate which patients would benefit from IGF-1R inhibitors by measuring levels of the activated receptor. We also provide evidence that BMS-536924 is a P-IGF-1R inhibitor that shows promising pre-clinical activity in Tamoxifen-sensitive and Tamoxifen-resistant and aggressive triple negative breast cancer models. These findings suggest that inhibiting P-IGF-1R with

small molecule inhibitor might be a potential solution for some of the obstacles in treatment of patients with breast cancer.

Figure 1. Optimization of P-IGF-1R/IR immunostaining. A) Validation of P-IGF-1R antibody by cells in culture, B) validation of P-IGF-1R antibody on paraffin embedded cell pellets, C) Validation of P-IGF-1R antibody by immunoblotting.

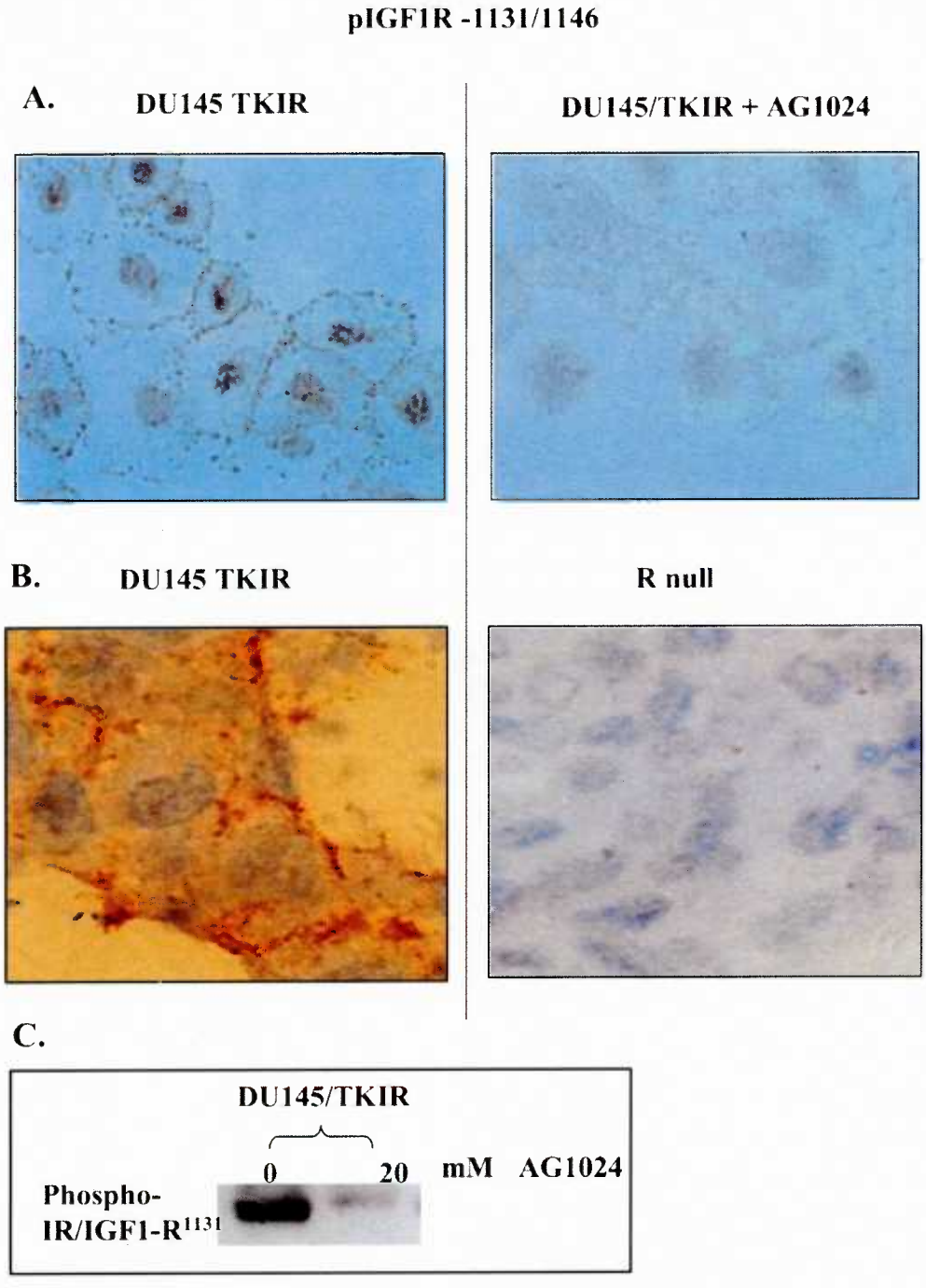


Figure 2. Panel of immunohistochemical stainings on the primary breast tumours. Examples of immunostaining with A) Phospho-IGF-1R antibody B) Phospho-S6 ribosomal protein antibody C) Insulin receptor antibody D) Total-IGF-1R antibody.

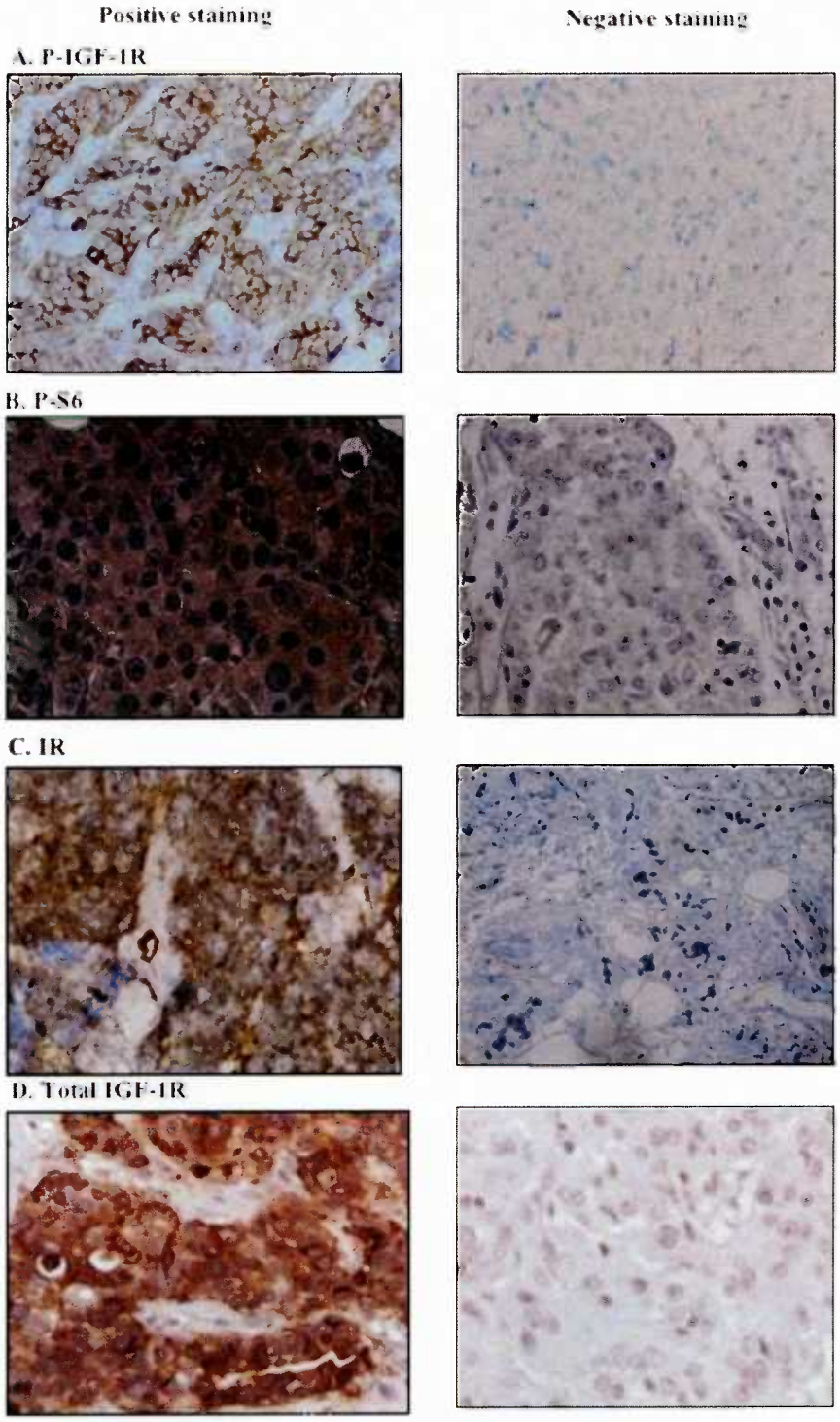


Table 1. Frequency of P-IGF-1R expression in the cohort of 438 primary breast tumours.

Binarized P-IGF1R

		Frequency	Valid Percent
Valid	Negative	208	51.1
	Positive	199	48.9
	Total	407	100.0

Figure 3. Effect of P-IGF-1R expression on breast cancer specific survival. P-IGF1-R was stained positive in 47% (n=173/361) of the patients that had BCSS data. Patient survival was based on deaths specifically related to breast cancer and not other causes Univariate survival analysis after A) 15 years B) 20 years. N represents the number of patients that died of breast cancer (N of event).

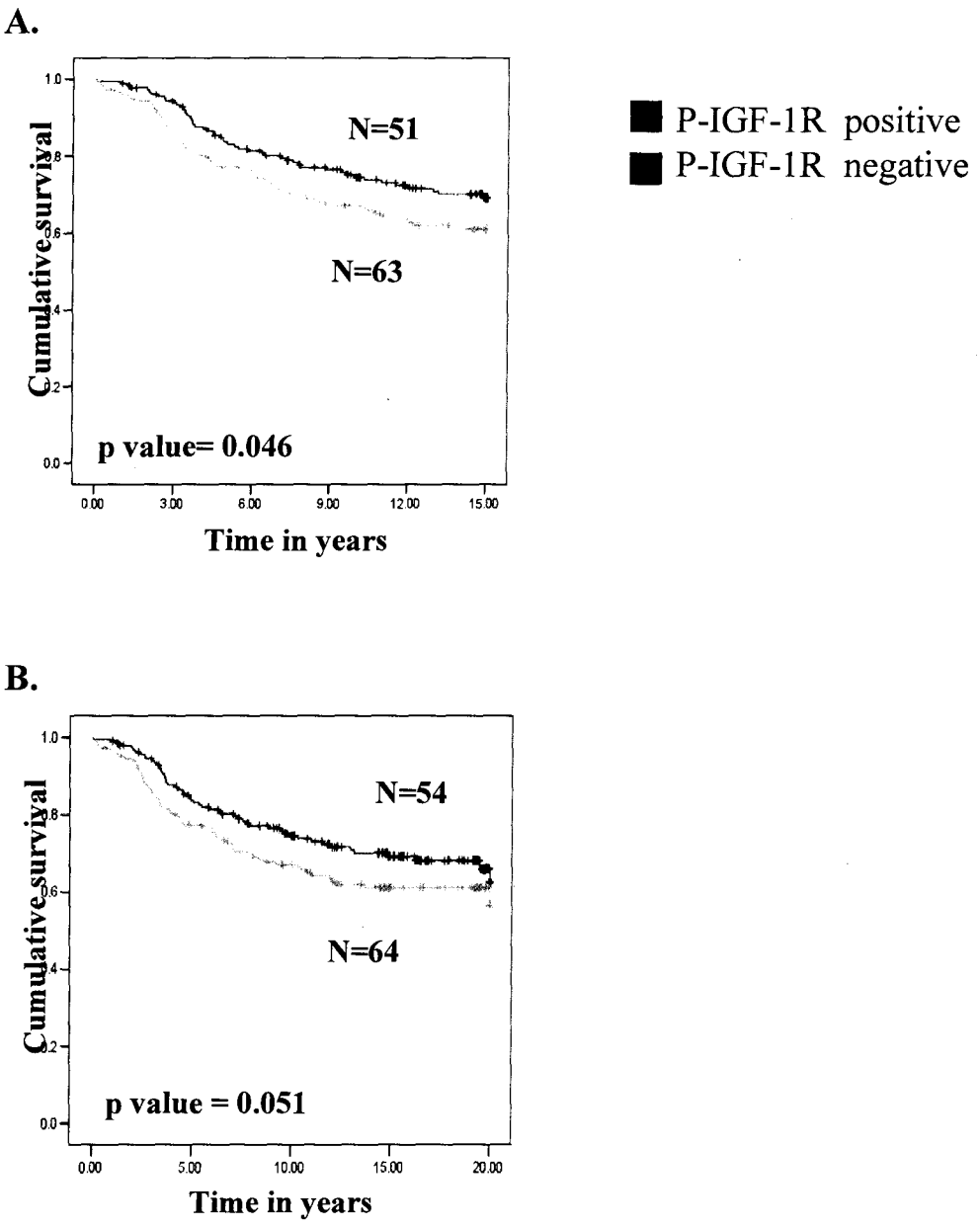


Table 2. Crosstab analysis between P-IGF-1R and P-S6 in 389 primary breast tumours.

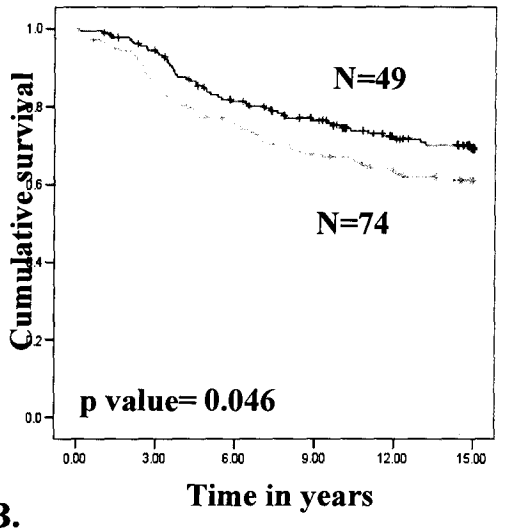
		P-IGF1R	
P-S6		Negative	Positive
	Negative	83/155 (53%)	72/155 (46%)
	Positive	46/144 (31%)	98/144 (68%)

Table 3. Correlation between P-IGF-1R/IR and IGF-1R, IR, ER, P-AKT, YB-1, Ki 67, and P-S6 in primary tumours. Spearman's correlation was used to analyze 438 cases of primary breast tumours.

	Spearman's correlation	**P value**
P-S6	0.218	1.454×10^{-4}
P-Akt	0.012	0.836
Ki67	0.094	0.070
YB-1	0.041	0.456
ER	-0.057	0.348
IR	0.065	0.206
IGF-IR	-0.085	0.123

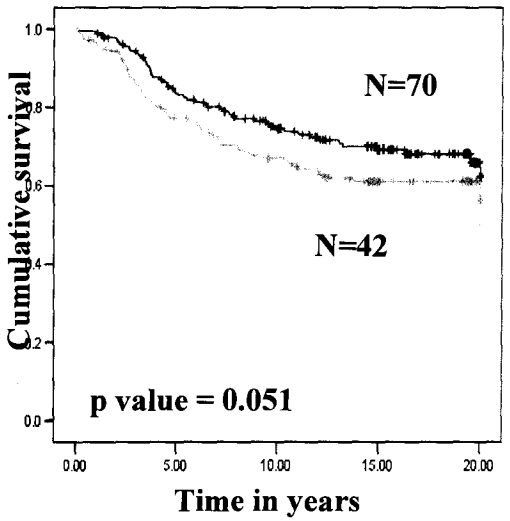
Figure 4. Effect of Insulin receptor and IGF-1R expression on breast cancer specific survival in the cohort of 438 primary breast tumours. A) IR is expressed in 48% (179/366) of this cohort and it's expression is correlated with poor outcome. B) IGF-1R is expressed in 31% (103/326) of primary breast tumours and it's expression in is not associated with shorter survival time this cohort.

A.



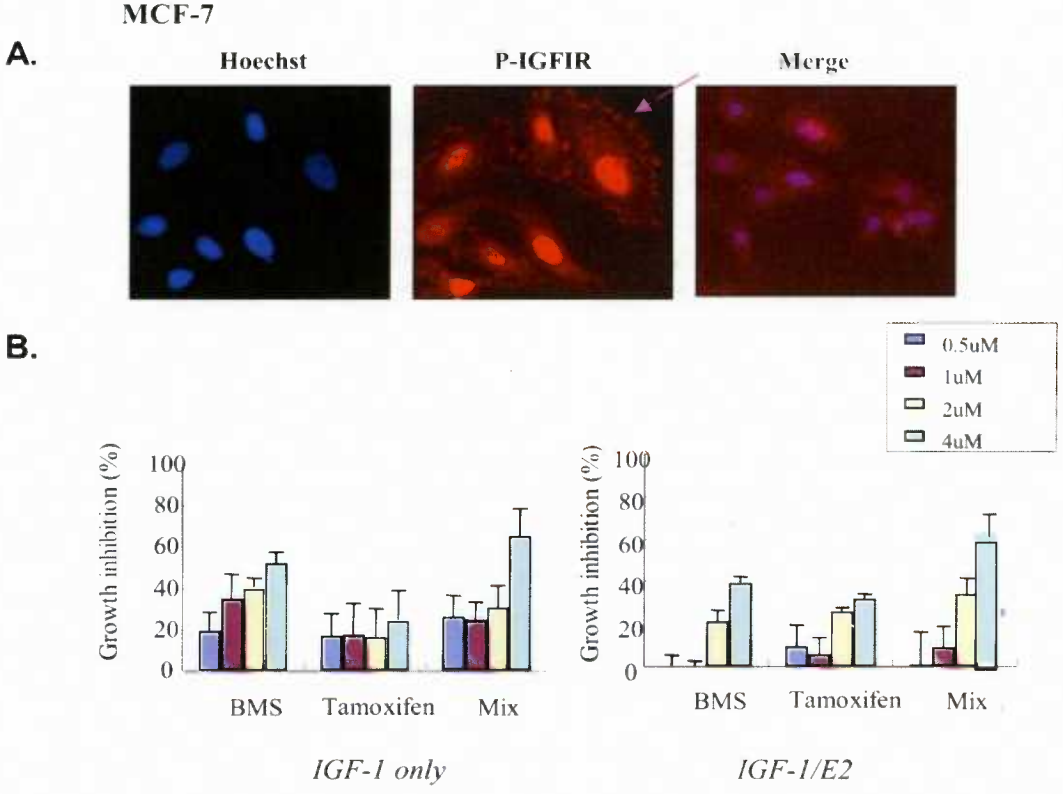
1R positive
IR negative

B.



IGF-1R positive
IGF-1R negative

Figure 5. High content screening for P-IGF-1R/IR and the effects of BMS-536924 on growth. A) P-IGF-1R/IR immunofluorscence in MCF-7 cells counterstained with DAPI. B) MCF-7 cells were serum starved for 24 hours and then IGF-1 (left panel) or IGF-1 plus estradiol (right panel) was added in the presence of increasing amounts (0.5, 1.0, 2.0 and 4.0 μM) of drug (BMS-536924, Tamoxifen, and combination of both drugs) growth inhibition measured 72 hours later. C) MCF-7 cells were serum-starved then pretreated with BMS-536924 (0-2 uM) then IGF-1/E2 was added for 30 min. Levels of P-S6 were evaluated by immunoblotting. Actin served as a loading control. D) MCF-7 TamR cells were treated as described in (B) and evaluated for changes in cell growth based on Hoechst staining to enumerate the number of viable cells.



C.

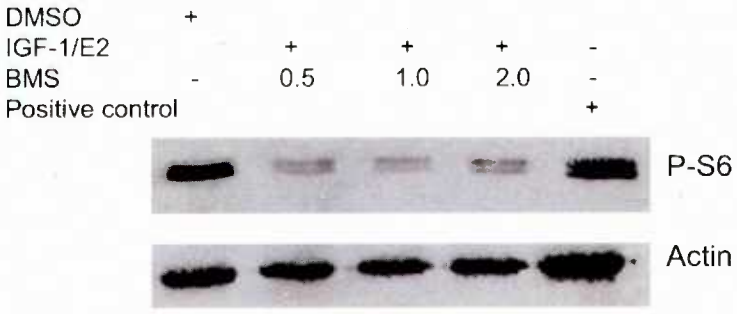

DMSO +
IGF-1/E2 + + + -
BMS - 0.5 1.0 2.0 -
Positive control +
P-S6
Actin

Figure 5

D.

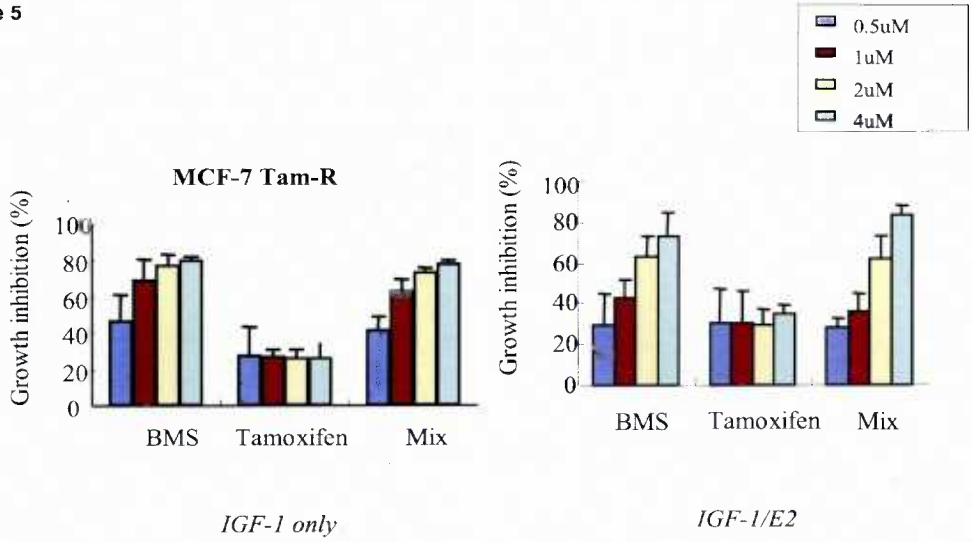
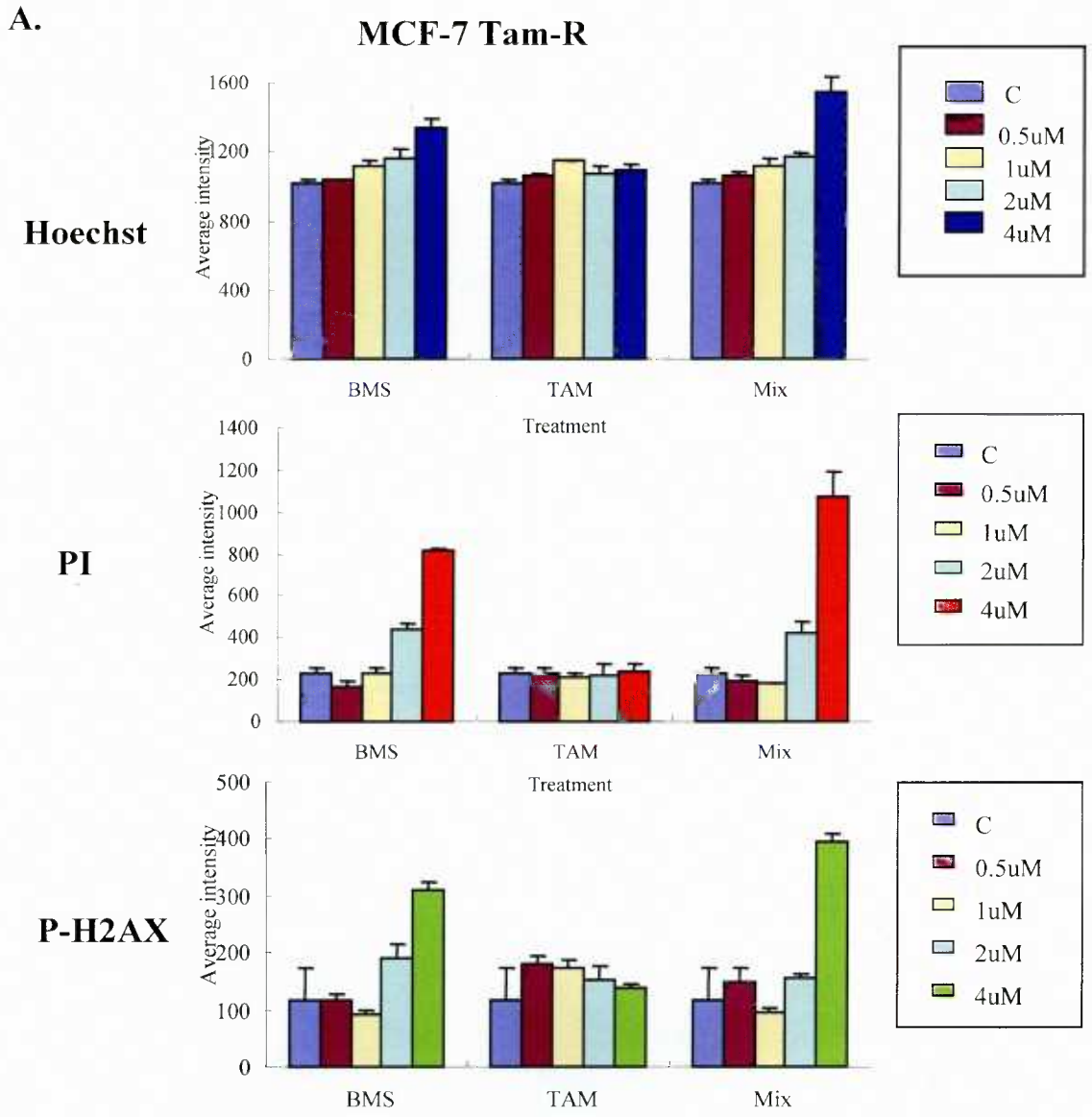

0.5uM
1uM
2uM
4uM
MCF-7 Tam-R
Growth inhibition (%)
100
80
60
40
20
0
BMS
Tamoxifen
Mix
IGF-1 only
IGF-1/E2

Figure 6. The effects of BMS-536924 on apoptosis using high content screening. MCF-7 Tam-R were serum starved for 24 hours and IGF-1 plus estradiol was added in the presence of increasing amounts (0.5, 1.0, 2.0 and 4.0 μM) of drug (BMS-536924, Tamoxifen, and combination of both drugs) apoptosis measured 72 hours later. A) Average intensity level of the Hoechst, Propidium Iodide and Phospho-H2AX in the cells treated as mentioned above. B) Representative image of the cells in the absence (top panel) or presence of BMS-536924. The merged imaged illustrates the three channel screen for apoptosis where chromatin condensation (Hoechst intensity), propidium iodide and P-H2AX can be simultaneously imaged.



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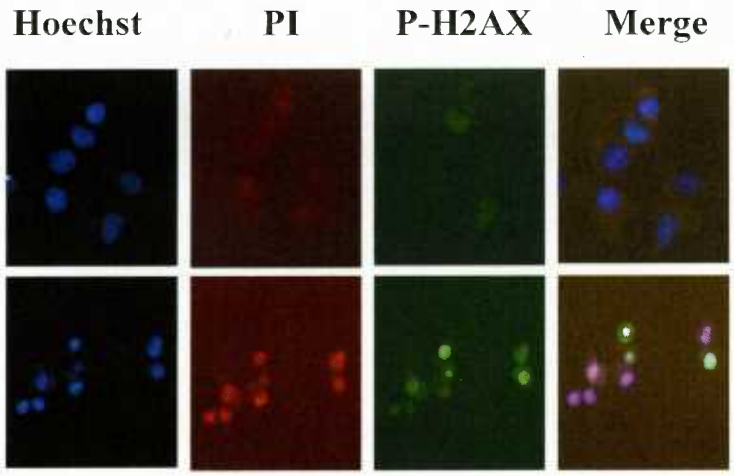
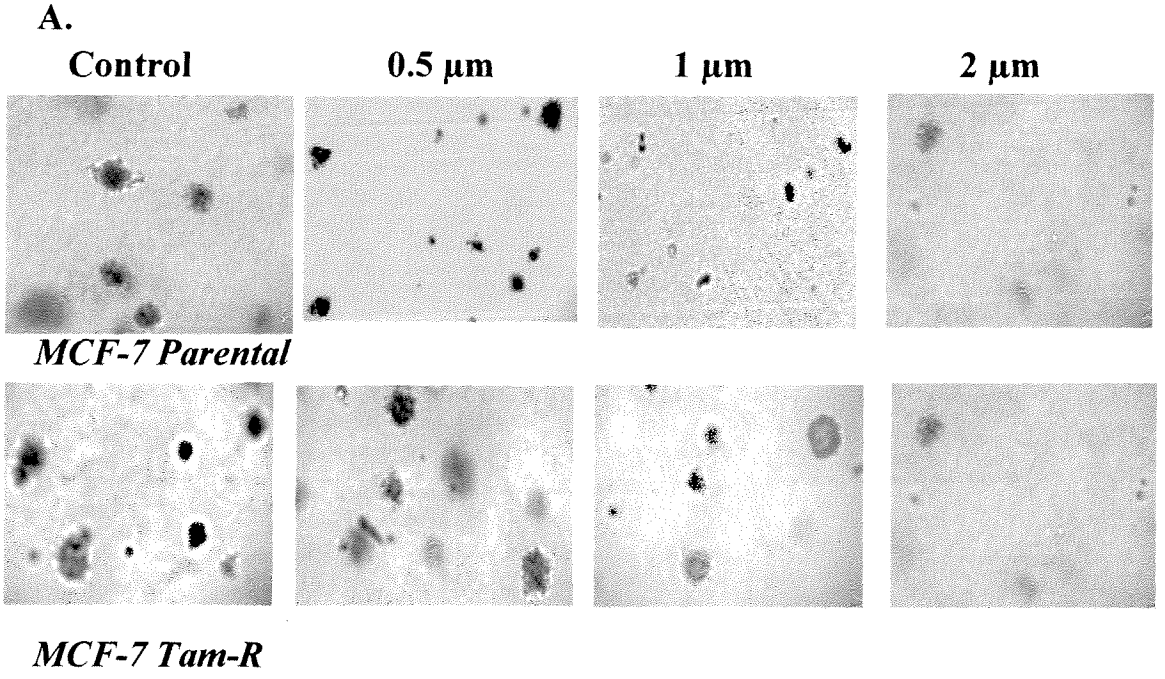


Figure 7. BMS-536924 inhibits the growth of MCF-7 and MCF-7 Tam-R cells in soft agar. A) MCF-7 and MCF-7 Tam-R cells were plated into soft agar and colony formation was enumerated after 28 days in the presence of BMS-536924. Colony formation was inhibited by ~50% in when MCF-7 and MCF-7 Tam-R were treated with 0.5 or 2.0 μM, respectively. **B)** Bar chart diagram showing the dose dependent growth inhibiting effect of BMS-536924 in MCF-7 and MCF-7 Tam-R cells.



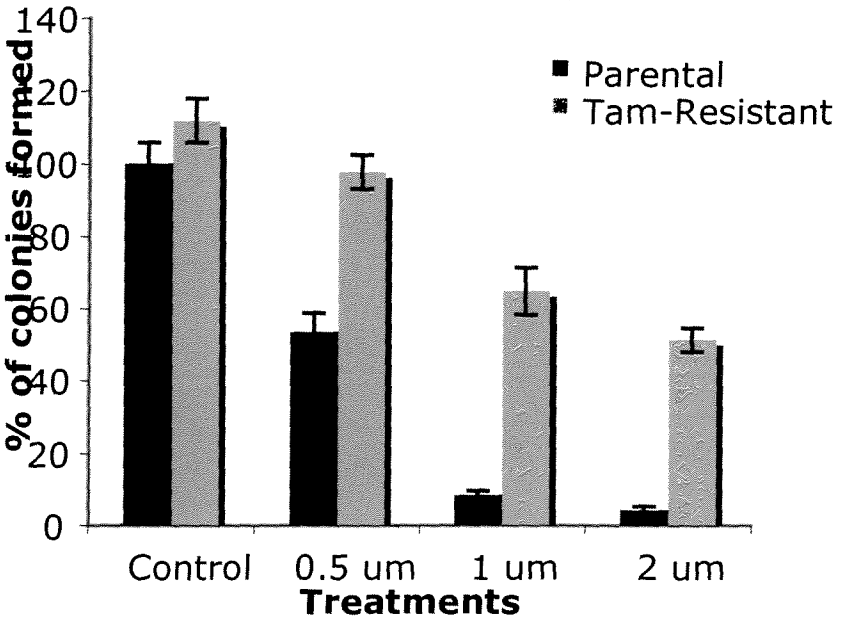
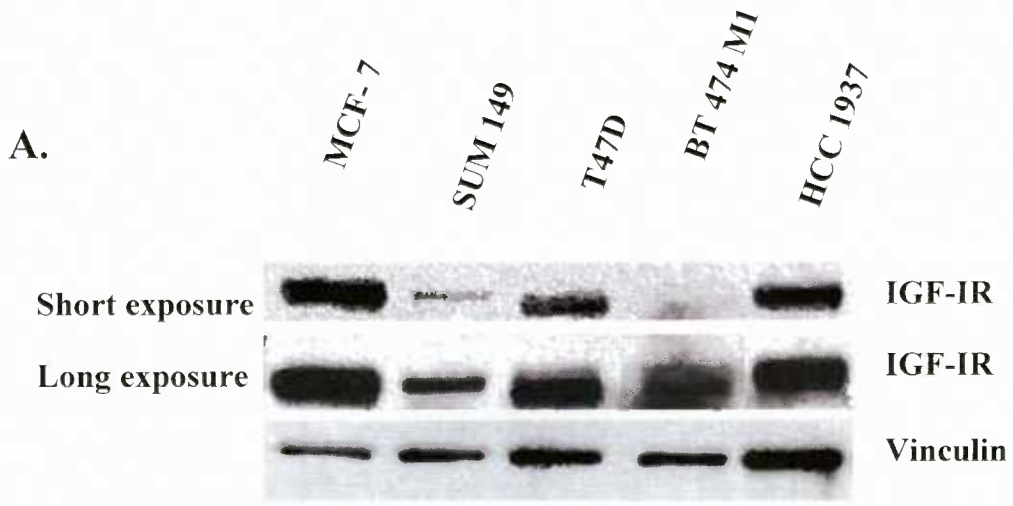


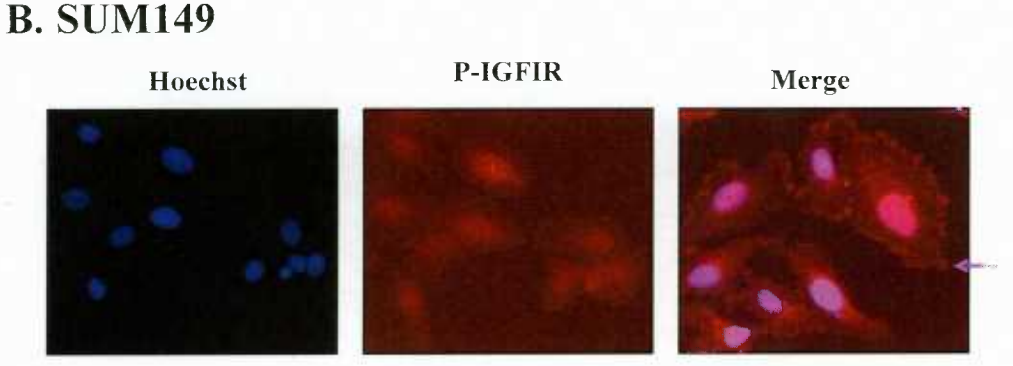
Table 4. The expression pattern of P-IGF-1R in subtypes of breast cancer defined by hormone receptor and HER-2 status. Activated IGF-1R is expressed in all breast cancer subtypes.

Breast tumor subtype	**Markers**	**Correlation for VGH set**
Luminal	ER(+)or PR(+)	n= 94/199 (47.2%)
Triple negative	ER, Her-2, and PR(-)	n=15/33 (45.5%)
Her-2	ER(-ve) and Her-2 (+ve)	n=10/16 (62.5%)

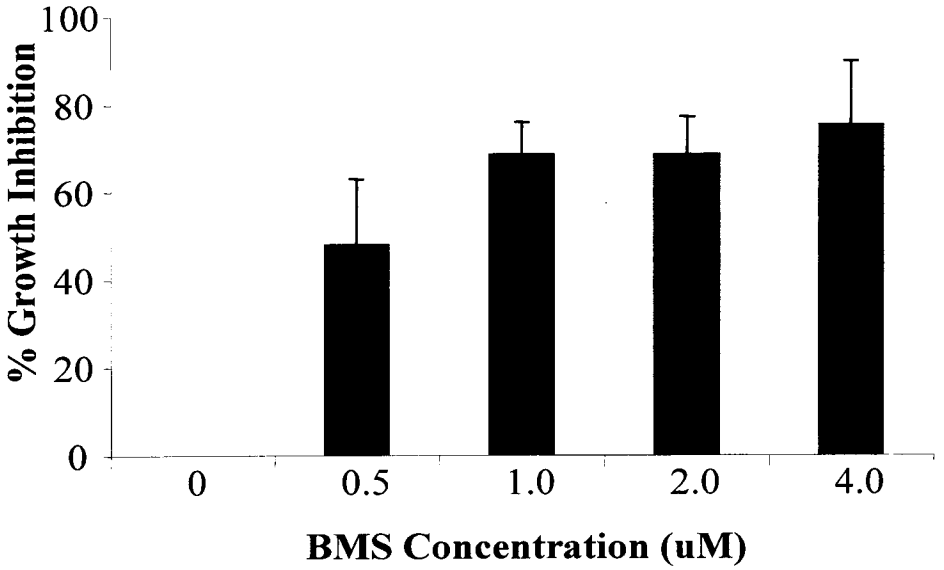
Likelihood ratio=1.489
P value=0.47

Figure 8. Evaluation of basal-like breast cancer cells response to the inhibitory effects of BMS-536924. A) A panel of breast cancer cell lines were evaluated for IGF-1R levels by immunoblotting. The basal-like breast cancer cell lines, SUM149 and HCC1937 had moderate to high levels of IGF-1R expression. **B)** High content screening confirmed activated P-IGF-1R in the SUM149 cells. **C)** SUM149 cells were treated with increasing concentrations of BMS-536924 for 72 hours and cell viability was evaluated using Hoechst staining.





C. SUM 149


100
80
60
40
20
0
% Growth Inhibition
0
0.5
1.0
2.0
4.0
BMS Concentration (uM)

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CHAPTER 6

DISCUSSION

Cancer recurrence is one of the greatest challenges in the management of breast cancer. Currently, scientists are searching for molecular markers that can help select patients that may have poor outcome and increased risk of relapse. In this study, we demonstrated the impact of Y-Box binding protein-1 (YB-1) as a marker of aggressiveness and disease recurrence in breast cancer by screening one of the largest TMAs in North America. In a pilot study, we first examined 438 cases of primary breast carcinoma and asked whether YB-1 expression is correlated with a particular subtype of breast cancer. Our attention was drawn to the basal-like breast cancers (BLBC), known as "triple negative", because currently they are very difficult to control and there is no targeted treatment available for this subtype of breast cancer. Subsequently, we screened 4049 primary breast tumours and asked whether YB-1 expression is correlated with poor survival in all subtypes of breast cancer (chapter 3). We illustrated that YB-1 is not only expressed in triple negative breast cancer but all subtypes of breast cancer defined by hormone receptor and HER-2 status. YB-1 expression is correlated with poor patient outcome. Consistent with univariate survival analysis, using a multivariate Cox regression model, we were able to show that YB-1 expression is independent of subtypes. In another multivariate survival analysis, we tested the effect of YB-1 expression as an independent marker compared to markers that are currently being used for diagnosis in the clinic. Interestingly, we found that the hazard ratio for YB-1 was higher than tumours size, age, grade, ER and HER-2 status. This suggesting that YB-1 is a strong independent prognostic indicator compared to already established bio-markers in clinic (chapter 3). At this point, we were keen to determine whether YB-1 expression is associated with earlier cancer recurrence. We discovered that YB-1 expression is correlated with earlier relapse in the overall cohort and in every subtype of breast cancer. These findings were confirmed using multivariate and univariate analysis. Consistently, YB-1 is a stronger marker compared to tumour size, age, grade, ER and HER-2 status (chapter 3). Considering our interest in studying triple negative breast cancers, we asked how YB-1

could be involved in aggressiveness of this particular subtype of breast cancer. Using correlation analysis in this TMA, we found that there is an association between YB-1 and uPA, an established marker for metastasis, in triple negative subtype. ChIP on chip studies on SUM149 cells also suggested that uPA might be one of the down stream YB-1 target genes which is involved in relapse and metastasis. Subsequently, using siRNA, we discovered that YB-1 is involved in the regulation of uPA expression in SUM149 cells (chapter 4). The use of siRNA for cancer treatment in the clinic is currently not a feasible option; therefore we decided to evaluate the effect of a new small molecule inhibitor to phospho-IGF-1R, BMS-536924, on SUM149 cells. Firstly, we screened the 438 primary breast tumours and determined that P-IGF-1R expression is correlated with poor patient outcome and is in fact expressed in 45% triple negative breast cancers. Both high content screening and Western blot analysis confirmed that SUM149 cells express moderate levels of P-IGF-1R. Interestingly, we discovered that BMS-536924 has a robust effect on inhibiting the growth of these cells. Finally, this small molecule inhibitor not only inhibits uPA expression but also inhibits YB-1 expression at the mRNA and protein level and may prove to be a potential drug for treatment of triple negative breast cancer.

6.1 YB-1 is a marker of poor survival and aggressiveness

A pilot study of 83 primary breast tumours by Janz et al. (2002) reported that high expression of YB-1 in tumour tissue and surrounding benign breast epithelial cells was significantly associated with poor patient outcome (Janz et al. 2002). They established YB-1 as a marker with predictive significance independent of currently available tumour biologic factors; HER-2, uPA (urokinase-type plasminogen activator) and PAI-1 (plasminogen activator inhibitor type 1) (Janz et al. 2002). More recently, our team screened a cohort of 438 cases of primary breast tumours with longer follow-up (20 years), and we were able to validate this trend (Wu et al. 2006). In this study (chapter 2) we were able to further investigate the expression of YB-1 in regard to the subtypes of breast cancer defined by hormone receptor and HER-2 status. Although we were unable to associate YB-1 expression with outcome of each breast cancer subclass, this work provided the rationale for additional studies.

Recently, Perou and Sorlie et al. categorized sporadic breast tumours into distinct

subtypes by using DNA microarrays and their "intrinsic" gene list (Perou et al. 2000). Although gene expression profiling had a large impact on the categorization of patients in to different breast cancer subtypes, using this technique on a routine basis is very costly and not practical in the clinical setting. Therefore, we have assessed a number of potential molecular markers for the subtypes of breast cancer by immunohistochemistry, a much faster and easier method for use in the clinic. We have also optimized the procedure for automated staining and are working towards robotic scoring.

We first used this cohort as a pilot screen and asked whether YB-1 is expressed in a particular subtype of breast cancer. In this screen, we found that YB-1 expression is skewed towards ER-negative breast cancers especially the triple negative breast cancers (chapter 2). Subsequently, we examined the expression of YB-1 in 4049 primary breast tumours both within the overall cohort and in the individual subtypes of breast cancer. Our data indicated that YB-1 is a strong prognostic marker for all subtypes of human breast cancer. Consistent with our data from the 438 series, there was a particular bias toward the ER-negative breast cancers (chapter 3). These results suggest a new strategy for targeted therapy of breast cancers that currently cannot be treated effectively. In a multivariate survival analysis, Cox regression model, we were also able to show that YB-1 expression is independent of hormonal receptors and HER-2 status (chapter 3). In another Cox regression analysis, we compared YB-1 expression as an independent variable with other markers currently used for clinical decision-making. Interestingly, YB-1 hazard ratio was higher than age, grade, ER and HER-2 status suggesting that the value of YB-1 as a prognostic indicator could be superior to these markers that are established in clinic for diagnosis and treatment of breast cancer patients. Although YB-1 is highly expressed in more aggressive ER-negative subtypes the expression of this protein is still an indicator of aggressiveness even in the patients that are considered as "low risk" and did not receive any systemic treatment (chapter 3). In this cohort, YB-1 prognostic value was further upheld in a multivariate Cox regression model, in comparison to age, grade, ER and HER-2 status (chapter 3).

6.2 YB-1 is a maker for cancer recurrence

Our data from the "low risk" subgroup were consistent with the preliminary results from Janz et al. where they examined YB-1 expression in a cohort of 42 patients that were not treated with chemotherapy and found that YB-1 was expressed in 76% (32/42) of the cases. None of the patients that had low YB-1 expression relapsed; however, 30% of those with high levels did ($p<0.011$) (Janz et al. 2002). Soon after, Huang et al also reported in a study of 42 patients that YB-1 was associated with shorter relapse free survival however long-term follow-up was not evaluated (Huang et al. 2005). Based on these studies, and since the value of YB-1 in regard to relapse was not deeply understood, we assessed the effect of YB-1 expression on cancer recurrence. In this cohort, we found that YB-1 expression is associated with shorter relapse free survival time in both the overall cohort and in each subtype of breast cancer. Importantly, in a Cox regression model we found that YB-1 is a much stronger independent marker for earlier relapse compared to other established markers such as age, grade. ER and HER-2 status (chapter 3).

We now can state that YB-1 is a strong marker cancer recurrence as well as poor survival. This finding is very valuable since there is a desperate need for prognostic markers to help in early identification of patients with high risk of relapse and metastasis. Given that YB-1 staining is optimized on an automated immunostainer it would be very feasible to use this marker in routine clinical practice for diagnostic purposes as similar equipment is employed.

6.3 Role of YB-1 in relapse and metastasis

There have been several reports on the role of YB-1 in metastasis and relapse. YB-1 was shown to elevate the expression of matrix metalloproteinase-2 (MMP-2) (Mertens et al. 1997; Mertens et al. 2002), matrix metalloproteinase-12 (MMP-12) (Samuel et al. 2005), and suppress that of collagen α1(I) (Norman et al. 2001), and collagen α2(I) (Dooley et al. 2006; Higashi et al. 2003). The above mentioned genes have all been reported to be involved in cell adhesion, motility, invasion and thus metastasis. In this work, we found an association between YB-1 and urokinase plasminogen activator (uPA), an important biomarker for cancer recurrence and metastasis, in the aggressive

triple negative breast cancers (chapter 4). Subsequently, we also found that uPA expression is associated with poor outcome and earlier time to relapse in this cohort of patients. This work was consistent with the findings by Nielsen et al. where they screened 930 breast cancer patients and reported that uPA expression was also associated with poor survival (Nielsen et al. 2004).

We then performed a ChIP on chip experiment using SUM149 cells, which are an *in vitro* model for triple negative breast carcinoma. We found that uPA might be one of the down stream target genes for YB-1 (chapter 4).

6.3.1 YB-1 regulates uPA expression

Using siRNA and shRNA we were able to determine that YB-1 is involved in regulating uPA expression in SUM149 cells. More importantly, we were able to significantly inhibit invasion of cancer cells through matrigel when we silence YB-1 for 72 hours. We have seen the same effect on cell invasion when we inhibit uPA with uPA siRNA for 72 hours. The regulation of uPA by YB-1 further confirms the impact of YB-1 on metastasis and relapse.

The results that we obtained from a large-scale tumour tissue microarray (TMA) study have confirmed the significance of YB-1 as an important prognostic marker in breast cancer. Collectively, these studies suggest that over-expression of YB-1 may be an important predictor for poor outcome and relapse. Thus, targeted disruption of YB-1 function may be a novel strategy to treat human malignancies.

6.4 Targeting YB-1 for anti-cancer therapy

6.4.1 Direct inhibition of YB-1

Direct inhibition of molecules such as YB-1 is now possible either by designing small molecule inhibitors or developing molecular techniques so that they can be used in clinic. An important experimental technique is knockdown of YB-1 using small interfering RNA (siRNA). By knocking down YB-1 using siRNA we previously reported that the growth of breast cancer cells is inhibited by 48% (Wu et al. 2006). Recently, we illustrated that silencing YB-1 using siRNA results in suppression of cell growth in monolayer and/or soft agar and a delay in formation of tumours *in vivo* in HER-2 over-

expressing breast cancer cells BT474-m1 and MDA-MB-453 (Chapter 3). In this study we have been able to inhibit uPA expression and cell invasion in SUM149 triple negative breast cancer cells using YB-1 siRNA and shRNA (chapter 4). These are very promising results that could translate into the clinic. Recently, clinical trials using siRNAs directed against the vascular endothelial growth factor (VEGF) have been initiated for the treatment of age-related macular degeneration (Grunweller et al. 2005). However, using this technique in clinic is relatively new and has yet to be applied to anti-cancer therapy. In addition, although siRNA is a convenient method to study the function of a specific protein, however, there are major downsides in that they are short-lived and relatively unstable. The concentration of siRNA becomes "diluted" out when cells divide and the effect lasts for approximately one week (Pai et al. 2006). Therefore, we specifically employed a short-hairpin RNA (shRNA) technique to overcome the downsides of YB-1 siRNA. A short hairpin RNA (shRNA) is a sequence of RNA that forms a tight hairpin loop that can be introduced into the cell by vectors carrying the U6 and H1 promoter to ensure that the shRNA is always expressed. This vector is passed on to daughter cells, allowing the gene silencing to be inherited. The shRNA hairpin structure is cleaved by an enzyme called DICER into siRNA, which is then bound to the RNA-induced silencing complex (RISC). This complex binds to the mRNA which is complementary in sequence to the siRNA and cleaves it (Paddison et al. 2002). Recently, our team has established a stable shYB-1 cell line by transfecting SUM149 cells with pSUPER-DUPER YB-1. This vector produces short-hairpin RNA targeting YB-1 transcripts. In this study we have been able to reduce the expression of YB-1 by 98% in a stable manner and subsequently inhibit uPA expression by approximately 80% in the SUM149 cells (chapter 4). These results suggest using shRNA against YB-1 might be a good strategy for targeting this protein and is potentially useful in the clinic. However, this system requires more investigation and optimization of the delivery of the shRNA in patients; thus, it does not fulfill our immediate need in treatment of patients that express high level of YB-1 in their tumours. Therefore, we are testing other commercially available inhibitors to indirectly target YB-1.

6.4.2 Indirect inhibition of YB-1

The phosphoinositol 3-kinase/Akt/mammalian target of Rapamycin (mTOR) pathway is the main signal transduction pathway that leads to the activation of YB-1. Inhibition of Akt would be a great approach to suppress YB-1 phosphorylation at serine 102. This strategy, would suppress YB-1 nuclear localization and inhibit its ability to bind and up-regulate growth promoting genes such as *EGFR* and *HER-2* (Wu et al. 2006). Preventing Akt activation through phosphoinositide-dependent kinase-1 (PDK-1) inhibition (Crowder et al. 2005; Kucab et al. 2005) is also a possible way of ultimately suppressing YB-1's function. The PDK-1 inhibitor OSU03012 is an excellent candidate because not only does it inhibit Akt activation, but it is orally available and does not lead to overt toxicity in mice (Kucab et al. 2005). Following these studies, our team also showed OSU03012 can inhibit the growth of the aggressive BLBC by blocking YB-1 binding to EGFR promoter (To et al. 2007). In addition, Evdokimova, et al. recently reported that Rapamycin, a clinically approved immunosuppressant that inhibits the mammalian target of rapamycin (mTOR) pathway, reduces the phosphorylated level of YB-1 (Evdokimova et al. 2006). Therefore, it could suppress the growth promoting effect of YB-1.

6.4.2.1 Inhibition of phospho-IGF-1R/IR

Previously it was reported that activated IGF-1R results in activation of PI3K/Akt signaling cascade leading to up-regulation of uPA expression in breast cancer cells (Dunn et al. 2001). In chapter 4, we also showed that YB-1 regulates uPA expression in SUM149 cells. We could also significantly inhibit the invasion of these cancer cells through matrigel using YB-1 or uPA siRNA (chapter 4). Extensive studies in our laboratory illustrated that Akt phosphorylates YB-1 at S102 through IGF stimulation, resulting in nuclear localization of YB-1 (Sutherland et al. 2005). Considering this evidence and the fact that currently there are no YB-1 inhibitors commercially available, we investigated the effect of BMS-536924, a small molecule inhibitor of activated P-IGF-1R, on the expression of uPA and YB-1 (chapter 4).

BMS-536924 is small molecule inhibitor, which targets both the IGF-1R and IR (Hofmann et al. 2005). This compound can be used orally (Hofmann et al. 2005), and has

been shown to inhibit tumour growth *in vivo* (Kim et al. 2007). More recently, Kim et al. demonstrated that constitutive activation of IGF-1R results in epithelial to mesenchymal transition (Chinnaiyan et al.; Kim et al. 2007). Yang et al (2004) indicated that both invasion and metastasis might be dependent on acquiring features of EMT by primary cancer cells (Yang et al. 2004). It has been shown by Kim et al that BMS-536924 can partially reverse the EMT process and its features (Kim et al. 2007).

Taking these evidences into account, we initially screened 438 cases of primary breast tumours and found that phospho-IGF-1R/IR is associated with increased rates of breast cancer-related deaths (chapter 4). Subsequently, we showed that P-IGF-1R/IR is expressed in all subtypes of breast cancer and more specifically, 45% of triple negative breast cancers. By Westren blot analysis, we then confirmed that SUM149 cells have moderate levels of P-IGF-1R. Treating these cells with BMS-536924 resulted in inhibition of cancer cell growth in a dose dependent manner. Interestingly, uPA and YB-1 expression were significantly reduced (at mRNA and protein level) after 72 hours treatment with this inhibitor suggesting that BMS-536924 might be a great candidate for treatment of aggressive triple negative breast cancers, which are currently very challenging to treat in clinic.

We therefore suggest that uPA is a potential downstream target of YB-1. This leads us to believe that YB-1 fits into the already published pathway of IGF-1R activating Akt leading to an increase in uPA, since Akt phosphorylates YB-1 and we now show that YB-1 induces uPA.

6.5 Summary and future directions

This study led us to a deeper understanding of YB-1 as a prognostic marker for aggressiveness, poor survival and cancer recurrence in subtypes of breast cancer defined by hormone receptor and HER-2 status. YB-1 is a stronger independent bio-marker compared to established clinico-pathological bio-markers such as tumour size, age, grade, ER and HER-2 status. This finding suggests that YB-1 should be in a priority list of biomarkers for selection of the patients with increased risk of relapse and poor outcome. We have also proposed that the association of YB-1 with the higher rate of relapse might

be through it's regulation of uPA expression. More importantly, silencing YB-1 results in a significant reduction in cancer cell invasion.

Since there are no commercially available YB-1 inhibitors, we examined the response of basal-like breast cancer cells to BMS-536924, a small molecule inhibitor for activated IGF-1R/IR. We first demonstrated that activation of IGF-1R is associated with poor survival in primary breast tumours and secondly that BMS-536924 reduces uPA expression through inhibition of YB-1 in SUM149 cells.

This study has provided a deeper understanding of the prognostic value of YB-1 and how it contributes to the aggressive nature of breast cancer cells. However, undoubtedly, many experiments are still required to further verify these findings. Firstly, it will be very beneficial to understand the mechanism behind the regulation of uPA expression by YB-1. Since, we found a significant reduction in uPA transcript after silencing YB-1 for 48 and 72 hours it is more likely that YB-1 regulates uPA expression at the transcriptional level. YB-1 as a transcription factor could directly bind to Y-boxes and related sequences by itself or with other transcription factors. For instance, our team reported that YB-1 can directly bind to *egfr* promotor and regulate its expression (Wu et al. 2006). It could also interact with other transcription factors and functions as co-activator or co-repressor. The transcriptional activity of YB-1 on MMP-2 was demonstrated to be dependent on the interaction with two other transcription factors, namely the activating protein-2 (AP-2) and p53 (Mertens et al. 2002). In order to investigate whether YB-1 directly binds to the uPA promoter we searched for YB-1 binding sites on the first 2 kb upstream of the start site. We have found 12 potential YB-1 binding sites in this region and designed 4 primer sets to include all these (Figure 1). We firstly tried to optimize the PCR conditions of the primer sets. We performed PCR based on the conditions that were described previously (Wu et al. 2006). We observed PCR product with the right size using primer set 1 and 3. Unfortunately, we were unable to obtain the correct PCR product with primer sets 2 and 4. We performed chromatin immunoprecipitation (ChIP) using YB-1 antibody on SUM149 cells. The immunoprecipitated DNA was amplified using primer set 1 and 3. No binding was observed at these regions (Figure 2 A and B). EGFR primer 2a (chapter 1) was used to confirm the ChIP had worked (Figure 2C). At this point, it would be very useful to check

for direct interaction between YB-1 and uPA by redesigning primer sets, and also to assess a large portion of the promoter for YB-1 binding sites. Samuel et al. (2005) demonstrated that YB-1 could also bind to AP-1 DNA binding sites of its downstream target genes and regulate their gene expression (Samuel et al. 2007; Samuel et al. 2005). We could also use DNA-affinity-chromatography-based assay termed NAPSTER (nucleotide-affinity preincubation specificity test of recognition) to see if YB-1 binds to AP-1 DNA binding sites on the uPA promoter. Another way to determine if YB-1 interacts with the uPA promoter is the ChIP sequencing method as described by Robertson et al. 2007. It is a relatively new method, has greater sensitivity and specificity than ChIP on chip (Robertson et al. 2007). As mentioned above, there is also another possibility that YB-1 indirectly regulates uPA expression via interaction with other transcription factors. In this case, using co-immunoprecipitation (CoIP) prior to gel electrophoresis and mass spectrometry will enable us to identify other potential transcription factors that might interact with YB-1. In both scenarios, performing ChIP, electrophoretic mobility shift assay (EMSA) and reporter assays, is necessary to evaluate the mechanism behind the regulation of uPA by YB-1.

Secondly, we showed that BMS-536924 reduces uPA expression through inhibition of YB-1 in SUM149 cells. Ideally, we will study the effect of BMS-536924 on cell migration using an invasion assay.

We have recently developed cell-permeable peptides and we are in the process of developing small molecule inhibitors for YB-1. These inhibitors are designed to interfere with YB-1 function by blocking the serine 102 phosphorylation site, which is important for YB-1 nuclear translocation and DNA binding. We are currently evaluating the efficacy of the inhibitors in a panel of cancer cell lines using various monolayer and soft agar assays. We will then also examine the effect of these inhibitors on tumour formation *in vivo*.

Figure 1. First 2kb upstream of start site on uPA promoter.

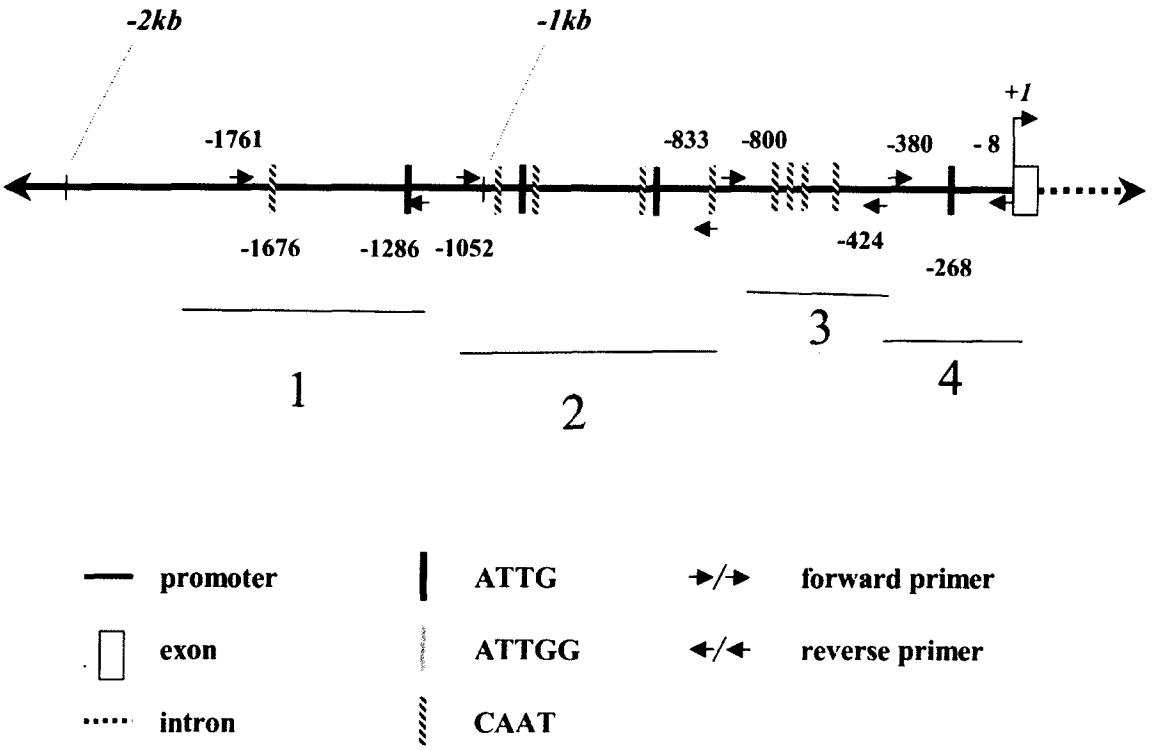
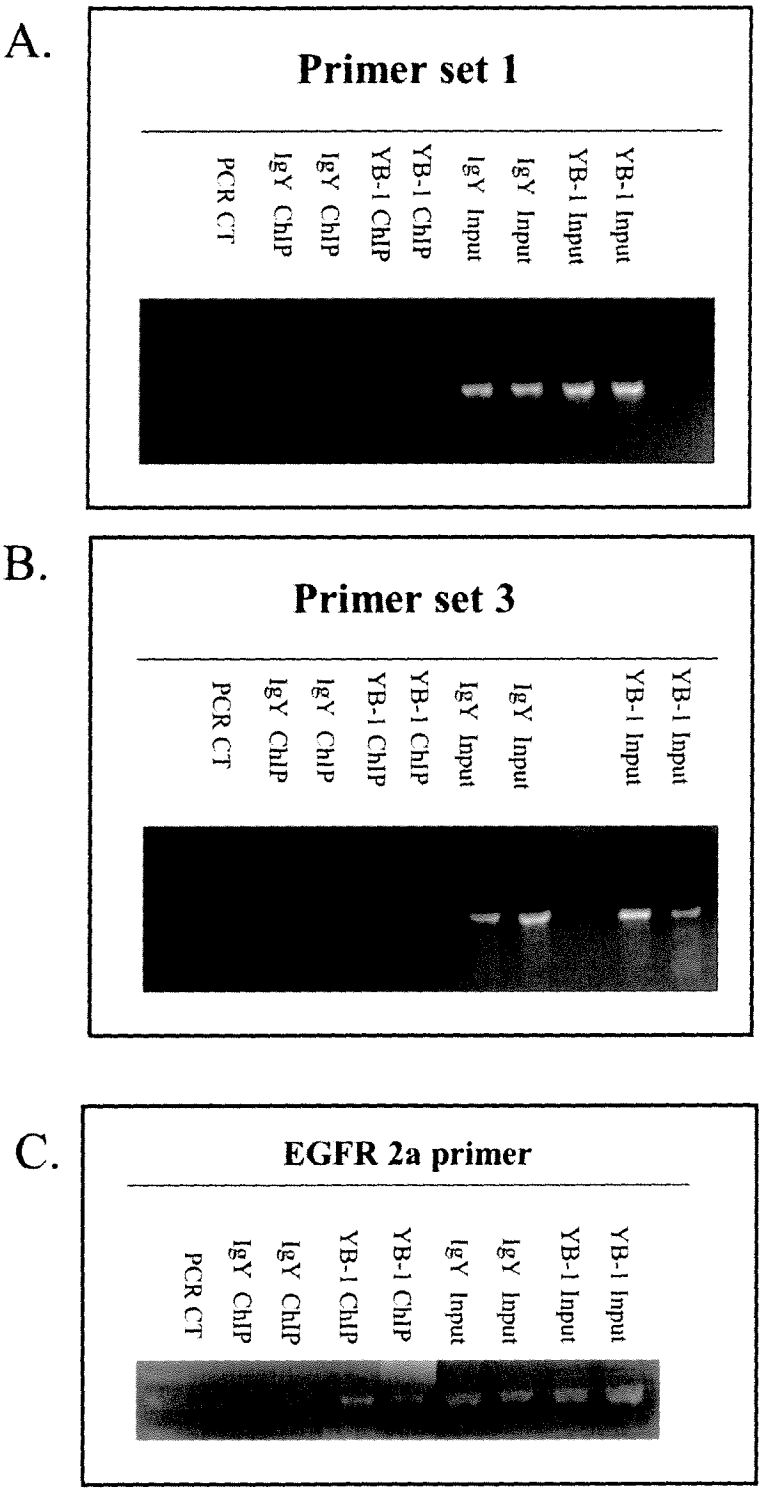


Figure 2. Chromatin immunoprecipitation using YB-1 antibody.

PCR products from primer set 1 (A) primer set 3 (B) and *egfr* primer set 2a (C).



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