The Role of the Apoptotic Pathway in Hormone Resistant Breast Cancer

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Abstract

Activation of the PI3K/Akt signal transduction pathway has been linked to endocrine resistance in tamoxifen treated breast cancer patients. Activation of the PI3K/Akt pathway causes phosphorylation of Bad leading to modulation of cellular apoptosis. This study was carried out to test the hypothesis that disruption of apoptosis in breast cancer, via Akt activation, is linked with hormone resistance.

Immunohistochemistry (IHC) was performed on 402 oestrogen receptor (ER) positive breast cancers using antibodies against PTEN, Bad, pBad (ser 112), Bax, Bcl-2, Bcl-xl, P70S6K, pP70S6K (thr 389) and pP90RSK (thr 359/scr363). In addition FISH was performed on the same cohort for HER2. This was compared with the results for the herceptest performed on the same tumours. I also performed pilot studies looking at the amplification and deletions of PI3K and PTEN respectively in this cohort of patients.

Patients whose tumours had high levels of PTEN expression had a favourable outcome compared to those patients with a low PTEN expression. Patients, whose tumours had high levels of Bad expression, had a significantly improved disease-free survival when compared to patients whose tumours had low levels of Bad expression. There were no associations with disease free or overall survival in the remaining antibodies investigated. Pilot studies did not suggest that it would be fruitful to perform FISH on the whole tumour cohort for either PI3K or PTEN.

Data presented here shows that reduced Bad expression is associated with relapse in tamoxifen-treated breast cancer patients, supporting our hypothesis that the apoptosis pathway is involved in tamoxifen resistance. My results also show an association between increased expression of PTEN and a reduced overall and disease free survival.

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Cannings E, Kirkegaard T, Tovey SM, Dunne B, Cooke TG, Bartlett JMS (2006). Bad expression predicts outcome in patients treated with Tamoxifen. Breast Cancer Res Treat. 2006 Sep 27; [Epub ahead of print]. PMID: 17004114

List of Abbreviations

AF-1, AF-2	transcription-activating function 1,2
ATAC	Arimidex, Tamoxifen, alone or in combination Trial
AIBI	Amplified in breast cancer 1
BH3-	BCL-2 homology 3 domain
DBD	DNA binding domain
DFS	Disease free survival
ERα	Oestrogen Receptor α
ERE	oestrogen response elements
FISH	Fluoresence in situ hybridyzation
H&E	hematoxylin and cosin
HER	Human epidermal growth Factor Receptor
HSP	heat shock proteins
4ICD	HER4 intracellular domain
IES	Intergroup Exemestane Study
IHC	Immunohistochemistry
LBD	ligand-binding domain
MAPK	Mitogen-activated protein kinase pathway
mTOR	Mammalian target of Rapamycin
NEAT	National Epirubicin Adjuvant Trial
NTD	N-terminal domain
OS	Overall survival
pP70S6K(thr389)	Phosphorylated P70S6K at Threonine 389

pP90RSK (thr 359/ser 363)	Phosphorylated P90RSK at Threonine 359 and Serine 363
pBad(ser 112)	Phosphorylated Bad at Serine 112
PDK	Pyruvate dehydrogenase kinase
pERαSer118	Phosphorylated ERα at Serine 118
pERαSer167	Phosphorylated ERα at Serine 167
pHER2	Phospho specific HER2
PI3K	phospatidylinositol-3 kinase
PR	Progesterone Receptor
PTEN	Phosphatase and tensin homolog
TACE	TNFα-converting enzyme
TEAM	Tamoxifen Exemestane Adjuvant Multinational Trial
TMA	Tissue microarray

Chapter 1

Introduction

1.1 Breast Cancer

Breast cancer is the second major cause of cancer related death among women in the United States and Europe (www.uicc.org). The incidence is increasing and the lifetime risk for a woman to develop breast cancer is 1 in 9 (www.statistics.gov.uk). Breast cancer is uncommon in women under 25 years of age. Thereafter the incidence steadily increases until the menopause when the rate slows again. There is a familial disposition in 10% of cases but for the majority the cause is not known. The risk factors for developing breast cancer, in addition to familial factors include increased age, early menarche, late menopause, exogenous oestrogens, increased alcohol intake, dietary factors, late first pregnancy and nulliparity.

1.2 Oestrogen Receptor

Oestrogen (E2) is a steroid hormone. It is mainly produced in the ovary in premenopausal patients with a switch to production in the peripheral adipose tissue through aromatisation of androgens (Korach KS, 1996) in post menopausal women. In men the main site of sythesis are the Leydig cells of the testis. E2 is essential for reproductive function (testis, ovary, endometrium), anabolic functions (bone and muscle mass) and also plays a central role in the central nervous system and the cardiovascular system(Hess et al., 1997). The oestrogen receptor was identified in the 1960s[3] and shown to control expression of oestrogen responsive genes. Further

studies investigating the mechanism of action of other steroid hormones (especially gluco-corticosteroids) led to the classification of the ER as a member of the steroid receptor superfamily(Yamamoto, 1985). In 1996 a second oestrogen receptor, ERβ was found(Kuiper et al., 1996). Despite the identification of ERβ, most of the knowledge about the role played by ER in breast cancer relates to ERα.

ERα is a 65kDa nuclear transcription factor receptor expressed in 46-77% of breast cancers (Robertson, 1996). ERα exists as an inactive monomer bound by heat shock proteins (especially Hsp90) in the cell cytoplasm or nucleus. Subsequent ligand binding causes dissociation of the heat shock proteins and alteration of receptor conformation. The activated receptors then homodimerize, translocate to the nucleus, form a complex with co-regulatory molecules and bind to oestrogen response elements (ERE) in the promoter region of target genes, to alter transcription of ERα regulated genes (Klein-Hitpass et al., 1988; Kumar and Chambon, 1988) (*figure1*).

The targets of proliferative E2/ERα action include genes such as fos, jun-B and myc(Weisz and Bresciani, 1993), cell cycle-regulators such as cyclin D1 (Musgrove et al., 1993), cyclin-dependent kinase inhibitors (Planas-Silva and Weinberg, 1997) and growth factors and their signalling pathways (Migliaccio et al., 1996; Dickson and Lippman, 1995).

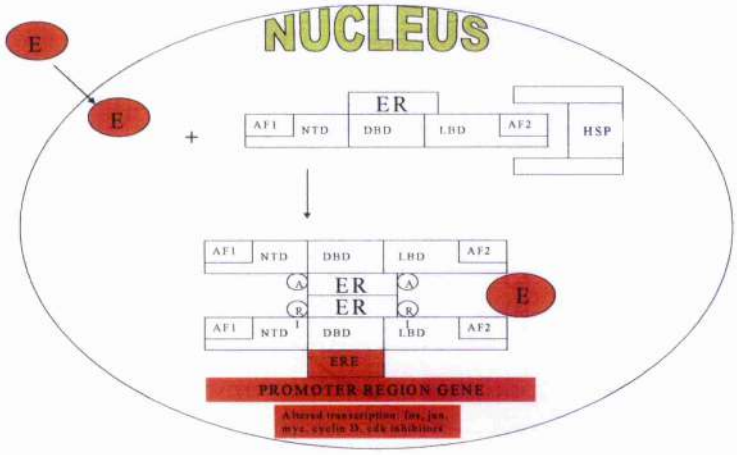


Figure 1

The ER is composed of an N-terminal domain (NTD), a central DNA binding domain (DBD) and a C-terminal ligand-binding domain (LBD). Oestrogen binding to the ER causes dissociation of the heat shock proteins (HSP). The activated receptor subsequently homodimerize and form a complex with co-activators (A) and co-repressors (R), which binds to oestrogen response elements (ERE) in the promoter region of target genes.

ERα is composed of different domains, i.e. the ligand binding domain (LBD) which contains the binding region for oestrogens and anti-oestrogens(Brzozowski et al., 1997; Shiau et al., 1998; Tanenbaum et al., 1998). The LBD also contains a ligand activated transcription activation function, AF-2, as well as sequences required for ligand-dependent dimerisation(Ali and Coombes, 2000c). The N-terminal 180 amino acids contain transcription activation function AF-1(Ali and Coombes, 2000b). Extensive studies using ERE-containing reporter genes have shown that AF-1 and

AF-2 can act both independently and synergistically in a promotor and cell specific manner(Gronemeyer, 1991; Tsai and O'Malley, 1994; Beato et al., 1995).

1.2.1 Receptor Phosphorylation

ERα is phosphorylated and consequently activated at several sites (Le Goff et al., 1994b) (*Figure 2*). Growth factor signal transduction pathways or cyclin dependent kinases can activate ERα in a ligand-independent manner by phosphorylation at specific serine residues(Joel et al., 1998) . Phosphorylation of ERα at Ser-167 is mediated by the phosphoinositide 3-kinase (PI3K)/Akt pathway, whilst the mitogen-activated protein kinase (MAPK) pathway phosphorylates ERα at Ser-118; both sites are in the hormone-independent AF-1 region. Phosphorylation is an important mechanism by which the activity of transcription factors is modulated(Shao and Lazar, 1999). Phosphorylation of ERα at serines 104, 106, 118, 167, 236 and tyrosine 537 has been demonstrated using deletional or point mutation analysis(Ali et al., 1993; Castoria et al., 1993; Le Goff et al., 1994a; Chen et al., 1999a). (*Figure 2*) (Campbell et al., 2001a) (Chen et al., 2000; Rogatsky et al., 1999) The DNA binding domain (DBD) can be phosphorylated by protein kinase A (PKA) (Chen et al., 1999b) to inhibit transcriptional activity via decreasing DNA binding and regulation of dimerization. In addition the AF2 region can be phosphorylated by Src kinases on tyrosine 537 (Arnold et al., 1997) to enhance dimerization, ligand binding and co-regulator recruitment.

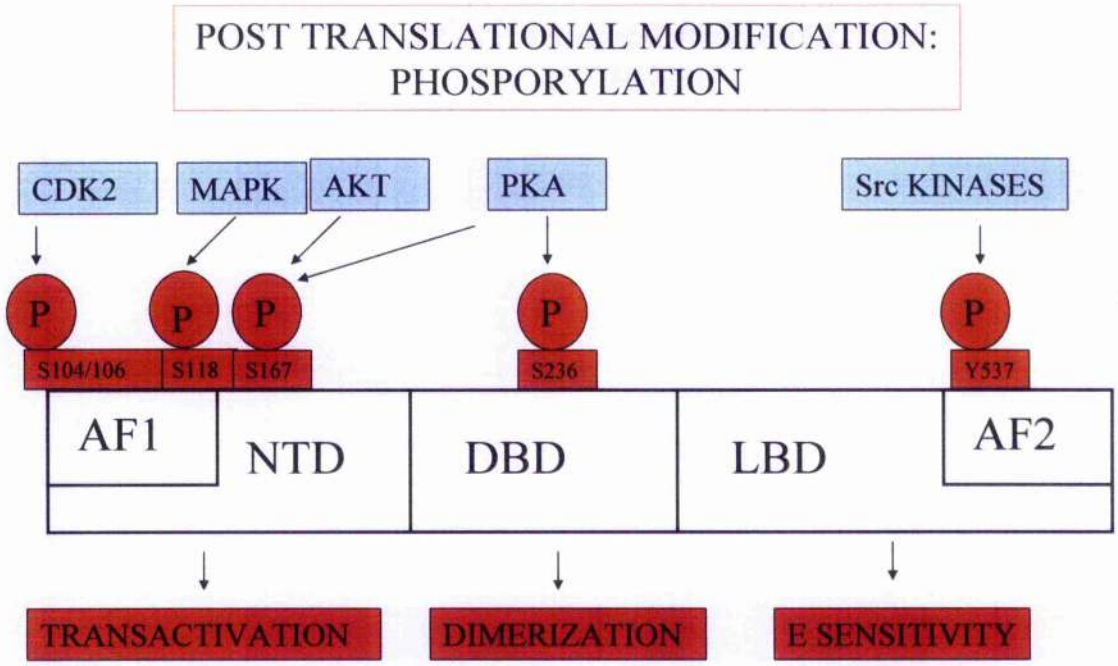


Figure 2

The AF1 region can be phosphorylated by MAPK kinases, Akt or cyclin dependent kinases. The DBD has been shown to be phosphorylated by PKA and the AF2 region by Src kinases.

1.2.2 Ligand Independent Action

The AF-1 region controls ligand-independent receptor activation. Cells with high levels of ER expression may demonstrate ligand independent AF-1 transactivation and growth (Fowler et al., 2004).

1.2.3 Non-genomic Action

There is increasing evidence for "non-genomic" actions of ERα, particularly to explain its rapid non-nuclear effects, such as activation of the calcium channel and the MAPK pathway(Simoncini et al., 2000). These non-genomic, extranuclear, membrane mediated effects take place outside the nucleus. There is evidence that that the non-genomic actions of oestrogen are mediated by membrane associated ERα which functions as an atypical G-protein coupled receptor(Evinger, III and Levin, 2005; Watson and Gametchu, 1999). Functionally, oestrogen initiated non-genomic events like those induced by many cytokines, have important physiological consequences leading to DNA synthesis, cell proliferation and protection against cell death(Castoria et al., 1999; Kousteni et al., 2001; Song et al., 2005a). O'Malley suggested that activation of cytoplasmic kinases through membrane initiated events results in the phosphorylation and activation of co-activators, which enhance nuclear transcriptional events(Song et al., 2005b; O'Malley, 2005). Because ERα has no intrinsic kinase domain and therefore is not capable of phosphorylating other proteins, the signalling molecules must function directly downstream of, and physically associate with ERα. At the same time, the signalling molecule must transduce ERα signal to downstream cascades, leading to rapid activation of MAPK and Akt(Cheskis, 2004). Some evidence exits that this membrane receptor may directly signal via activation/cross talk with the membrane growth factor EGFR (Razandi et al., 2000). This would also provide a mechanism for a positive feedback loop as downstream growth factor pathway members such as MAPK and Akt may then phosphorylate ERα

1.2.4 ERβ

ERβ is homologous to ERα, particularly in the DNA-binding domain (95% amino acid identity). The human ERβ gene has been mapped to band q22-24 of chromosome 14(Enmark et al., 1997) and the human ERα gene has been mapped to the long arm of chromosome 6, excluding the possibility of differential splicing to explain the formation of ERβ(Enmark and Gustafsson, 1999). E2 binds to ERα and ERβ with similar affinities(Kuiper et al., 1998) and activates expression of reporter genes containing estrogen response elements in an estrogen-dependent manner. However, in addition to acting as a homodimer, ERß also heterodimerizes with ERα(Pace et al., 1997). Evidence suggests that the expression of functional ERα/β heterodimers, may result in unique patterns of gene regulation, many of which are distinct from the genes regulated by the ER homodimers(Monroe et al., 2005).

1.3 Endocrine Therapy

In 1898 Beatson discovered the benefits of oophorectomy in young women with locally advanced breast cancer and ERα expression in breast tumours is today a strong predictor of response to endocrine therapy. There are several well-known types of endocrine therapy. Aromatase inhibitors act by blocking conversion of androgens to oestrogens. Lutenising hormone releasing hormone analogues (such as goserlin) prevent the release of LH from the pituitary thus depriving the thecal and granulosa cells of the ovary of stimulation and reducing oestrogen synthesis in the ovary of oestrogen from the ovaries. Alternatively, there is the 'selective oestrogen receptor modulator (SERM)', Tamoxifen. Tamoxifen works by competing with E2 to bind to ERα receptor(Jordan and Dowse, 1976) and was originally developed as an oral

contraceptive, but its potential anti-oestrogenic properties were subsequently recognised(Harper and Walpole, 1966) and it was introduced as an endocrine agent for the treatment of breast cancer. Initially hormone treatment was given to all patients but trial evidence confirmed responsiveness in the ERα positive patients only(1998).

Studies have shown that the agonist activity of Tamoxifen derives from activation of the AF-1 and antagonist activity derives from inhibition of AF-2(Ali and Coombes, 2000a).

The primary mechanism of action of tamoxifen is via competitive inhibition of E2 binding to ERα. This disrupts a series of cellular mechanisms that regulate cellular replication. This disruption caused by tamoxifen changes the growth factor profile in responsive tissues and causes cells to be held in the G1 phase of the cell cycle(Osborne et al., 1983; Colletti et al., 1989; Gradishar, 2004). This produces changes in tumour cell proliferation and cell death and consequently the observed anti-tumour responses(Cameron et al., 2000d; Cameron et al., 2001) and improvement in overall survival(Yao and Jordan, 1998; Gradishar, 2004). Tamoxifen is not a pure antagonist, but rather a partial agonist therefore exerting both agonist and antagonist effects. It is an agonist in bone(Love et al., 1992b) and endometrium(Kedar et al., 1994). It is used for its antagonist effect on genes important for cell proliferation or survival in the breast.

In the Early Breast Cancer Trialists' collaborative Groups overviews of 1998 and 2005, there was a 50% proportional reduction in the recurrence rate and a 28% proportional mortality reduction for ERα positive patients taking adjuvant tamoxifen for 5 years. These overviews demonstrated a long term and increasing benefit of Tamoxifen with survival benefits maximised at 5 years of treatment. Tamoxifen,

taken for 5 years, has been the standard adjuvant endocrine treatment for post-menopausal women with primary ERα positive breast cancer since the 1970's. Tamoxifen can also achieve tumour regression in patients with locally advanced or metastatic disease. Its side effects include those related to its anti-oestrogen effects (flushes, vaginal dryness) and those related to its agonist effects (risk of endometrial cancer(Bergman et al., 2000) and increased risk of thromboembolic events(Falkson et al., 1990)). A positive side effect of Tamoxifen's partial agonist activity is its effect on bone mineralisation(Love et al., 1992a). It produces an increase and stabilisation of bone mineral density. Unfortunately, approximately one fourth of ERα breast cancers exhibit either *de novo* (present before tamoxifen treatment) or acquired resistance during the course of therapy(Gradishar, 2004). Those patients that relapse on tamoxifen therapy generally retain their nuclear steroid receptors(Clarke et al., 2001). However it appears that a proportion of ERα positive tumours are intrinsically hormone independent(McGuire, 1980)

Recently alternative endocrine therapies, such as treatment with Aromatase Inhibitors (AI), have shown to be effective after disease relapse following tamoxifen treatment since most tumours remain ERα-positive(Coombes et al., 2004d) and thus retain the potential to respond to anti-oestrogen therapies. Aromatase is a cytochrome p450-dependent enzyme, which acts at the last step in the synthesis of E2 by catalysing the conversion of androgens to oestrogens. Aromatase inhibitors work by inhibiting this peripheral conversion of androstenedione to oestrone and oestradiol (the primary source of oestrogen in post menopausal women). The Arimidex versus Tamoxifen alone or in combination (ATAC)(Baum et al., 2002b) trial compared five years of therapy using anastrazole (Arimidex) alone with five years of tamoxifen alone. With a combination of the two drugs, there was a small but significant DFS for the

anastrazole arm compared with the tamoxifen arm (89.4% versus 87.4% respectively p=0.013). Results from the combination arm were not significantly different from the tamoxifen arm and this arm was discontinued(Baum et al., 2002a). The ATAC trial was updated in 2005 with 68 months follow up and the benefit of anastrazole over tamoxifen was maintained(Howell et al., 2005b) but only in terms of disease free survival No increase in overall survival has yet been demonstrated. The Intergroup Exemestane Study (IES) randomised ER positive postmenopausal women who had completed 2-3 years of tamoxifen to either complete 5 years of tamoxifen or switch to exemestane for the remaining 2-3 years of therapy(Coombes et al., 2004c). After a median follow up of 36 months switching to exemestane resulted in a 4.7% decrease in the absolute risk of recurrence (p=0.00005). A recent update of this trial showed a significant advantage in terms of overall survival in ER positive cases recruited to this trial. The National Cancer Institute of Canada Clinical Trials Group M.A 17 evaluated the benefit of 5 years of letrozole or placebo after completion of five years of tamoxifen therapy. After a median follow up of 2.4 years, the use of letrozole resulted in a 43% proportional reduction in the risk of recurrence when compared with placebo (p=0.00008)(Goss et al., 2003). As a result of these trials, patients whose tumours are of higher risk (higher grade, nodal metastasis, PR negativity, HER2 positivity) may get an aromatase inhibitor as first line adjuvant therapy or switch from tamoxifen to an aromatase inhibitor after 3 years or giving 5 years of an aromatase inhibitor after 5 years of tamoxifen. There is much debate about when to start AIs but at present those post-menopausal patients who are Her 2 positive are treated from the outset with an AI as the main effect of Her2 positivity is seen within the first three years (early effect)(Tovey et al., 2005b) Patients whose tumours are Her 2 negative but with a higher grade and nodal positivity either have extended adjuvant therapy (AI for 5

years after 5 years Tamoxifen therapy or a switch to an AI after 2-3 years Tamoxifen therapy. The rationale behind 2-3 years of tamoxifen therapy, then a switch to an AI is that women would have increased bone protection from initial exposure to Tamoxifen, and consequently may have a reduced risk of fracture whilst on aromatase inhibitor therapy(Spicer and Ellis, 2006). A study by Kennecke(Kennecke et al., 2006) et al has suggested that there is a subset of post-menopausal women who are at increased risk of relapse after 5 years standard Tamoxifen therapy and those include node negative grade 2 or 3 tumours (10% risk of relapse after 5 years Tamoxifen treatment), and node positive grade 2 tumours (15% risk of relapse after 5 years Tamoixfen therapy). Continuing Tamoxifen therapy for longer than 5 years is no longer recommended as it has been shown that its agonist activity starts to predominate and therefore the risks associated with its use increase whilst the benefits decrease(Fisher et al., 2001). Further data is expected from the ATAC and IES trials soon. Whilst data suggests that AIs are also of value in the adjuvant treatment of breast cancer, switching therapeutic agents in early breast cancer may be unnecessary since about 75% of patients treated with tamoxifen remain alive and disease-free up to 15 years after treatment(2005) . AIs are not without risk such as the increased risk of bone demineralisation and increased cholesterol levels(Perez, 2006).

Indiscriminate implementation of AIs in early breast cancer may both remove viable treatment options from patients who subsequently relapse and lead to over treatment of many women for whom tamoxifen is essentially curative. There is a need to identify the molecular mechanisms involved in development of tamoxifen resistance. The aim is the selective targeting of patients with early breast cancer at high risk of developing tamoxifen resistance, who would derive maximum benefit from selective targeting of other drugs such as AIs(Howell et al., 2005a).

1.4 Potential mechanisms of tamoxifen resistance

Selective ERα modulators (SERMs) have been the most commonly used neoadjuvant therapy for hormone-dependent breast cancer. Resistance to endocrine therapy (either *de novo* or acquired) is a major challenge in the management of breast cancer. The causes of resistance to hormone therapy have been a subject of intensive investigations but are still not well understood. Several mechanismsm have been suggested to promote tamoxifen resistance(Shou et al., 2004b; Jordan et al., 2004b; Knuefermann et al., 2003) : modulation of ER activity via phosphorylation by growth factor receptors (eg HER2)(Shou et al., 2004a; Schiff et al., 2004; Tovey et al., 2004a); phosphorylation/overexpression of steroid receptor cofactors(Osborne et al., 2003; Fleming et al., 2004; McKenna et al., 1999), non-genomic ER activity(Johnston et al., 2003) and increase in cell survival independent of ER(Zhou et al., 2000b; Treeck et al., 2004d; Jordan et al., 2004a), . It is thought decreasing sensitivity of ERα positive breast cancer cells to anti-oestrogens is caused by several factors: Each of these activities may contribute to the tamoxifen resistance phenotype(Clark et al., 2002a; West et al., 2002a; Tsuruo et al., 2003a). Previous work in our group shows Her 1-3(Tovey et al., 2004a), Akt(Kirkegaard et al., 2005e) and AIBI (in press) are linked to de novo tamoxifen resistance.

1.4.1 Loss of ERα

Loss of ERα expression, particularly in metastasis, has been proposed as a mechanism for the development of resistance to hormonal therapy(Graham et al., 1992b) . Most ERα positive breast cancers will also contain populations of ERα negative cells. Under the selective pressure of anti-oestrogen treatment, ERα negative cells may come to predominate so accounting for an apparent switch in their tumour receptor status (Graham et al., 1992a)Two thirds of tumours that become resistant to tamoxifen continue to express ERα (Encarnacion et al., 1993). Tamoxifen resistant cell lines frequently demonstrate sustained ERα content and remain responsive to pure anti-oestrogen therapy (Brunner et al., 1993). ERα positive tumours that have become resistant to Tamoxifen as an initial endocrine therapy will often go on to respond to a 2nd line anti-hormonal treatment such as an aromatase inhibitor or oophorectomy (Murray and Pitt, 1982; Robertson et al., 1989; Sawka et al., 1986), indicating the continued presence of a functional Erα.

1.4.2 Expression of ERβ

In breast cancer archives, ERβ positivity was reported to be approximately 50-70% by immunohistochemistry(Speirs et al., 2004). Recent progress in cellular experiments has shown that ERβ works as a counter partner to ERα through inhibition of the transactivating function of ERα by heterodimerisation, distinct regulation on several specific promoters by ERα or ERβ, and ERβ-specific regulated genes which are probably related to its anti-proliferative properties(Saji et al., 2005). When ERα works as a transcriptional activator on ERE, the function of ERα is suppressed by

dimerization with ERβ(Lindberg et al., 2003). The activity of the AF-1 region in the ERβ receptor is weak compared with the ERα receptor(Cowley and Parker, 1999). Microarray analysis has demonstrated the presence of oestrogen-regulated genes that are only enhanced by ERα or ERβ(Stossi et al., 2004). The expression of ERβ appears to be reduced during carcinogenesis (Roger et al., 2001; Shaaban et al., 2003; Skliris et al., 2003) although levels are high in normal mammary tissue. At present the exact role of ERβ in breast cancer is not clear.

1.4.3 ER mutations

Re-arrangements and/or amplifications of the ERα gene are rare in breast cancer. Some variants have been found that lack specific exons that code for active forms of ERα. However analysis of clinical material failed to find a significant role for these variants in disease progression and/or resistance to endocrine therapies(Hopp and Fuqua, 1998)

1.4.4 PR Expression

PR expression is thought to reflect a functional ER transcription pathway. The IES study(Coombes et al., 2004b) showed benefit in DFS with exemestane regardless of PR status. Data from Tovey et al(Tovey et al., 2004a) showed significant differences in relapse rates on tamoxifen when patients with ER positive and PR negative tumours were compared to those with ER positive and PR positive tumours after 3 years of tamoxifen therapy. This supports the conclusion that PR negativity identifies a group of patients whose tumours have de novo resistance to tamoxifen. The ATAC

(Baum et al., 2002c) trial also supports this conclusion by showing a superior disease free survival for patients whose tumours were ER positive/PR negative with anastrazole as compared to Tamoxifen treatment. However in contrast to the findings of the ATAC trial the Breast International Group (BIG) 1-98(Thurlimann et al., 2005) (which looked at Letrozole as an initial therapy as compared to Tamoxifen) showed that the hazard ratio in favour of the use of letrozole was similar in both the Er+/PR+ and ER+/PR- groups.

1.4.5 Modulation of apoptosis

There is increasing evidence that hormone resistance is linked with the modulation of apoptosis(Treeck et al., 2004c; Fernando and Wimalasena, 2004g). This is further explored in 1.5, 1.7 and 1.8

1.5 Apoptosis and Tamoxifen resistance

1.5.1 Apoptosis

There are 3 different mechanisms by which a cell commits suicide by apoptosis.

1. Generated by signals arising within the cell;
2. Triggered by death activators binding to receptors at the cell surfaces
3. Triggered by reactive oxygen species

There is some evidence that the modulation of apoptosis triggered by internal signals: (the intrinsic or mitochondrial pathway) is linked to endocrine resistant breast cancer. In a healthy cell, the outer membranes of its mitochondria display the proteins Bcl-2/Bcl-xl on their surface.

Internal damage to the cell (e.g., from reactive oxygen species) causes Bcl-2/Bcl-xl to activate a related protein, Bax (see 1.7), which punches holes in the outer mitochondrial membrane, causing cytochrome c to leak out.

The released cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) using energy provided by adenosine tri-phosphatases (ATPs). These complexes aggregate to form apoptosomes. Apoptosomes bind to and activate caspase-9.

Caspase-9 is one of a family of over a dozen caspases. They are all proteases, which cleave other proteins (mainly other caspases). Caspase-9 cleaves and, activates other caspases (caspase-3 and -7).

These caspases create an expanding cascade of proteolytic activity, which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA, and phagocytosis of the cell.

The apoptosis pathway is, in part under the control of the PI3K/AKT and RAF-1 pathways(Tan et al., 1999e) (*figure 3*) which are, in turn under the control of the

Human Epidermal growth factor Receptor (HER) family of receptors. The PI3K/AKT and RAF-1 pathways, activate (phosphorylate) p70S6K (Tan et al., 1999d) and p90RSK respectively which in turn modulate apoptotic activity via phosphorylation of members of the Bcl-2 family.

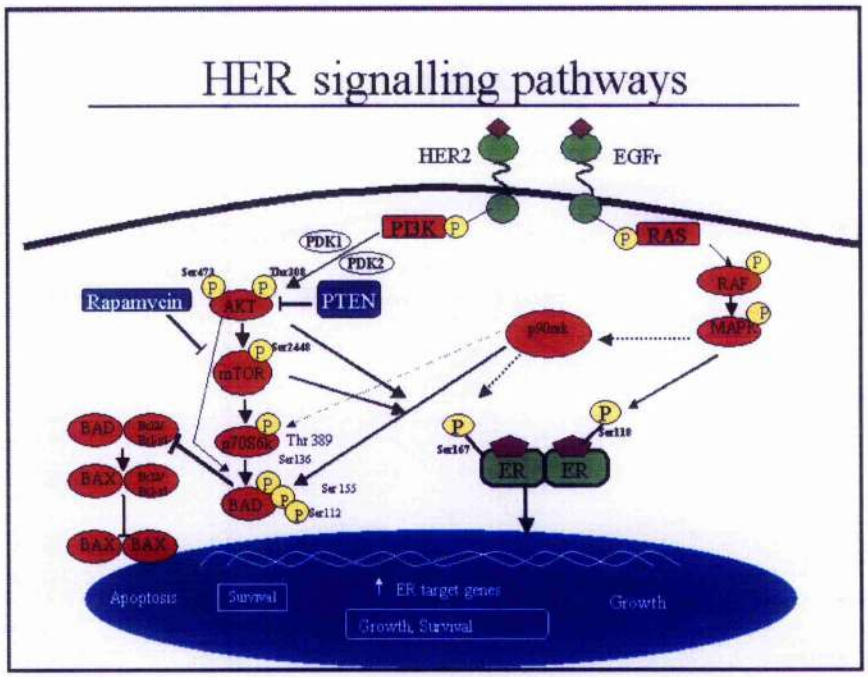


Figure 3. HER signalling pathways showing the proposed apoptotic pathways and activation of the oestrogen receptor.

1.6 HER 1-4 Family

The Human Epidermal growth factor Receptor (HER) family of type-1 receptor tyrosine kinases consists of four members, EGFR/HER1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4 that modulates cell proliferation, survival, adhesion, migration and differentiation. Growth factor induced signalling is essential for normal cellular processes and plays a key role in the aberrant growth and development of tumour cells(Yarden, 2001c). The HER receptors are trans-membrane receptors. Each HER family member is composed of an extracellular ligand binding domain, a lipophilic transmembrane segment and cytoplasmic tyrosine kinase region with a regulatory carboxy-terminal segment (Ullrich and Schlessinger, 1990; van der et al., 1994). Her 2 has no ligands. Ligand binding to the extracellular domain induces the receptors to form hetero- or homo-dimers. Cross-phosphorylation of the tyrosine residues then occurs resulting in the docking sites for signalling complexes. The subsequent release of enzymes and adapter proteins into the cytoplasm stimulates multiple signal transduction cascades.

Three layers of interaction have been identified as being key factors in the generation of the diverse pattern of signalling messages (Alroy and Yarden, 1997).

Firstly, 2 main groups have been identified as HER ligands (Mendelsohn and Baselga, 2000). There are 6 direct ligands of HER1. Epidermal growth factor (EGF), amphiregulin and transforming growth factor alpha (TGFα) only bind to HER1. Heparin binding EGF (HB-EGF), betacellulin, and epiregulin can bind to both HER1 and HER4. A second class of ligand called neuregulins bind to HER3 and HER4. Neuregulin-1 and –2 bind to either receptor, while neuregulin-3 and -4 bind to HER4 only. Recent work has shown that 2 different ligands activating the same receptor can have a differential impact on transcriptional outcome (Amin et al., 2004b).

Secondly, each of the ligands has a different preference for stabilising particular receptor dimers. It appears that the most potent dimer is HER2-HER3 (Yarden, 2001a).

Thirdly, each ligand receptor dimer directs its signal through a unique but overlapping set of signalling pathways (Olayioye et al., 2000). These signal transduction cascades include the MAPK pathway, PKC, phosphoinositol kinase, Akt and several transcription regulators. Ultimately these signals reach the nucleus and lead to nuclear gene activation.

Further complexity is highlighted by the fact that HER2 enhances and stabilises dimerization but apparently has no ligand (Klapper et al., 1999a) whereas HER3 has no inherent kinase activity so must be dimerised with another HER family member to exert an influence on downstream signalling (Guy et al., 1994b).

1.6.1 HER1

HER1 is a 170kD transmembrane receptor that is expressed on the surface of most cell types and is encodeed by a gene on human chromosome 7q21. HER1 is expressed in a variety of human cancers, including non-small cell lung cancer, head and neck, gastric, colorectal, oesophageal, prostate, bladder, renal, pancreatic and ovarian cancers (Salomon et al., 1995). Osborne et al showed that the HER1 ligand EGF was a potent mitogen in the breast cancer cell line MCF-7 (Osborne et al., 1980). The first reports of HER1 expression in human breast cancers were in 1985 (Sainsbury et al., 1985).

Sainsbury et al were the first to identify a link between HER1 and prognosis in breast cancer (Sainsbury et al., 1987). Since then, many studies have examined this relationship, not always with conclusive results (reviewed (Klijn et al., 1992) and

(Nicholson et al., 2001)). One of the largest studies was recently performed using immunohistochemistry in 1029 patients (Tsutsui et al., 2002). Multivariate analysis demonstrated that HER1 was an independently significant prognostic factor for disease free and overall survival. Certainly we know there is a significant negative correlation between EGFR positivity and ER status (Koenders et al., 1991). Tovey et al(Tovey et al., 2004a) showed that Her 1 expression was predictive for early relapse on Tamoxifen which increased when increased associated with over-expression of Her 2 and 3. There has also been speculation as to the future of HER1 as a predictive factor alone for selection of various treatment modalities. ZD1839 (Iressa) is an orally active selective HER1 tyrosine kinase inhibitor which has shown promise in preclinical trials, and is currently undergoing phase II clinical trials in breast cancer (Morris, 2002). High expression of HER1 has been associated with resistance to radiotherapy (Sartor, 2000) .

1.6.2 HER 2

The HER2 proto-oncogene encodes an 185kD transmembrane glycoprotein and is mapped to chromosome 17q21. Despite apparently having no ligand, HER2 homodimers form avidly and HER2 is the preferred dimerization partner for other HER members (Graus-Porta et al., 1997; Tzahar et al., 1996). This may help explain the high oncogenic potential of HER2. HER2 heterodimers have particularly high ligand-binding and signalling potency (Sliwkowski et al., 1994) with the most potent dimer in terms of cell growth and transformation being HER2-HER3 (Pinkas-Kramarski et al., 1996). It has been proposed (Klapper et al., 1999b) that HER2 may be the ultimate co-ordinator of the signalling network, amplifying signalling by multiple ligands.

HER2 is normally expressed in a variety of cell and tissue types and is frequently overexpressed in a number of human cancers (Hynes and Stern, 1994). HER2 overexpression and amplification is found in lung, gastric and ovarian cancers (Schneider et al., 1989; Yoshida et al., 1989; Berchuck et al., 1990).

Both preclinical and clinical studies indicate that HER2 amplification and overexpression is involved in oncogenic transformation and tumourigenesis in breast cancer. Cell lines that overexpress the HER-2 gene product display a highly transformed and tumourigenic phenotype as compared with control cells (Chazin et al., 1992; Di Fiore et al., 1987). In addition HER2 gene amplification or protein overexpression has been demonstrated in 15-30% of invasive breast cancers (Berger et al., 1988; Slamon et al., 1987c). The majority of cases of HER2 overexpression appear to be as a consequence of HER2 gene amplification (Pauletti et al., 1996). Slamon et al (Slamon et al., 1987b) first linked HER2 with poor prognosis in breast cancer in 1987. Since then there have been multiple studies investigating this relationship. A meta-analysis of 47 trials (Ross and Fletcher, 1998) demonstrated HER2 was an independent predictor of prognosis in 60% of the trials involving 67% of patients. Of note all the studies that used FISH to detect gene HER2 amplification concluded that there was a clear association between HER2 status and prognosis.

Herceptin (trastzumab) is a HER2 monoclonal antibody against its extracellular domain. The HERA trial (a large multi centre trial of Herceptin) (Piccart-Gebhart et al., 2005a) showed a significant improvement in disease free survival in Her2 positive breast cancer patients. Tovey et al(Tovey et al., 2004a) demonstrated that those patients with over-expression of Her 2 had an increased chance of early relapse on Tamoxifen treatment compared with those with a low expression of Her 2. They also showed that those patients whose tumours had increased levels of Her 1-3 exhibited

de novo resistance as evidenced by early relapse on Tamoxifen compared to those with reduced expression levels of Her 1-3(Tovey et al., 2004a).

1.6.3 HER3

In 1989 a 3rd member of the HER family was identified (Kraus et al., 1989b) as a 148-kDa transmembrane polypeptide encoded to a gene located on chromosome 12q13. Due to substitutions in critical residues in its catalytic domain, HER3 has an impaired kinase (Guy et al., 1994a), and thus can only process its signalling in the context of a receptor heterodimer. HER3 is found in cells of the developing gastrointestinal, reproductive and urinary tracts as well as the skin, endocrine and nervous systems (Prigent et al., 1992). HER3 is expressed in tumours of the skin, ovary and gastrointestinal tract (Bodey et al., 1997; Maurer et al., 1998; Simpson et al., 1995). There is also evidence for its role in breast cancer. Markedly elevated HER3 mRNA levels have been demonstrated in certain human mammary tumour cell lines (Kraus et al., 1989a). In addition HER3 is overexpressed in 15 –52% of breast cancers (Lemoine et al., 1992; Naidu et al., 1998a; Travis et al., 1996a).

Conflicting evidence has been produced on the prognostic significance of HER3. Some (Witton et al., 2003a; Naidu et al., 1998b; Tovey et al., 2004b; Travis et al., 1996b) have demonstrated a relationship between HER3 and markers of poor prognosis in breast cancer. Others have concluded that HER3 overexpression is consistent with a good prognostic outlook (Pawlowski et al., 2000b). Again Tovey et al(Tovey et al., 2004a) demonstrated that over-expression of Her 1-3 predicted *de-novo* resistance to Tamoxifen (as evidenced by early relapse on Tamoxifen).

1.6.4 HER 4

The HER4/erbB4 gene encodes a 180-kDa transmembrane tyrosine kinase (Plowman et al., 1993b) on chromosome 2 (Zimonjic et al., 1995). HER4 is widely expressed in many adult and fetal tissues and is generally found in the differentiated compartments (Srinivasan et al., 1998a). In normal breast tissue HER4 appears to play a critical role in the late differentiation of mammary gland function (Jones et al., 1999) especially during pregnancy and lactation (Tidcombe et al., 2003). This is consistent with data from cell lines showing that whilst HER4 can support proliferation and transformation, in some lines it has also been shown to induce growth arrest and differentiation (Sartor et al., 2001b; Williams et al., 2003b). Evidence from cell lines using agonistic monoclonal antibodies, showed the HER4 antibody inhibited cell growth in contrast to the HER2 antibody which stimulated growth (Amin et al., 2004a). HER4 is expressed in several breast cancer cell lines (Plowman et al., 1993a). Studies have demonstrated the expression (29 –82%) of the HER4 protein in breast cancers (Srinivasan et al., 1998b; Srinivasan et al., 2000b; Suo et al., 1998; Suo et al., 2002b) although they also suggested that this expression may be decreased when compared to normal breast tissue. Gene amplification has been demonstrated in 13% of breast cancers (Vogt et al., 1998).

It has been shown that, in contrast to other HER family members, HER4 expression is associated with increased survival and lower proliferation indices (Tovey et al., 2004b; Witton et al., 2003b). These results are supported by data linking HER4 to established good prognostic indicators such as a lower grade of tumour (Kew et al., 2000; Srinivasan et al., 2000a), ER positivity (Pawlowski et al., 2000a) and low proliferation indices (Knowlden et al., 1998). Other groups have demonstrated a link between HER4 positivity and a longer disease free interval (Suo et al., 2002a) .

However there have also been conflicting reports associating HER4 with an adverse prognostic significance (Lodge et al., 2003). More recently there has been evidence from a large series of patients suggesting that the prognostic value of HER4 overexpression is dependent on coexpression with other HER family members (Abd El-Rehim et al., 2004d). In this study, when the group was looked at as a whole, HER4 status was not related to survival. However in cases showing expression of one family member only (homodimers), they found a significant association between HER4 homodimer-expressing tumours and improved disease free survival. . Tovey et al did not show any association between expression of the HER4 receptor and disease-free survival on tamoxifen. Previous studies have linked HER4 to improved survival and good prognostic markers(Abd El-Rehim et al., 2004c; Witton et al., 2003c). However, the study from Abd El-Rehim(Abd El-Rehim et al., 2004b) also suggests that the "protective" effect of HER4 may be abrogated if expression of other members of the type I receptor tyrosine kinases is also present. This is consistent with data from cell lines showing that whereas HER4 can support proliferation and transformation, in some lines, it has also been shown to induce growth arrest and differentiation(Sartor et al., 2001a; Williams et al., 2003a). Conversely, when coexpressed with other receptors, such as HER2 and HER3, signaling through these receptors promotes proliferation and overrides the effects seen when HER4 is expressed in isolation(Yarden, 2001b). In Tovey et al's study, few tumours expressed HER4 in isolation and this may have precluded the identification of a distinct HER4 effect.

The role of HER4 in tumour biology as well as its clinical relevance is still emerging.

1.6.5 HER 1-4 and Tamoxifen resistance

The role of the HER family in tamoxifen resistance was initially shown by in-vitro studies, where ER positive hormonally sensitive MCF-7 cells transfected with very high levels of HER2 became resistant to tamoxifen(Benz et al., 1993). MCF-7 cells derived in vitro have increased levels of both HER1 and HER2(Nicholson et al., 2002; Knowlden et al., 2003). Restoration of tamoxifen sensitivity can be achieved by HER1 inhibitors (Iressa) and HER2 blockade (Herceptin (Kurokawa et al., 2000; Pietras et al., 1995)).

HER1-3 expression is associated with high proliferation indices(Tovey et al., 2004a) . There is an increased risk of relapse if ER positive with Her 1-3 expression than if PR negative(Tovey et al., 2004a)'Tovey et al(Tovey et al., 2004a) showed that HER1-3 predicts tamoxifen resistance in hormone responsive breast cancer. This applies in the first three years of tamoxifen therapy only (early effect).

1.7 PI3K/Akt Pathway

1.7.1 PI3K Activation

Upon ligand activation, the HER receptors form homo- or heterodimers and subsequently activate downstream signal transduction pathways such as the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways.

Activated Her receptors activate PI3K by phosphorylating specific tyrosines on the cytoplasmic tails of their partner's receptors. The p85 subunit binds to these receptors and undergoes conformational changes, which lead to PI3K activation. PI3K is a heterodimer consisting of a 110 kDa catalytic subunit and a 55-85 kDa regulatory subunit(Vanhaesebroeck and Waterfield, 1999; Fry, 1994). This leads to the production of PIP3 and activation of PDK's, which then phosphorylate Akt(Franke et al., 1995c) (*figure 3*).

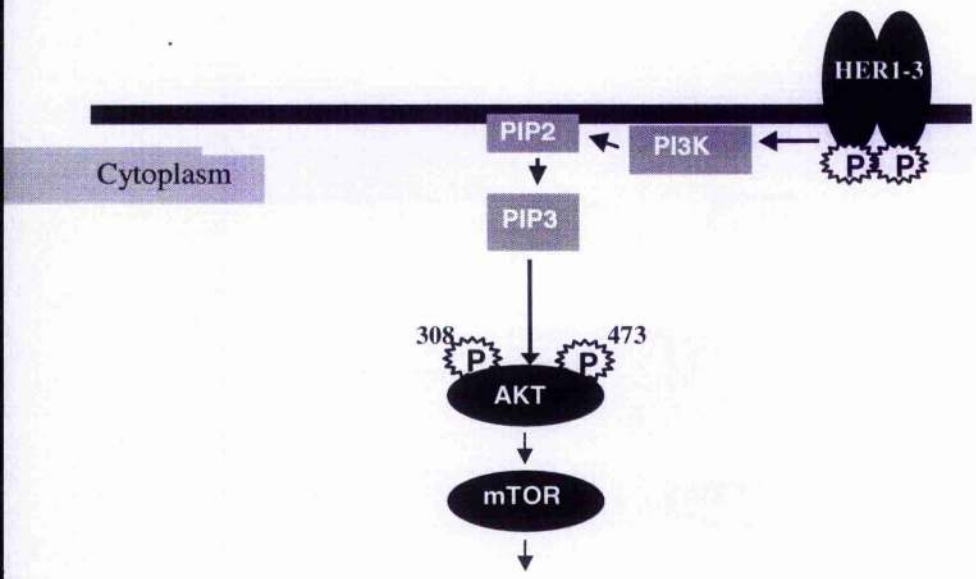


Figure 4. Activation of Akt via phosphorylation of PI3K which catalyses the formation of PIP3 and leads to the phosphorylation of Akt

Mutations in the PIK3CA gene which encodes the PI3K p110α catalytic subunit are seen in 20-40% of breast tumours(Crowder and Ellis, 2005; Samuels and Velculescu, 2004a; Bachman et al., 2004c; Campbell et al., 2004a; Saal et al., 2005b).

Akt is first phosphorylated at Thr 308 but additional phosphorylation at Ser473 is necessary for full activation. Once activated, Akt can phosphorylate other downstream members of the pathway such as mTOR, p70S6K, which in turn activate ER by phosphorylation at Ser167 which directly or indirectly phosphorylates substrates that directly or indirectly regulate apoptosis such as Bad (Figure 3).

Recent research has highlighted the central involvement of the PI3K/AKT pathway in multiple resistance mechanisms(Clark et al., 2002b; West et al., 2002b; Tsuruo et al., 2003b). Previous work in this laboratory(Kirkegaard et al., 2005d) has demonstrated that up-regulation of this pathway is associated with endocrine resistance.

1.7.2 PTEN

PTEN encodes a protein product that has sequence homology with dual specificity phosphatases capable of dephosphorylating both tyrosine phosphates and serine threonine phosphate residues on proteins(Li et al., 1997b; Steck et al., 1997; Li and Sun, 1997). PTEN dephosphorylates PIP2 and PIP3; lipids that are normally absent from quiescent cells but appear on stimulation with growth factors. They are involved in the activation of AKT(Franke et al., 1997) via the activation of PDK's, which then phosphorylate Akt(Franke et al., 1995b) (*figure 3*). Panigrahi et al's study(Panigrahi et al., 2004a) has indicated that loss of PTEN expression is infrequent in breast cancer. It did not show a prognostic significance of PTEN. This study used IHC on wildtype PTEN it did not use mutation analysis.

It has been shown that PTEN activation contributes to tumour inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients(Nagata et al., 2004).

The PTEN gene is frequently mutated or inactivated in a high proportion of human cancers(Risinger et al., 1997; Tashiro et al., 1997), including up to 30% of human breast cancers, resulting in hyper-activation of the PI3K/Akt pathway. Germline mutations have been associated with three autosomal dominant cancer syndromes: Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndromes(Liaw et al., 1997c; Marsh et al., 1998). The significance of PTEN mutations and loss of PTEN expression in primary sporadic breast cancer is at present unclear. Estimates of the mutation and loss of expression rate range from 1-48% (various IHC and mutational analysis studies(Depowski et al., 2001b; Freihoff et al., 1999)). The estimated rate of PTEN mutations in sporadic breast cancer is only 5%. Promoter hypermethylation has been identified as an alternative mechanism of PTEN inactivation. One study(Garcia et al., 2004b) found that 48% of sporadic breast cancers had hypermethylation of the PTEN promotor. PTEN hypermethylation was also associated with HER2 overexpression, larger size and higher histiologic grade.

It has been demonstrated that PTEN mutations derived from sporadic tumours as well as Cowden disease patients, retain protein phosphatase activity but loose the ability to dephosphorylate inositol phospholipids(Myers et al., 1998). This provides strong evidence that the loss of ability to dephosphorylate inositol phospholipids is vital to the tumour suppressor role of PTEN.

1.7.3 AKT

AKTs (protein kinase B or RAC-PK) are a family of serine/threonine protein kinases with three mammalian isoforms (AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBϒ), activated by growth factors in a PI3K-dependent manner(Franke et al., 1995a). Three AKT isoforms, AKT1-3, are encoded by distinct genes localized on different chromosomes(Hill and Hemmings, 2002a; Thompson and Thompson, 2004c). They have similar structures with approximately 80% amino acid identity(Thompson and Thompson, 2004a; Hill and Hemmings, 2002b), but their expression patterns and biological activities differ. In normal physiology, AKT1 is involved in placental development and maintenance; AKT2 in glucose metabolism, adipogenesis, and β-cell function; whereas AKT3 plays a role in postnatal development of the brain (reviewed in (Yang et al., 2004b)). They also act co-operatively, since double knockouts (AKT1/2 and AKT1/3) exhibit severe developmental abnormalities(Yang et al., 2004a).

Whilst tissue distribution and function of the AKT proteins are not identical, activation of all three isoforms occurs in a similar fashion (reviewed in (Datta et al., 1999)). AKT activation occurs at the plasma membrane, through PI3K and 3-phosphoinositide-dependent kinases (PDKs)(see 1.7.1). AKT is first phosphorylated at Thr-308 but additional phosphorylation at Ser-473 is necessary for full activation (reviewed in (Luo et al., 2003a)). Once activated, AKT activates ERα by phosphorylation at Ser-167(Campbell et al., 2001b; Stoica et al., 2003) or phosphorylates substrates that directly or indirectly regulate apoptosis, such as Bad(Datta et al., 1997), caspase 9(Cardone et al., 1998) or forkhead transcription family members(Brunet et al., 1999).

AKT1 is overexpressed in 20% of gastric tumours(Staal, 1987), and AKT3 is over expressed in hormone-independent breast and prostate cancer cell lines(Nakatani et al., 1999b). AKT activity is present in primary carcinomas of the breast, colon, ovary, and prostate(Nakatani et al., 1999a; Sun et al., 2001; Yuan et al., 2000; Roy et al., 2002).

In a study looking at MCF-7 cells that expressed a constituitively active form of Akt, and were therefore able to proliferate under reduced oestrogen conditions and were resistant to the growth inhibitory effects of tamoxifen, co-treatment with the mTOR inhibitor rapamycin inhibited mTOR activity and restored oestrogen sensitivity(deGraffenried et al., 2004a). Kirkegaard et al(Kirkegaard et al., 2005c)performed a study using immunohistochemistry (IHC) with antibodies against AKT1-3, pan-AKT, pAKT (Thr-308), pAKT (Ser-473), pER (Ser-167), and pHER2 on the same 402 ER α positive breast carcinomas used in this study. High pAKT (Ser-473) activity ($p = 0.0406$) and low AKT2 expression ($p = 0.0115$) alone, or in combination [high pAKT (Ser-473)/low AKT2; 'high-risk' patient group] ($p = 0.0014$), predicted decreased overall survival in tamoxifen-treated patients with ERα-positive breast cancers. There was no significant association between tumour levels of AKT expression or activity and disease-free survival (DFS); however, the 'high-risk' patient group was significantly more likely to relapse ($p = 0.0491$). During tamoxifen treatment, neither AKT2 nor pAKT predicted DFS. They also showed that activation of AKT, via phosphorylation, was linked to activation of both HER2 and ERα in this patient cohort. This reinforces the differential functions of the AKT isoforms. The mechanism by which increased AKT2 expression may modulate breast cancer survival is, however, unclear. High AKT2 expression did not predict AKT activity.

1.7.4 mTor

mTor is a member of the phosphoinositide kinase-related kinase (PIKK) family, consisting of large serine/threonine kinases including ATM, ATR and DNA-PK.(Abraham, 2004). mTor activates P70S6K and the eukaryotic initiation factor 4E-binding protein-1(Mita et al., 2003). mTor activates P70S6K by phosphorylating it at thr 389and thr 229(Weng et al., 1998a). PTEN-negative cell lines have been shown to elevate P70S6K activation that is abrogated in the presence of the mTOR inhibitor CCI-779, an ester of rapamycin. This supports the theory that mTOR is an important downstream signalling mediator in PTEN-negative tumours(Podsypanina et al., 2001; Neshat et al., 2001). Inhibition of mTOR by rapamycin results in cell-cycle arrest, p53-dependent and independent apoptosis and tumour growth inhibition(Huang et al., 2001; Hosoi et al., 1999; Grewe et al., 1999; deGraffenried et al., 2004b)

1.8 Apoptotic Pathway

As described previously the apoptotic pathway is partly under control of the PI3K/Akt pathway The PI3K/AKT and RAF-1 pathways, activate (phosphorylate) p70S6K (Tan et al., 1999c) and p90RSK respectively which in turn modulate apoptotic activity via phosphorylation of members of the Bcl-2 family *(figure 3).*

1.8.1 P70S6K

P70S6K is a mitogen activated Ser/Thr protein kinase that is required for cell growth and cell cycle progression(Pullen and Thomas, 1997d). There are multiple phosphorylation sites on P70S6K located within the catalytic, linker and pseudosubstrate domains(Pullen and Thomas, 1997a). P70S6K is activated by mTor via phosphorylation at thr 389/229(Pullen and Thomas, 1997b). Thr 389 is located in P70S6K's linker domain and thr 229 is located in its catalytic domain (Pullen and Thomas, 1997c). Phosphorylation of thr 389 is thought to most closely correlate with P70S6K activity (Weng et al., 1998b). Activated P70S6K phosphorylates Bad at Ser136(Harada et al., 2001g). Lin et al(Lin et al., 2005b)showed that pP70S6K expression was increased in primary breast cancers. They also showed negative expression in normal breast epithelial tissues. Enhanced phosphorylation was positively associated with disease progression from normal breast epithelial tissue to invasive breast carcinoma ($p<0.05$). P70S6K was shown to be amplified in approximately 10% of the breast cancers looked at by Barlund et al (Barlund et al., 2000a; Andersen et al., 2002b) In another study(van der Hage et al., 2004a) that looked at 452 node negative tumours from pre-menopausal women (drawn from the EORTEC trial) over-expression of P70S6K was found to be associated with an increased risk of loco-regional recurrence.

1.8.2 P90RSK

P90RSK is composed of two functional kinase domains that are activated in a sequential manner by a series of phosphorylations(Frodin and Gammeltoft, 1999). Phosphorylation at Ser 380, Thr 359, Ser 363 and Thr 353 are important for its activation(Dalby et al., 1998). P90RSK is phosphorylated via the RAF-1 pathway (see *figure3*). Activated P90RSK phosphorylates BAD at Ser112(Harada et al., 2001f). There is very little in the literature looking at P90RSK expression and survival in breast cancer. Most of the studies concentrate on looking at its role in phosphorylating the pro-apoptotic protein Bad(Harada et al., 2001e).

1.8.3 Bcl-2 Family

The Bcl-2 family of proteins control a critical intra-cellular checkpoint in the apoptotic pathway. Bcl-2 expression blocks cell death following multiple physiological and pathological stimuli.(Danial and Korsmeyer, 2004b) Pro-survival members of the bcl-2 family include Bcl-2 and Bcl-xl. There are two pro-apoptotic groups: the Bax group (Bax, Bak and Bok) and the BH-3 only proteins (which include Bim, Bad, and Bmf).

The BH-3 only proteins monitor cellular health and, when activated by cytotoxic signals, engage pro-survival relatives by inserting the BH-3 domain, an amphipathic α helix, into a groove on their surface(Willis and Adams, 2005b). This primes the cell for apoptosis but commitment also requires the activation of Bax or Bak(Willis and Adams, 2005a). Once activated, Bax and Bak form oligomers in intracellular membranes, including the mitochondrial outer membrane. The resulting membrane

permeabilization releases pro-apoptotic proteins, such as cytochrome c, that provokes activation of the caspases mediating cell demolition. Bad and Bmf bind only Bcl-2, Bcl-xl and Bcl-w(Willis and Adams, 2005c).

Bad is a BH-3 only pro apoptotic, member of the Bcl-2 family of proteins. Its structure has not yet been fully mapped, however it is known to have multiple phosphorylation sites

Phosphorylation of P70S6K and P90RSK leads to phosphorylation of Bad via various sites: P90RSK phosphorylates BAD at Ser112(Harada et al., 2001d), whilst p70S6K phosphorylates BAD at Ser136(Harada et al., 2001c). Other phosphorylation sites, include Ser 155, that is phosphorylated by protein kinase A, RSK1 and survival factor signalling have also been identified.(Tan et al., 2000a; Zhou et al., 2000c).

Phosphorylation (activation) of Bad blocks the binding of Bad to Bcl-2/Bcl-xl (*figure5*). When Bad is in complex with Bcl-2 or Bcl-xl it is in its pro-apoptotic state. However, when Bad is phosphorylated, Bcl-2/Bcl-xl are released from the complex allowing Bcl-2/Bcl-xl to form a complex with Bax. The Bax:Bax homodimer is required for apoptosis. Bcl-2:Bax hetrodimerisation therefore prevents apoptosis. This is the key and all other interactions modulate levels of Bax:Bax and thus apoptosis. The ratio of anti-apoptotic to pro-apoptotic members such as Bcl-2/Bax constitutes an internal rheostat that sets the threshold of susceptibility to apoptosis for the intrinsic pathway, which uses organelles such as mitochondrion to amplify death signals.(Danial and Korsmeyer, 2004a)

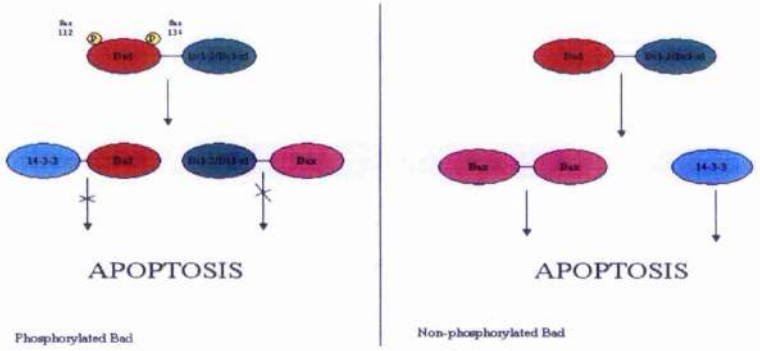


Figure 5. Figure illustrating the effects of phosphorylation of the pro-apoptotic protein Bad

Increased expression of Bcl-2 may therefore also be implicated in the resistance of cancer cells to apoptosis. Bad is released from its heterodimer with Bcl-2/Bcl-xl after it is phosphorylated. Phosphorylated Bad then forms a complex with 14-3-3, which also inhibits cell death.

RSK1 phosphorylates Bad at both Ser 112 and ser-155 and prevents Bad mediated cell death in a manner dependent upon phosphorylation at both sites. The BAD protein acts as a switch that integrates the anti-apoptotic effects of the EGFR/MAPK and PI3K/Akt pathways. Inhibition of both pathways is required to release Bad from 14-3-3 and activate its pro-apoptotic functions(Antonsson et al., 2001)

Activation of the PI3K/Akt signal transduction pathway has been implicated in endocrine resistance(Kirkegaard et al., 2005b). Activation of the PI3K/Akt pathway causes phosphorylation of Bad, which causes release of Bcl-2/Bcl-xl and disruption of

the Bax:Bax homodimer and prevents apoptosis The phosphorylated Bad binds to 14-3-3 and this also prevents apoptosis. Previous in-vitro studies suggest that long term treatment with tamoxifen modulates the expression of apoptotic key genes impairing the apoptotic response of MCF-7 breast cancer cells(Treeck et al., 2004b). *In vitro* studies have shown that oestrogen-mediated phosphorylation of Bad prevents apoptosis(Fernando and Wimalasena, 2004f). The ability of oestrogen to prevent apoptosis was blocked by over expression of Bad where the phosphorylation sites have been mutated (S112A/S136A) but not by the wild type Bad. Bad S112A/S136A, which lacks the phosphorylation sites, was not phosphorylated in response to oestrogen *in vitro*(Fernando and Wimalasena, 2004e).

The anti-apoptotic proteins Bcl-2 and Bcl-xl are thought to play important roles in inhibiting mitochondria-dependent pathways(Kim, 2005d). Some studies suggest that these two proteins have distinct functions for inhibiting extrinsic and intrinsic cell death pathways. The over expression of Bcl-2 has been shown to be associated with cell cycle arrest in the G1 phase, which may promote cellular senescence. The over expression of Bcl-2 may also have the ability to enhance cell death in the interaction of Bcl-xl with other factors(Kim, 2005a). An earlier study looking at the prognostic significance of p53, Bcl-2 and Bax expression in early breast cancer(Linjawi et al., 2004b), did not find a correlation between Bax or Bcl-2 and OS or DFS. However Bcl-2 correlated significantly with favourable tumour features such as DNA diploid status and expression of oestrogen and progesterone receptors(Linjawi et al., 2004a). This has also been seen in other studies(Baccouche et al., 2003b). Another study showed that reduced expression of Bax (p=0.03) and Bcl-2 (p=0.03) was associated with lymph node metastasis in multivariate analysis(Bukholm et al., 2002b). Expression of Bcl-2 was associated with improved patient survival in univariate

analysis (p=0.04) but lost its predictive value in multivariate analysis (p=0.2)(Cameron et al., 2000c).

The present study was carried out to test the hypothesis that disruption of apoptosis in breast cancer, via Akt activation is linked with hormone resistance.

1.9 Statement of Aims

Hypothesis:

The present study was carried out to test the hypothesis that activation of the PI3K/Akt pathway and the part of the apoptotic pathway activated by the PI3K/Akt pathway is linked to Tamoxifen resistance

1. To test the hypothesis that disruption of the apoptotic pathway is linked with hormone resistance. The PI3K/Akt and MAPK pathways appear to inhibit the apoptotic pathway by phosphorylation of Bad. Expression levels of Bad, pBad (Ser112), Bax, Bcl-2, Bcl-xl, P70S6K, pP70S6K (Thr389) and pP90RSK (Thr359/Ser363) are to be assessed in the tumour samples using antibodies against these proteins with IHC. The expression levels of these proteins will then be correlated against data previously obtained on the level of apoptosis (TUNEL data).
2. To investigate the role of specific members of the PI3K/Akt pathway in mediating hormone resistant breast cancer (in conjunction with other researchers in our unit). Tumour samples will analysed for PTEN and PI3K expression (IHC) and preliminary studies using FISH will be carried out on a limited number of the samples to assess gene copy number status. This data

will then be correlated with other proteins in the pathway (as investigated by our lab).

3. To investigate using fluorescence in situ hybridisation (FISH) on the tissue microarrays (TMA's) made by Sian Tovey HER2 gene amplification status, and to compare these results with previously obtained results on HER2 over-expression in this patient cohort. This is to look at the number of false positives/negatives obtained using the HercepTest (IHC) and to see how many of them are picked up by the current standard of performing FISH on all patients whose tumours were categorized as 2+ using the HercepTest.

A summary of aims would therefore be

1.IHC on proteins in the part of the apoptosis pathway controlled by the PI3K/Akt and RAF-1 pathways (Bad, Bax, Bcl-2, Bcl-xl, P70S6K, p90RSK and their phosphorylated versions where applicable). Correlation of these results with the clinical progression of these patients on tamoxifen plus correlation with other proteins in the pathway. In addition correlation of the expression level of these proteins with previously obtained data (TUNEL data) on the level of apoptosis in the tumours.

2.IHC for PTEN, phospho PTEN, PI3K and FISH for PTEN and PI3K . Correlation of these results with the clinical progression of these patients on tamoxifen plus correlation with other proteins in the pathway.

3.FISH for HER2 to compare with HerceptTest results

Chapter 2. Methodology

2.1 Patient Selection

2.1.1 Ethical clearance

Ethical clearance was obtained from the Glasgow Royal Infirmary LREC committee (LREC 02SG007).

2.1.2 Patient Database

Details of sequentially diagnosed breast cancer patients, suitable for surgical excision, from years 1980 –1999 were kept in a registered database within the University Department of Surgery. These patients had surgery and subsequent adjuvant therapy according to protocols at the time of diagnosis. Patients who had neo-adjuvant treatment in terms of endocrine treatment, chemotherapy and radiotherapy prior to their surgery were also included in the database. The total number of patients in the database is 1116. Patient follow-up details have been entered prospectively since diagnosis. These entries include information on attendances, recurrence and metastasis details, date and cause of death and adjuvant therapy details (including tamoxifen treatment duration).

From this database, patients were selected who had been treated with adjuvant tamoxifen therapy and who were either known to be ER positive or who did not have a record of oestrogen receptor (ER) status being tested. Patients with an unknown ER status were included to increase the numbers available for analysis, on the basis that all patients in the study would have their ER status re-tested. The number of tamoxifen treated ER positive or ER unknown tumours were 685. For full patient details see appendix I.

2.1.3 Patient Exclusion

From this group of 685 patients, further exclusions were made on the following basis:

1. If their follow up and full tamoxifen history was not complete in terms of details of any relapse or duration of tamoxifen treatment (n=52).
2. If the tumour blocks from pathology were not available or deemed unsuitable by the pathologist (Barbara Dunne; BD) because of limited tissue availability, which may have compromised further diagnostic testing (n=177).

456 patients were therefore eligible for the study. 75 of these patients were ER non-specified, with the remainder having being tested positive for ER by either ligand binding (n=130) or immunohistochemistry (IHC) (n=251).

2.2 TMA Construction

TMAs were made by Sian Tovey using the following methods:

2.2.1 Technique

This department has been using TMA technology for some time in IHC and fluorescence in situ hybridisation (FISH) analysis of breast, ovarian and prostate tissue. We are currently involved in the construction of TMAs for the TEAM and NEAT (National Epirubicin Adjuvant Trial) trials. The TMAs were constructed for this study under guidance from pathology MLSOs working within the department.

Formalin fixed paraffin embedded tissue blocks, prepared after surgical excision of the tumours, were obtained from the department of pathology. Bouins was not used in the fixation of these specimens. A tissue section from each block was stained using haematoxylin and eosin (H&E) to identify areas of tumour. Representative areas on

the H&E were identified by a pathologist (BD) and marked on the slide. This is essential as it ensures the core taken contains representative tumour cells.

Prior to removing the cores, 5 × 3μM sections were cut from each pathology block by a MLSO in the laboratory. These sections have been stored and may be used in the case of any missing samples from the TMA slides or to validate the TMAs in any cases where there are doubts about the heterogeneity of tissue staining for a particular antibody. Subsequently 3 x 0.6mm cores of tissue were removed from each block at the area marked by BD using a tissue arrayer (Beecher instrument). This precision instrument uses 2 separate core needles for punching the donor and recipient blocks, together with a precise co-ordinate system to place and locate the tissue samples in the array. These cores of tissue were then transferred to 3 recipient blocks (80-120 cores per block) to form triplicate tissue arrays. In total, 5 different arrays each in triplicate (total 15 donor blocks) were constructed.

2.2.5 TMA Layout

The cores were placed at 1 mm intervals, with regular larger gaps as to ensure ease of identification of core position during analysis (Figure 4).

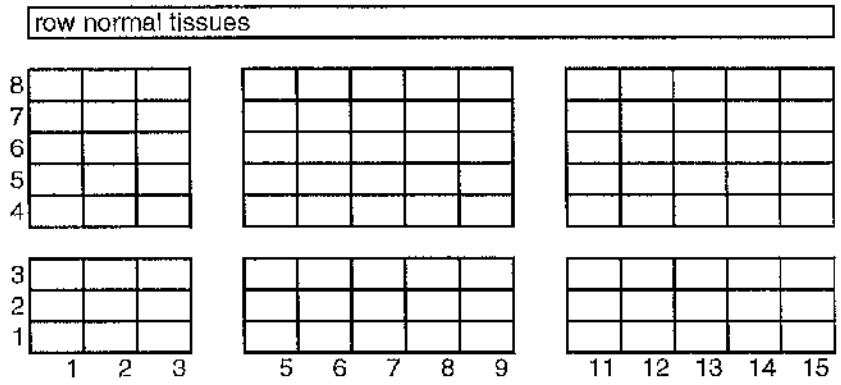


Figure 6

Illustration of typical core placement layout in TMA.

2.2.2 Control Tissues

Cores were taken from 10 blocks containing samples of normal tissue (sourced from USA commercial). These normal tissues consisted of skeletal muscle, smooth muscle, normal breast, lung, placenta, prostate, tonsil, lymph node, skin and testis. A core from each of these was placed in order, in a row, above the tumour cores in each of the 15 donor blocks. This provides the basis of controls for each of the antibodies, which have known staining patterns in normal tissues. This row of normal tissues also provided a mechanism for correct orientation of the TMA section after sectioning.

The completed tissue array blocks were heated at 37° for 10 minutes to ensure the tissue cores adhere to the recipient paraffin block.. Serial 3μM sections were then cut from each TMA by a MLSO and placed on silanised slides to improve section contact with the slide during high temperatures used in some antigen retrieval protocols.

2.3 Immunohistochemistry

Immunohistochemistry (IHC) is the localization of antigens in tissue sections by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as stain. Advantages of IHC include the ability to detect location of staining (i.e. membranous, cytoplasmic or nuclear) as well as providing a semi quantitative assessment of intensity (0, 1, 2, or 3). Difficulties with IHC can occur in relation to the specificity of the antibody as well as the need to expose antigenic sites to the antibody, particularly in formalin fixed tissues.

The 10 antibodies I have chosen to work with for this project are PTEN, PI3K, Bad, phospho-Bad (Ser 112), Bax, Bcl-2, Bcl-xl, P70S6K, phospho-P70S6K (Thr 389) and phospho-P90RSK (Thr 359/Ser 363).

2.3.1 General Principles of IHC and Protocols

All of the antibodies were fully worked up by E.C and all of the IHC procedures were carried out by E.C. During the work up of the antibodies I followed a set of general principles described below which were subsequently tailored to each antibody as described in Table 1. .

A. Dewax, rehydration

Prior to any immunohistochemical staining on formalin fixed, paraffin embedded tissue, paraffin must be removed from the tissue to allow the water-based buffers and antibodies to penetrate. For all antibody protocols this was done using the following treatment: dewax in xylene (Fishers chemicals) (2 x 2 minutes). Rehydrate in

graduated alcohol washes: 100% alcohol (2 x 2 minutes), 90% alcohol (2 x 2 minutes), 70% alcohol (2 x 2 minutes). Finally rinse in water.

B. Antigen retrieval

As a result of formalin-fixation and wax embedding, the antigen under investigation may lose its immunoreactivity, thus either binding weakly to the appropriate primary antibody or not at all. This is due to the formation of methylene bridges during fixation, which cross-link proteins and therefore mask antigenic sites. To facilitate the antibody - antigens reactions in fixed tissue, it may be necessary to unmask or "retrieve" the antigens through pre-treatment of the specimens. This antigen-retrieval step breaks the protein cross-links and exposes the antigen-binding site. Antigen retrieval has been shown to increase reactivity of the majority of antigens in tissues. This was done by one of the below mentioned methods under routine use in the lab, depending on the antibody used. These methods were:

1. Sections were microwaved under pressure (approximately 15 psi) in TE solution (5mM Tris base pH 8.0 (VWR International Ltd) and 1mM Sodium EDTA (BDH Laboratory Supplies)).

2. Sections were microwaved under pressure (approximately 15 psi) in 10mM citrate buffer (Epitope retrieval solution x 10, K-5205, Dako or Antigen unmasking solution (Vector)).

Microwave irradiation of formalin-fixed, paraffin-embedded specimens in buffer has been found to markedly enhance the retrieval of antigens. During this procedure the energy provided helps break some of the bonds formed during fixation, thus increasing the intensity of reactions.

3. Sections were incubated in Tris EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA) or 1:10 citrate buffer (Antigen unmasking solution (Vector)) for 20, 25 or 40 minutes depending on the specific antibody protocol at 96°C in a standard water bath.

C. Block of endogenous peroxidase activity

Breast tissue is known to contain endogenous peroxidase. To minimise background staining from this, TMA's were treated with hydrogen peroxidase (VWR International Ltd; supplied at concentration ~ 30%), which is known to irreversibly inactivate endogenous peroxidase. This was done after antigen retrieval at a concentration of 0.3%, 1% or 3% for 10 minutes depending on the protocol determined for each antibody.

D. Blocking non-specific background staining

Any non-specific background staining was blocked using one of the following methods:

Applying normal horse serum (Vector) to the tissue at a concentration of 15 µl/ml for 1 hour or 0.1 % casein (Vector) for 1 hour. Following incubation with blocking agent the solution was tapped off the tissue and the TMA's were blotted and ringed with Dako pen to create a barrier.

E. Incubation with Primary antibody

The TMA's were incubated with the primary antibodies at optimum concentrations and conditions determined for the individual antibodies (as described later in Table 1). Antibodies (including the negative control antibodies) were always diluted in

antibody diluent (Dako). Slides were washed twice in TBS buffer for 5 minutes following incubation.

F. Quality Assurance

For each IHC experiment, it is essential to have both a positive and a negative control. These controls were performed on the same tissue. The positive control confirmed that the chosen IHC method gives the same level of staining in every run, whilst the negative control (performed exactly the same way as the other slide except that the primary antibody is substituted with isotype matched control reagent (Dako)) was done to substantiate that the staining was not due to unspecific binding. Tonsil tissue, placenta tissue or breast tissues previously shown to have strong expression of the desired antigen were incubated with the appropriate antibody and used as positive controls.

G. Visualisation Methods

EnVision™ System from Dako was used as visualisation. The EnVision™ System is a simple, two-step visualization system of very high sensitivity. It is based on a unique enzyme-conjugated polymer backbone, which, in addition, also carries secondary antibody molecules. Endogenous biotin will not affect EnVision™ staining results. Slides were washed in TBS buffer (10x: 0.1M Tris/HCL and 1.5M NaCL per 1L dH_20 (pH to 7.5). Before use 10x TBX is diluted 10 times in dH_2O) between and after incubations with the link antibody and Strepavidin peroxidase solutions.

Detection was then completed with incubation with a 3, 3'diaminobenzidine (DAB) solution (Vector Laboratories) for 2-10 minutes. The DAB solution was washed off in running water for 10 minutes.

H. Counterstaining, Dehydration and Mounting

The sections were counterstained, and then dehydrated using the following method: Haemoxylin 45 seconds followed by rinse in running H_20. Then wash with Scotts tap water substitute (about 45 seconds), this produced a blue colour, in contrast to the brown positive staining of the antigen. Rinse in running H_20. The last steps involved dehydrating the tissues through a series of alcohol washes: 70% (1x1minute), 90% (1x1minute), 100% (2x1minute) and xylene (2x1 minute, and then mounting the slides onto coverslips using DPX mountant (Dibutyl Phtalate containing Xylene).

Protein	Antibody	Antigen Retrieval	H_2O_2 Conc^n	Blocking Solution	Antibody Conc^n	Incubation Time & Temperature
PTEN	PTEN (2689) Cell Signalling Technology	Citrate Buffer (microwave)	1%	1:10 1% Casein solution	1:100 20µl/ml	Overnight at 4°C
Bad	Bad (9292) Cell Signalling Technology	Citrate Buffer (water bath for 25 mins)	0.3%	1.5% normal horse serum	1:25 7.8µl/ml	Overnight at 4°C
Bax	Bax (A3533) DAKO	Citrate Buffer (water bath for 40 mins)	1%	1.5% normal horse serum	1:1500 0.46µl/ml	Overnight at 4°C
Bcl-2	Bcl-2 (MO887) DAKO	TRIS EDTA (microwave)	1%	1.5% normal horse serum	1:100 28µl/ml	One hour at 25°C
Bcl-xl	Bcl-xl (2762) Cell Signalling technology	TRIS EDTA (microwave)	3%	1.5% normal horse serum	1:500 0.46µl/ml	Overnight at 4°C
pBad (Ser 112)	pBad (Ser 112) (7E11) Cell Signalling Technology	Citrate Buffer (microwave)	1%	1.5% normal horse serum	1:25 40µl/ml	Overnight at 4°C
pP90RSK (Thr 359/ ser 363)	PP90RSK (Thr 359/ser 363) (9344)Cell Signalling technology	Citrate Buffer (water bath for 25 mins)	3%	1.5% normal horse serum	1:25 2.4µl/ml	Overnight at 4°C
P70S6K	P70S6K (2708) Cell Signalling Technology	Citrate Buffer (microwave)	3%	1.5% normal horse serum	1:50 2µl/ml	Overnight at 4°C
pP70S6K (Thr 389)	PP70S6K (Thr 389) (9206) Cell Signalling Technology	Citrate Buffer (microwave)	1%	1.5% normal horse serum	1:1000 1µl/ml	Overnight at 4°C
PI3K	PI3K (4254) Cell Signalling Technology	Citrate Buffer (microwave)	1%	1.5% normal horse serum	1:50 3.8µl/ml	Overnight at 4°C

Table 1. IHC antibody information

2.3.2 Scoring Principles

Two observers using a semi-quantative weighted histoscore method(McCarty, Jr. et al., 1986) independently evaluated the staining of the TMA's. The interobserver

variation within the scoring of tumour cell immunoreactivity was assessed by measuring interclass correlation coefficient (ICCC) statistics by using SPSS statistical package (version 9.0 for Windows). ICCC is a measurement of reliability assessing the amount of overall data variance by comparing the variability of each independent observer for each case to the overall variation between all scorings. An ICCC value of 1 representing a perfect correlation, an ICCC value below 0.40 regarded as poor, an ICCC value between 0.40 and 0.59 regarded as fair, an ICCC value between 0.6 and 0.74 regarded as good and an ICCC above 0.74 regarded as excellent correlation between observers (Kirkegaard et al., 2006b). ICCC were calculated and were greater than 0.80 for all stainings. ICCC was used as opposed to kappa as we were using a continuous scoring system. Kappa depends on a categorical system and a published study from our laboratory supports the use of ICCC in this context(Kirkegaard et al., 2006a).

2.3.3 Statistical Analysis

All statistics were analysed using the SPSS statistical package (version 9.0 for windows). Kaplan-Meier life tables with log-rank testing were plotted to assess overall (OS), disease-free survival (DFS), DFS on and off tamoxifen and OS and DFS after 3 years tamoxifen treatment. Spearman rank tests were conducted to test the association between the expressions of the various antibodies. Cox's multivariate analysis (Cox regression) and hazard ratio analysis were performed with inclusion of biological markers alongside known predictive factors, size, nodal status and grade. A value of $p < 0.05$ was considered statistically significant.

2.4 FISH

2.4.1 General Principles of FISH

Fluorescence *in situ* hybridisation (FISH) uses fluorescent molecules to vividly paint genes or chromosomes. FISH involves the preparation of short sequences of single stranded DNA, called probes, which are complimentary to the sequences that we wish to examine. These probes hybridise (bind) to the complementary DNA and because they are labelled with fluorescent tags allow us to see the location of those sequences of DNA. FISH does not need cells to be actively dividing (unlike most other techniques to study chromosomes) and can be used on paraffin embedded tissue. The FISH procedures were fully worked up by E.C and performed by E.C except where stated.

2.4.2 Her2

The PathVysion HER-2 DNA Probe Kit (PathVysion Kit) is designed to detect amplification of the HER-2/*neu* gene via fluorescence *in situ* hybridisation (FISH) in formalin fixed, paraffin-embedded human breast cancer tissue specimens. The HER-2/*neu* gene codes for a 185 kd transmembrane cell surface receptor that is a member of the tyrosine kinase family.

The PathVysion HER-2 DNA Probe Kit consists of two labelled DNA probes. The LSI HER-2 probe that spans the entire HER-2 gene is labelled in SpectrumOrange. The CEP 17 probe is labelled in SpectrumGreen and hybridizes to the alpha satellite DNA located at the centromere of chromosome 17 (17p11.1-q11.1). Inclusion of the CEP 17 probe allows for the relative copy number of the HER-2 gene to be determined.

Results on enumeration of 20 interphase nuclei from tumour cells per target are reported as the ratio of average HER-2/*neu* copy number to that of CEP 17. The manufacturers clinical study found that specimens with amplification showed a LSI HER-2/*neu* and CEP 17 signal ratio of ≥ 2.0; normal specimens showed a ratio of less than 2.0. The manufacturers suggest that results at or near the cut-off point (1.8-2.2) should be interpreted with caution.

The tissue was incubated for one hour at 56°C prior to being dewaxed and rehydrated through a series of xylene (2x5 minutes) and alcohol washes ((95% (2x5 minutes)). The tissue was then washed in 0.2N hydrochloric acid (HCl) for 20 minutes followed by 3 minutes wash in water. The tissue was then treated with protease K for 29 minutes. The protease buffer should be pre-heated to 37°C, and the protease added immediately before the addition of the slides. Slides were then washed for 2 minutes in water dehydrated through a graded series of ethanol washes (70% for 2 minutes, 85% for 2 minutes and 99% for 2 minutes) and subsequently air-dried. The slides were mounted in DAPI and the tissue digestion was checked under the microscope. If digestion was sufficient then the coverslips were removed, the tissue washed for 2x3 minutes in distilled water and subsequently placed in 10% formalin for 10 minutes. Following formalin treatment, the tissue was washed (2x3 minutes), dehydrated in graded series of ethanol (70% for 2 minutes, 85% for 2 minutes and 99% for 2 minutes) and subsequently air-dried. 100µl of denaturing solution (49mls formamide, 14mls distilled water, 7mls 20x SSC (3 M NaCl, 0.3M Na citrate, pH5.3); pH 7.0-8.0) was added and the slides covered with parafilm and incubated for 5 minutes at 72°C. The slides were then dehydrated through a graded series of ethanol's (70% for 2 minutes, 85% for 2 minutes and 99% for 2 minutes) and air-dried. 10µl of probe was

added to each slide, covered with a coverslip and sealed with rubber sealant. The slides were then placed in a Hybaid omnislide at 37°C overnight.

The following morning the coverslips were removed the slides placed in Post-Hybridisation Wash Buffer (PHWB) (100 ml 20 x SSC, 2 ml NP-40 make up to 1L with distilled water (pH 7-7.5)) at room temperature (see below). Care was taken to protect the slides from the light. PHWB was heated to 72°C and the slides added for 2 minutes. The slides were then removed, allowed to air dry in the dark, and mounted onto coverslips using Vectashield mounting media with 4'-6-Diamidino-2-phenylindole (DAPI) (135µl Vectashield with DAPI: 565µl Vectashield) (Vector Laboratories). Prior to viewing the slides were sealed with clear nail varnish.

2.4.3 PTEN and PI3K

The PTEN and PI3K probes were prototypes made at Dako, and are not commercially available. The PTEN gene is located on 10q23.31 and PIK3CA (p110 catalytic subunit of PI3K) is located on chromosome 3 band q26.32.

FISH was performed on the TMA's using a probe mix consisting of Texas Red-labeled DNA cosmid clones covering PIK3CA or PTEN (Dako Denmark A/S, Glostrup, Denmark) and fluorescein isothiocyanate (FITC)-labeled DNA cosmid probes directed against the centromeric region of chromosome 10 or 3, respectively (Dako Denamrk A/S, Glostrup, Denmark). To confirm the specificity of the probe mix, it was tested on metaphase spreads of normal cells and showed hybridization to 10q23 and 3q26, respectively (These control experiments were performed at Dako). FISH was performed using buffers from Dako's Histology FISH Accessory Kit (Dako A/S, Glostrup, Denmark).

Prior to the FISH experiments, the tissue was incubated at 56°C for 1 hour. Tissue was then dewaxed and rehydrated in series of ethanol and incubated in pre-treatment solution for 10 minutes at 96°C. After 2x3 minutes wash in wash buffer (Dako A/S, Glostrup, Denmark), the tissue was treated with pepsin for 18 minutes at 25°C, washed, dehydrated and air-dried. Ten µl of probe mix was applied, denatured at 82°C for 22 minutes and tissue incubated overnight at 45°C in a humidified hybridization chamber. Tissue was washed in stringent wash buffer (Dako A/S, Glostrup, Denmark) for 10 minutes at 65°C, dehydrated, air-dried and the slides mounted in 0.5 µg/ml 4,6-diamindino-2 phenylindole-2 hydrochloride (DAPI) in Vectashield antifade (Vector Laboratories, CA, USA). Gene copy number status was determined as the ratio of red signals (PTEN or PIK3CA) over the number of green signals (chromosome) in 20 cancer cell nuclei for each TMA core. The tumour was considered amplified if the gene/chromosome ratio in at least one of the cores was more than 2 and deleted if the ratio was below 0.8.

The FISH experiments were performed for the PI3K and PTEN probes using buffers from Dako's Histology FISH Accessory kit. Initially the tissue was incubated for one hour at 56°C before being dewaxed and rehydrated through a series of xylene (2x5 minutes) and alcohol washes ((100% (2x2 minutes)), 90% (1x2 minutes), 70% (1x2 minutes)).

Following this the slides were rinsed twice in wash buffer (diluted 1:20) (DAKO), for three minutes. The TMA's were then incubated for 10 minutes at 96°C in pre-treatment buffer (diluted 1:20) (DAKO), followed by a 15-minute cool down period. Next the tissue was rinsed twice in wash- buffer (diluted 1:20) for three minutes,

before treatment with cold ready to use Pepsin reagent (Dako) for 19 minutes at 25°C. The pepsin was removed and the slides rinsed twice in wash buffer. Slides were again rinsed twice in wash buffer for three minutes, dehydrated through a graded series of ethanol washes (70% 1x2 minutes, 85% 1x2 minutes and 99% 1x2 minutes) and subsequently air dried. The tissue digestion was checked under the microscope. If the digestion was sufficient 10µl of probe was added, and the tissue sections concealed with a coverslip and sealing agent. The probe and tissue were denatured at 82°C for 22 minutes and then the probe allowed to bind to its complimentary sequence, by placing the slides in a pre-heated humidified chamber for 14-20 hours at 45°C.

The following morning the tissue was rinsed in cold stringent wash buffer (diluted 1:20) (Dako) and subsequently washed in stringent wash buffer (diluted 1:20) (DAKO) for 10 minutes at 65°C. Slides were rinsed twice in wash buffer for three minutes before being dehydrated through a series of alcohol washes (70% 1x2 minutes, 85% 1x2 minutes and 99% 1x2 minutes). Finally slides were allowed to air dry before being mounted onto coverslips using Vectashield mounting media with 4'-6-Diamidino-2-phenylindole (DAPI) (135µl Vectashield with DAPI: 565µl Vectashield) (Vector Laboratories). DAPI is known to form fluorescent complexes with natural double-stranded DNA, making it a nuclear counterstain in fluorescent techniques. Signals corresponding to both gene and chromosome were visualised using a fluorescent microscope.

2.4.4 Scoring Principles

For each TMA core analysed, red (gene) and green (chromosome) signals were counted in 20 cells and the results reported as the ratio of average gene copy number to that of the corresponding chromosome. As recommended by Dako, a ratio of

greater than two was considered an amplification whilst a ratio of less than 0.8 was considered a deletion. All of the FISH experiments were scored by E.C and all of the HER2 FISH samples were double scored by T.K whilst 20% of the PI3K and PTEN FISH samples were doubled scored by E.C. In all cases an ICCC greater than 0.85 was achieved.

2.4.5 Statistical Analysis

All statistics were analysed using the SPSS statistical package (version 9.0 for windows). For HER2 Kaplan-Meier life tables with log-rank testing were plotted to assess overall (OS) and disease-free survival (DFS). Spearman rank tests were conducted to test the association between the 2+ and 3+ on the Hereeptest and amplification of HER2 as analysed by FISH. Cox's multivariate analysis (Cox regression) and hazard ratio analysis were performed with inclusion of biological markers alongside known predictive factors, size, nodal status and grade. A value of $p < 0.05$ was considered statistically significant. FISH was performed only on TMA B with PI3K and PTEN and this was compared with the results from IHC on the same TMAs.

2.5 Western Blotting

2.5.1 General Principles of Western Blotting

Western blotting is a technique that enables the detection and quantification of the levels of a specific protein in tissue or cell samples. Essentially proteins are separated by electrophoresis in 10% SDS gels and transferred to nitrocellulose membranes by electroblotting. After blocking in milk the blots are incubated first with primary antibody overnight at 4°C and then with a secondary antibody, recognizing the antibody-antigen complex. Proteins are detected using a chemiluminsecent method. Western blotting was used to verify the specificity of all the antibodies used. The details of the primary and secondary antibodies used can be found in Table 5. We used Western blotting instead of siRNA knockdown as siRNA knockdown is a technique that silences RNA and although, very accurate would not detect the phosphorylated proteins (as it looks at the RNA stage before this occurs). It is also a very time consuming and expensive technique that was outwith the scope of this project.

2.5.2 Western Blotting of Protein Samples

Preparation of the SDS-PAGE (Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis) gels.

All western blots were performed using the Bio-Rad Mini-PROTEAN 3 Electrophoresis system. For all the proteins analysed, 10% resolving gels (10% Acrylamide/Bis-Acrylamide (Sigma), 3.3mM EDTA, 0.3M Tris, pH 8.9, 0.1% SDS, 0.06% APS and 0.06% TEMED) were used. Gels are formed from the

polymerisation of the acrylamide and N-N-methylene-bis-acrylamide (bis). Bis acts as the cross linking agent for the gel, and the TEMED and APS are the catalysts for gel polymerisation. The separation of proteins within the gel is governed by the size of the pores within the gel, which is determined by the amount (percentage) of acrylamide-bis present. As the percentage of acrylamide present increases, the pore size decreases. Generally a higher percentage of resolving gels are used for smaller proteins and *vice-versa*.

The gels were prepared as follows: A short plate was placed on top of the larger spacer Plate (0.75mm used). The two plates were slid into the casting frame keeping the short plate facing front. The pressure cams were locked to secure the glass plates and the casting frame secured in the casting stand by engaging the spring-loaded lever. The resolving gel was slowly poured in between the two plates. Isopropanol was then poured along the top of the gel to remove any air bubbles and flatten it. The gel was left to set for approximately 20 minutes. Once set, the isopropanol was removed, and the 4.5% acrylamide stacking gel (4.5% Acrylamide/Bis-Acrylamide (Sigma), 4mM EDTA, 0.1M Tris, pH 8.9, 0.1% SDS, 0.006% APS and 0.006% TEMED). The stacking gel was carefully poured on top of the resolving gel until it reached the top of the plates. The 10 well gel comb was then inserted and the gel allowed to polymerise for 30 minutes.

Protein denaturation

To enable the primary antibody to recognise and bind to its epitope, it is necessary to denature the proteins. Denaturing the proteins also allowed them to run more efficiently through the gels. Having previously measured the concentration of protein within each sample, the same amount of protein (25µg) was removed from each

sample and transferred to a new tube. This was stored on ice and 2x Laemmli's sample reducing buffer (60mM Tris-HCL pH 6.8, 10% Glycerol, 20% sodium dodecyl sulphate (SDS), 5% 2-Mercaptoethanol, 2.5% Bromophenol Blue (0.5% w/v)) was added to each sample. The samples were thoroughly mixed and boiled for 2 minutes. The SDS within the sample buffer is a detergent, with a highly negative charge that has a hydrophobic tail that binds to the proteins, causing them to become negatively charged. Additionally SDS disrupts the tertiary structure of the proteins, resulting in their unfolding. The 2-Mercaptoethanol prevents the reformation of the disulphide bonds and helps maintain the protein in its denatured state. Boiling of the samples also contributes to the denaturing of the proteins by unfolding them completely. Boiled samples were immediately stored back on ice. The molecular weight marker (Biotinylated Protein Ladder (Cell Signalling Technology), used to determine the size of the proteins, was boiled together with the samples.

Gel Electrophoresis

The principal behind electrophoresis is that an electrical charge moves the proteins down the polyacrylamide gel. This is made possible by the SDS, which transfers a negative charge onto the proteins, thus allowing them to be attracted towards the positive anode. The pores result in the smaller proteins travelling through the gel faster than the larger molecules, therefore they progress further down the gel. The proteins are therefore separated according to their weight.

To load the samples onto the gel, the comb was carefully removed and the wells rinsed with running buffer. The gel cassette sandwich was removed from the casting frame and placed gel into the electrode assembly with the short plate facing inward. The gel cassette sandwiches and electrode assembly were then slid into the clamping

frame. This inner chamber was then lowered into the mini tank and the tank filled with 1x running buffer (details in Table 2). Denatured protein samples and ladder (7µl) were then loaded into the wells using a fine tipped pipette and the gel was run at 40mA for one hour.

Protein Transfer

The transfer was achieved using the Mini-Trans Blot Cell (Bio-Rad). The membrane was pre-treated in methanol for 1 minute and then placed in 1x transfer buffer for 15 minutes (see Table 2) together with 2 fiber pads and 6 sheets of Whatmann 3M paper. The gels were then carefully removed from the glass plates and the stacking gel discarded. The remaining resolving gel was then equilibrated, for approximately 5 minutes in transfer buffer. This step prevented the gel from shrinking during the transfer process. The transfer sandwich was assembled on the blotter cassette surface in transfer buffer as follows: One fiber pads was placed on the black side of the cassette, next 3 sheets of filter paper, the gel, the membrane and the sandwich was completed by placing a further 3 sheets of filter paper on the membrane followed by the last fiber pad and any air bubbles removed using a glass tube. The assembled sandwich was then slotted into the electrode assembly and placed in the mini-tank which was filled with transfer buffer. To reduce the temperature of the buffer during the transfer process, the Bio-Ice cooling unit was used. Additionally a magnetic stirrer was used to maintain even buffer temperature and ion distribution. Proteins were transferred from the gel (negative/cathode) to the membrane (positive/anode) overnight (approximately 18 hours) at 10volts.

Blocking of Membrane

Since the proteins transferred from the gel bind to the PVDF membrane, it was equally likely that the antibody used to detect the protein would also bind non-specifically to the membrane. Therefore to prevent these interactions between antibody and membrane it was necessary to mask the membrane. This was achieved by incubating the membrane in 5% Non-Fat Dry Milk (Marvel) blocking solution, prepared in TBS-Tween (TTBS) (see table 2) for 1 hour at room temperature. This step and all further steps were performed on an orbital shaker.

Buffer	**Reagents**
10x Running Buffer	200mM Tris, 2M Glycine, 1% SDS
10x Transfer Buffer	248mM Tris, 1.3M Glycine, 20% Methanol
10x TBS	0.1M Tris/HCL, 1.5M NaCl, pH=7.4 (for 1x dilute in dH_2O)
0.001% TTBS	1000µl Tween 20 per litre of 1x TBS

Table 2. Buffers used in Western Blotting

Incubation of the membrane with primary antibody

Having sufficiently blocked the membrane, the next step was incubation with primary antibody. All antibodies were prepared in 5% Non-Fat Dry Milk/TTBS solution to further reduce non-specific binding. Membranes were incubated with primary antibody overnight (approximately 18 hours) at 4°C. For the size of membrane being used 10mls of antibody was sufficient to cover the entire membrane. Table 3 lists the dilutions/concentrations of the antibodies used in western blotting.

Antibody	Clone	Supplier	Cat no	Positive Control	Concentration	Species	2°Ab
Bad		Cell Signalling technology	#9292	HELA, Jurkat, Bad (control cells)	1:1000 0.2 µl/ml	Rabbit	Anti-rabbit, 1:5000
PBad (ser112)	7E11	Cell signalling Technology	#9296	MCF-7, MDA-MB-361-unstim/stim	1:1000 1 µl/ml	Mouse	Anti-mouse, 1:10000
Bax		Dako	A3533	MCF-7, MDA-MB-361	1:2000 0.35 µl/ml	Rabbit	Anti-rabbit, 1:5000
Bcl-2	124	Dako	M0877	Jurkat, HELA	1:1000 2.8 µl/ml	Mouse	Anti-mouse, 1:10000
Bcl-xl		Cell signalling Technology	#2762	MCF-7, MDA-MB-361	1:500 0.46 µl/ml	Rabbit	Anti-rabbit, 1:5000
P70S6K	49D7	Cell Signalling Technology	#2708	MDA-MB-361	1:2000 0.05 µl/ml	Rabbit	Anti-rabbit, 1:5000
PP70S6K (thr 389)	1A5	Cell Signalling Technology	#9206	MCF-7, MDA-MB 361 unstim/stim	1:2000 0.5 µl/ml	Mouse	Anti-mouse, 1:10000
PP90RSK (thr 359/ser 363)		Cell signalling Technology	#9344	MDA-MB-361, BT474 unstim/stim	1:500 0.12 µl/ml	Rabbit	Anti-rabbit, 1:5000
PI3K		Cell signalling Technology	#4254		1:1000 0.19 µl/ml	Rabbit	Anti-rabbit, 1:5000
PTEN	26H9	Cell signalling Technology	#9556		1:1000 2 µl/ml	Mouse	Anti-mouse, 1:10000

Table 3. Table showing the primary and secondary antibodies and proteins used in the Western Blots

In addition Bad, pBad (Ser 112), bax, bcl-2, Bcl-xl and P70S6K were run on membranes that were probed with the antibody to them, in MDA-MB-361 breast cancer cells stimulated in the presence or absence of heregulin and oestrogen. Before treatment, cells were starved in phenol red free, serum free Dulbeccos modified Eagle media (DMEM) for 24 hours. To study endogenous gene expression, cells were stimulated in the presence or absence of heregulin (10 nM) and oestrogen (10 nM)

alone or in combination for 15 hours. After stimulation, cells were washed in PBS and lysed immediately in lysis buffer (Cell signalling Technology, Beverly, USA).

Incubation of the membrane with secondary antibody

Following incubation with primary antibody, the membrane was washed in TBST three times to remove any excess antibody. Detection of the protein required a secondary antibody bound to either a biotin or enzyme conjugate, such as horseradish peroxidase (HRP) that was species specific to the primary antibody. The secondary antibodies used were HRP-linked anti-mouse IgG (Cell Signalling Technology) or anti-rabbit IgG (Cell Signalling Technology). The anti-mouse antibody was used at a dilution of 1:5000 and the anti-rabbit antibody at a dilution of 1:10000 concentration. For each experiment, a secondary antibody was also needed for detection of the biotinylated ladder. The anti-biotin HRP linked antibody (Cell Signalling Technology) was used at a dilution of 1:1000 concentration. The anti-mouse/rabbit and anti-biotin secondary antibodies were prepared together in 10mls of 5% Non-Fat Dry Milk/TTBS solution and were incubated with the membranes for one hour at room temperature. Table 3 lists the secondary antibodies used for each primary antibody.

Protein visualisation

The protein was detected using a chemiluminescent method. Luminescence is the emission of light due to the dissipation of energy from a substance in an excited state. Horseradish peroxidase catalyses the oxidation of luminol, a chemiluminescent substrate, in alkaline conditions. Oxidation results in the luminol being in an excited state which then decays to ground state via a light-emitting pathway. For this method

ECL Plus (Amersham) was used. The principle behind this is that horseradish peroxidase, conjugated to the secondary antibody, oxidises the ECL Plus chemiluminescent substrate Lumigen PS-3 Acridan, which produces thousands of acridinium ester intermediates per minute. These intermediates then interact with the peroxidase to produce a sustained, high intensity chemiluminescence with a maximum emission at 430nm. This light is then detected on autoradiology film.

Following incubation with the secondary antibody, membranes were washed three times in TTBS for 10 minutes. While the membranes were washing the ECL Plus reagents were warmed to room temperature. Once the reagents had warmed sufficiently, the 2 solutions (A and B) were mixed in a ratio of 40:1. A total of 3 mls of solution was adequate for each membrane. When using ECL reagents all steps were performed in semi-darkness. Membranes were placed protein side up on a sheet of Saran wrap. The ECL solution was pipetted onto the membranes ensuring complete coverage. The membrane was incubated with the reagents for 5 minutes, excess solution removed and the membrane wrapped in Saran wrap. Finally the membrane was exposed to autoradiology film for various times. Generally the incubation times were 1, 15 and 30 minutes. The film was then developed and both the marker and protein bands visualised.

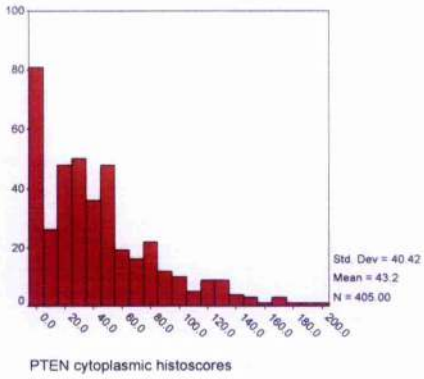
Chapter 3 Results

In the survival and disease free analyses only those tumours that were ER positive were analysed (402 out of the 456 available).

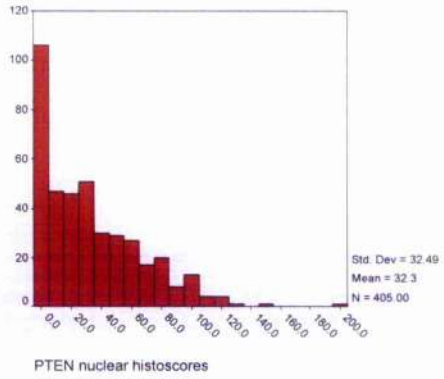
3.1 IHC for PTEN

3.1.1 PTEN expression and staining patterns using IHC

Initially, the specificity of the antibody was confirmed by Western Blotting, a clear single band at 54 kDa, the size of PTEN, was seen. A total of 51 out of 456 (11.2%) cases were unavailable for analysis for PTEN expression either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Both nuclear and cytoplasmic immunohistochemical staining was noted (see Figure 5a-c). Staining was found in both the normal and invasive tumour components of the breast tissue. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.K achieving an ICCC of 0.96 (excellent). 18% of the cores were negative for PTEN and 6% had low expression.



a)



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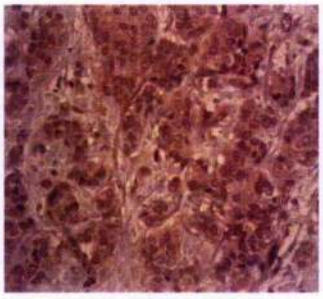

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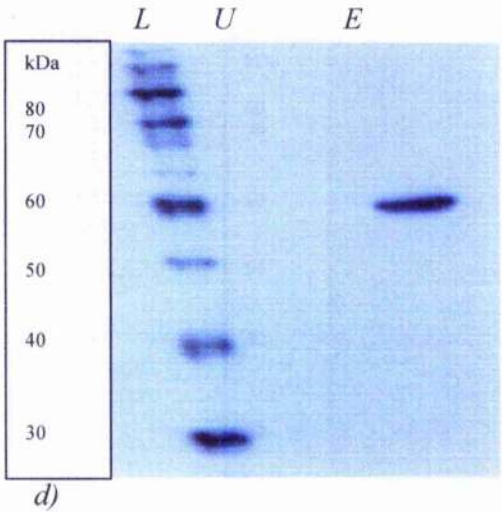


d)

Figure 7. a) Histogram showing distribution of cytoplasmic scores for PTEN IHC b) Histogram showing distribution of nuclear scores for PTEN IHC c) Breast tumour stained with PTEN antibody. Staining was seen in both the nucleus and cytoplasm d) Western Blot for PTEN antibody. The protein used was MDA-361 cells (L=ladder, US= unstimulated, E= stimulated with oestrogen. Band at 54 kDa (ladder slightly skewed)

3.1.2 PTEN expression and patient outcome

The PTEN scores were categorised as high (above median histoscore) or low (below median histoscore) for subsequent analysis. Staining was seen in both the nucleus and cytoplasm.

Cytoplasmic PTEN histoscores

Survival Category	P Value
Overall survival	0.013
Disease free survival	0.018
Disease free survival on tamoxifen	0.233
Disease free survival off tamoxifen	0.022
Disease free survival after 3 years tamoxifen	0.048
Overall survival after 3 years tamoxifen	0.009

Table 4. Table showing OS and DFS p-values for PTEN cytoplasmic expression. P values represent log-rank testing of the difference in survival

Using these cut offs, patients whose tumours had high levels of PTEN cytoplasmic expression had a reduced OS (p = 0.013, log rank test, Figure 6) compared to those patients whose tumours had low levels of PTEN cytoplasmic expression. The median OS for patients whose tumours had low PTEN cytoplasmic expression is 14.84(12.02, 14.96) years compared to 14.84(13.98, 15.71) years for patients whose tumours had high PTEN cytoplasmic expression.

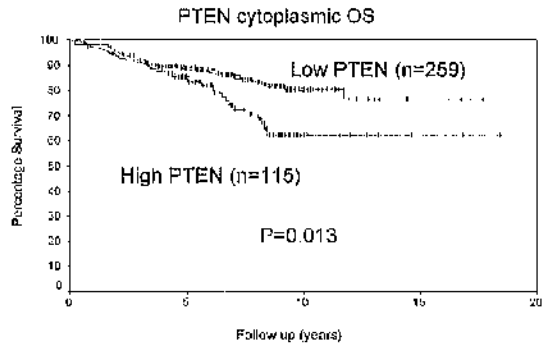


Figure 8. Kaplan-Meier survival plots showing percentage OS in patients whose tumours had low versus high cytoplasmic PTEN expression. The cut off for high and low levels of PTEN is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival

Patients whose tumours had high levels of PTEN expression had a reduced DFS (p=0.018, log rank test, Figure 7) compared to those patients whose tumours had low levels of PTEN expression. The median DFS for patients whose tumours had low PTEN cytoplasmic expression is 12.80 (11.61, 13.98) years compared to 10.43 (8.87, 11.99) years for patients whose tumours had high PTEN cytoplasmic expression.

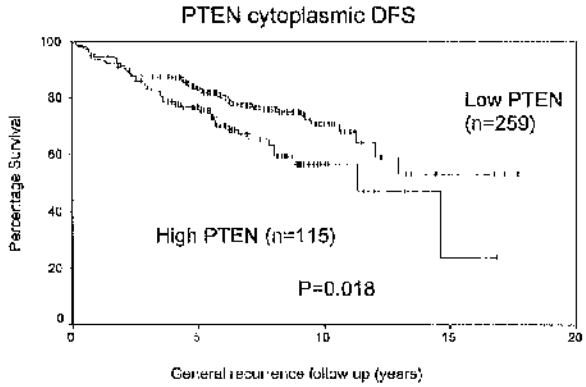


Figure 9. Kaplan-Meier survival plots showing percentage DFS in patients whose tumours had low versus high cytoplasmic PTEN expression. The cut off for high and low levels of PTEN is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival

Patients whose tumours had high levels of PTEN expression had a reduced DFS after finishing tamoxifen treatment (p=0.022, log rank test, Figure 8) compared to those patients whose tumours had low levels of PTEN expression. The median DFS after finishing tamoxifen treatment for patients whose tumours had low PTEN cytoplasmic expression is 10.83(9.88, 11.78) years compared to 8.14 (6.35, 9.94) years for patients whose tumours had high PTEN cytoplasmic expression. We need to assume that as the OS survival curves separate and the DFS curves do not, that gain of PTEN is a late event that occurs following relapse.

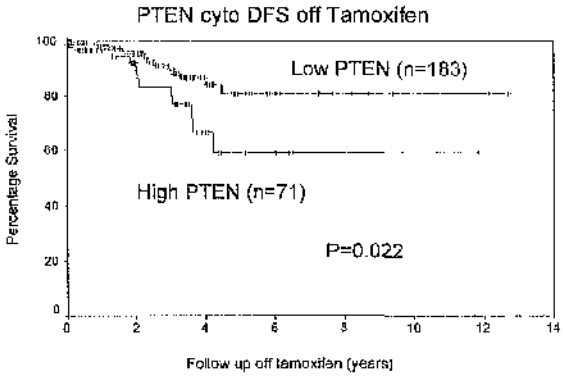


Figure 10. Kaplan-Meier survival plots showing percentage DFS after end tamoxifen treatment in patients whose tumours had low versus high cytoplasmic PTEN expression. The cut off for high and low levels of PTEN is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival

Patients whose tumours had high levels of PTEN expression had a reduced DFS after 3 years Tamoxifen treatment (p=0.048, log rank test, figure 9) compared to those patients whose tumours had low levels of PTEN expression. The median DFS after

three years tamoxifen treatment for patients whose tumours had low PTEN cytoplasmic expression is 11.45 (10.23, 12.66) years compared to 9.43 (7.72, 11.13) years for patients whose tumours had high PTEN cytoplasmic expression.

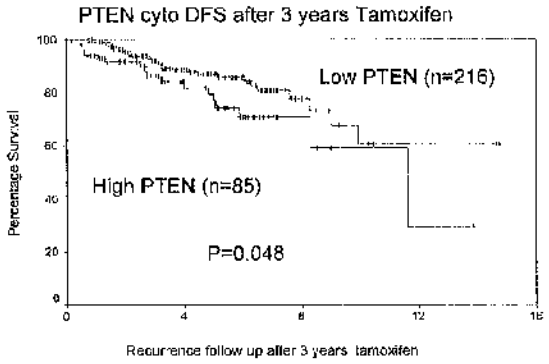


Figure 11. Kaplan-Meier survival plots showing percentage DFS after 3 years tamoxifen treatment in patients whose tumours had low versus high cytoplasmic PTEN expression. The cut off for high and low levels of PTEN is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival.

Patients whose tumours had high levels of PTEN expression had a reduced OS after 3 years Tamoxifen treatment (p=0.009, log rank test, figure 10) compared to those patients whose tumours had low levels of PTEN expression. The median OS after three years tamoxifen treatment for patients whose tumours had low PTEN cytoplasmic expression is 13.83(13.31, 14.36) years compared to 11.64 (10.46, 12.83) years for patients whose tumours had high PTEN cytoplasmic expression.

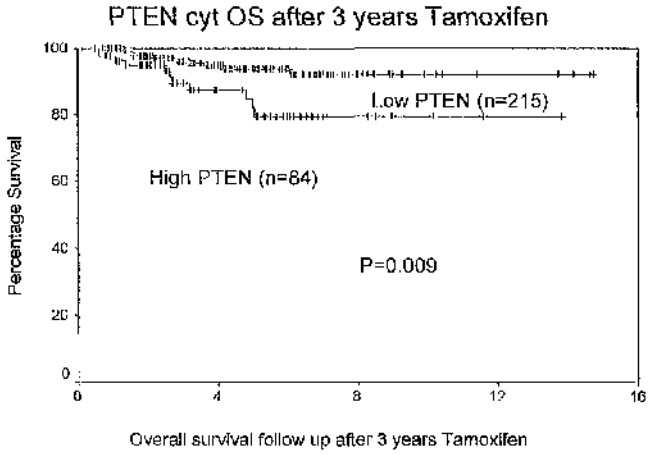


Figure 12. Kaplan-Meier survival plots showing percentage OS after 3 years tamoxifen treatment in patients whose tumours had low versus high cytoplasmic PTEN expression. The cut off for high and low levels of PTEN is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival.

Patients whose tumours had high levels of PTEN expression had a 0.55-fold (95% CI 0.35-0.89, p=0.01) chance of survival as compared to those patients whose tumours had low levels of PTEN expression and a 0.62-fold (95% CI 0.42-0.93, p=0.02) risk of relapse as compared to those patients whose tumours had low levels of PTEN expression.

Patients whose tumours had high levels of PTEN cytoplasmic expression had a 1.82-fold increased risk of dying from their cancer compared to patients whose tumours had low levels of PTEN cytoplasmic expression and a 1.62 increase risk of relapse as compared to those patients whose tumours had low levels of PTEN

Ab	Median histoscore (inter-quartile range)	OS hazard ratio	DFS hazard ratio	DFS on tamoxifen hazard ratio	DFS off tamoxifen hazard ratio	DFS after 3y tamoxifen hazard ratio	Survival after 3y tamoxifen hazard ratio
PTEN	33.33 (11.25-60.83)	0.55 (0.35-0.89) p=0.01	0.62 (0.42-0.93) p=0.02	0.75 (0.47-1.20) (NS)	0.42 (0.20-0.90) p=0.03	0.56 (0.31-1.00) p=0.05	0.34 (0.15-0.79) p=0.01

Table 5. Median histoscore and relative risk (hazard ratio) for PTEN cytoplasmic expression.

Multivariate analysis suggested that PTEN cytoplasmic expression was an independent predictor of decreased DFS after 3 years of tamoxifen treatments only ($p = 0.01$, Cox regression) and OS after 3 years tamoxifen therapy ($p = 0.0443$, Cox regression) when analyzed alongside known prognostic markers such as tumor size, grade and nodal status. There was a trend towards significance for OS (p=0.08) and DFS off tamoxifen (p=0.06).

Nuclear PTEN histoscores

When patients were divided into those whose tumours exhibited high or low PTEN nuclear scores, there was no statistically significant association between tumour levels of nuclear PTEN and patient OS or DFS.

Survival Category	P Value
Overall survival	0.997
Disease free survival	0.762
Disease free survival on tamoxifen	0.313
Disease free survival off tamoxifen	0.515
Disease free survival after 3 years tamoxifen	0.843
Overall survival after 3 years tamoxifen	0.558

Table 6. Table showing OS and DFS p-values for PTEN nuclear expression. P values represent log-rank testing of the difference in survival

3.1.3 Correlations between PTEN scores and other member of the PI3K/Akt pathway

High levels of cytoplasmic PTEN were significant associated with high levels of cytoplasmic PI3K ($p<0.0001$, $R=0.51$, Spearman Rank Test) and cytoplasmic pAkt (473) ($p<0.0001$, $R=0.364$; Spearman Rank Test) (Figure 11 and 12)

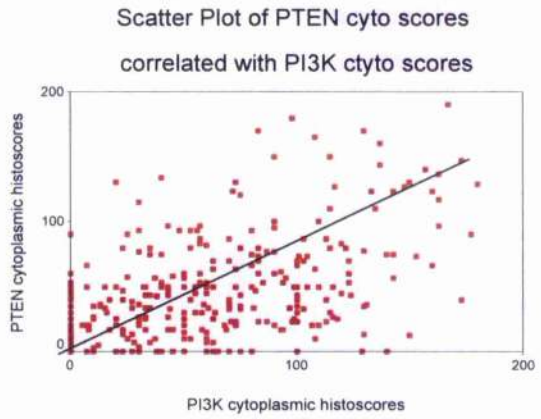


Figure 13. Scatter plot of the association between cytoplasmic PTEN and PI3K scores

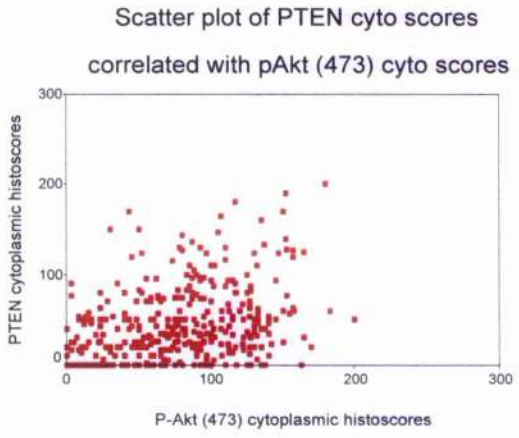


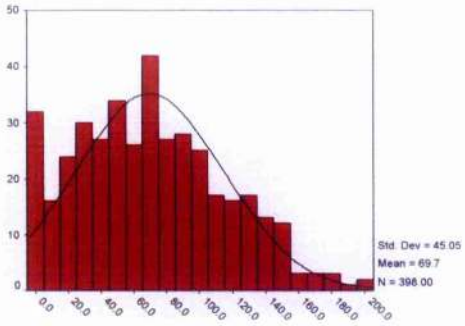
Figure 14. Scatter plot of the association between cytoplasmic PTEN and pAkt (473) scores

3.2 IHC for Bad

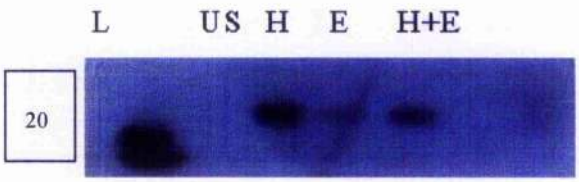
3.2.1 Bad expression and staining patterns using IHC

To check the specificity of the Bad antibody, Western Blotting was carried out. A single band was seen at 21 kDa corresponding to the size of Bad (Figure 13b). Using western blot, the expression levels of Bad in growth-factor- or hormone-stimulated cell lines (MDA-361) were investigated. No changes in Bad expression was observed in non-stimulated cells compared to cells stimulated with oestrogen or heregulin alone or in combination

For immunohistochemical analyses of Bad, 58 out of 456 (12.7%) cases were unavailable for analysis either due to core loss or insufficient tumour material in the cores and these patients were therefore excluded from further analysis. Only cytoplasmic staining was noted (Figure 13c), and the staining was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.K achieving an ICCC of 0.94 (excellent).



a)



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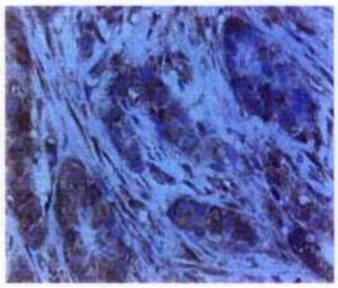


c)

Figure 15. a) Histogram showing distribution of cytoplasmic scores for Bad IHC (curve shows normal distribution) b) Western Blot for Bad antibody. The protein used was MDA-361 cells (L=ladder, US= unstimulated, H= stimulated with heregulin, E= stimulated with oestrogen, H+E= stimulated with heregulin and oestrogen. Band at 21kDa, c) Breast tumour stained with Bad antibody

3.2.2 Bad expression and patient outcome

Survival Category	P Value
Overall survival	0.051
Disease free survival	0.049
Disease free survival on tamoxifen	0.278
Disease free survival off tamoxifen	0.252
Disease free survival after 3 years tamoxifen	0.019
Overall survival after 3 years tamoxifen	0.010

Table 7. Table showing OS and DFS p-values for Bad cytoplasmic expression

When patients were divided into those with high (above median histoscore) and low (below median histoscore) Bad tumours expression, patients whose tumours had high levels of Bad expression had a trend toward improved OS (p = 0.051, log rank test, Figure 16) as compared to those patients whose tumours had low levels of Bad

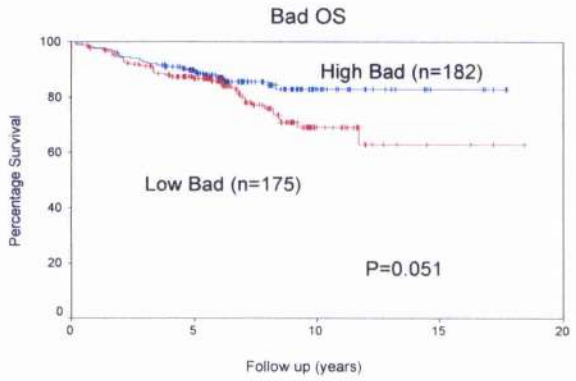


Figure 16. Kaplan-Meier survival plots showing percentage OS in patients whose tumours had low versus high Bad expression. The cut off for high and low levels of Bad is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival.

Patients whose tumours had high levels of Bad expression had improved DFS (p = 0.049, log rank test, Figure 17) with a 1.49-fold (95% CI 1.00-2.22, p=0.05) decreased risk of relapse as compared to those patients whose tumours had low levels of Bad expression. The median DFS for patients whose tumours had low Bad cytoplasmic expression is 11.31(10.02, 12.61) years compared to 12.83 (11.44, 14.21) years for patients whose tumours had high Bad cytoplasmic expression.

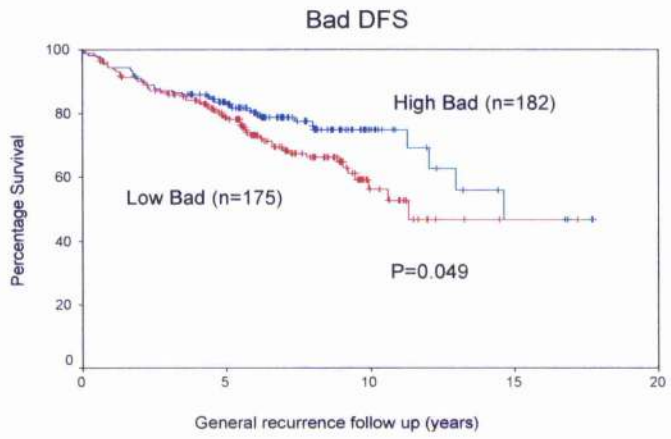


Figure 17. Kaplan-Meier survival plots showing percentage DFS in patients whose tumours had low versus high Bad expression. The cut off for high and low levels of Bad is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival.

Further analysis showed that patients whose tumours had high levels of Bad expression had a significantly improved DFS and OS after three years tamoxifen treatment (p=0.019, log rank test; Figure 18 and p=0.010, log rank test, Figure 19 respectively) as compared to patients whose tumours had low levels of Bad expression. Patients with high Bad tumour levels had a 1.98-fold (95% CI 1.10-3.55) decreased risk of relapse after 3 years of tamoxifen treatment as compared to patients with low tumour levels of Bad (p=0.05) (Table 6). The median DFS after three years

Tamoxifen treatment for patients whose tumours had low Bad cytoplasmic expression is 10.00(8.62, 11.36) years compared to 11.55 (10.14, 12.96) years for patients whose tumours had high Bad cytoplasmic expression.

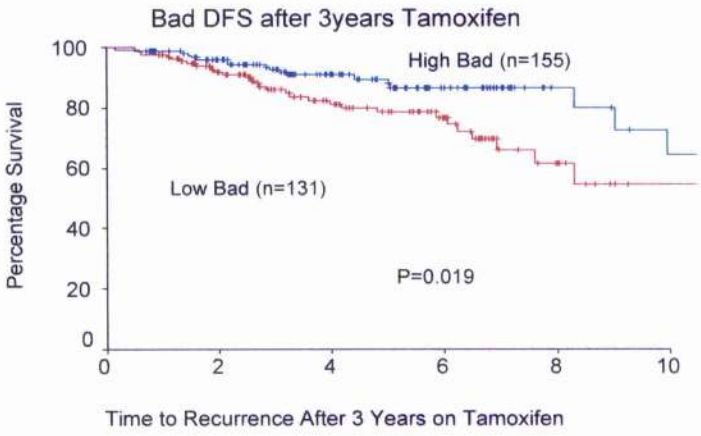


Figure 18. Kaplan-Meier survival plots showing percentage DFS after 3 years of tamoxifen treatment in patients whose tumours had low versus high Bad expression. The cut off for high and low levels of Bad is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival

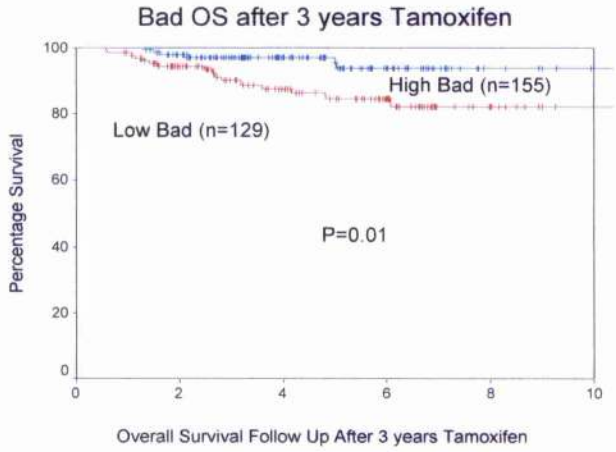


Figure 19. Kaplan-Meier survival plots showing percentage OS after 3 years of tamoxifen treatment in patients whose tumours had low versus high Bad expression. The cut off for high and low levels of Bad is defined as above and below median histoscore. P values represent log-rank testing of the difference in survival

In multivariate analysis the level of Bad expression was not an independent prognostic marker for disease free survival or overall survival (p = 0.083 and p=0.113 respectively, Cox regression) when analysed alongside known conventional prognostic indicators of overall survival such as tumour size, grade and nodal status. We need to assume that as the OS survival curves separate and the DFS curves do not, that gain of Bad is a late event that occurs following relapse.

3.2.3 Correlations between Bad and other members of the PI3K/akt pathway

High tumour levels of Bad expression were weakly but significantly associated with high cytoplasmic tumour levels of Akt 1 (p = 0.029; R= 0.117) and Akt 3 (p = 0.032; R= 0.116). However scatter plots revealed that the main reason for correlation was the

significant number of tumours with no staining (histoscore = 0). There were no significant correlations between bad expression and apoptosis, as measured by TUNEL assay (data not shown).

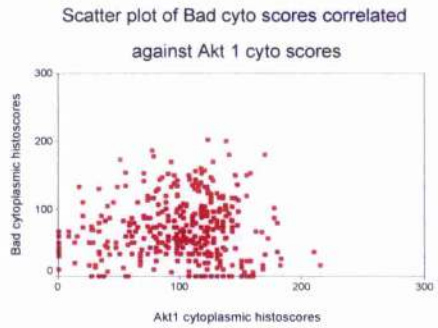


Figure 20. Scatter plot of the association between cytoplasmic Bad and Akt 1 cytoplasmic histoscores.

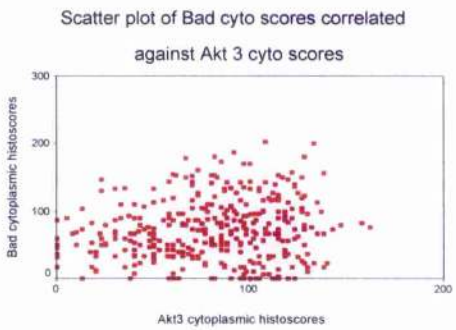
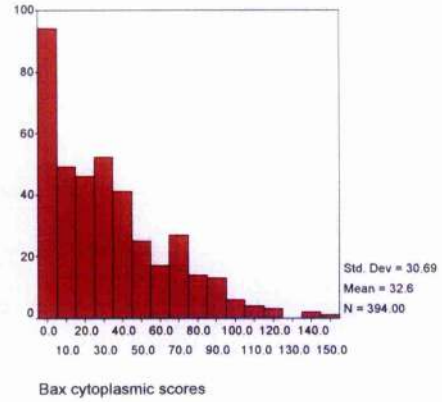


Figure 21. Scatter plot of the association between cytoplasmic Bad and Akt 3 cytoplasmic histoscores.

3.3 IHC for Bax

3.3.1 Bax expression and staining patterns using IHC

The specificity of the antibody was checked using Western Blotting. A single band was seen at 21kDa corresponding to the size of Bax (Figure 19b). For immunohistochemical analyses of Bax, 62 out of 456 (13.6%) cases were unavailable for analysis either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Only cytoplasmic staining was noted (Figure 22c). Staining for the antibody was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.K achieving an ICCC of 0.89 (excellent).



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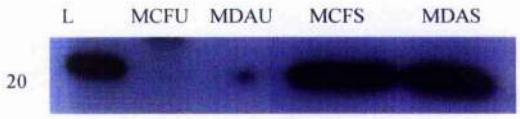

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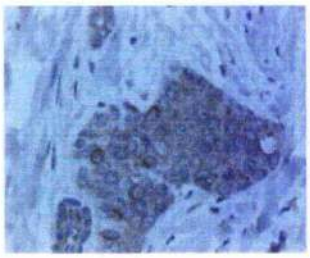


c)

Figure 22. a)Histogram showing distribution of cytoplasmic scores for Bax IHC, b) Western Blot of Bax antibody showing band at 21kDa (MCFU ==MCF-7 cells unstimulated, MDAU = MDA-MB-361cells unstimulated, MCFS ==MCF-7 cells stimulated with oestrogen, MDAS = MDA-MB-361cells stimulated with oestrogen) , c)breast tissue stained with Bax antibody

3.3.2 Bax expression and patient outcome

When patients were divided into those with high or low levels of Bax (above or below median histoscore) there was no significant association with overall or disease free survival.

Survival Category	P Value
Overall survival	0.433
Disease free survival	0.778
Disease free survival on tamoxifen	0.892
Disease free survival off tamoxifen	0.530
Disease free survival after 3 years tamoxifen	0.952
Overall survival after 3 years tamoxifen	0.103

Table 8. OS and DFS p-values for cytoplasmic expression of Bax

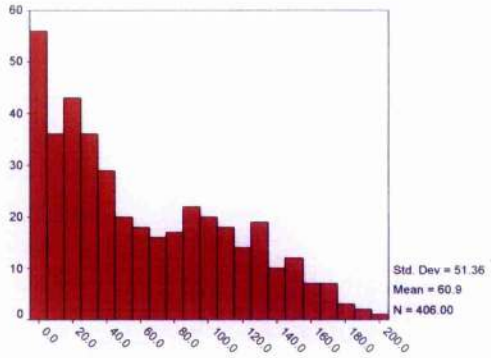
3.3.3 Correlations between Bax and other members of the PI3K/Akt pathway

There were no significant correlations between Bax and pAkt or between Bax and apoptosis, as measured by TUNEL assay (data not shown).

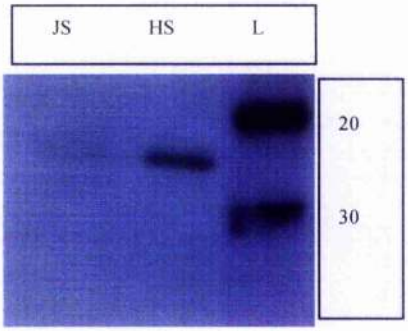
3.4 IHC for Bcl-2

3.4.1 Bcl-2 expression and staining patterns using IHC

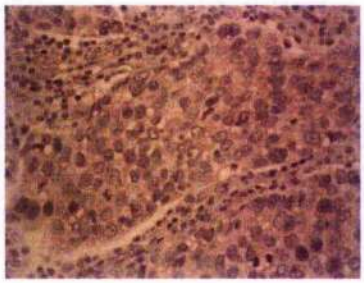
The specificity of the antibody was checked using Western Blotting. A single band was seen at 26kDa corresponding to Bcl-2 (Figure 20b). For immunohistochemical analyses of Bcl-2, 50 out of 456 (11.0%) cases were unavailable for analysis either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Only cytoplasmic staining was noted (Figure 23c). Staining for the antibody was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.C achieving an ICCC of 0.96 (excellent).



a)



b)



c)

Figure 23. a) Histogram showing distribution of cytoplasmic scores for Bcl-2 IHC, b) Western blot of Bcl-2 showing a single band at 26kDa (JS=Jurkat cells stimulated with oestrogen, HS=Hela cells stimulated with oestrogen, L=ladder), c) Breast tissue stained with Bcl-2 antibody

3.4.2 Bcl-2 expression and patient outcome

When patients were divided into those with high or low levels of Bcl-2 (above or below median histoscore) there was no significant association with overall or disease free survival.

Survival Category	P Value
Overall survival	0.893
Disease free survival	0.267
Disease free survival on tamoxifen	0.068
Disease free survival off tamoxifen	0.309
Disease free survival after 3 years tamoxifen	0.869
Overall survival after 3 years tamoxifen	0.244

Table 9. OS and DFS p-values for Bcl-2 cytoplasmic expression

3.4.3 Correlations between Bcl-2 and other member of the PI3K/Akt pathway

High tumour levels of Bcl-2 expression were significantly associated with high cytoplasmic tumour levels of Akt 1 ($p < 0.0001$; $R= 0.362$) (figure 19), Akt 3 ($p <0.0001$; $R= 0.429$) (Figure 20) and p Akt (Ser 473) nuclear scores ($p<0.0001$; $R= 0.233$) (Figure 24). There was no significant association between apoptosis as, measured by TUNEL assay and Bcl-2 expression.

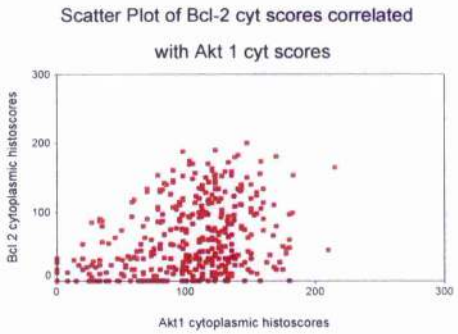


Figure 24. Scatter plot of Bcl-2 cytoplasmic histoscores correlated with Akt 1 cytoplasmic histoscores

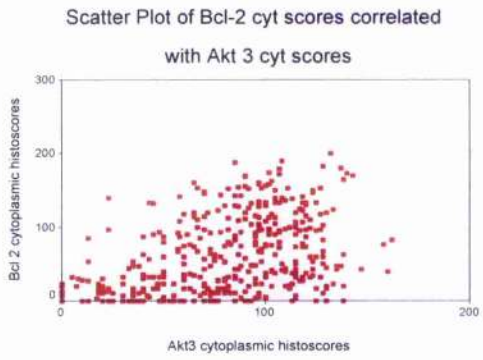


Figure 25. Scatter plot of Bcl-2 cytoplasmic histoscores correlated with Akt 3 cytoplasmic histoscores

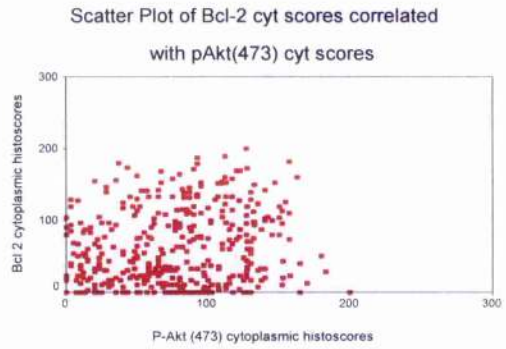
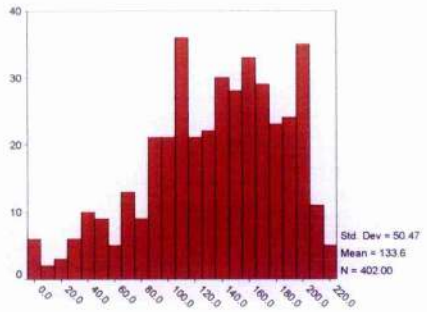


Figure 26. Scatter plot of Bcl-2 cytoplasmic histoscores correlated with p Akt (473) cytoplasmic histoscores

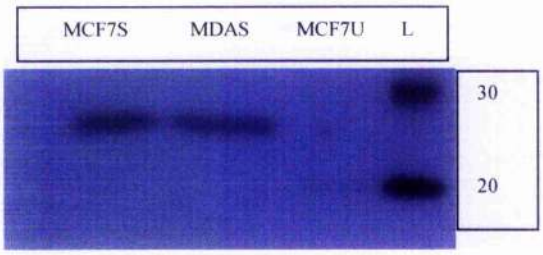
3.5 IHC for Bcl-xl

3.5.1 Bcl-xl expression and staining patterns using IHC

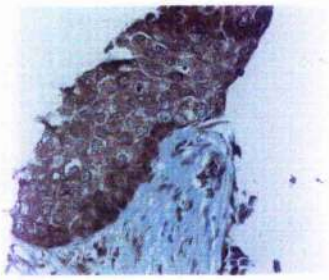
The specificity of the antibody was checked using Western Blotting. A single band was seen at 30kDa corresponding to Bcl-xl (Figure 24b). For immunohistochemical analyses of Bcl-xl, 54 out of 456 (11.8%) cases were unavailable for analysis either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Only cytoplasmic staining was noted (Figure 27c). Staining for the antibody was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.K achieving an ICCC of 0.95 (excellent).



a)



b)



c)

Figure 27. a) Histogram showing distribution of cytoplasmic scores for Bcl-xl IHC, b) Western blot showing single band at 30kDa (MCFU ==MCF-7 cells unstimulated, MCFS ==MCF-7 cells stimulated with oestrogen, MDAS = MDA-MB-361cells stimulated with oestrogen, L=ladder) c)Breast tissue stained with Bcl-xl antibody

3.5.2 Bcl-xl expression and patient outcome

When patients were divided into those with high or low levels of Bcl-xl (above or below median histoscore) there was no significant association with overall or disease free survival.

Survival Category	P Value
Overall survival	0.411
Disease free survival	0.284
Disease free survival on tamoxifen	0.594
Disease free survival off tamoxifen	0.151
Disease free survival after 3 years tamoxifen	0.751
Overall survival after 3 years tamoxifen	0.978

Table 10. OS and DFS p-values for Bcl-xl cytoplasmic expression

3.5.3 Correlations between Bcl-xl and other members of the PI3K/Akt pathway

High tumour levels of Bcl-xl expression were (weakly) associated with high cytoplasmic tumour levels of Akt 2 ($p = 0.014$; $R = 0.133$) (figure 25) and Akt 3 ($p = 0.033$; $R = 0.114$) (figure 26). However scatter plots revealed that the main reason for correlation was the significant number of cores with weak staining. There was no significant association between apoptosis as, measured by TUNEL assay and Bcl-2 expression

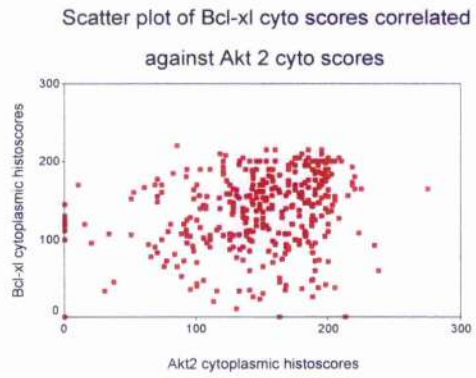


Figure 28. Scatter plot of Bcl-xl cytoplasmic histoscores correlated with Akt 2 cytoplasmic histoscores

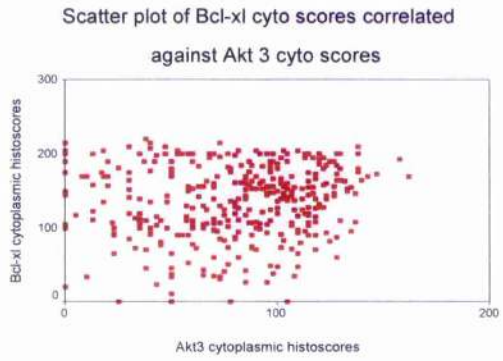
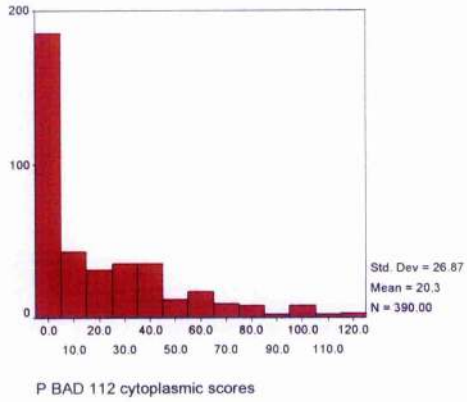


Figure 29. Scatter plot of Bcl-xl cytoplasmic histoscores correlated with Akt 3 cytoplasmic histoscores

3.6 IHC for pBad (Ser112)

3.6.1 pBad (Ser112) expression and staining patterns using IHC

Western blot analysis was used to assess the specificity of the pBad (Ser 112) antibody. A single band was seen at 23kDa corresponding to pBad (Ser112). Stimulation with heregulin alone or in combination with oestrogen induced phosphorylation of Bad at Ser 112 whilst oestrogen alone had no effect (Figure 23b). For immunohistochemical analyses of pBad (ser 112), 66 out of 456 (14.5%) cases were unavailable for analysis either due to core loss or insufficient tumour material in the cores and these patients were therefore excluded from further analysis. Only cytoplasmic staining was noted (Figure 30c). Staining for the antibody was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.C achieving an ICCC of 0.96 (excellent).



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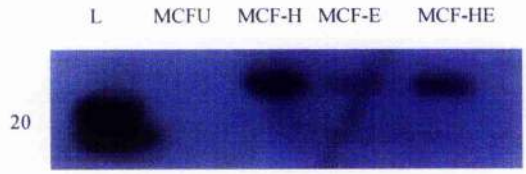
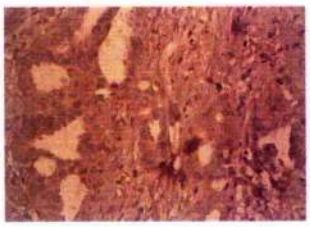


b)



c)

Figure 30. a) Histogram showing distribution of cytoplasmic scores for p-Bad (ser 112) IHC, b) Western blot showing single band at 23kDa (L=ladder, MCFU= MCF-7 cells unstimulated, MCF-H=MCF-7 cells stimulated with heregulin, MCF-E= MCF-7 cells stimulated with oestrogen, MCF- HE= MCF-7 cells stimulated with heregulin plus oestrogen

3.6.2 Bad activity and patient outcome

When patients were divided into those with high or low levels of p-Bad (Ser112) (above or below median histoscore) there was no significant association with overall or disease free survival.

Survival Category	P Value
Overall survival	0.778
Disease free survival	0.830
Disease free survival on tamoxifen	0.783
Disease free survival off tamoxifen	0.966
Disease free survival after 3 years tamoxifen	0.236
Overall survival after 3 years tamoxifen	0.583

Table11. OS and DFS p-values for pBad (ser 112) cytoplasmic expression

3.6.3 Correlations between pBad (Ser-112) and other members of the PI3K/Akt pathway

High pBad (Ser 112) was associated with high levels of apoptotic activity ($p<0.0001$ $R=0.211$) as measured by TUNEL assay. However scatter plot (figure 28) revealed that the main reason for correlation was the significant numbers of low scores.

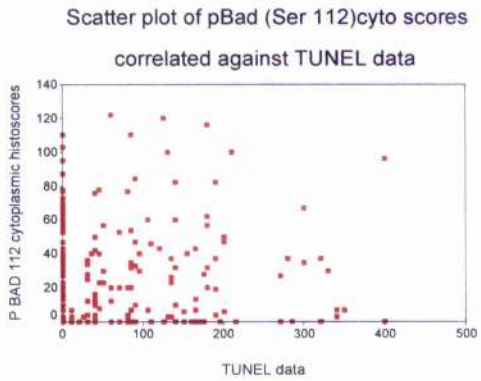


Figure 31. Scatter plot of pBad (Ser 112) cytoplasmic histoscores correlated with TUNEL data (measure of apoptotic activity)

	Median Histoscores (inter-quartile range)	Disease Free survival hazard ratio	Disease free survival after three years tamoxifen hazard ratio
Bad	67 (34-100)	1.49 (1.00-2.22), p=0.05	1.98 (1.10-3.55), p=0.022
PBad (ser 112)	7 (0-33)	1.00 (0.67-1.48), NS	1.26 (0.70-2.26), NS
Bax	27 (7-50)	1.06 (0.71-1.57), NS	1.02 (0.57-1.83), NS
Bcl-2	53 (20-104)	1.24 (0.85-1.82), NS	1.05 (0.60-1.82), NS
Bcl-xl	143 (103-175)	1.13 (0.77-1.68), NS	1.10 (0.62-1.97), NS

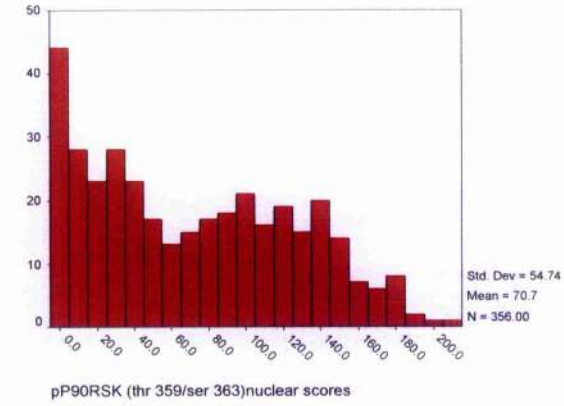
NS=non significant

Table 12. Median histoscore and relative risk (hazard ratio) for Bad, pBad (Ser112), Bax, Bcl-2 and Bcl-xl.

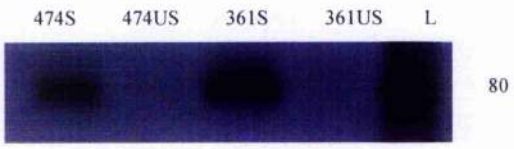
3.7 IHC for P-P90RSK (Thr 359/Ser 363)

3.7.1 p-P90RSK (Thr 359/Ser363) expression and staining patterns using IHC

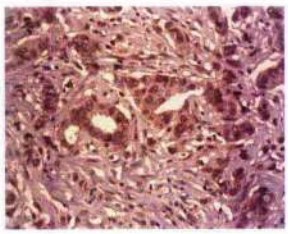
The specificity of the antibody was checked using Western Blotting. A single band was seen at 80kDa corresponding to p-P90RSK (Figure 29b). For immunohistochemical analyses of pP90RSK (Thr359/Ser 363), 100 out of 456 (21.9%) cases were unavailable for analysis either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Only nuclear staining was noted (Figure 32c). Staining for the antibody was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.K achieving an ICCC of 0.94 (excellent).



a)



b)



c)

Figure 32.a) Histogram showing distribution of nuclear histoscores for pP90rsk Thr 359Sser363) IHC, b) Western blot showing single band at 80kDa,(474S=BT474 cells stimulated by oestrogen, 474US =BT474 cells unstimulated, 361S=MDA-MB361 cells stimulated by oestrogen, 361U= MDA-MB361 cells unstimulated c)Breast tissue stained with pP90RSK (Thr 359/Ser 363)

3.7.2 pP90RSK and patient outcome

When patients were divided into those with high or low levels of p-P90RSK (Thr359/Ser363) (above or below the median) there was no significant association between p-p90RSK expression and overall or disease free survival.

Survival Category	P Value
Overall survival	0.450
Disease free survival	0.676
Disease free survival on tamoxifen	0.607
Disease free survival off tamoxifen	0.734
Disease free survival after 3 years tamoxifen	0.993
Overall survival after 3 years tamoxifen	0.880

Table 13. OS and DFS p-values for pP90RSK nuclear expression

3.7.3 Correlations between pP90RSK (thr 359/Ser 363) and other members of the PI3K/Akt pathway

There was a weak but significant correlation between phosphorylated MAPK (42/44) cytoplasmic expression and pP90RSK nuclear expression ($P<0.0001$, $R=0.211$)(figure 30). There was also a weak but significant correlation between pP90RSK nuclear expression and pBad (Ser112) expression ($R=0.384$, $p<0.0001$)(figure 31), and between pP90RSK nuclear expression and p-mTOR cytoplasmic expression ($R=0.340$, $p<0.0001$)(figure 32) (Immunohistochemistry for mTOR was performed by T.K). Scatter plots revealed that the main reason for the correlations was the significant number of tumours with weak staining.

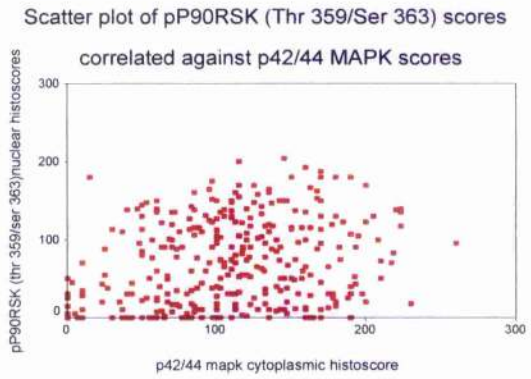


Figure 33. Scatter plot showing the association between cytoplasmic expression pP90RSK (Thr 359/Ser 363) and phosphorylated MAPK (42/44) cytoplasmic expression.

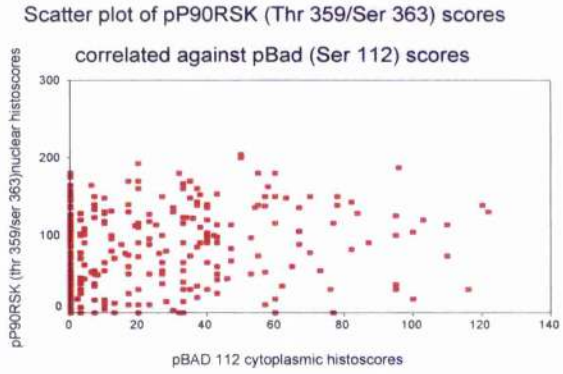


Figure 34. Scatter plot showing the association between cytoplasmic expression pP90RSK (Thr 359/Ser 363) and pBad (Ser 112) cytoplasmic expression.

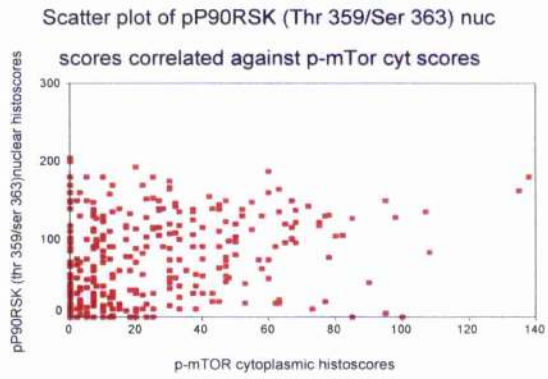
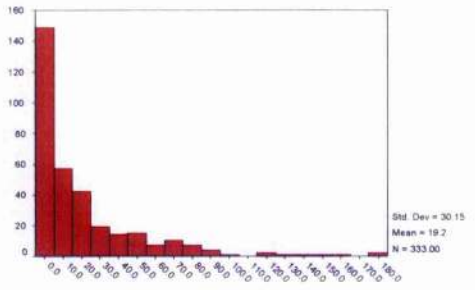


Figure 35. Scatter plot showing the association between cytoplasmic expression pP90RSK (Thr 359/Ser 363) and p-mTor cytoplasmic expression.

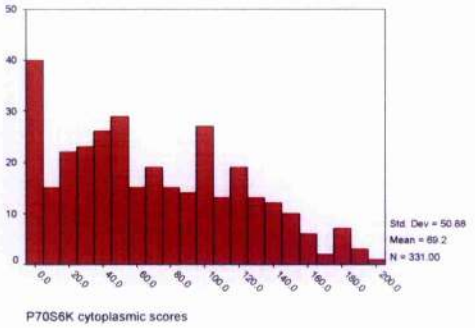
3.8 IHC for P70S6K

3.8.1 P70S6K expression and staining patterns using IHC

The specificity of the antibody was checked using Western Blotting. Two bands were seen at 70kDa and 85kDa respectively (Figure 33c). Both bands corresponded to P70S6K; the 85kDa band corresponded to P85S6K, a second isoform that is identical to P70S6K except for 23 extra amino acids at the amino-terminus. For analyses of P70S6K, 125 out of 456 (27.4%) cases were unavailable for analysis either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Nuclear and cytoplasmic staining was noted (Figure 36d). Staining for the antibody was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.K achieving an ICCC of 0.85(excellent).



a)



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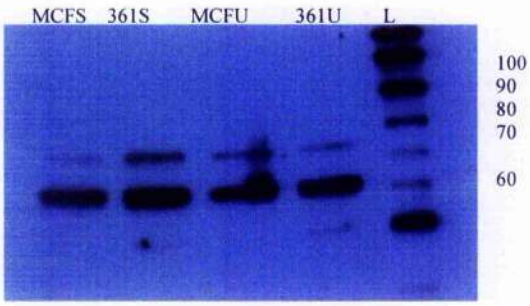
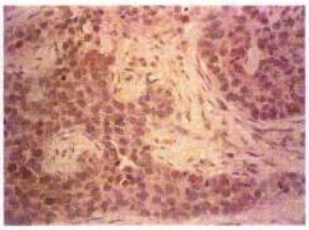


c)



d)

Figure36. a) Histogram showing distribution of nuclear scores for P70S6K IHC b) Histogram showing distribution of cytoplasmic scores for P70S6K IHC, c) Western blot of P70S6K antibody showing two bands at 70kDa and 80kDa (L=ladder, MCFS=MCF-7 cells stimulated by oestrogen, MCFU =MCF-7 cells unstimulated, 361S=MDA-MB361 cells stimulated by oestrogen, 361U= MDA-MB361 cells unstimulated), d) Breast tissue stained with P70S6K antibody

3.8.2 P70S6K and patient outcome

Nuclear scores

When patients were divided into those with high or low levels of nuclear P70S6K (above or below median histoscore) there was no significant association with overall or disease free survival.

Survival Category	P Value
Overall survival	0.463
Disease free survival	0.436
Disease free survival on tamoxifen	0.455
Disease free survival off tamoxifen	0.676
Disease free survival after 3 years tamoxifen	0.450
Overall survival after 3 years tamoxifen	0.118

Table 14. OS and DFS p- values for P70S6K nuclear scores

Cytoplasmic scores

When patients were divided into those with high or low levels of cytoplasmic P70S6K (above or below median histoscore) there was no significant association with overall or disease free survival. However there was a trend towards significance (p=0.065) for those patients whose tumours had high levels of P70S6K expression as compared to those with low levels of P70S6K expression for overall survival after three years of tamoxifen treatment.

Survival Category	P Value
Overall survival	0.540
Disease free survival	0.580
Disease free survival on tamoxifen	0.683
Disease free survival off tamoxifen	0.848
Disease free survival after 3 years tamoxifen	0.848
Overall Survival after 3 years tamoxifen	0.065

Table 15. OS and DFS p-values for P70S6K cytoplasmic scores

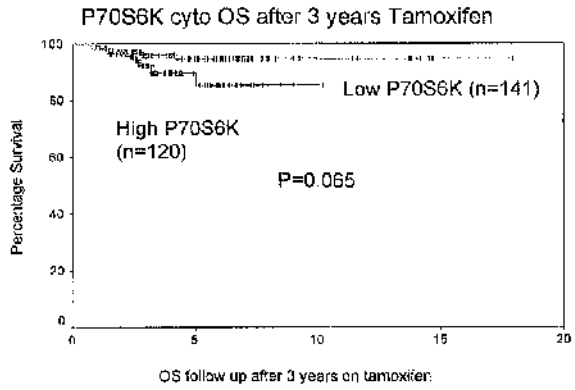


Figure 37. Graph showing percentage survival after 3 years Tamoxifen (y axis) plotted against time in years (x axis) for P70S6K cytoplasmic scores.

3.8.2 Correlations between P70S6K and other members of the PI3K/Akt pathway

Nuclear and cytoplasmic P70S6K scores weakly but significantly correlated with Akt 2 expression (R=0.129, p=0.024 and R=0.213, p<0.0001, respectively). Cytoplasmic P70S6K scores weakly but significantly correlated with pAkt (473) (R=0.114, p=0.044). However scatter plots revealed that the main reason for the correlations was the significant number of tumours with low expression. There was no correlation between P70S6K scores and p-mTOR scores (p-mTOR scores obtained from other member of our group, Dr T Kirkegaard).

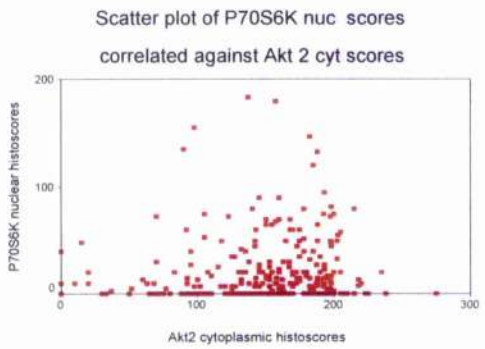


Figure 38. Scatter plot showing the association between nuclear expression of P70S6K and Akt 2 cytoplasmic expression.

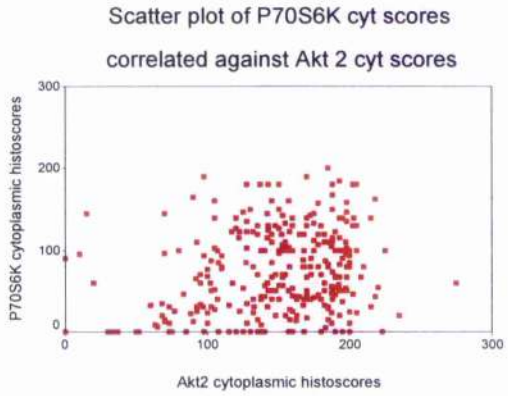


Figure 39. Scatter plot showing the association between cytoplasmic expression of P70S6K and Akt 2 cytoplasmic expression.

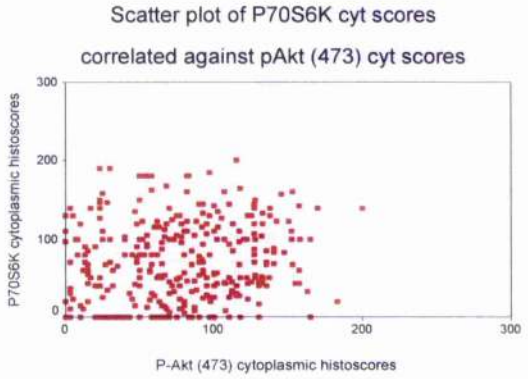
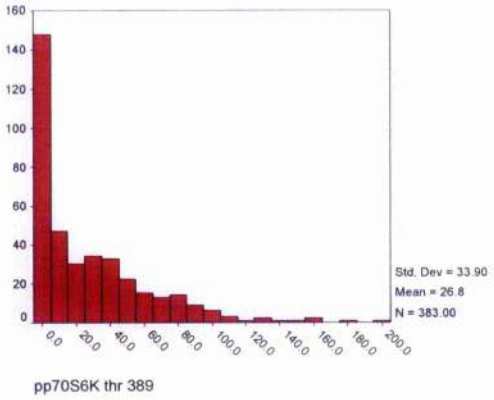


Figure 40. Scatter plot showing the association between cytoplasmic expression of P70S6K and pAkt (473) cytoplasmic expression.

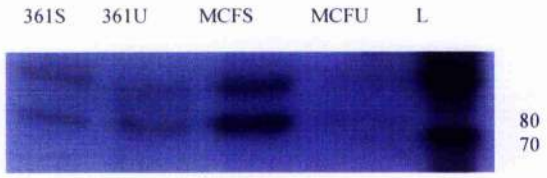
3.9 IHC for pP70S6K (Thr 389)

3.9.1 pP70S6K (Thr 389) expression and staining patterns using IHC

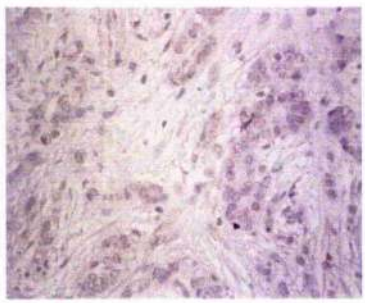
The specificity of the antibody was checked using Western Blotting. Two bands were seen at 70kDa and 85kDa respectively (Figure 38b). Both bands corresponded to P70S6K. The 85kDa band corresponded to pP85S6K, a second isoform that is identical to P70S6K except for 23 extra amino acids at the amino-terminus. For analyses of pP70S6K (Thr389), 73 out of 456 (16.0%) cases were unavailable for analysis either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Only nuclear staining was noted (Figure 41c). Staining for the antibody was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.K achieving an ICCC of 0.94 (excellent).



a)



b)



c)

Figure 41. a) Histogram showing distribution of nuclear scores for pP70S6K (Thr 389) IHC, b) Western blot of pP70S6K (Thr 389) antibody showing a bands at 70kDa and 80kDa, (L=ladder, MCFS=MCF-7 cells stimulated by oestrogen, MCFU =MCF-7 cells unstimulated, 361S=MDA-MB361 cells stimulated by oestrogen, 361U= MDA-MB361 cells unstimulated) c) Breast tissue stained with pP70S6K (thr389) antibody

3.9.2 p-P70S6K (thr 389) and patient outcome

When patients were divided into those with high or low levels of nuclear pP70S6K (Thr389) (above or below median histoscore) there was no significant association with overall or disease free survival. However there was a trend towards significance (p=0.082) for those patients whose tumours had a high (above the median) level of expression of pP70S6K (Thr389) as compared to those with a low level (below the median) expression for overall survival after three years tamoxifen treatment.

Survival Category	P Value
Overall survival	0.214
Disease free survival	0.149
Disease free survival on tamoxifen	0.426
Disease free survival off tamoxifen	0.205
Disease free survival after 3 years tamoxifen	0.140
Overall survival after 3 years tamoxifen	0.082

Table 16. OS and DFS p-values for pP70S6K (Thr389)

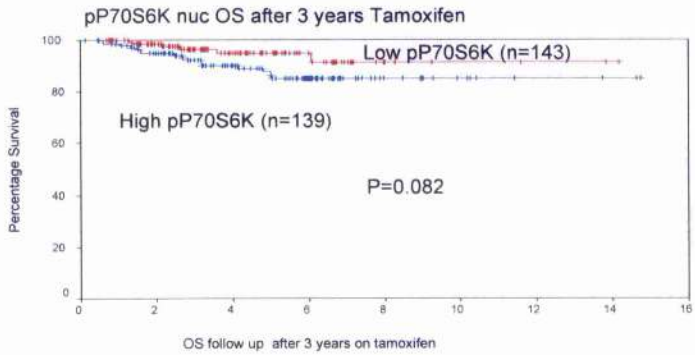


Figure 42. Graph showing percentage survival after 3 years Tamoxifen (y axis) plotted against time in years (x axis) for pP70S6K (Thr389).

3.9.3 Correlations between p-P70S6K and other members of the PI3K/Akt pathway

P-P70S6K scores correlated weakly but significantly with Akt1 and pAkt (473) (R=0.113, p=0.034 and R=0.189, p<0.0001, respectively). PP70S6K scores also correlate weakly with p-mTOR cytoplasmic expression, R=0.213, p<0.0001 (p-mTOR scores obtained from other member of our group, Dr T Kirkegaard). However scatter plots revealed that the main reason for the correlations was the significant number of cores with low expression. There was no correlation between P70S6K and HER2 expression.

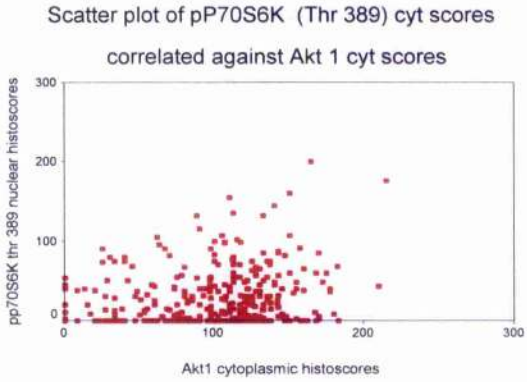


Figure 43. Scatter plot showing the association between nuclear expression of pP70S6K (Thr 389) and Akt 1 cytoplasmic expression

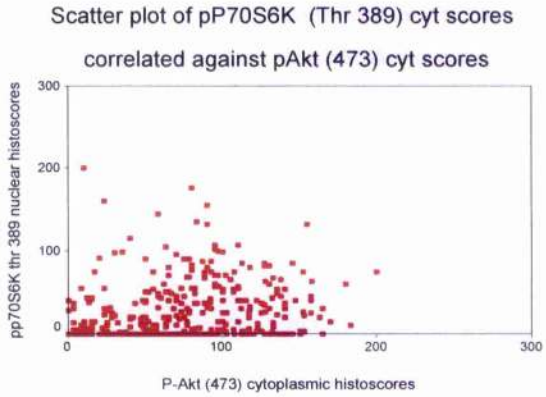


Figure 44. Scatter plot showing the association between nuclear expression of pP70S6K (Thr 389) and pAkt (473) cytoplasmic expression

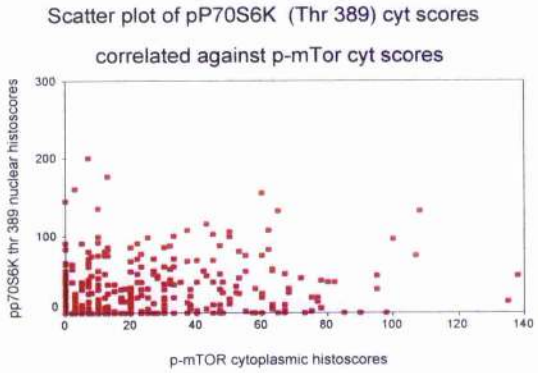
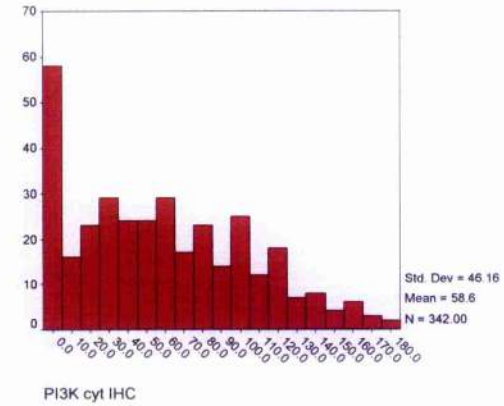


Figure 45. Scatter plot showing the association between nuclear expression of pP70S6K (Thr 389) and p-mTor cytoplasmic expression

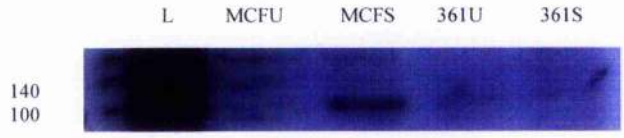
3.10 IHC for PI3K

3.10.1 PI3K expression and staining patterns using IHC

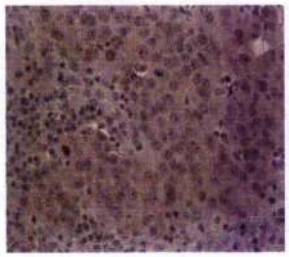
To test the specificity of the antibody Western Blotting was performed. A single band was obtained at 110kDa, corresponding to PI3K (Figure 43b). For analyses of PI3K, 114 out of 456 (25.0%) cases were unavailable for analysis either due to core loss or insufficient tumour material in cores and these patients were therefore excluded from further analysis. Only cytoplasmic staining was noted (Figure 46c). Staining for the antibody was confined to the invasive tumour components, with no staining in normal breast epithelial cells. The staining was scored as described previously and scores for each core were then averaged. 15% of the cores were double scored by T.K achieving an ICCC of 0.83 (excellent).



a)



b)



c)

Figure 46. a) Histogram showing distribution of cytoplasmic scores for PI3K IHC, b)Western Blot of PI3K antibody showing a single band at 110kDa , (L=ladder, MCFS=MCF-7 cells stimulated by oestrogen, MCFU =MCF-7 cells unstimulated, 361S=MDA-MB361 cells stimulated by oestrogen, 361U= MDA-MB361 cells unstimulated c) Breast tissue stained with PI3K antibody showing cytoplasmic staining only

3.10.2 PI3K and patient outcome

When patients were divided into those with high or low levels of cytoplasmic PI3K (above or below median histoscore) there was no significant association with overall or disease free survival.

Survival Category	P Value
Overall survival	0.254
Disease free survival	0.614
Disease free survival on tamoxifen	0.897
Disease free survival off tamoxifen	0.289
Disease free survival after 3 years tamoxifen	0.712
Overall survival after 3 years tamoxifen	0.459

Table 17. OS and DFS p-values for PI3K cytoplasmic scores

3.10.3 Correlations between PI3K and other members of the PI3K/Akt pathway

PI3K cytoplasmic histoscores weakly but significantly correlated with Akt1 and Akt3 (R=0.187, p=0.001 and R=0.189, p<0.0001 respectively). The scatter plot for these correlation showed that the main reason for the correlation was the large number of cores with low PI3K expression. There was a stronger correlation between PI3K and pAkt (Ser-473), R=0.425, p<0.0001 (figure 47).

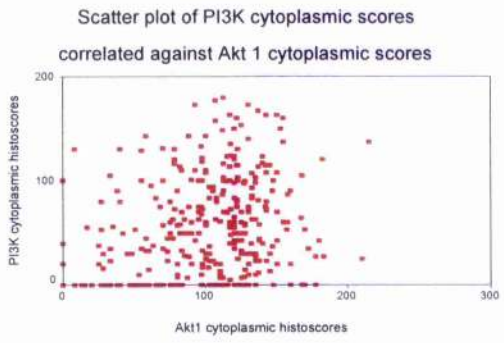


Figure 47. Scatter plot of PI3K cytoplasmic histoscores correlated against Akt 1 cytoplasmic histoscores

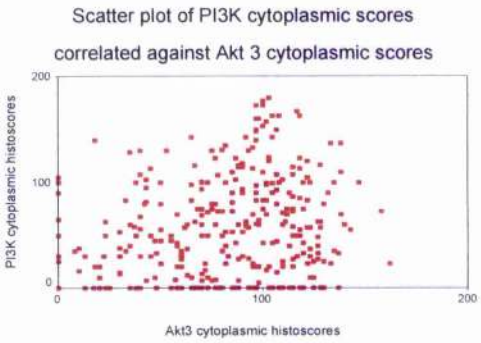


Figure 48. Scatter plot of PI3K cytoplasmic histoscores correlated against Akt 3) cytoplasmic histoscores

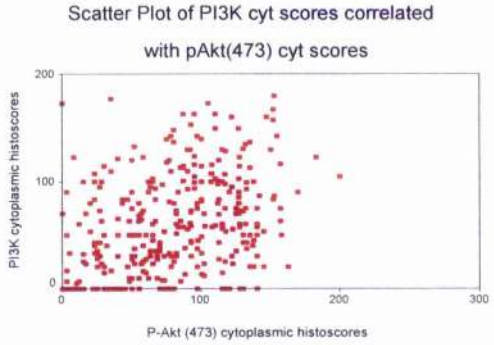


Figure 49. Scatter plot of PI3K cytoplasmic histoscores correlated against pAkt (473) cytoplasmic histoscores

3.11 FISH for HER2

3.11.1 HER2 Amplification

For analyses of HER2 gene amplification, 70 out of 456 (15.4%) cases were unavailable for analysis either due to core loss, poor penetration of probe or insufficient tumour material in cores and these patients were therefore excluded from further analysis. The number of orange (HER2) and green (chromosome) signals were counted in 20 cells of each TMA core (a total of 60 cells per pations). The ratio of green to orange signals was calculated. A ratio greater or equal to 2.0 was counted as an amplification. All of the cores were double scored by T.K achieving an ICCC of 0.93 (excellent). 26 cores showed amplification of Her 2.

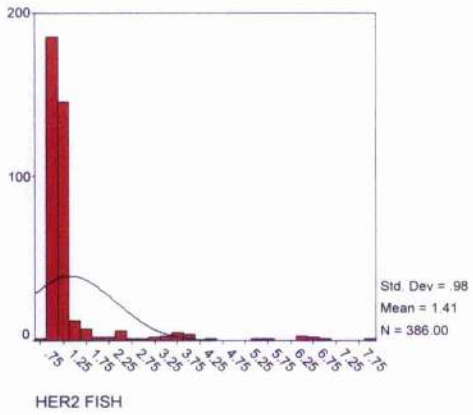


Figure 50. Histogram showing the distribution of amplifications for Her 2FISH (curve shows normal distribution)

3.11.2Her 2 and patient outcome

There was no significant correlation between OS and DFS and Her 2 amplification in this patient cohort.

Survival Category	P Value
Overall survival	0.6691
Disease free survival	0.5420
Disease free survival on tamoxifen	0.8758
Disease free survival off tamoxifen	0.2817
Disease free survival after 3 years tamoxifen	0.1696
Overall survival after 3 years tamoxifen	0.6287

Table 18. OS and DFS p-values for Her 2 amplification binary scores

3.11.3Correlations between Her 2 and other members of the PI3K/Akt pathway

When the HER2 FISH binary scores were correlated with the HER2 herceptest binary scores significant correlation was seen (CC=0.307, p<0.0001). HER2 Herceptest was taken as positive if 2+.

Correlations

			Her2 membrane binary scores	Her2 fish binary
Spearman's rank	Her2 membrane binary scores	Correlation Coefficient	1.000	.307(**)
		Sig. (2-tailed)	.	.000
		N	395	350
	Her2 fish binary	Correlation Coefficient	.307(**)	1.000
		Sig. (2-tailed)	.000	.
		N	350	355

** Correlation is significant at the 0.01 level (2-tailed).

Table 19. Table showing correlation between Her 2 FISH binary scores and Herceptest binary scores.

Herceptest score	Her 2 FISH amplified	Her 2 FISH not amplified	Total
0	0	275	275
1	6	40	46
2	4	21	25
3	16	8	24
Total	26	344	370

Table 20. Table showing the correlations between the various Herceptest and FISH scores.

There was a 100% correlation between those cases with a herceptest score of zero and no amplification with FISH. There were 6 cases that were amplified with FISH but only Herceptest 1+. Of the 25 HER2 2+ cases only 4 were amplified with FISH. Out of the 24 HER2 3+ cases 8 were not amplified with FISH.

3.12 FISH for PI3K

3.12.1 PI3K amplification

Initially, PI3K was only analysed in TMA B. If the percentage of PI3K amplifications had been above 10%, then the whole data set would have been analysed for PI3K gene alterations. For analyses of PI3K, 57 out of 119 (47.9 %) cases were unavailable for analysis either due to core loss, poor penetration of probe or insufficient tumour material in cores and these patients were therefore excluded from further analysis. The number of red (PI3K) and green (chromosome) signals were counted in 20 cells of each TMA core (a total of 60 cells per patient). All of the cores were scored by E.C, with 20% being double scored by F.C achieving an ICCC of 0.90 (excellent). The ratio of green to red signals was calculated and a ratio of greater or equal to 2.0 was counted as an amplification. Out of the 62 samples analysed only two were amplified

3.13 FISH for PTEN

3.13.1 PTEN deletions

Like FISH for PI3K, PTEN FISH was only analysed in TMA B. For analyses of PTEN, 55 out of 119 (46.2%) cases were unavailable for analysis either due to core loss, poor penetration of probe or insufficient tumour material in cores and these patients were therefore excluded from further analysis. The number of red (PTEN) and green (chromosome) signals were counted in 20 cells of each TMA core (a total of 60 cells per patient). The ratio of red to green signals was calculated and a ratio of less than or equal to 0.8 was counted as a deletion. All of the cores were scored by E.C and 20% of the cores were double scored by F.C achieving an ICCC score of 0.94 (excellent)

Out of the samples analysed only 3 were found to have deletions of PTEN.

Chapter 4 Discussion

4.1 PTEN

The PTEN data presented here shows that a high cytoplasmic PTEN expression is associated with a decreased overall (p=0.013, log-rank test) and disease free (p=0.018, log-rank test) survival. This is in contrast to the perceived wisdom(Panigrahi et al., 2004b; Lu et al., 1999; Sansal and Sellers, 2004) that loss of PTEN leads to an activation of AKT, which in turn promotes anti-apoptosis, essential in tumourogenesis. In a study by Depowski et al(Depowski et al., 2001a)looking at the tumours of 151 women a loss rate of 48% was seen and they found that loss of PTEN was associated with disease related death in univariate analysis. However the association was lost in multivariate analysis against known prognostic markers. In a study looking at several series of sporadic breast cancers(Perren et al., 1999a) the PTEN mutation frequency was less than 5%. In IHC analysis of the same tumours 15% were PTEN negative and 18% showed absent or reduced expression. This is in line with our data showing 18% had no PTEN expression and 22% reduced or absent expression. Another study on primary operable breast cancers(Panigrahi et al., 2004c).showed no association between patient, tumour and outcome variables such as tumour grade. No association of PTEN expression and overall survival was noted , supporting our data that showed no association between PTEN expression and overall or disease free survival in multivariate analysis. This study suggests that loss of PTEN is infrequent and argues against the current model of a simple linear tumourigenic PTEN-PI3K-AKT-mTOR pathway in breast cancer. Our data however shows a positive link between high levels of PTEN and reduced survival after 3 years of tamoxifen treatment – a late effect (in multivariate analysis against known prognostic

indicators, p=0.01). We found both cytoplasmic and nuclear PTEN expression but only cytoplasmic PTEN expression was significant associated with both OS and DFS. Chung et al(Chung and Eng, 2005) have suggested that subcellular localization of PTEN may regulate its function. They found that cytoplasmic PTEN down-regulates phosphorylation of Akt and up-regulates p27(kip1), whereas nuclear PTEN down-regulates cyclin D1 and prevents the phosphorylation of MAPK. They also observed that nuclear PTEN is required for cell cycle arrest, but that cytoplasmic PTEN is required for apoptosis. They concluded that nuclear-cytoplasmic partitioning differentially regulated the cell cycle and that nuclear import of PTEN may play a role in carcinogenesis. To further clarify the role of PTEN in carcinogenesis, we intended to look at the expression and localisation of phospho-PTEN (activated PTEN) but unfortunately we were not able to find an antibody that was specific in IHC. Another small study(Bose et al., 2002b) (34 patients)showed a PTEN loss rate of 38%. The study found an association between reduced PTEN expression and stage and ploidy. Initially it was thought that PTEN mutations were the key to disease progression, however the PTEN mutation rate in sporadic breast cancer is around 5%(Perren et al., 1999b), and this is also confirmed in analysis of breast cancer cell lines(Li et al., 1997a). In hereditary cancer syndromes such as Cowden disease the rate is much higher(Liaw et al., 1997b). We did not assess the mutation rate in our tumours as this theory seems to have been discredited with numerous studies showing a low rate of mutation in sporadic cancers(Liaw et al., 1997a; Perren et al., 1999c). There was also a series of studies(Bose et al., 2002a; Leslie and Downes, 2004) looking at loss of heterozygosity within the PTEN gene – the small study by Bose et al showed no correlation with reduced PTEN expression, It seems clear that mutation or loss of hererozygosity alone is not sufficient to reduce PTEN expression. Our data further

adds to the confusion around PTEN by showing significant associations between high levels of PTEN expression and overall and disease-free survival. It would be interesting to look at the level of loss of heterozygosity in the tumour samples we analysed.

There is evidence(Garcia et al., 2004a; Perren et al., 1999d; Salvesen et al., 2001; Soria et al., 2002; Baylin and Herman, 2000) that expression of tumour suppressing genes can be greatly decreased in some tumours by promoter methylation and it has been a matter of debate if this is the case for PTEN. To further investigate the hypothesis that methylation of PTEN could be responsible for lack of expression, we are currently doing methylation studies on a similar cohort of tumours. There are no studies in the literature looking at FISH for PTEN. Our pilot study looking at PTEN deletions using FISH showed so few deletions that it was not considered worthwhile performing FISH on the whole tumour cohort so further comment cannot be made. In view of the differing results associated with decreased PTEN expression, ranging from a positive, to no effect through to a negative effect on overall or disease free survival, it is still controversial if PTEN is a prognostic marker and further and larger studies need to be performed. It may be that the current model of a simple linear tumourigenic PTEN-PI3K-AKT-mTOR pathway in breast cancer is not so simple after all and has many other areas of crosstalk and interaction.

4.2 Bad, Bax, Bcl-2 and Bcl-xl

In the current study we report that patients whose tumours have high Bad expression have a significantly increased DFS when compared to those with low expression, in univariate analysis. This supports our hypothesis that high levels of Bad result in the sequestration of Bcl-2/Bcl-xl which allows Bax:Bax homodimerisation leading to a higher apoptotic index and improved DFS. Our results show an association between over-expression of Bad and DFS. This appears to be a late effect, which has greater significance after three years of tamoxifen treatment but is not significant in multivariate analysis (p=0.083). We have also shown ans association between over-expression of Bad and improved OS after 3 years Tamoxifen treatment (p=0.010) but again this is not significant in multivariate analysis. In the current climate of debate about switching endocrine treatment from tamoxifen to aromatase inhibitors it would appear that further studies of Bad in a larger patient population are warranted Increased expression of Bad leads to increased sequestration of Bcl-2 (heterodimerisation) and therefore increases the amount of available Bax to promote apoptosis. The improved disease free survival after three years on tamoxifen in those tumours with increased expression of Bad is interesting in the context of the Intergroup Exemestane Study (IES) trial. The IES trial was designed to investigate if there was a survial advantage in giving postmenopausal women Exemestane after 2-3 years of tamoxifen therapy as compared to continuing tamoxifen therapy for 5 years. Exemestane therapy after 2-3 years of tamoxifen therapy significantly improved disease free survival as compared with the standard 5 year of tamoxifen treatment. Overall survival was not initially significantly different between the two groups (Coombes et al., 2004a) however it was in a later update of the trial.

Previous in-vitro studies suggest that long term treatment with tamoxifen modulates the expression of apoptotic key genes impairing the apoptotic response of MCF-7 breast cancer cells(Treeck et al., 2004a). In this study we performed Western Blots using cells treated with oestrogen alone, heregulin alone and oestrogen and heregulin combined and found no changes in the expression levels of Bad, Bcl-2, Bcl-xl and Bax. In contrast, stimulation with heregulin alone or in combination with oestrogen induced phosphorylation of Bad at Ser 112 whilst oestrogen alone had no effect. Expression of pBad (ser 112) positively correlated with apoptosis (as measured by TUNEL assay), however this correlation was shown to be mainly due to the signigicant number of negative results when a scatter graph was drawn. As there are multiple phosphorylation sites on Bad and the literature suggests that more than one site is reqiured to be activated to prevent apoptosis it is perhaps not particularly surprising that pBad (ser 112) alone is not associated with DFS/OS.

In vitro studies have shown that oestrogen-mediated phosphorylation of Bad prevents apoptosis(Fernando and Wimalasena, 2004d). The ability of oestrogen to prevent apoptosis was blocked by over expression of Bad where the phosphorylation sites have been mutated (S112A/S136A) but not by the wild type Bad. Bad S112A/S136A, which lacks the phosphorylation sites, was not phosphorylated in response to oestrogen *in vitro*(Fernando and Wimalasena, 2004c).

The anti-apoptotic proteins Bcl-2 and Bcl-xl are thought to play important roles in inhibiting mitochondria-dependent pathways(Kim, 2005b). Some studies suggest that these two proteins have distinct functions for inhibiting extrinsic and intrinsic cell death pathways. The over expression of Bcl-2 has been shown to be associated with cell cycle arrest in the G1 phase, which may promote cellular senescence. The over expression of Bcl-2 may also have the ability to enhance cell death in the interaction

of Bcl-xl with other factors(Kim, 2005c). Consistent with our findings an earlier study looking at the prognostic significance of p53, Bcl-2 and Bax expression in early breast cancer(Linjawi et al., 2004c), did not find a correlation between Bax or Bcl-2 and OS or DFS. However Bcl-2 correlated significantly with favourable tumour features such as DNA diploid status and expression of oestrogen and progesterone receptors(Linjawi et al., 2004d). This has also been seen in other studies(Baccouche et al., 2003a).Our results do not demonstrate a link between expression of Bcl-2 and Bcl-xl and apoptosis measured by TUNEL experiment. Possibly due to the interaction of these proteins with Bad being complicated and involving cross talk with other pathways (in addition to PI3K/Akt and Ras/RAF-1).

Imbalance between pro-apoptotic and anti-apoptotic proteins, causing altered apoptosis, may lead to tumour development and tumour progression, and reduced response to adjuvant therapy(Cameron et al., 2000b). Our results do not demonstrate a link with Bcl-xl and Bcl-2 and OS or DFS.

Whilst this study represents one of the larger cohorts used to investigate apoptotic markers, it failed to clearly identify a predictive marker of response in this cohort. This may be related to the low event rate (22% over >10 years) in this low risk population. Results form the literature suggest that reduced expression of Bax (p=0.03) and Bcl-2 (p=0.03) was associated with lymph node metastasis in multivariate analysis(Bukholm et al., 2002a). Expression of Bcl-2 was associated with better patient survival in univariate analysis (p=0.04) but lost its predictive value in multivariate analysis (p=0.2) (Cameron et al., 2000a). Both this previous study and our own are suggestive of a link between apoptotic proteins and patient outcome. Based on our data (and that from the literature on Bax/Bcl2), it is now possible, for the first time to estimate the appropriate sample size to definitively test this. Based on

an assumption that the hazard ratio for the Bad cytoplasmic binary scores, adjusted for nodal status, tumour size and grade remains unchanged with increased patient numbers, the required patient population is estimated to be 1100 (for an 80% powered study).

Here we highlight a possible link between the apoptotic parts of the PI3K/Akt/mTOR pathway by linking Bad expression to disease free survival of tamoxifen treated breast cancer patients. It would be interesting to test these markers in a larger data set to see if low Bad expression is a marker of late (after 3 years), relapse of breast cancer. In this centre we are currently constructing a TMA that will consist of tumour samples from 1700 patients to allow us to further test this hypothesis.

4.3 pP90RSK (Thr 359/Ser 363)

In this study we report that phosphorylated P90RSK (Thr 359/Ser 363) expression is not associated with changes in OS or DFS in this patient cohort

P90RSK expression is not widely reported in the literature, and the data from the current study does not support a role for phospho-P90RSK influencing patient survival. Activated (phosphorylated) p90RSK phosphorylate Bad at Ser112 and thereby influence apoptosis (Harada et al., 2001b). The majority of studies (Tan et al., 1999b; Fernando and Wimalasena, 2004b; She et al., 2005a; Bonni et al., 1999b) reporting on the relationship between P90RSK and phospho- Bad are therefore also looking at the phosphorylation of Bad at Ser112 by P90RSK and the effect on apoptosis and not the expression of P90RSK per se in breast cancer. The literature(Tan et al., 1999a; Fernando and Wimalasena, 2004a; She et al., 2005b; Bonni et al., 1999a) suggests that P90RSK phosphorylates Bad at Ser112, and we also found a correlation, although weak, between pP90RSK expression and Bad phosphorylated at Ser112. That expression of p-P90RSK also correlates with phospho-MAPK (CC=0.231, $p<0.0001$) also fits with the proposed structure of the EGFR/MAPK pathway. That both P70S6K and P90RSK phosphorylate Bad (at sites ser 136 and 112 respectively) and that both are needed to maximally prevent the apoptotic action of Bad indicates that they are unlikely to be the control steps in the process. Other studies have shown that there are other phosphorylation sites on Bad that may be important(Berndtsson et al., 2005; Dramsi et al., 2002; Tan et al., 2000b). The role of these sites has however, not been fully established. In this study we have only investigated a single phosphorylation site on p90RSK, however, to more fully assess the role of Bad it would be necessary to look at all of the phosphorylation sites alone and in combination as activation of Bad may require a specific sequence of

phosphorylation. Again the current model of the PI3K/Akt and MAPK pathways may be too simplistic with a greater degree of crosstalk than previously thought

4.4 P70S6K/pP70S6K (Thr 389)

In this study we found that there was no significant correlation between expression of either P70S6K or pP70S6K (Thr 389) and overall or disease free survival. This is in contrast to an earlier study (Lin et al., 2005a) showing that pP70S6K was increased in primary breast cancers and missing in normal breast epithelial tissues. In this study, enhanced phosphorylation was positively associated with disease progression from normal breast epithelial tissue to invasive breast carcinoma ($p<0.05$)(Lin et al., 2005c). In line with our data showing 52% P70S6K over expression, 56%of the breast tumours from this study had p70S6K over-expression The RPS6KB1 gene encodes for the mTOR effector P70S6K and is amplified in approximately 10% of breast cancers(Barlund et al., 2000b; Andersen et al., 2002a). RPS6KB1 gene amplification has been found to correlate with Her2 over expression in breast tumours possibly due to co-amplification of RPS6KB1 with Her2 (both genes reside on 17q). In another study(van der Hage et al., 2004b) that looked at 452 node negative tumours from pre-menopausal women (drawn from the EORTEC trial) over-expression of P70S6K was found to be associated with an increased risk of loco-regional recurrence (HR 2.50, 95% CI 1.30 – 4.8, P=0.006) and distant metastasis (HR 1.80, 95% CI 1.08 – 3.01, p=0.025). On multivariate analysis over-expression of P70S6K was independently predictive for loco-regional recurrence only (HR 2.67, 95% CI 1.39 – 5.14, p=0.003). It also found a significant association between p70S6K and Her2 expression (p=0.01). This was not borne out by our study in which there was no significant correlation between the two. It is known that P70S6K is important for cell growth and survival. Activation of P70S6K requires sequential phosphorylation of multiple serine and threonine sites often triggered by growth factors and hormones(Le et al., 2003). In a study by Le et al Paclitaxel was found to induce phosphorylation of P70S6K at Thr

421 and Ser 424 in a time and concentration manner. Phosphorylation at these sites caused INACTIVATION of P70S6K. Paclitaxel did not induce phosphorylation at sites Thr 389 and Ser 411. Phosphorylation of these sites leads to activation of P70S6K. Inhibitors of mTOR impair P70S6K activity. Inhibitors of PKC and JNK prevent paclitaxel induced P70S6K inactivation. We looked at the phosphorylation of P70S6K at Thr 389 – this is an activation site. As there are many phosphorylation sites on P70S6K it may be that there is a specific sequence of activation and that the site we looked at is not the rate limiting site. Rapamycin has been found to inhibit mitochondrion bound P70S6K, this prevents the phosphorylation of Bad at Ser 136 and hence blocked cell survival induced by insulin-like growth factor 1 (IGF-1)(Harada et al., 2001a). This proved that P70S6K signals cell survival as well as growth inactivating the pro-apoptotic molecule Bad. Unfortunately we were unable to obtain a pBad (ser 136) antibody. It would have been interesting to see how pP70S6K (Thr 389) over-expression correlated with expression of p-Bad (ser 136). When we correlated the expression levels of pP70S6K (Thr389) with p-mTOR there was a weak but significant correlation (CC 0.213, $p<0.0001$). This is supported by the literature that shows that elevated levels of pP70S6K (Thr 389) are indicative of increased mTOR activity(Klos et al., 2006). The same study also demonstrated that tumours with increased levels of pP70S6K expression were more likely to develop metastasis ($p<0.05$). This study looked at 155 tumour samples. Our results do not show any link with metastasis and over-expression of pP70S6K although there is a trend toward significance for recurrence after 3 years Tamoxifen treatment in patients with elevated levels of phosphorylated P70S6K ($p=0.085$). Although our study did not find any link between expression of P70S6K and pP70S6K (thr 389) it did find similar levels of over-expression in tumour cells. It is becoming increasingly apparent that the

PI3K/Akt pathway is not a simple linear pathway and that there are feed back loops and cross talk involved. Activation of P70S6K involves multi-site phosphorylation and phosphorylation of some sites (Thr 421 and Ser 424) causes inactivation. More work needs to be done on the sequence of activation of these sites and the role of feedback loops before this part of the pathway is fully understood.

4.5 PI3K

In this study we have investigated the role of PI3K in mediating tamoxifen resistance, and found no association between over expression of PI3K and disease free or overall survival.

In addition we also investigated a subset of the patients for genetic changes (gene amplifications and deletions) of PI3K and observed only 2 (3.2%) tumours with PI3K gene amplifications The literature suggests that the PI3K is amplified in breast cancer however no studies have suggested a role for PI3K as predictive marker of hormone relapse. Mutations in the PIK3CA gene which encodes the PI3K p110α catalytic subunit are seen in 20-40% of breast tumours(Crowder and Ellis, 2005; Samuels and Velculescu, 2004b; Bachman et al., 2004b; Campbell et al., 2004b; Saal et al., 2005c). This study was not set up to look at the levels of PI3K in breast tumour cells compared with normal tissues – we were looking specifically at ER positive tumours and looking for proteins that would be predictive of relapse on hormonal treatment. One study involving a large cohort of breast tumour samples(Saal et al., 2005a) reported that PIK3CA mutations are mutually exclusive with PTEN loss, and that PIK3CA mutations correlate with Her2 expression and ER positive status in breast tumours although similar correlations were not seen in other studies involving smaller sample sizes(Crowder and Ellis, 2005; Bachman et al., 2004a; Saal et al., 2005d) Considering the small number of cases where genetic status for both PTEN and PI3K were investigated, PTEN loss and PI3K amplification were mutually exclusive though it should be noted that the small number of cases prevents this from being anything more than an interesting observation. We also did not look at PI3K mutation just amplification. Looking at the IHC data for Her 2 and PI3K expression there is no association between Her 2 and PI3K over-expression. Activated PI3K generates

membrane bound phosphoinositides which act as second messengers and serve to recruit second messengers such as Akt(Thompson and Thompson, 2004b). PI3K has also been found to act as a viral oncoprotein(Thompson and Thompson, 2004d; Chang et al., 1997). Previsouly, we found that Akt activation predicts outcome in breast cancer patients treated with tamoxifen (Kirkegaard et al., 2005a). PI3K is crucial for the activation of Akt via the production of membranous phosphoinositides , which phosphorylate (activate) Akt. . AKT is first phosphorylated at Thr 308 but additional phosphorylation at Ser 473 is necessary for full activation(Luo et al., 2003b). Supporting the pathway we found that PI3K protein expression significantly correlated with pAkt (Ser 473). Looking at the mechanism of PI3K and its involvement at the initial stages of the PI3K/Akt pathway it is more likely that amplification/over-expression of PI3K is involved in initial oncogenic transformation rather than being related to hormonal resistance. The therapeutic use of PI3K is more likely to be as a target for prevention of activation of the PI3K/AKT pathway and indeed there is ongoing research into this area(Daub et al., 2004). It is increasingly being thought that there is a negative feedback loop in the PI3K/AKT pathway(Manning, 2004b). It is thought that this loop involves S6K protein inhibiting IRS proteins, which in turn inhibit PI3K. This leads to the question of whether inhibition of mTOR (with Rapamycin) could have a detrimental effect on tumours. A further area of study would be to look at PI3K activation and the association between activated Akt and PI3K. Previous results do not suggest that PI3K is likely to prove predictive for hormone resistance in breast cancer. Our results do, however support the current model of the PI3K/AKT pathway.

4.6 Her2 FISH

Her 2 gene amplifications taken as a single marker did not have a role in predicting overall or disease free survival in this patient cohort of tamoxifen treated breast cancer patients. It should be noted that our cohort of tumours were low malignant as shown by the total number of events (74 deaths). There is a fair correlation between FISH and the herceptest but these results show that some Her 2 positive cores may be missed by the accepted practice of performing FISH on all Her 2 2+ cores only. There may also be some false positives with the Her 2 3+ cases. In the published literature Bartlett et al(Bartlett et al., 2001) compares herceptest with FISH and CB11 IHC. 12.6% of the tumours were positive using herceptest (2+/3+), 19.4% were positive using FISH and 28.5% were positive using CB11 IHC. Two experienced scorers scored all the tumours and the IHC methods were more susceptible to intra-observer variation (κ=0.67 and 0.74 for the herceptest and CB11 respectively) than FISH (κ= 0.973). Overall test accuracy for CB11 IHC was 83.8%, herceptest was 87.1% and FISH was 93.2%. FISH predicted p185 Her 2 overexpression (determined by quantitative IHC) (concordance index 0.90) better than CB11 IHC (0.85 or herceptest (0.81). Of the 42 cases with gene amplification measured by FISH, 67% were positive with the herceptest (2+/3+). Of the 174 cases negative by FISH, 96% were negative when using the herceptest. Another study(Birner et al., 2001)showed that of 35 cases that had gene amplification by FISH, 97.1% were positive with the herceptest (although interestingly no cases were scored as 2+ that were also FISH positive). Of the 172 cases negative by FISH 76.2 % were negative with the herceptest. 16.9% of the tumour specimens analysed by FISH showed amplification of Her 2, 26.8% were overexpressed with the Herceptest (2+ or 3+). The Her2 herceptest data from our group shows 13.4% positivity 2+ or 3+) whilst with FISH I have a positivity rate of

7%. Of the 26 cases with gene amplification by FISH 76% were positive with the herceptest (2+ or 3+). Of the 344 cases negative by FISH, 92% were negative with the herceptest. Another study(Press et al., 2002) found the accuracy of Vysis FISH to be 97.4% and that of the herceptest to be 88.9%. In a correlation(Dowsett et al., 2003) between immunohistochemistry (HercepTest) and FISH for Her 2 in 426 breast carcinomas from 37 centres 0.7% of IHC 0/1+ tumours were FISH positive and 5.9% of IHC 2+ were FISH negative. The results from our tumour group are similar for the Her 2 0/1+ group, 1.8% were FISH positive. However many more of the 3+ group were FISH negative (33%). 48% of the study's Her 2+ tumours were FISH positive compared with 16% of mine. With Dowsett et al's study 29% of their tumours showed gene amplification with FISH compared to 7% of mine. It has been shown that Her2 *neu* gene amplification or over expression predicts shorter disease free or overall survival in both axillary lymph node negative(Gusterson et al., 1992) and node positive breast cancer(Slamon et al., 1987a; Slamon et al., 1989; Slamon, 1990). In a study from Nottingham(Abd El-Rehim et al., 2004a) 31.8% of the tumours studied were Her 2 positive. Her 2 had an independent prognostic effect on overall and disease free survival however in lymph node negative patients it lost its predictive power for overall survival but still predicted disease free survival. This study used IHC on TMAs. It has been shown(Zhou et al., 2000a) that Her2/*neu* constitutively activates the Akt/NF-κβ anti-apoptotic cascade to confer resistance to TNF on cancer cells and to decrease host defences against neoplasia. Tovey et al(Tovey et al., 2005a) showed that Her 1-3 (but not Her 4) over expression predicts for early relapse on tamoxifen (p=0.0060). PR negative cancers were also more likely to relapse on tamoxifen (p=0.017). Her 1-3 positive and/or PR negative patients combined as a 'high risk' group were significantly more likely to relapse on tamoxifen. This applied

to early relapse only, as any relapse after 3 years of tamoxifen treatment was unrelated to PR/Her status. The data for Her2 FISH does not show any significant correlation between gene over expression and OS or DFS, despite the fact that the same tumour set was used in both studies. Following publication of the first large multi centre trial of adjuvant Herceptin(Piccart-Gebhart et al., 2005b) which showed a significant improvement in disease free survival in Her2 positive breast cancer patients it is now increasingly important to accurately identify those patients who are likely to benefit from treatment with Herceptin. The current standard of HercepTest on all tumours followed by FISH of all Her2 2+ tumours has been shown to be accurate however there will always be some patients who are either falsely positive or negative. The HercepTest has greater intra-observer variation than FISH and it may be possible (and financially viable) in the future to FISH all specimens, as missing out on a highly effective treatment is obviously undesirable (false negatives) as is being exposed to a potentially dangerous drug if you are not going to respond to it (false positives). This is especially relevant in an increasingly well-informed and litigious society.

Chapter 5

Conclusions

The importance of the main body of this work lies in the fact that there has been limited research so far, looking at the role of the distal part of the PI3K/Akt and Raf-1 pathways in hormone resistant breast cancer. Most of the work in the published literature has looked at the more proximal end of the pathway or used cell line work to establish a hypothetical model for the pathway. We used a large (402) cohort of patients on whom we had pathological and survival data. I worked as part of a team looking at various proteins within the pathway and this enabled me to correlate expression levels of the proteins, I was investigating with others in the pathway. I have highlighted a possible link between the apoptotic parts of the PI3K/Akt/mTOR pathway by linking Bad expression to disease free survival of tamoxifen treated breast cancer patients (and OS after 3 years tamoxifen treatment). It would be interesting to test these markers in a larger data set to see if low Bad expression is a marker of late (after 3 years), relapse of breast cancer. In this centre we are currently constructing a TMA that will consist of 1700 patients that will allow us to further test this hypothesis. That the other proteins in the lower end of the pathway did not correlate with overall or disease free survival supports the hypothesis that the PI3K/Akt pathway is more complicated than first thought and unfortunately is not a simple, linear model and that crosstalk between the various steps in likely. The proteins looked at, have multiple activation sites and it is may be (as in the case of P70S6K) that it is the sequence of activation that determines the activity rather than simply the levels of phosphorylated and unphosphorylated proteins.

Increased PTEN expression was shown to be significantly associated with decreased OS and DFS. This association was maintained in multivariate analysis for DFS after 3 years Tamoxifen treatment. There are many conflicting reports in the literature (see chapter 4.2) ranging from increased expression having a positive effect on OS and DFS through to having no or (as in our results) a negative effect. Expression of PTEN does not seem to be a reliable prognostic marker and the role of PTEN remains poorly understood. Our group is in the process of starting methylation studies on PTEN in the hope that the role of PTEN may be further elucidated.

The FISH studies on Her 2 did not show a significant correlation between gene over-expression and OS or DFS. It should be noted that there is a low level of recurrence in the cohort we looked at. This result is interesting in the context of the results for HercepTest on the same cohort of tumours which showed a significant association between increased expression of Her 2 and reduced OS and DFS (Tovey et al., 2004a). The current standard is to perform FISH on all samples that are 2+ using the HercepTest. According to my data using this approach, may miss some Her positive patients (false negatives) and expose others to a potentially dangerous drug (false positives). There is a greater degree of intra-observer variation with the HercepTest than with FISH and in an increasingly litigious society it may be possible and financially viable to perform FISH on all specimens.

From this study it has become increasingly clear that the PI3K/Akt pathway is not as simple as first thought. It is likely that IHC and FISH alone are not going to be adequate to fully investigate it and that further cell line work and alternate investigations such as methylation may be required. The phosphorylation sites of the individual proteins need to be more closely examined and the degree of cross-talk

between the Pi3K/Akt and Raf-1 pathways need to be more fully investigated. Negative feedback loops may also play a role (Manning, 2004a).

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Appendix 1

Patient Clinical & Pathological Variables

		Number/Total	%
GRADE	1	99/391	25.32
	2	193/391	49.36
	3	99/391	25.32
	Unknown	11	
NODAL STATUS	0	193/369	53.1
	1-3	107/369	29.1
	4+	69/369	17.8
	Unknown	33	
Size	T1 (<20mm)	154/380	40.53
	T2 (20-50mm)	204/380	53.68
	T3 (>50mm)	22/380	5.79
	Unknown	22	
NPI	<3.5	128/344	37.21
	3.5-5.5	106/344	30.81
	5.5+	110/344	31.98
	Missing	58	
AGE	<50 years	73/402	36.5
	>50 years	328/402	63.5

Grade = Bloom and Richardson grade
Nodal Status = Number of positive nodes
NPI = Nottingham Prognostic Index = (size in cm)0.2 + Grade + Nodal Status

Appendix 2

Raw data for Her 2 FISH and Bcl-2, Bax, pBad (Ser 112), Bcl-xl, pP70S6K (Thr 389), P70S6K, PI3K and pP90RSK (Thr 363/Ser 359) histoscores

Key:

Path no.	Pathology number
Her 2 F	Her 2 FISH scores
PTEN n	PTEN nuclear histoscores
PTEN c	PTEN cytoplasmic histoscores
Bcl-2 c	Bcl-2 cytoplasmic histoscores
Bax c	Bax cytoplasmic histoscores
Bad c	Bad cytoplasmic histoscores
pBad c	pBad (Ser 112) cytoplasmic histoscores
Bcl-xl c	Bcl-xl cytoplasmic histoscores
pP70S6K	pP70S6K nuclear histoscores
P70S6K n	P70S6K nuclear histoscores
P70S6K c	P70S6K cytoplasmic histoscores
PI3K	PI3K cytoplasmic histoscores
pP90RSK	pP90RSK cytoplasmic histoscores

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
8008137.		0	0	20	0	14	8	13.33
8207635	0.96	10	33	35	66	94	4	124
8304707	0.98	65	75	11	58	96	8	116
8310775	0.98	10	13.33	60	13	72	0	41
8400199	1.02	45	36.67	129	70	142	60	87.5
8400387	1.23	0	16.67	97	0	34	0	112.5
8408902	0.96	35	10	36	14	54	0	93.33
8501076	0.89	0	15	23	35	86.67	13	145
8505021	1.25	16.67	33	74	6	95	4	47
8505709	0.97	60	60	28	30	20	6	73.33
8507604.	.	.	.	.	.	.	.	
8507901	1.16	40	36.67	0	51.67	133.33	13	141.67
8510490	1.11	35	36.67	33	33	87	53	175
8610595	1.01	75	50	98	30	96	62	91.25
8612132	1.06	20	60	0	55	56.67	35	143.33
8700790	1.06	5	45	53	0.		0	112.5
8701087	2.11	68.33	93.33	87	89	90	84	91.25
8704950	1.04	0	50	7	0	60	43	165
8705943	1.15	0	0	60	40	48	0	65
8706410.	.	.		0.	.	.	.	
8708872	0.85	0	0	10.		36.67.		110
8709046	1.17	70	56.67	33	45	80	28	23.33
8709490	1.24	13.33	26.67	30	17	55	12	117.5
8711800	1.02	20	30	0	10	33.33	3	150
8800033	1.13	55	70	44	42	110	100	72.5
8802163	0.99	20	10	80	0	24	7	10
8803618.		55	50.	.		33.33.	.	
8804484	1.05	73.33	90	85	0	0	0	111.67
8806919	1.2	0	20	50	60	10	0	150
8808120	1.19	0	0.		33	134	32	106
8808858	1.06	66.67	110	112	82	153	6	140
8809213	1.02	10	30	0	33	93	40	190
8900716	1.2	36.67	56.67	126	90	101	47	98.75
8901273	1.13	93.33	190	95	97	56.67	67	195
8901400	1.15	81.67	140	113	43	23.33	37	166.67
8904878	1.13	10	25	42	30	74	57	35
8907245	1.17	80	120	20	40.	.		40
8907716	1.55	5	0	17	60	7	43	190
8908418	1.27	78.33	66.67	128	0	0	0	176.67
8909319.		2.5	5	30	10	20	20.	
8909417.	.	.	.	.		52	28	0
8909430	1.12	80	123.33	153	26.67	10	7	166.67
8910355	0.9.	.		0	7.		0	110
8910865.	.	.	.	.		0.	.	
8911078.	.	.		0.		53.	.	
8911083	1.29	25	95.	.	.	.		0
8911515.		25	110	74	15	42	116	72.5
8912036.	.	.	.	.	.	.	.	
9000072.		0	0.		43.33	0	0.	

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9000073.	.	.	.	.	.	.	.	
9003516	2.64	16.67	30	66	26	66	4	37.5
9003988	0.92.	.		0	33.33	50	0.	
9004119	0.95	50	75	7	13	70	10	190
9004589	1.09	96.67	136.67	140	7	66.67	0	125
9005059	1.06	93.33	93.33	80	0	180	33	205
9005575	1.09	70	126.67	95	10	93.33	10	156.67
9005576	1.1	11.67	128.33	20	43.33	0	3	156.67
9005607	1.25	23.33	43.33	76	40	96	4	82.5
9006603	1.	.		20.	.	.	.	
9006722	1.1	85	170	104	90	60	0	153.33
9007890	1.08	25	25	17	3.33	53.33	17	182.5
9007905	1.07.	.		20	60	50	40	200
9007996	1.12	0	33	44	34	82	4	116.25
9010055	1.14	16.67	33	179	34	58	4	71.25
9010281	1.17	0	0	0	0	33.33	0	150
9011029.	.	.	.	.	.	.	.	
9011054	6.41	13.33	26.67	4	40	46	20	121.25
9011814	0.98	50	50	4	38	143	96	74
9012080	1.09	60	30	23	50	133	33	190
9100036.		150	50	0	23	37	17	175
9100823	1.03	33.33	16.67	20	14	92	12	82
9101094.	.	.	.	.	.	.	.	
9101357	1.18	10	25	58	26	134	82	70
9101891.	.	.	.	.	.	.	.	
9102250	1.05	16.67	33	142	38	144	26	52
9102372.	.	.	.	.		0.	.	
9102602	1.2	30	45	86	26.67	10	0	170
9102889	1.09.	.		55	10	105	42	82.5
9103112	1.15	3.33	23.33	132	68	107	35	81
9103262	1.08	0	0	0	33	50	0	170
9103744	1.39.	.	.	.	.	.	.	
9104030.		0	0	0	17	50	0.	
9104337	1.12	11.67	60	75	0	0	3	97.5
9105576	1.12	25	65	32	62	36	54	128.75
9106709	4.55	60	50	10	88	80	51	0
9106712.	.	.	.	.	.	.		0
9106880	1.09	0	50	48	4.	.		0
9107442	1.17	100	83.33	10.		150.		185
9108014	1.13.		10	90	5	73	4	76.67
9109046	0.97	0	0	17.	.	.		170
9109185	2.59	66.67	53.33	148	66	105	40	96.25
9109446	1.12	48.33	53.33	128	17	16.67	0	190
9109520	1.1	16.67	36.67	152	40	87	54	106
9110219	2.35	0	96.67	0	33.33	66.67	0	183.33
9110376	1.04	45	60	23	63	87	33	200
9110864.		130	150	13	67	67	67	205
9200106	1.26	30	40	4	70	144	29	110
9200946.	.	.	.	.	.	.	.	

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9201756	1.13	0	43.33	130	7	84	8	130
9201866	1.2	10	25	150	88	134	78	141.25
9202007	1.05	0	0	13.		30.		215
9202261	1.1	52.5	95	103	0	0	3	190
9202681	0.98	0	0	17	40	73.33	8	200
9202922	1.1	81.67	123.33	70	17	123.33	95	106.67
9203176.	.	.	.		33.33.		0.	
9203184	1.13	10	73.33	133	27	0	0	186.67
9203254	1.03	40	25	52	20	60	40	128.75
9203590	1.1	5	20	13	40	46.67	0	215
9203954	3.93	0	0	5	4	75	0	45
9204054	1.11	0	20	30	33.33	40	38	190
9204266	0.96	0	0	0	10	0	7	220
9204364	1.13	98.33	180	158	3	10	23	195
9204491	1.14	61.67	130	113	33	0	30	188.33
9204693	1.14	10	2.5	23	27	77	20	200
9204716	0.93	16.67	6.67	27	73	57	0	198.33
9204717	1.18	65	83.33	163	33	66.67	0	146.67
9205324	1.14.	.	.	.	.	.	.	
9205594	1.11	100	115	35	0	0	0	185
9205687	1.02	21.67	35	0	13.33	116.67	0	200
9205835	1.14	0	0	17	20	53	50	205
9206189	0.99	0	30	28	4	69	0	33.33
9207128	6.96	6.67	26.67	67	96	132	89	139
9207359.	.	.	.	.	.	.	.	
9207829	0.97	25	23.33	144	45	88	16	136
9208073	1.35	122.5	105	85.		36.67.		200
9208160	1.14	10	10	64	34	72.		33.33
9208255	0.97.	.		10	16.67	33.33	27	170
9208691	1.1	15	12.5	95	0	6.67	30	170
9208751.		0	0.		40.		0.	
9208764.		0	50	0.	.	.	.	
9208915	1.05	75	80	23	40	70	32	120
9209466	1.41	0	10	143	52	58	20	41.67
9209746	1.19	30	0	3	6.67	40	13	175
9210214	1.15	0	0	17	16.67	76.67	32	210
9210338	1.76	10	5	37	116.67	40	35	156.67
9211015	1.32	33.33	16.67	169	28	108	0	108
9211284	1.16	33.33	33.33	88	53	106	36	118
9211307	1.22	16.67	13.33	4	24	114	2	63
9300038	1.18	50	40.		10	30	4	78.33
9300203	1.2	75	115	5	44	82	68	105
9300414	1.21	40	100	138	92	139	122	95
9300505	1.06	0	10	7	20	86.67	0	195
9300517	1.11	30	0	0	33	80	0	200
9300806	1.03.	.	.	.	.	.	.	
9300946	1.02	6.67	43.33	36	50	154	110	78
9301463	1.11	50	20	0	23	23	3	200
9301496	1.18	10	25	10	46.67	80	0	181.67

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9301709	1.16	33.33	23.33	136	62	67	34	47.5
9301742	1.11	25	25	68	0	38	12	37.5
9302004	1.21	3.33	10	37	46.67	96.67	30	183.33
9302281	1.3	30	30.		63	60	27	175
9303058.	.	.		7.	.	.	.	
9303305	1.15	40	16.67	172	22	101	12	103.75
9303334	1.16	0	0	0	37	17	0	200
9303835	1.04	0	0.	.	.	.		0
9303848	1.02	92.5	75	30	16.67	66.67	0	170
9303995	0.97	58.33	73.33	146	60	152	20	106.25
9304892	1.04	57.5	77.5	0	33	43	63	200
9305263	1.05	45	75	158	13	0	37	178.33
9305697	1.07	55	35	33	70	46.67	20	200
9305719.	.	.	.	.	.	.	.	
9305937	6.75	35	20	17	73	35	17	215
9305972	0.98	0	0	0	0.		0	200
9306294	6.49.	.		73	0	0	0	192.5
9306938.	.	.	.	.	.	.	.	
9308293	0.93.	.		13.		0.		60
9308295	1.03	30	38.33	47	23.33	101.67	33	163.33
9309731	1.01	25	55	40	71	110	58	63.75
9309739	1.23	42.5	75	114	20	80	76	43.33
9310797.	.	.	.	.	.	.	.	
9310816.		0	0	0.		0.	.	
9310859	1.11	3.33	23.33	161	17	96	10	36
9311333.		0	30	40	0	37	0	36.67
9311343	0.99	51.67	80	114	108	138	46	97.5
9312194	1.11	63.33	70	133	3	23.33	0	115
9312208	1.13	3.33	23.33	152	14	122	19	94
9312498	6.69	40	23.33	120	8	82	25	100
9400251	1.15	35	25	20	0	13	10	170
9401076	1.06	43.33	46.67	5	73.33	50	80	198.33
9401443.		30	50	17.	.	.	.	
9401646	1.08	30	35	13	53	67	43	207.5
9401940	1.48	10	40	104	17	6.67	0	148.33
9403539	1.11	0	10	92	35	86	20	26.67
9403796	1.14	98.33	93.33	32	63.33	43.33	60	177.5
9404046	1.05	100	100	0.		33.33.		157.5
9404065	1.41	113.33	90	156	37	53.33	53	180
9404346	1.18	47.5	50	77	40	56.67	33	142.5
9404349	1.08	0	0.		0	0	0	110
9404604	1.1	33.33	26.67	33	57	33	27	200
9405719	1.07.	.	.		0	33	0.	
9405958	1.01	81.67	116.67	105	35	23.33	55	190
9405960.	.	.	.	.	.	.	.	
9406536	1.05.	.		0.		0.		142.5
9406665	0.99	60	50	34.		64	2	26.67
9406692	1.1	28.33	33.33	134	27	135	12	48
9406790.		60	50.		16.67	36.67	0	215

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9406805	1.12	23.33	26.67	153	51	178	82	106
9407111	1.16	46.67	66.67	190	86	202	100	137
9408178	1.14	102.5	125	0	20	56.67	70	205
9408557.		0.		0	3	17	10	205
9408685	5.56	80	50	0	23.33	60	13.	
9409104	1.09	41.67	83.33	99	10	16.67	0.	
9409109	1.05	100	130	10	33.33	16.67	23	170
9409112	1.11	75	50.		0	33.33	0	210
9409346.	.	.	.	.	.	.	.	
9409784	1.04	45	25	0	43.33	50	0	190
9410051.		10	53.33	53	70	73.33	95	122.5
9410252.		0	25	27	0.		0	200
9410430	1.2	63.33	10	155	3	0	3	153.33
9410568	1.1	0	0	0	6.67	103.33	0	158.33
9410701	1.13	0	0.		0.		0	20
9410706	1.27	0	23.33	23	0	83.33	10	135
9410823.		0	0	0	0	100	3	130
9410890.		25	15	7.	.	.		200
9410906	1.04	0	0	17	0	50	0	92.5
9410953	1.04	35	150	113	17	0	3	190
9411100.		10	0	0	0	60	33	65
9411449	0.98	0	3.33	63	50	110	38	135
9411476	1.15	0	50	13	30	60	27	120
9411819	2.6	0	0	0	0	13.33	0	130
9412034.		0	0.		10	50	0	100
9412265	3.63	23.33	20	97	40	130	0	90
9412269	1.85	0	20	13	27	50	37	120
9412313	1.14	105	70	133	10	93.33	7	166.67
9412422	3.65	25	20	10	0	53.33	33	135
9412614	3.88	50	50	0	3.33	56.67	0	110
9413539	1.01	72.5	100	108	115	170	57	120
9500073.		20	140	33	37	0	0.	
9500228	2.15.	.		0.	.	.	.	
9500763	1.27	0	0	17.	.	.		170
9500955.		0	0	0	0	7	13.	
9501106	6.42	68.33	80	118	20	46.67	3	141.67
9501108	1	40	20	47	53.33	66.67	55	70
9501958	1.01	43.33	33.33	0	7	90	17	113.33
9501978	3.2	20	16.67	37	67	113	67	170
9502003	3.9.	.	.	.	.	.	.	
9502205	1.13	0	0	33	27	90	55	117.5
9502318	1.24	0	0	7	0	47	0	105
9502659	1.09	53.33	43.33	139	0	0	3	137.5
9502985	1.23	0	0	33	30	30	20	180
9503407	0.98	0	0	17	0	43.33	0	100
9503884	1.13	35	110	10	77	123	40	165
9504207	1.19	80	123.33	76	50	40	20	146.67
9504214	3.53	10	35	10	47	73	17	110
9504389	3.8.	.	.	.	.	.		160

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9505535	1.15	20	16.67	93	0	66.67	37	100
9505600	1.16	31.67	16.67	92	13	50	7	130
9506010	0.95	26.67	33.33	67	10	43.33	0	110
9506050	1.12	0	0	33	0	33.33	33	110
9507243	1.11	50	70.		17	33.33	0	85
9507433.		2.5	0	17.		33.33	7	23.33
9507574	1.2	95	123.33	38	43	33.33	23	153.33
9507835	2.39	60	50	57	33.33	90	57	103.33
9509140	3.8	26.67	58.33	7	90	116.67	7	90
9510723.		85	96.67	90	63	153.33	20	72.5
9510764	1.14	0	16.67	33	35	100	27	90
9511536	0.99	30	46.67	100	77	80	10	125
9511736	8.03	16.67	53.33	13	67	103.33	0	101.67
9511896	1.12	0	0.		3	60	27	53.33
9512307	1.06	26.67	16.67	108	17	90	3	90
9512526	1.16	10	20	93	26.67	100	0	176.67
9512539	1.06	0	13.33	153	23	26.67	0	156.67
9512595	1.05	16.67	23.33	105	67	153.33	23	175
9513175	1.18	13.33	6.67	98	37	120	0	93.33
9514124	1.16	0	50	100	33	0	0	107.5
9514572	1.34	68.33	35	63	48	40	7	125
9600346	1.09	26.67	23.33	77	10	83.33	0	161.67
9601048	1.11	20	40	123	40	100	0	126.67
9601571	1.21	76.67	120	73	26.67	90	43	127.5
9601591	1.03	0	0	0	0.		0	90
9601927.		0	0	17	7	33.33	0	125
9602066	1.15	5	0	53	0	73	0	155
9602566.		55	165	133	23.33	16.67	0	142.5
9602691	1.1	23.33	20	30	0	153	3	161.67
9603454	1	20	50	20.		13.		200
9603640	1.18	30	25	0	67	187	3	160
9603856	1.29	15	30	0	36.67	93.33	57	130
9603877	1.14	0	20	20	27	67	0	210
9603882	1.44	0	10	63	7	97	0	153.33
9604092	1.08	16.67	0	13	0	67	0	106.67
9604189	1.25	5	0.	.		0.	.	
9605089	1	75	86.67	154	33	20	0	158.33
9605413	1.05	30	20	20	3.33	73.33	33	140
9605556	1.78	3.33	43.33	0	32	160	0	196.67
9606350.		0	0	50	0	73	0	165
9607449	1.13	0	0	37	20	73.33	0	200
9608209	1.07	25	15	43	43.33	43.33	0	155
9608671	1.03	0	110	109	96	130	77	125
9609194	1.13	13.33	20	90	36.67	130	0	160
9609268	0.97.	.	.		0	0.	.	
9609341	1.55.	.		0.	.	.	.	
9609370	0.98	115	146.67	129	140	26.67	120	186.67
9610658	1.06	20	46.67	83	30	76.67	35	170
9610680	1.08	10	20	97	20	93.33	0	190

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9610687	1.16	95	66.67	80	30	116.67	38	150
9611280	1.24	10	6.67	0	0	97	38	183.33
9611545	1.09	0	25	42	20.		10	205
9611561	1.11	5	0	17	83	87	17	95
9612201	3.6	16.67	13.33	0	33.33	50	7	105
9612530	1.69	35	25	73	0	33.33	0	156.67
9612556	1.1	0	33.33	7	13	120	3	145
9612761	1.08	16.67	13.33	110	30	103.33	23	180
9613408	1.18.	.		38.	.	.	.	
9613574	1.23	43.33	70	83	20	23	33	185
9613576	1.05	0	0	33.		23.		120
9613866	1.04	0	0	17	0	33	28.	
9613959	1.08	26.67	73.33	60	97	157	60	195
9614053	1.18	43.33	50	97	10	120	40	190
9614055	1.18	0	0	50	3	143	0	155
9614063	1.07	5	80	111	0	0	0	170
9614565	1.25	10	0	90	0	83	0	160
9614775	1.09.	.	.		0	6.67.		205
9614882.		200	200	50.	.	.	.	
9615087	1.45	0	0	30	3	53	40	145
9615245.	.	.	.		3	10	7.	
9615255	1.08	26.67	16.67	120	13	57	0	170
9615632	1.31.	.		40	0.		0.	
9615643	1.02	20	16.67	67	0	76.67	0	186.67
9700110	1.16	25	50	87	10	50	0	200
9701798	1.04	0	10	17.	.	.		180
9702089	1.02	0	30	17	0	110	0	180
9702111	1.08	6.67	33.33	115	23.33	146.67	70	186.67
9702489	1.11	0	0	10	0	66.67	0	45
9702830	1.08	75	50	33	50	150	0	143.33
9703425	3.51	40	80	23	83	200	58	150
9703943	1.06	40	43.33	47	13	100	77	146.67
9704092	1.28	25	57.5	45	43	36.67	0	165
9704237	1.05	6.67	40	63	90	156.67	78	200
9704247	1.29	0	0	43	13	113	0	155
9704740	1.18	35	35	43	0	80	47	173.33
9704850	1.13	0	0	27	0	50	0	185
9704894	1.23	10	26.67	63	72	133	67	176.67
9705111	1.1	5	50	0	61.67	96.67	0	165
9705250	1.06	63.33	76.67	27	67	150	73	146.67
9705308	1.16	0	25	0	17	117	0	195
9705652	1.06	43.33	50	23	27	67	0	200
9706167	2.6	70	50	0	0	66.67	0	180
9706176.		0	20	0	16.67	83.33	0	170
9706414	1.16	0	0	53	0	73.33	0	200
9706430	1.4	30	25	40	20	66.67	0	120
9707062	1.31	3.33	0	0	3.33	73.33	0	163.33
9707685	2.46	0	0.		0.		0	135
9707724.	.	.	.	.		33.33.		100

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9707729	3.77	23.33	20	120	0	70	0	170
9708066	1.18	0	0	27	10	66.67	0	50
9708349.	.	.		0.	.	.	.	
9708498	3.29	42.5	70	147	0	60	33	160
9708537	0.98	0	0	20	0	10	0	200
9708552	1.14	26.67	20	133	0	86.67	0	140
9709182	1.23	23.33	40	30	123	173	43	166.67
9709188	1.14	36.67	50	77	36.67	133.33	47	136.67
9709380	1.07	61.67	170	174	113	60	3	140
9709398	1.17	16.67	50	77	36.67	83.33	37	193.33
9709804.	.	.		23	0	33.33	0	140
9709871	1.23	10	20	43	17	50	0	140
9710196	1.91	0	0	33	0	90	10	126.67
9710733	1.17	6.67	0	140	93	147	30	100
9711814	1.24	30	53.33	87	63.33	96.67	95	156.67
9712045	1.21	0	0	33	10	47	0	135
9712067	1.15	50	80	38	50	143.33	65	126.67
9712386	1.1	83.33	50	60	90	120	95	126.67
9712389	1.1	45	25	40	30	87	0	143.33
9712913	1.22	0	0	83	16.67	76.67	0	143.33
9713464	1.74	0	0	47	13	127	0	130
9714535	1.03	0	0	28	20	43	0	100
9714864	1.27	0	0	93	20	80	0	153.33
9715190	1.21	0	33.33	10	106.67	170	103	183.33
9715210	1.01	105	160	124	53	23.33	37	153.33
9715546	1.23	0	0	23	63	143	40	156.67
9715565	1.38.	.	.	.	.	.	.	
9715848	0.95	30	42.5	0	70	67	60	152.5
9800088	1.31	70	75	38	40	0	50	120
9800098.		15	50	60	0.		0	50
9800394.	.	.	.	.	.	.	.	
9800402.		60	60	5	40	16.67	0	125
9800752	1.2	46.67	100.		73	133.33	33	113.33
9800760.		0	0	60	0	0	3.	
9801850	1.04	26.67	20	53	33.33	110	0	85
9802148	1.12	75	20	70	10	33.33	0	115
9802155	1.14	36.67	40	92	10	86.67	17	85
9802172	4.06	23.33	40	10	33	143.33	7	160
9802362	1.15	20	70	21	10	0	0	140
9802721	2.55	33.33	56.67	125	97	56.67	3	155
9802841	1.11	58.33	81.67	70	75	110	57	148.33
9803066	1.01	30	33.33	72	20	56.67	0	106.67
9804234	1.02	53.33	61.67	93	100	100	110	145
9804284	1.23	65	50	20	26.67	33.33	20	65
9805156	1.11	10	10	40	0	30	0	97.5
9805159	1.28	3.33	6.67	27	7	53.33	0	90
9806071	1.13	0	0	7	0	33.33	0	30
9806110	1.19	0	0	160	7	83.33	0	110

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9806239	1.09	23.33	63.33	107	6.67	116.67	43	117.5
9806264.		17.5	35	53	50	73.33	0	93.33
9806475.		19.5	25	80	0	16.67	0	105
9806498	1.08	73.33	86.67	100	80	126.67	0	153.33
9807077.		45	50	20	0	30	0	60
9807270	1.14	0	50.	.	.	.	.	
9807703	1.28	16.67	0	75	60	120	0	105
9808040	1.86	6.67	0	90	0	3.33	0	103.33
9808389	1.06	6.67	43.33	43	10	96.67	0	106.67
9808452	1.03	6.67	6.67	153	0	116.67	0	80
9808762	1.03	36.67	40	93	20	70	0	73.33
9809087	1.45	10	0	17	0	50.		87.5
9809105	1.17	16.67	53.33	27	70	100	37	108.33
9809341	1.23	23.33	43.33	133	77	113.33	7	132.5
9809686	1.11	68.33	73.33	110	87	16.67	0	158.33
9810005	1.06	6.67	20	180	3.		0	108.33
9810111	1.1	53.33	60	33	45	90	0	128.33
9810371	1.11	58.33	60	170	66.67	106.67	40	100
9810382	1.15	56.67	40	30	0	33.33.		95
9810399.	.	.		0	33.33.	.	.	
9810674	1.04	51.67	76.67	129	33	43.33	7	160
9810735	5.66	20	63.33	105	80	13.33	10	200
9811712	1.33	27.5	35	13	16.67	40	0	105
9811724	1.15	56.67	66.67	57	76.67	81.67	0	117.5
9811988	1.06	53.33	56.67	150	76.67	136.67	10	140
9812002	1.15	63.33	46.67	97	66.67	36.67	3	128.33
9812059	1.01	83.33	86.67	43	66.67	140	0	143.33
9812603	1.12	105	143.33	165	153	16.67	40	176.67
9812688	1.37	26.67	23.33	120	66.67	50	3	105
9813075	1.14	15	0	57.	.		0	35
9814086	1.52	26.67	26.67	117	0	86.67	10	153.33
9814456	1.11	20	0	23	0	73.33	0	156.67
9814740	1.11	60	36.67	48	13	116.67	17	93.33
9814760	2.91	0	50	60.		80	0	87.5
9815130	1.06	78.33	26.67	95	33.33	63.33	3	81.67
9815137	1.03	101.67	86.67	115	13.33	0	23	155
9815874	1	90	80	47	50	66.67	33	95
9815885	1.32	60	42.5	133	83.33	0	0	200
9815921.		30	130	67.	.	.		60
9815991	1.05	45	30	142	43.33	36.67	0	70
9816264	1.32	46.67	53.33	103	40	50	3	200
9816428.	.	.	.	.	.	.	.	
9816701.		115	60	120	16.67	16.67	0	110
9816797	1.2	31.67	76.67	43	73.33	60	15	163.33
9816834	1.14	18.33	10	143	36.67	90	0	106.67
9900235	1.24	33.33	93.33	123	0	66.67	17	200
9900879.		27.5	30	120	50	123.33	43	163.33
9901023.		7.5	25	107	0	43.33	10	115
9901042	1.04	85	83.33	132	17	126.67	17	121.67

Path no.	Her2 F	PTEN n	PTEN c	Bcl-2 c	Bax c	Bad c	pBad c	Bcl-xl c
9901655	1.22	66.67	96.67	77	10	133.33	27	150
9901847	1.36	62.5	50	170	0	73.33	3	170
9902012	1.27	78.33	93.33	117	77	105	37	146.67
9902576	1.24	86.67	133.33	33	135	100	87	145
9902708	1.67	96.67	130	188	77	23.33	20	151.67
9903328.		40	0	173	67	23.33.		165
9903476	1.13	95	90	133	87	126.67	7	190
9903477	1.05	53.33	126.67	183	113	180	43	173.33
9903684.		50	20	200	17	76.67	0	163.33
9905581	1.05	47.5	45	73	0	33.33	0	90
9905633	1.05	20	40	87	46.67	56.67	0	200
9905838	1.09	33.33	40	97	27	50	0	111.67
9906151	1.24	40	16.67	87	33	66.67	0	33.33
9906165	1.05	123.33	96.67	78	53	43.33	0	173.33
9906168	1.17	58.33	63.33	110	43	150	47	163.33
9910349	1.03	0	0	47.	.	.		140

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
8008137.		40	10	0	0
8207635	6.67	0	18	123	50
8304707	0	0	2	101	0
8310775	0	0	40	130	75
8400199	81.67	18	45	131	17.5
8400367	0	25	33	69	73
8408902	75	10	8	108	75
8501076	50	0	0	50	70
8505021	43.33	53	51	75	108
8505709	10	0	20	123	165
8507604.		20.	.	.	
8507901	37	75	160	30	13
8510490	37	73	123	13	50
8610595	67.5	0	47	99	35
8612132	27	0	103	0	30
8700790	42.5.	.		60	45
8701087	0	0	30	56	128
8704950	43	0	115	15	25
8705943	55.		0.	.	
8706410.	.	.	.	.	
8708872.		0.	.	.	
8709046	105	20	20	95	80
8709490	15	20	50.		80
8711800	45	0	30	0	70
8800033	5	5	25	83	18
8802163.		0	0.	.	
8803618.	.	.	.	.	
8804484	13.33	0	0	0	0
8806919	30.	.	.	.	
8808120	32.5.		0	129	0
8808858	0	180	104	135	52
8809213	25	50	115	58	30
8900716	132.5	0	43	143	83
8901273	75	7	110	167	135
8901400	26.67	0	100	157	138
8904878	0	0	33.		10
8907245	0	0	23.		0
8907716	98	75	100	20	113
8908418	40	20	100	160	60
8909319	40.	.		0	90
8909417.	.	.	.	.	
8909430	40	3	83	160	50
8910355	0.	.	.	.	
8910865.	.	.	.	.	
8911078.	.	.	.	.	
8911083.	.	.	.	.	
8911515	40	35	55	115	30
8912036.	.	.	.		20
9000072	0.	.	.	.	

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9000073.		20.	.	.	
9003516	10	10	58	64	101
9003988	0	0	140	0	25
9004119	55	10	110	80	65
9004589	86.67	0	105	163	147
9005059	85	52	157	53	167
9005575	76.67	0	57	148	128
9005576	3.33	0	160	180	45
9005607	0	13	50	124	62
9006603.	.	.	.	.	
9006722	3.33	7	123	130	52
9007890	10	0	20	0.	
9007905	83	0	80	25	95
9007996	0	17	70	103	90
9010055	0	0	24	44	44
9010281	90	20	180	0.	
9011029.	.	.	.	.	
9011054	70	38	25	80	118
9011814	75	50	43	116	188
9012080	8	0	130	55	30
9100036	75	0	140	105	170
9100823	0	7	22	59	35
9101094.		0.	.	.	
9101357	90	30	48	118	82
9101891.	.	.	.	.	
9102250	3.33	0	22	70	77
9102372.	.	.	.	.	
9102602	10	0	130	120	88
9102889	75	0	13	80	100
9103112	25	20	46	113	73
9103262	73	10	35	38	95
9103744.	.	.	.	.	
9104030	0.	.	.	.	
9104337	0	0	0	50	83
9105576	10	0	27	0	0
9106709.		120	60	83	160
9106712.	.	.	.	.	
9106880.		15	43.	.	
9107442.		12.	.		123
9108014	0.		0	50	107
9109046.	.	.	.	.	
9109185	107.5	7	28	94	90
9109446	55	17	80	123	22
9109520	56.67	0	50	59	135
9110219	0.	.	.		0
9110376	63	0	100	50	160
9110864	0.	.		115.	
9200106	0	0	8	95	136
9200946.	.	.	.	.	

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9201756	20	5	50	75	49
9201866	101.67	10	40	99	138
9202007.	.	.	.	.	
9202261	60	0	50	90	25
9202681	0	0	20	0.	
9202922	30	0	17	72	100
9203176.	.	.	.	.	
9203184	75	15	133	153	17
9203254	115.		0	50	30
9203590	15	0	140	90	110
9203954	0	3	0	80	0
9204054	53	0	70	20	110
9204266	0.	.		0	0
9204364	26.67	7	67	98	95
9204491	15	45	145	150	130
9204693	10	0	180	10	170
9204716	48	48	103	35	127
9204717	0	15	60	120	63
9205324.	.	.	.	.	
9205594	55.	.		30	155
9205687	5	0	0	13	0
9205835	53.	.		140	205
9206189	0	0	0	16	0
9207128	50	40	49	98	100
9207359.	.	.	.	.	
9207829	6.67	33	46	73	77
9208073	67.5	80.	.		135
9208160	0.		0.	.	
9208255	10	15	100	0	150
9208691	35	20	140	150	130
9208751	0.	.	.	.	
9208764.	.	.	.	.	
9208915	30	135	165	53	180
9209466	0	45	50.		110
9209746	15	17	67	0.	
9210214	45	20	130	45	180
9210338	70	70	100	12	170
9211015	0	7	14	94	106
9211284	30	27	23	81	122
9211307	0	0	4	51	33
9300038	0	30	20	15	75
9300203	25	0	0	120	133
9300414	135.		0	90	130
9300505	8	40	80	0	105
9300517	5	95	185	20	165
9300806.	.	.	.	.	
9300946	0	0	6	35	73
9301463	0	70	100	8.	
9301496	3	20	105	0	27

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9301709	87.5	10	56	71	123
9301742	0	0	23	33	100
9302004	70	50	130	37	135
9302281	15	0	40	100	65
9303058.	.	.	.	.	
9303305	132.5	73	53	93	95
9303334	80	0	50	0	90
9303835.	.	.	.	.	
9303848	13	0	0	35	105
9303995	0	0	8	101	10
9304892	80	20	70	130	148
9305263	45	15	130	140	143
9305697	53	35	68	75	193
9305719.	.	.	.	.	
9305937	53	30	105	68	140
9305972	0.	.		0.	
9306294.	.	.		0	0
9306938.	.	.	.	.	
9308293.		0.	.	.	
9308295	40	0	80	27	90
9309731	0	0	38	43	30
9309739	0	0	10	143	30
9310797.	.	.	.		40
9310816.	.	.	.	.	
9310859	30	27	34	98	5
9311333	0	0	0.		10
9311343	0	10	27	75	44
9312194	70	0	87	103	137
9312208	53.33	23	44	100	112
9312498	17.5	60	42	83	113
9400251	13	65	150	10	148
9401076	50	20	143	82	150
9401443.	.	.	.	.	
9401646	38	0	115	130	50
9401940	40	73	97	173	28
9403539	155	15	53.		160
9403796	30	30	145	30	115
9404046.	.	.	.		0
9404065	98.33	60	65	177	97
9404346	55	8	110	60	123
9404349	0	0	0	0.	
9404604	50	0	115	30	55
9405719.	.	.	.	.	
9405958	106.67	3	93	163	138
9405960.	.	.	.	.	
9406536.	.	.	.	.	
9406665	25	10.		110	18
9406692	41.67	10	73	61	131
9406790	40	120	200	100	180

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9406805	56.67	42	40	116	143
9407111	40	0	45	100	104
9408178	30	50	100.		150
9408557.	.	.	.	.	
9408685	0	0	0	0	0
9409104	68.33	0	40	120	145
9409109	90	10	95	20	10
9409112	20.	.	.	.	
9409346.	.	.	.		180
9409784	50	30	100	30	110
9410051	37.5	10	90	0	37
9410252	20.	.	.		0
9410430	91.5	0	40	53	100
9410568	8.33.	.		30	0
9410701.	.	.	.	.	
9410706	0	0	180.		115
9410823	42.5	90	150.		78
9410890.	.	.	.	.	
9410906	0	20	20	0	35
9410953	37.5.	.		90	120
9411100	20.	.		0.	
9411449	68.33	0	120	30	90
9411476	10	0	100	40	50
9411819	0.	.	.		180
9412034.	.	.	.	.	
9412265	21.67	60	190	50	10
9412269	0	70	180	50	140
9412313	48.33	23	67	123	140
9412422	5	0	100.		55
9412614	0.	.		100.	
9413539	31.67	48	145	110	137
9500073	73.33.	.	.	.	
9500228.	.	.	.	.	
9500763.	.	.	.	.	
9500955	0	0	0	0	0
9501106	18.33	0	103	122	28
9501108	20	0	77	50	73
9501958	13.33	65	110	35	10
9501978	40	13	68	45	105
9502003.	.	.	.	.	
9502205	0	40	90.		180
9502318	0	0	100	65.	
9502659	60	30	40	43.	
9502985	45.	.	.	.	
9503407	2.5	0	93	0.	
9503884	0	0	135.		120
9504207	50	7	123	133	72
9504214	36.67	10	100	0.	
9504389.	.	.	.	.	

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9505535	0	0	10	33	20
9505600	0	10	40	8	0
9506010	0.	.	.		60
9506050	0	0	0	30	15
9507243	0.	.	.	.	
9507433.	.	.	.		0
9507574	100	13	80	143	117
9507835	0	40	70	7	52
9509140	3.33	0	20	45	55
9510723	0	0	13	57	115
9510764	0	0	70	58	100
9511536	20.	.		0	118
9511736	3.33	0	27	60	13
9511896	0	0	10	30	30
9512307	21.67	0	45	43	70
9512526	20	17	97	107	117
9512539	0	0	30	27	20
9512595	0	0	123	87	127
9513175	1.67	0	43	60	122
9514124	0.	.		20.	
9514572	15	15	25	75	123
9600346	3.33	0	50	30	5
9601048	23.33	0	67	113	70
9601571	25	0	60	75	60
9601591	0.	.	.		0
9601927	36.67.	.		75	40
9602066	5	58	130	0	0
9602566	5	0	100	108	20
9602691	31.67.	.		73.	
9603454.	.	.	.		30
9603840	0	5	0	63	7
9603856	40	90	120	37	80
9603877	0.	.		110	100
9603882	1.67	0	0	0	103
9604092	0	0	0	30	0
9604189.	.	.	.	.	
9605089	40	0	25	103	90
9605413	0	50	60	7	0
9605556	20	20	123	100	20
9606350	0.	.		0.	
9607449	30	0	10	0	40
9608209	20	0	15	60	10
9608671	0	0	0.		0
9609194	28.33	3	20	0	7
9609268	0.	.		70	0
9609341.	.	.	.	.	
9609370	65	7	127	173	138
9610658	75	15	140	23	148
9610680	5	0	15	5	0

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9610687	85	0	120	80.	
9611280	30	0	0	28	152
9611545	0	0	15	20	130
9611561	6.67	10	60	23	75
9612201	15	0	0	0	133
9612530	7.5	0	0	0	0
9612556	0	133	168	27	30
9612761	16.67	5	95	23	88
9613408	200.	.	.	.	
9613574	3.33	0	90	63	115
9613576.	.	.	.	.	
9613866	5	10	55	0	15
9613959	48.33	12	112	83	180
9614053	28.33	82	158	33	140
9614055	6.67	20	80	0	0
9614063	25.	.		115	150
9614565	15	0	50	70	117
9614775	65.	.		20.	
9614882	60.	.	.	.	
9615087	0	0	35	23	65
9615245	0.	.	.	.	
9615255	5	5	0	17	22
9615632.		80	140.	.	
9615643	3.33	75	100	0	25
9700110	0	0	70	0	30
9701798.	.	.	.	.	
9702089	0	68	100	0	7
9702111	0	13	123	33	78
9702489	0	30	40	0	10
9702830	8.33	15	90	63	38
9703425	15	25	100	23	163
9703943	55	10	50	33	115
9704092	42.5	0	60	25	60
9704237	48.33	50	180	70	150
9704247	5	20	85	100	10
9704740	80.	.		100.	
9704850.	.	.		0	85
9704894	5	3	147	43	88
9705111	0	0	0	80	20
9705250	8.33	7	52	30	55
9705308	0.	.	.	.	
9705652	10.	.		0	20
9706167	0.	.	.		100
9706176	0.	.		0	25
9706414	3.33	5	25	20	10
9706430	0	45	70	50	95
9707082	0	155	190	0	2
9707685	10.	.		0.	
9707724.	.	.	.	.	

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9707729	5	60	180	30	30
9708066	0.	.	.	.	
9708349.	.	.	.	.	
9708498	20.	.		100	40
9708537	0	50	110	0	50
9708552	0	0	80	0	10
9709182	26.67	13	33	53	98
9709188	31.67	27	67	43	113
9709380	36.67	0	30	83	128
9709398	31.67	8	163	73	35
9709804	0	40	130	0	0
9709871	0	10	70.		0
9710196	23.33	65	150	40	17
9710733	25	35	147	63	3
9711814	13.33	7	60	40	125
9712045	0	0	120	0	158
9712067	30	0	127	83	60
9712386	41.67	30	43	32	30
9712389	36.67	55	140	0	90
9712913	0	10	10	0	0
9713464	0	80	120	10	8
9714535	0.	.	.		0
9714864	3.33	17	37	7	53
9715190	18.33	10	125	97	120
9715210	48.33	0	85	137	140
9715546	11.67	10	135	20	103
9715565.		0.	.	.	
9715848	95	0	0	38	0
9800088	145	20	80	40	200
9800098	0.	.	.		20
9800394.	.	.	.	.	
9800402	85	7	120	83	90
9800752	31.67	0	47	95	30
9800760	0.	.		0	35
9801850	0	0	0	50	17
9802148	0.	.	.	.	
9802155	5	0	20	0	25
9802172	50	90	160	100	150
9802362	35	10	80	70	113
9802721	0	0	0	50	0
9802841	63.33	7	53	60	150
9803066	0.	.		15	0
9804234	13.33	0	50	93	113
9804284	20	0	0	0	0
9805156	0	50	30	25	35
9805159	0	0	0	0	10
9806071	0	0	40	50	10
9806110	20	32	33	20	78
9806239	0	7	107	73	153

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9806264	30	60	120	10	5
9806475	0.	.	.	.	
9806498	3.33	13	57	70	0
9807077	30.	.		50	15
9807270.	.	.	.		0
9807703	0	0	27	27	30
9808040	0	3	5	0	50
9808389	0.	.		0	5
9808452	0	20	53	63	8
9808762	0	0	0	17	17
9809087	0	0	0.	.	
9809105	0	0	87	85	160
9809341	3.33	35	85	43	38
9809686	60	40	107	105	175
9810005	1.67	0	0	0	0
9810111	0.	.		57	8
9810371	0	0	100	90	100
9810382	0	0	30.	.	
9810399.	.	.	.	.	
9810674	1.67	0	30	90	17
9810735	160	23	40	108	130
9811712	0	0	10	10	5
9811724	0	30	67	7	88
9811988	0	3	133	37	12
9812002	8.33	20	120	43	102
9812059	0	10	50	57	2
9812603	176.67	5	15	137	135
9812688	0	0	0.		0
9813075.	.	.	.		40
9814086	25	15	57	33	55
9814456	56.67.	.	.		75
9814740	25	90	130	70	130
9814780.	.	.	.		15
9815130	0	30	80	10	38
9815137	7.5	20	85	113	50
9815874	3.33	0	45	35	65
9815885.	.	.	.	.	
9815921.	.	.	.	.	
9815991	0	15	80	50	70
9816264	36.67	27	83	57	43
9816428.	.	.	.	.	
9816701	0.	.	.		0
9816797	21.67	0	67	87	88
9816834	0	25	88	60	48
9900235	0	0	30	80	50
9900879	26.67	20	150	115	90
9901023	40.	.		50	10
9901042	36.67	183	180	57	60
9901655	0	7	60	43	38

Path no.	pP70S6K	P70S6K n	P70S6K c	PI3K	pP90RSK
9901847	12.5	13	43	55	78
9902012	98.33	0	110	48	85
9902576	0	0	50	40	90
9902708	5	7	93	73	30
9903328.		0	100	60.	
9903476	6.67	0	110	25	35
9903477	45	17	143	117	43
9903684	81.67	35	145	90	33
9905581	0	0	78	55	18
9905633	0	0	140	100	30
9905838	11.67	0	43	23	40
9906151	8.33	10	23	23	38
9906165	70	147	120	163	135
9906168	40	13	43	63	67
9910349.	.		40.	.	