ROLE OF OESTROGEN RECEPTOR BETA (ERβ) IN HORMONE RESPONSIVENESS IN BREAST CANCER

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine

By

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August 2010

Dedicated to my father and brother

DECLARATION

The material contained in this thesis has not been presented for any other degree or other qualification. This is my own work, except where technical assistance and collaboration with colleagues is acknowledged. The work was carried out at the JK Douglas Cancer Research Laboratory, Clatterbridge Cancer Research Trust, Wirral, during my appointment as a Research Fellow.

This work has been presented in the following meetings:

Correlation of mRNA for oestrogen receptor beta variants with outcome in tamoxifen treated breast cancer (*ASGBI Scientific Conference, Glasgow, Apr 2005).*

Quantitative analysis of oestrogen receptor beta splice variants in tamoxifen treated breast cancer (*BASO the Association for Cancer Surgery Annual Scientific Conference, London, Nov 2005*).

Expression of estrogen receptor beta variant mRNA in primary breast cancer – a potential role in predicting hormone responsiveness (*28th Annual San Antonio Breast Cancer Symposium, San Antonio, USA, Dec 2005).*

Some aspects of the work was published in the following paper:

Association of oestrogen receptor beta 2 (ERbeta2/ERbetacx) with outcome of adjuvant endocrine treatment for primary breast cancer – a retrospective study. (Original research article)
BMC Cancer.2007 July18;7(1):131

CONTENTS

ABSTRACT

Purpose: Oestrogen receptor beta (ERβ) has several isoforms which can act as modulators of ERα. Here ERβ isoforms were quantitated in breast cancer patients and related to outcome.

Experimental Design: mRNA of ERβ1 (full-length), C-terminal truncations (ERβ2/ERβcx, ERβ5) and exon deletions (ERβΔ5, ERβΔ3) were quantitated in 100 cases (70 ERα+ and 30 ERα-). ERβ2 immunostaining was carried out in 141 cases (98 ERα+ and 43 ERα-). All the breast cancer patients in this study were postmenopausal women treated with surgery and adjuvant endocrine therapy, but not chemotherapy or primary endocrine therapy.

Results: ERβ isoform mRNAs were differentially expressed in ERα+ and ERα- breast cancers; ERβ2 levels were higher whilst, ERβ5 and ERβ1 levels were lower in ERα+ cases. ERβ deletion variants were only detected in 20 cancers, mostly ERα-. In ERα- cases, high ERβ5 was associated with good outcome, independent of nodal status. In ERα+ cases, high ERβ2 mRNA levels were associated with reduced relapse and improved survival (Log-Rank P=0.01), independent of grade, size or nodal status (Cox P=0.02). High ERβ2 mRNA was also associated with better outcome in the node negative cases (Log Rank P<0.001). High ERβ2 immunostaining was associated with better outcome across the whole cohort (Log-Rank relapse P=0.018), but not in the ERα+ subgroup. There was no clear association between levels of ERβ2 mRNA and protein, but cases with both high mRNA and protein had significantly better outcome.

Conclusions: High ERβ2 protein levels were associated with ERα expression. Although most cases with high ERβ2 mRNA had strong ERβ2 immunostaining, mRNA levels but not protein levels were independently predictive of outcome in tamoxifen-treated ERα+ tumours. Post-transcriptional control needs to be considered when assessing the biological or clinical importance of ERβ proteins. ERβ5 mRNA measurements may be useful in targeting endocrine therapy in ERα- cases.

ACKNOWLEDGEMENT

I am grateful to many people for their help and support at JK Douglas Cancer Research Laboratory, Wirral. I would like to thank Mrs Wendy Prime, Cancer Tissue Bank Research Centre, Liverpool for providing sections. I am grateful to Mr Andy Dodson for technical help with immunohistochemistry and Dr Vijay Aachie for Immunohistochemistry analysis, Department of Pathology, Royal Liverpool University Hospital NHS Trust, Liverpool. At Clatterbridge Cancer Research Trust, I am especially grateful to Dr Michael Davies for his unfailing reserves of patience and enthusiasm in teaching scientific methods and principles to a surgeon, and help with day to day lab-work and preparation of the manuscript. I would also like to thank Ms Ceri Stewart for help with polymerase chain reaction. Finally I wish to thank my supervisors; Mr Christopher Holcombe and Professor Ross Sibson for their calm and encouraging attitude and sound advice throughout the project. This work was funded by Clatterbridge Cancer Research Trust.

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ABBREVIATIONS

AP-1	Activator protein 1
BCS	Breast cancer survival
BSA	Bovine serum albumin
cAMP	cyclic Adenosine monophophate
CCRT	Clatterbridge Cancer Research Trust
cDNA	Copy Deoxyribonucleic acid
CTBRC	Cancer Tissue Bank Research Centre
DAB	Diaminobenzidine
DNase`	Deoxyribonuclease
dNTPs	Deoxynucleoside triphosphates
ddNTPs	Dideoxynucleoside triphoshates
dsDNA	double-strand deoxyribonucleic acid
E2	Oestrogen
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Oestrogen receptor
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
ERE	Oestrogen Response elements
ESR	Oestrogen receptor gene
FISH	Fluorescence in-situ hybridisation
IDC	Invasive ductal carcinoma
IGF-1	Insulin like growth factor 1
IHC	Immunohistochemistry
KM	Kaplan Meier survival

LVI	Lymphovascular invasion
MW	Man Whitney U Test
mRNA	messenger RNA
OS	Overall survival
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PgR/PR	Progesterone receptor
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RFS	Relapse free survival
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RT-PCR	Reverse transcriptase-polymerase chain reaction
SP-1	Specificity protein 1
Tris	tris(hydroxymethyl)aminomethane
TBS	Tris buffered saline

CHAPTER 1 INTRODUCTION

1.1 Epidemiology

Breast cancer is the most common cancer in women worldwide accounting for 23% of all cancers. The incidence is the highest in Europe and North America and lowest in parts of Asia and Africa, and in between in Eastern Europe, South America and South Africa (Parkin *et al*, 2005). The high incidence in industrialised countries is thought to be partly due to the early diagnosis of invasive cancers by mammographic screening programmes. Genetic variations and differences in lifestyle, including diet and environmental exposures were thought to play a significant role in the occurrence of breast cancer in different parts of the world (Dumitrescu & Cotarla, 2005). This concept is supported by the study of migrants from low-risk country to high-risk countries, which has shown that the incidence of breast cancer assumes the rate of the host country within one or two generations (McPherson *et al*, 2000).

Breast cancer is the most common cancer in women in England. One in nine women will develop breast cancer at some point in their lives. Incidence rates for breast cancer increased by more than 80 percent between 1971 and 2007. The age-standardized incidence rate increased by 5 percent in the ten years to 2007. The introduction of national screening programme in the 1988 and the increasing use of hormonal replacement therapy in the 1990s were thought to have contributed to this increased incidence. However, mortality rates have fallen by 30 percent since 1971. Falls occurred in all age groups, but were greatest in women aged 55 to 69 years (Office for National Statistics, 2009) (Figure 1.1).

1.2 Risk factors

1.2.1 Sex and age

Sex and age are among the strongest risk factors associated with breast cancer. Breast cancer is mainly a disease of the women. It is 100 times more common in women than men (Thomas, 1993).

Increasing age is an important risk factor for breast cancer as the incidence is very low before the age of 25 (less than 10 new cases per 100,000 women) and this increases up to hundred times by the age 45, with four out of every five new cases are diagnosed in women aged 50 and over (Office For National Statistics, 2009). This breast cancer risk continues to increase after menopause until age 75 in Western countries. In contrast, the incidence exhibits a plateau followed by slow decrease in Japan after 45 years of age (Hulka & Moorman, 2001).

1.2.2 Family history

A family history of breast cancer increases the risk of developing the disease. The risk depends upon the number of relatives affected, as well as whether they are first degree or second-degree relatives. A meta-analysis of 52 individual epidemiological studies showed that compared to women with no family history of breast cancer, women with one, two and three or more first degree relatives had relative risks of 1.8 (99% CI: 1.69 - 1.91), 2.93 (99% CI: 2.36 - 3.64) and 3.90 (CI: 2.03 - 7.49) respectively (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). Another meta-analysis showed that relative risk of having one or more second-degree relatives was 1.5 (99% CI: 1.4 to 1.6) (Pharoah *et al*, 1997). It is also interesting to note that the Collaborative group meta-analysis has highlighted that eight out of nine women who develop breast cancer do not have any affected first-degree relatives and among women, who may have affected first-degree relatives, most will never develop breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2001). This observation supports the notion that most of the breast cancers are sporadic.

1.2.3 Breast cancer susceptibility genes

BRCA1 and BRCA2 (breast cancer one and two) are located in chromosome 17 and 13 respectively. These are tumour suppressor genes and mutations in them account for approximately 80 to 90% of hereditary breast cancers (de Jong *et al*, 2002). The cumulative risk of breast cancer in women is 60% for BRCA 1 mutation carriers and 40% for BRCA 2 mutation carriers (Antoniou *et al*, 2003).

Other high penetrance genes implicated in breast cancer include *p53, PTEN, ATM* and *STK11*. The location of these genes in chromosomes and associated syndromes are summarised in the table (Table 1.1). Mutations in these genes account for 5-10% of all breast cancers (Oesterreich & Fuqua, 1999).

Recently, a number of genes have raised interest and are thought to play a role in breast cancer risk. These are called low penetrance cancer susceptibility genes and they are relatively common. Many encode enzymes of different metabolic pathways. These genes along with endogenous and lifestyle risk factors may contribute to the occurrence of sporadic breast cancers, which comprise the majority of all breast cancers (Johnson-Thompson & Guthrie, 2000). Examples of these genes include *CYP1A1*, *CYP2D6*, *CYP19*, *GST1* and *GST P1, ADH1C, MTHFR, XRCC1* and *XRCC3, ERCC4/XPF*, *ESR1*, *TNFα* and *HSP70* (Coutelle *et al*, 2004; Ergul *et al*, 2003; Goode *et al*, 2002; Smith *et al*, 2003). More recent genome-wide association studies (Easton *et al*, 2007; Turnbull *et al*, 2010) have identified further loci implicated in breast cancer susceptibility including four further plausible genes (*FGFR2*, *TNRC9*, *MAP3K1* and *LSP1*).

1.2.4 Previous breast disease

Atypical epithelial hyperplasia is related to an increased risk of developing breast cancer. Women who had severe atypical epithelial hyperplasia have a four to five times higher risk of developing breast cancer than women who did not have proliferative changes in their breast. Moreover, the risk of breast cancer increases to nine times if the woman has an affected first-degree relative in their family (McPherson *et al*, 2000).

1.2.5 Reproductive factors

The reproductive history including the age at menarche, age at first full term pregnancy as well as number of pregnancies and age at menopause indirectly provides information about the amount of endogenous sex hormone exposure in premenopausal years.

Menarche at an early age (less than 12 years of age) has been shown to be associated with 10-25% increased risk of breast cancer compared to women who had menarche after 12 years of age (Bernstein, 2002; Kelsey *et al*, 1993). This increased breast cancer risk is attributed to prolonged oestrogen and progesterone exposure because of early onset of menstrual cycles (Bernstein, 2002). Similarly delayed menopause is associated with increased risk of breast cancer. Women who have natural menopause after 55 years of age are twice as likely to develop breast cancer compared to women who have menopause before 45 years (McPherson *et al*, 2000). In contrast, bilateral oophorectomy before the age of 35 results in 40% decrease in breast cancer risk, compared to women experiencing natural menopause (Kreiger *et al*, 1999).

Women, who had their first baby before the age of 20 had low risk of breast cancer, compared to women who were pregnant after 30 years of age (MacMahon *et al*, 1970). It has also has been shown that multiple pregnancies provide a strong protective effect against breast cancer (Yuan *et al*, 1988). Nulliparous women had a 30% increased chance of breast cancer, compared to parous women (Ewertz *et al*, 1990; Kelsey *et al*, 1993). One explanation for the protective effect of childbirth on breast cancer is that during pregnancy the breast epithelial cells mature and they are less prone to malignant transformation in the latter years (Gadducci *et al*, 2005). Breast feeding has been postulated to be protective against breast cancer and there is 4.3% decrease in relative risk of breast cancer for every 12 months of breast feeding (Collaborative Group on Hormonal Factors in Breast Cancer, 2002). This decrease of breast cancer risk is thought to be due to reduction in the total number of menstrual cycles that occur as a result of breast feeding (Bernstein, 2002).

These findings imply that cumulative endogenous oestrogen exposure is a significant risk factor for breast cancer. At the cellular level oestrogen exerts its effect via oestrogen receptors. The role of oestrogen receptor alpha (ERα) in breast cancer has been established, but the importance of oestrogen receptor beta (ERβ) is still not fully understood.

1.2.6 Exogenous hormones

Oral contraceptive use and the risk of breast cancer have been investigated by several studies. The meta-analysis published by the collaborative group on hormonal factors showed a significant increase in breast cancer risk in women taking combined oral contraceptive pills. There was 24% increase in breast cancer risk for current users, and this risk reduces after stopping pills and disappears at 10 years (Collaborative Group on Hormonal Factors in Breast Cancer, 1996a; Collaborative Group on Hormonal Factors in Breast Cancer, 1996b). In contrast, two large studies did not show association between oral contraceptive pills and increased breast cancer risk (Hannaford *et al*, 2007; Marchbanks *et al*, 2002). These inconsistent findings show that association between oral contraceptive pills and risk of breast cancer, if any, is likely to be modest.

Hormonal replacement therapy has been implicated as a risk factor for breast cancer in postmenopausal women. The risk depends upon the duration of therapy, as well as whether the therapy contained oestrogen alone or both oestrogen and progestin (Ross *et al*, 2000). The risk was higher for oestrogen and progesterone combined therapy than for the oestrogen only preparations (Santen, 2003). The meta-analysis conducted by collaborative group on hormonal factors in breast cancer concluded that HRT users had 14% increased risk of breast cancer, compared to women who never used it. The risk increased by 2.3% for each year of use for current users. Increased risk was limited to current or recent use and was not significant for previous users (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). Despite the increased incidence of breast cancer in women receiving HRT, the overall mortality among these women is reduced, because there are fewer deaths related to cardiac disease or osteoporosis (Col *et al*, 1999; Grodstein *et al*, 1997).

1.2.7 Lifestyle factors

Epidemiological studies have shown association between alcohol intake and increased risk of breast cancer (Longnecker, 1994; Longnecker *et al*, 1995). The risk increases linearly in a dose-dependent manner. There was no apparent increase in breast cancer risk in women who drank less than one standard unit per day, compared

to non-drinkers. However, the relative risk of breast cancer increased by 7% for every additional standard drink of alcohol (i.e. ~10 g of alcohol per day) (Hamajima *et al*, 2002).

The association between high fat intake and breast cancer risk is controversial. The majority of cohort studies have failed to find an association between dietary fat intake and breast cancer risk (Hunter *et al*, 1996; Willett *et al*, 1992). However, a recent pooled analysis suggests that high intake of saturated fat increases the breast cancer risk (Smith-Warner *et al*, 2001).

Obesity has a complex relationship with breast cancer risk. In postmenopausal women, obesity is associated with increased breast cancer risk, and in premenopausal women there is decrease in breast cancer risk (Cleary & Maihle, 1997; Huang *et al*, 1997; Potischman *et al*, 1996; Trentham-Dietz *et al*, 1997). The possible explanation is that obese postmenopausal woman may have low serum concentration of sex hormone binding globulin and high serum concentration of the oestrogen leading to increased risk of breast cancer (Thomas *et al*, 1997). However, premenopausal obese women are more likely to have anovulatory cycles and longer menstrual cycles, resulting in less cumulative oestrogen exposure and reduced breast cancer risk (Henderson *et al*, 1985).

1.3 Prognostic and predictive factors

A prognostic factor is any measurement available at the time of surgery that correlates with disease-free or overall survival in the absence of systemic adjuvant therapy and, as a result, is able to correlate with the natural history of the disease. In contrast, a predictive factor is the measurement associated with response to a given therapy (Cianfrocca & Goldstein, 2004).

1.3.1 Histological type

Breast cancer is classified into various histopathologic types depending upon microscopic morphology. Invasive ductal carcinoma of no special type (NST) accounts for 70 to 80% of breast cancers. The next common breast cancer is invasive

lobular carcinoma accounting for 5 to 10% of breast cancers. Even though they have some differing disease pattern, a study by Sastre-Garau *et al.* has shown that both ductal and lobular carcinoma had similar prognosis in terms of disease-free and overall survival (Sastre-Garau *et al*, 1996).

Tubular, papillary, medullary and mucinous cancers are other rare varieties, and these cancers have better prognosis than invasive ductal and lobular carcinoma (Cianfrocca & Goldstein, 2004). Another rare type is inflammatory breast cancer. This is an aggressive form of breast cancer and the patients are younger at diagnosis and have worse prognosis compared to other types (Chang *et al*, 1998).

1.3.2 Tumour size

Tumour size is one of the strongest prognostic factors along with axillary lymph node status (Vorgias *et al*, 2001). Larger tumours were associated with more positive nodes (Weiss *et al*, 2003). In lymph node negative patients, tumour size helps to make decisions about adjuvant treatment options (Cianfrocca & Goldstein, 2004). Carter *et al.* analysed over 13000 node negative breast cancer patients from the SEER database (Surveillance, Epidemiology, and End Results data of the National Cancer Institute). Tumour size was categorised into three groups and survival analysis calculated. The overall survival was close to 99% for tumours less than 1 cm in size and 89% for tumours between 1 to 3 cm in size and 86% for tumours between 3 to 5cm (Carter *et al*, 1989).

1.3.3 Histological grade

Breast cancers are graded using Scarff-Bloom-Richardson classification. According to the classification, tumours are classified into well differentiated, moderately differentiated and poorly differentiated cancers. Well differentiated cancers were associated with better prognosis (Bloom & Richardson, 1957). Tumour grade is mainly useful to make adjuvant treatment decisions in node-negative cancers with borderline tumour sizes (Cianfrocca & Goldstein, 2004).

1.3.4 Lymphovascular invasion

Lymphovascular invasion is the presence of neoplastic cells within the lymphovascular space in the peritumoural tissues. Rosen *et al.* have shown correlation between lymphovascular invasion and disease recurrence. In this study of 461 patients with stage one breast cancer, at 20 year follow-up lymphovascular positivity was associated with significantly higher disease recurrence (Rosen *et al*, 1989). In another study of 1275 patients, lymphovascular invasion was associated with 15% increase in disease recurrence at 5 years of follow-up and this effect was independent of whether or not they received adjuvant treatment (Neville *et al*, 1992).

1.3.5 Axillary lymph node status

The single most important prognostic factor for breast cancer is axillary node status. Node positive patients had much worse prognosis compared to node negative patients (Arriagada *et al*, 2006; Fisher *et al*, 2001). The number of positive nodes directly correlates with worse prognosis. In a study of node positive breast cancer patients, who were treated with chemotherapy and followed up for 20 years, the median disease free survival was 11.1 years for the patients with one to three nodes, 5.4 years for the patients with four to nine nodes, and 2.1 years for the patients with 10 or more positive nodes (Weiss *et al*, 2003).

1.3.6 ER/PR status

Oestrogen and progesterone receptor status serve as both prognostic and predictive factor. ER positive cancers had better relapse free survival than ER negative breast cancers (Fisher *et al*, 1988; Knight *et al*, 1977). However, this survival benefit was not maintained in other long term follow-up studies (Andry *et al*, 1989; Hilsenbeck *et al*, 1998; Raemaekers *et al*, 1985).

ER and PR status are powerful predictive factors for hormone therapy with tamoxifen (Early Breast Cancer Trialists' Collaborative Group, 1998) and aromatase inhibitors (Baum *et al*, 2002; Thurlimann *et al*, 2005). However, not all hormone receptor positive patients respond to anti-oestrogens; approximately 25% of

ER+/PR+, 66% of ER+/PR-, and 55% of ER-/PR+ breast tumours fail to respond to anti-oestrogens (Honig SF, 1996). The mechanism of resistance is not fully understood and is an area of intense research and will be discussed later.

1.3.7 HER2 status

HER2, also known as c-erbB2 is a member of the epidermal growth factor family with tyrosine kinase activity (King *et al*, 1985). It is amplified and overexpressed in 15%-30% of breast cancers (Slamon *et al*, 1987), although recent rates reported by standardized reporting in the UK are 15-17% (Walker *et al*, 2008), and the expression is associated with poor prognosis (Esteva & Hortobagyi, 2004). Currently, HER2 testing is part of a routine pathological workup in all newly diagnosed breast cancer patients (Wolff *et al*, 2007). HER2 expression is inversely correlated with ER/PR status and in one series it was expressed in 7% of postmenopausal patients who were ER positive (Rasmussen *et al*, 2008). HER2 is also a predictive marker for treatment with monoclonal antibody trastuzumab. Combination of trastuzumab and chemotherapy in breast cancer patients resulted in better relapse free and overall survival in the metastatic (Slamon *et al*, 2001) as well as in the adjuvant treatment setting (Pritchard *et al*, 2006).

1.3.8 Gene expression profile

Gene expression profiling is a new technology, which is revolutionising the understanding of various cancers. Using this microarray technology, the expression of thousands of genes can be measured in each sample and molecular patterns can be identified through bioinformatics analysis. In breast cancer, molecular profiling has identified molecular subtypes (Luminal A, Luminal B, HER2+ and Basal-like) that reflect intrinsic properties of the tumours (Sorlie *et al*, 2001) and expression patterns that are highly correlated with patient outcome (Sorlie *et al*, 2001; van 't Veer *et al*, 2002).

Oncotype DX and other gene expression studies like the Mamma Print assay (Agendia BV, Amsterdam, the Netherlands), Rotterdam Signature, and the Breast

Cancer Gene Expression Ratio may aid in accurately assessing prognosis as well as in allocating various treatment modalities to breast cancer patients.

For example, Oncotype DX (Genomic Health Inc., Redwood city, CA) measures the expression of 21 genes at the mRNA level in formalin-fixed paraffin embedded specimens and a "recurrence score" is calculated from this. The genes measured represent pathways including oestrogen (e.g. ER and PgR), proliferation (e.g. Ki67 and cyclin B), invasion (e.g. Stromelysin 3 and Cathepsin L2) and HER2. Each gene group is weighted to produce a combined recurrence score (0-100). This score is then divided into low, intermediate and high risk score that is used to plan treatment. Paik *et al.* validated this multi-gene assay in a cohort of 668 patients, who were node-negative, ER positive and tamoxifen treated, and enrolled in the NSABP-B14 trial. The 10-year distant recurrence rate was 6.8% in patients with low score, 14.3% for patients with intermediate score and 30.5% for patients with high score ($P<0.001$). The recurrence score was independent of age and tumour size in multivariate analysis (Paik *et al*, 2004). Similar results were also observed in a large population based study (Habel *et al*, 2006). Oncotype DX recurrence score was also able to identify patients, who may benefit from chemotherapy in a cohort of node-negative, ER positive and tamoxifen treated patients (Paik *et al*, 2006). This may be very useful in clinical setting to select patients for chemotherapy who are node negative and ER positive.

1.4 Hormonal manipulation

1.4.1 Ovarian suppression

In 1896, George Beatson, a surgeon from Glasgow showed that oophorectomy in premenopausal women resulted in breast cancer regression and improved prognosis; however, it benefited only one third of patients (Beatson, 1986). This was the earliest demonstrated link between breast cancer and ovarian hormones. Early endocrine therapy also included adrenalectomy and hypophysectomy, but they were later abandoned when tamoxifen was introduced in the treatment of advanced breast cancer in 1978 (Strasser-Weippl & Goss, 2005). A meta-analysis by EBCTCG showed that in women, aged under 50 with early breast cancer, ablation of

functioning ovaries significantly improves long-term survival (Early Breast Cancer Trialist's Collaborative Group, 1996). Ovarian suppression still remains as an important anti-hormonal treatment in premenopausal breast cancer patients. Ovarian suppression with LHRH agonist (e.g. goserelin) produces serum oestrogen and progesterone level similar to levels in women who have undergone oophorectomy (Pinder & Buzdar, 2008). In a trial comparing goserelin with chemotherapy, at a median follow-up of 7 years goserelin was similar to chemotherapy in terms of disease-free and overall survival in pre/peri-menopausal women who were ER positive and node positive (Kaufmann *et al*, 2003). In another trial comparing goserelin with tamoxifen to CMF chemotherapy, at median follow-up of five years, the combination of goserelin and tamoxifen were better in terms of disease-free survival in ER positive premenopausal woman (Jakesz *et al*, 2002). Another international study compared CMF on its own, goserelin on its own, and CMF followed by 18 months of goserelin. The patients treated with both goserelin and CMF chemotherapy had a better five-year overall survival than the other two groups in ER positive patients, and the benefit was significantly higher in young patients who are less than 40 yrs old (Castiglione-Gertsch *et al*, 2003).

1.4.2 Anti-oestrogens

In 1936, Professor Antoine Lacassagne proposed that breast cancer could be prevented by drugs that antagonise the effects of oestrogen (Lacassagne, 1936). In 1958, the first non-steroidal oestrogen antagonist 1-(p-2-diethylaminoethoxyphenyl)-1-phenyl-2-p-methoxyphenyl ethanol (MER-25) was discovered (Lerner *et al*, 1958), but in clinical trials, it showed low potency and high central nervous system toxicity (Herbst *et al*, 1964).

Tamoxifen

Tamoxifen is a trans isomer of substituted triphenylethylenes (ICI 46,474) and it was initially developed as an oral contraceptive, but found to have potential anti-oestrogenic action (Harper & Walpole, 1966). In 1960s oestrogen receptor was identified (Jensen, 1962), and tamoxifen was found to block oestrogen binding to oestrogen receptor (Jordan & Koerner, 1975). In a prospective trial of ER and PR

positive patients who had advanced breast cancer, 70% of patients responded to tamoxifen treatment, and in patients who were ER/PR negative the response rate was less than 10% (Ravdin *et al*, 1992). Tamoxifen was as effective as diethylstilbestrol in the management of metastatic breast cancer (Gockerman *et al*, 1986). Following these trials in metastatic and advanced breast cancer settings, tamoxifen was approved to treat patients in the adjuvant setting. An overview of 55 randomized trials showed that five years of tamoxifen therapy reduced breast cancer recurrence by 42% and mortality by 22% at 10 years (Early breast Cancer Trialist's Collaborative Group, 1998). Tamoxifen treatment for five years became the standard duration of therapy, as more than five years did not show any further improvement in disease-free survival or overall survival (Fisher *et al*, 1996; Tormey *et al*, 1996). Tamoxifen is still one of the first line hormone therapies in both premenopausal and postmenopausal women with breast cancer. Five years of tamoxifen therapy also reduced contralateral breast cancer by 47% (Early breast Cancer Trialist's Collaborative Group, 1998). Tamoxifen also reduced the frequency of invasive breast cancer and DCIS by about 50% in high-risk women in the NSABP chemoprevention trial (Fisher *et al*, 1998).

Tamoxifen is a mixed agonist and antagonist; it is beneficial in terms of reducing cholesterol concentrations, preserving bone density and reducing the risk of fractures in postmenopausal women. However, there are increased risk of endometrial cancer and thrombosis (O'Regan & Jordan, 2002).

Fulvestrant

Fulvestrant (ICI182,780) is a pure steroidal anti-oestrogen with none of the agonistic properties found in other selective oestrogen receptor modulators (Wakeling *et al*, 1991). Fulvestrant not only blocks ER receptor, it also induces ER degradation with a marked reduction in the cellular concentration of ER (Osborne *et al*, 2000). In animal models fulvestrant has been shown to be a more potent inhibitor of tumourigenesis and also produces regression of established tumours (Osborne *et al*, 1995). In a double-blind randomized multicentre trial, fulvestrant was as effective as tamoxifen as a first-line endocrine therapy in metastatic or locally advanced breast cancer patients (Howell *et al*, 2004). Additionally fulvestrant was beneficial as second line endocrine therapy in advanced breast cancer patients who develop resistance to

tamoxifen (Howell *et al*, 1995). In a double-blind randomized trial, the efficacy and tolerability of fulvestrant was compared with anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy, fulvestrant was as effective as anastrazole (Osborne *et al*, 2002) and the same effect was seen in another randomized controlled trial (Howell *et al*, 2002). These data confirm that fulvestrant is an additional, effective, and well-tolerated treatment for advanced breast cancer in postmenopausal women whose disease progressed on prior endocrine therapy.

1.4.3 Aromatase inhibitors

Aromatase inhibitors (AIs), especially the newer generation are an important addition to endocrine therapy in breast cancer. They inhibit the enzyme aromatase, which belongs to cytochrome P-450 superfamily and encoded by *CYP19* gene (Evans *et al*, 1986), hence reducing the production of oestrogen. Aromatase enzyme is present in subcutaneous fat, liver, muscle, brain, bone, normal breast and breast cancer tissue (Nelson & Bulun, 2001). The first generation AIs (e.g. aminoglutethimide) non-selectively block the enzymes of cytochrome 450 resulting in adrenal suppression as well. Because of this mechanism of action, aminoglutethimide had to be given with high-dose corticosteroids and also caused serious side effects (Howell & Buzdar, 2005). The second generation formestane was the first selective AI, but it had short half life and had to be given as an intramuscular injection (Pinder & Buzdar, 2008). The third generation AIs (anastrozole, letrozole and exemestane) selectively block aromatase and are widely used in endocrine treatment of breast cancer. Anastrozole and letrozole are non-steroidal inhibitors and competitively block aromatase in reversible manner (Vanden Bossche *et al*, 1994). On the other hand, exemestane is a steroidal inhibitor, it blocks aromatase irreversibly and is also known as suicidal inhibitor (Brueggemeier, 1994). In postmenopausal women, AIs suppress the circulating oestrogen level by over 95% (Demers, 1994; Geisler *et al*, 2002). In addition, AIs have the potential to block the oestrogen production by the peritumoural breast cancer tissue (Santen *et al*, 1999). AIs are mainly used in women with no ovarian function, i.e. postmenopausal women, as in women with intact ovarian function it causes a rise in gonadotrophin releasing hormone levels, with

resultant increases in oestrogen production (Pinder &Buzdar, 2008). So, AIs are contraindicated in premenopausal women with intact ovarian function (Smith & Dowsett, 2003).

Anastrozole

Anastrozole is a non-steroidal selective competitive aromatase inhibitor. It is administered orally and peak serum concentration is reached within two hours, with a plasma half-life of 30-60 hours (Plourde *et al*, 1994). In a combined analysis of two randomized controlled trials comparing anastrozole with megestrol acetate (a progesterone derivative used in the treatment of metastatic breast cancer) in women with advanced breast cancer who failed on tamoxifen, survival was significantly longer for patients treated with anastrozole with fewer side effects (Buzdar *et al*, 1998). Anastrozole was also shown to be beneficial in metastatic breast cancer in postmenopausal women, compared to tamoxifen as a first-line hormone therapy (Bonneterre *et al*, 2000). Anastrozole was then evaluated in early breast cancer as first line therapy. In the ATAC trial (Arimidex, Tamoxifen Alone or in Combination Trial) 9366 women were randomized to 5 years of tamoxifen, anastrozole or a combination of both therapies. After 68 months of median follow-up anastrozole significantly prolonged disease-free survival and time to recurrence, and there was also significant reduction in occurrence of distant metastases and contralateral breast cancer. In this trial, tamoxifen caused fewer musculoskeletal complaints and fractures, but was less tolerated with respect to endometrial cancer, vaginal bleeding and thromboembolic events. The authors concluded that anastrozole should be considered as the first line hormonal therapy in postmenopausal hormone positive breast cancer patients (Howell *et al*, 2005).

Letrozole

Letrozole is the other non-steroidal competitive aromatase inhibitor. It is more potent than anastrozole in suppressing aromatase enzyme (Santen & Harvey, 1999). On oral administration it is absorbed rapidly and reaches steady-state plasma concentrations in 4–8 hours and its half-life is approximately 45 hours (Mitwally & Casper, 2001). It was initially evaluated in advanced breast cancer treatment as a second-line endocrine treatment. In one European study, letrozole was superior to aminoglutethimide (Gershanovich *et al*, 1998) in disease control in post menopausal

women in advanced breast cancer. In another study letrozole was more effective and better tolerated than megestrol acetate in the treatment of postmenopausal women with advanced breast cancer previously treated with anti-oestrogens (Dombernowsky *et al*, 1998). Letrozole was superior to tamoxifen as first-line endocrine treatment in metastatic breast cancer (Mouridsen *et al*, 2003). The Breast International Group (BIG) 1-98 trial compared letrozole and tamoxifen as adjuvant treatment in postmenopausal women with hormone receptor positive early breast cancer. Women were randomly assigned to receive: tamoxifen alone for five years; letrozole alone for five years; tamoxifen for two years followed by letrozole for three years; and letrozole for two years, followed by tamoxifen for three years. At 76 months of median follow-up the letrozole arm showed better disease-free survival compared to tamoxifen, but there was no significant overall survival benefit (Coates *et al*, 2007). There was no significant benefit compared to upfront letrozole to sequential treatment with letrozole and tamoxifen (Mouridsen *et al*, 2009). Patients on tamoxifen experienced more thromboembolic events, endometrial pathology, hot flashes, night sweats, and vaginal bleeding, whilst patients on letrozole experienced more bone fractures, arthralgia, low-grade hypercholesterolemia and cardiovascular events (Coates *et al*, 2007). Another trial MA.17 looked into extended adjuvant therapy. Patients were randomized to letrozole or placebo after five years of tamoxifen therapy. This trial was stopped early as letrozole showed significantly improved disease-free survival. Although there was no overall survival advantage in the whole cohort, there was significantly improved overall survival in node positive patients (Goss *et al*, 2005).

Exemestane

Exemestane is an orally active, steroidal, irreversible aromatase inhibitor (di Salle *et al*, 1992). Its half life is 24 hours, comparatively less than that of non-steroidal inhibitors (Brueggemeier, 2002). In a randomized multicentre study (Kaufmann *et al*, 2000) exemestane was compared with megestrol acetate as a second line therapy in postmenopausal women with advanced breast cancer who were previously treated with tamoxifen. Time to progression, time to treatment failure and overall survival were better in the exemestane arm and it was also well tolerated. Another phase III randomized open-label clinical trial evaluated the efficacy and safety of exemestane

with tamoxifen as first-line treatment for metastatic breast cancer in postmenopausal women. There was better median progression-free survival in the exemestane arm, but there was no overall survival benefit (Paridaens *et al*, 2008). The Intergroup Exemestane Study (IES) compared tamoxifen for 5 years to tamoxifen for 2 to 3 years followed by exemestane for a total of five years of endocrine therapy in hormone positive early breast cancer patients. There was better disease-free survival at a median follow-up of 55 months as well as a non-significant reduction in death in the exemestane group (Coombes *et al*, 2007).

1.5 Oestrogen receptors in breast cancer

1.5.1 Oestrogen receptor alpha (ERα)

In 1950s, Jensen and Jacobson demonstrated that oestradiol was specifically retained by oestrogen target tissues and proposed that a receptor should exist for oestrogen (Jensen & Jacobson, 1962). After some years, oestrogen receptor was identified (Toft & Gorski, 1966) and it was eventually cloned in 1980s (Greene *et al*, 1986; Walter *et al*, 1985). The oestrogen receptor gene (now named *ESR1*) was found to be localised at chromosome 6q24-27 (Gosden *et al*, 1986; Ponglikitmongkol *et al*, 1988). Oestrogen receptor structure is similar to other nuclear receptor super family members (Green *et al*, 1986). It has six structural domains (domains A-F) and defined functional domains (Figure 1.2). The transactivation function domain AF-1 is located within the amino-terminal A and B domains and it is ligand independent (Kumar *et al*, 1987; Ribeiro *et al*, 1995). The C domain contains a DNA binding domain that is responsible for binding to specific oestrogen response elements (ERE) within the promoters of the oestrogen responsive genes (Klein-Hitpass *et al*, 1988). C and E domains contain the oestrogen receptor dimerization domain (Ribeiro *et al*, 1995). The carboxy terminal E and F are the ligand binding domain and contains the ligand dependant transactivation domain AF-2 (Norris *et al*, 1997). The ligand independent domain AF-1 can be activated by cAMP, dopamine, and growth factor receptors epidermal growth factor (EGF) and Insulin like growth factor 1 (IGF-1) (Herynk & Fuqua, 2004). This multi-domain structure allows oestrogen receptors to process multiple signals (e.g. oestrogen, growth factors), integrate them via cross

talk, and produce individualised responses (via ERE and/or SP1/AP1 promoter elements) (Figure 1.3). Several coregulatory proteins act as intermediary factors, which augment or suppress oestrogen receptor transactivation (Rosenfeld & Glass, 2001). These coregulators contribute to tissue specific actions of ERs, as they are expressed differentially in different cell types and tissues.

Gene splicing is a post-transcriptional modification due to differential inclusion or exclusion of exons resulting in multiple proteins (Black, 2003). Although splicing is a normal phenomenon it has been implicated in various diseases and cancers (Venables, 2004). ERα splice variants (mainly exon deletion variants) were identified in many normal tissues as well as in breast cancers (Herynk & Fuqua, 2004; Poola & Speirs, 2001). Most of the variants are transcriptionally inactive and some (ERαΔ3 and ERαΔ7) showed dominant negative activity to wild-type ERα (Garcia Pedrero *et al*, 2003; Wang & Miksicek, 1991). ERα splice variants were thought to play a role in tumourigenesis and response to various anti-cancer treatments (Poola & Speirs, 2001), however, as the splice variants were expressed in relatively few cancers and in relatively low levels compared to wild-type ERα the exact significance is unknown (Zhang *et al*, 1996).

Oestrogen receptor, present in 40-70% of breast cancers, is an independent prognostic marker (Knight *et al*, 1977) and also predicts response to endocrine therapy in breast cancer (Pertschuk & Axiotis, 1999). Anti-oestrogen strategies, such as inhibition of oestrogen-receptor binding and oestrogen deprivation, are effective for the management of hormone-dependent breast cancer (Brueggemeier, 2002), but up to 40% of tumours fail to respond to endocrine therapy (McGuire, 1975). The mechanism of this resistance is poorly understood. With the discovery of second oestrogen receptor in the 1990s there is huge interest in evaluating its role in hormone responsiveness.

1.5.2 Oestrogen receptor beta (ERβ)

In the 1990s, a second oestrogen receptor, named as ERβ (the classic oestrogen receptor being renamed ERα), was identified in a rat prostate cDNA library and it encoded a protein of 485 amino acids (Kuiper *et al*, 1996). ERβ was also found in

mouse (Tremblay *et al*, 1997) and human (Mosselman *et al*, 1996). Further studies identified a longer 530 amino acid sequence (Moore *et al*, 1998; Ogawa *et al*, 1998a) and this was universally accepted as the full length ERβ. ERβ was localised to chromosome 14q22-25 (Enmark *et al*, 1997). Like ERα, ERβ resides in cytoplasm and translocates to nucleus after ligand binding (Leung *et al*, 2006). The ERβ has structural domains similar to other nuclear receptor super family members, especially ERα, and has a high degree of homology with ERα in certain areas. There is 96% homology in the DNA binding domain and 53% homology in the ligand binding domain (Figure 1.2) (Weihua *et al*, 2003). There is less conservation between these two receptors in the amino terminal AF-1 and carboxy terminal AF-2, suggesting there may be functional differences.

ERα and ERβ are differentially expressed in breast and other tissues (Kuiper *et al*, 1997; Saunders *et al*, 1997). One study (Taylor & Al-Azzawi, 2000) looked at ERα and ERβ in a whole range of normal tissues in human. This study showed that distribution of ERβ appeared to present with ERα in most tissues, however ERβ did not appear to be linked with ERα expression as some ERα positive cells lack ERβ and *vice versa.* For example, prostate only expressed ERβ and liver only expressed ERα. In normal breast, ERα is only present in luminal epithelial tissues (Petersen *et al*, 1987) and is sparsely expressed (Clarke *et al*, 1997; Ricketts *et al*, 1991). In contrast, ERβ is widely expressed in normal breast and apart from epithelial cells also expressed in myoepithelial cells, stromal cells and endothelial cells (Palmieri *et al*, 2002; Speirs *et al*, 2002). However, the level of ERβ mRNA is much lower than ERα in breast cancers (de Cremoux *et al*, 2002; Speirs *et al*, 1999a).

1.5.3 Splice variants of ERβ

Many splice variants (exon deletion, insertions and c-terminal variants) of ERβ have been identified (Poola *et al*, 2002a; Poola & Speirs, 2001). Deletion variants are of unknown functional significance as it is unclear whether they are expressed as proteins or not. One variant, ERβΔ5 is detected in normal breast (Speirs *et al*, 2000) and in breast cancers (Poola *et al*, 2002b; Vladusic *et al*, 1998). ERβΔ5 lacks hormone binding and shows dominant negative activity towards both ERα and ERβ (Herynk & Fuqua, 2004). Poola *et al.* investigated ten ERβ exon deletion variants in

43 breast cancers and matched normal tissues by semi-quantitative RT-PCR. ERβΔ5-6 variant expression was significantly less in cancer tissues. ERβΔ5 expression was associated with postmenopausal status and tumour grade. ERβΔ2 and ERβΔ4 were expressed at low levels and ERβΔ7 was not detected (Poola *et al*, 2002b). ERβΔ3 was originally identified in ovary and has not been identified in normal breast or cancer (Poola *et al*, 2002b) but it is thought to encode part of the DNA binding domain and the assumption is that it may play a role with other transcription factors and further study has been recommended (Herynk & Fuqua, 2004).

The C-terminal splice variants are ERβ2, ERβ3, ERβ4 and ERβ5. The wild type was renamed as ERβ1. All five isoforms diverge at a common position within the predicted helix 10 of the ligand binding domain of ERβ, with nucleotide sequences consistent with differential exon usage (Moore *et al*, 1998) (Figure 1.2). These variants/isoforms are commonly known as C-terminal variants. ERβ2, ERβ4 and ERβ5 like ERβ1, are expressed in many normal tissues and breast cancers (Chi *et al*, 2003; Girault *et al*, 2004; Scobie *et al*, 2002). ERβ3 was thought to be testis specific but has been detected in low amount by qRT-PCR in normal and malignant breast (Chi *et al*, 2003; Girault *et al*, 2004). However, these variants are expressed differentially in many normal tissues (Moore *et al*, 1998). In breast, ERβ1, ERβ2 and ERβ5 were the three major isoforms in both normal and tumoural breast tissue (Girault *et al*, 2004). Within breast these isoforms are differentially expressed, while ERβ2 is less expressed, ERβ1and ERβ5 are predominantly expressed in terminal ductal lobular unit and luminal epithelial cells, respectively (Speirs & Shaaban, 2009).

ERβ1 binds oestradiol with high affinity and in transient transfection studies ERβ activates transcription in oestrogen-dependent manner (Hanstein *et al*, 1999). ERβ1 also forms heterodimers with ERα (Cowley *et al*, 1997). ERβ2 is also known as ERβcx and has a unique c-terminus with exon 8 replaced by an alternative exon of 26 amino acids (Ogawa *et al*, 1998b). The ERβ2 forms non-functional heterodimers with ERα and functions as a dominant negative inhibitor (Zhao *et al*, 2007). In *in vitro* analysis, ERβ2 has been found to be more expressed in the cytoplasm than in the nucleus and is modulated by 17β-oestradiol (Al-Madhoun *et al*, 2007). ERβ4 and ERβ5, like ERβ2 can also form heterodimers with ERα and negatively regulate

transcription (Poola *et al*, 2005a). The functional role of ERβ3 is unclear as it is expressed at very low levels and has been investigated less. It was thought that ERβ isoforms can form heterodimers with each other (Moore *et al*, 1998). However, recently through molecular modelling Leung *et al.* showed that ERβ1 is the only functional isoform and that ERβ2, ERβ4 and ERβ5 do not have innate activities in their homodimeric forms but can heterodimerize with ERβ1 and enhance ERβ1 induced transcription in ligand-dependent manner (Leung *et al*, 2006). This has functional significance in individual tissues as these variants are differentially expressed.

1.5.4 Oestrogen receptor β in breast cancer

ERβ is the dominant receptor in normal breast and unlike ERα is also expressed in myoepithelial cells, stromal cells and fibroblasts. In contrast to ERα there is no difference in expression level of ERβ during the menstrual cycle (Shaw *et al*, 2002). ERβ is anti-proliferative; when reintroduced into breast cancer cell lines it caused cell-cycle arrest. It caused tumour regression in a xenograft model (Paruthiyil *et al*, 2004). ERβ shows features of tumour suppressive effects; lowered levels are associated with malignant progression in breast (Bardin *et al*, 2004; Shaaban *et al*, 2003; Skliris *et al*, 2003). However, over 75% of breast cancers express ERβ, compared to the 85% of the normal breast (Shaw *et al*, 2002) but ERβ expression is significantly lower in breast cancer, compared to normal breast tissue (Girault *et al*, 2004; Iwao *et al*, 2000; Leygue *et al*, 1998), supporting the theory that ERβ expression is reduced/lost in tumourigenesis. Earlier studies looking at the role of ERβ in breast cancer at the mRNA level were inconsistent with each other (as reviewed elsewhere) (Speirs *et al*, 2004). However, many used non specific primers which may detect multiple C-terminal variants that have differing functional activities (Davies *et al*, 2004). Levels of ERβ2 and ERβ5 mRNA are recognised to be higher than those of the ERβ1 variant (Iwao *et al*, 2000; Leygue *et al*, 1999); hence, many of the non-specific RT-PCR studies may have measured these variants in varying amounts depending upon the primer selection and PCR methodologies, contributing to the inconsistencies in the reported findings. With the availability of the ERβ antibodies protein studies by immunohistochemistry was utilised. ERβ

mRNA level did not correlate with protein expression in many comparative studies (O'Neill *et al*, 2004; Omoto *et al*, 2002; Shaw *et al*, 2002). This may be partly due to measurement of non-tumoural expression of ERβ by PCR techniques. Another explanation may be that multiple splice variants that were not translated in to proteins were inadvently measured by PCR using nonspecific primers. The antibodies may also have had differing specificities.

The majority of ERβ protein studies have not found correlations with standard clinicopathological markers (Esslimani-Sahla *et al*, 2004; Palmieri *et al*, 2004; Skliris *et al*, 2006). However, some studies have shown some correlation. Saji *et al.* showed correlation between ERβ2 and PgR negative status (Saji *et al*, 2002). Chi *et al.* showed association between ERβ3 and ERβ5 with large tumour in a small study of 17 patients (Chi *et al*, 2003). Skliris *et al.* found correlation between ERβ and ERα/PgR status (Skliris *et al*, 2003). There were studies that measured outcome in association with ERβ but again these studies looked at either total ERβ or individual isoforms. In a recent large population based study (Nurse's Health Study) ERβ1 expression was assessed in women who developed breast cancer (2170 cancers) and correlations were sought between molecular subtypes of breast cancer. ERβ1 expression was significantly related to molecular subtypes and was more common in luminal A (73%) and luminal B (68%) than in HER2 or basal-like types. However, ERβ1 expression was found in 55% of HER2 type and 60% of basal-like subtypes (Marotti *et al*, 2010). This large population-based study is of great importance as it has clearly shown association between ERβ positivity and good prognostic molecular subtypes. Moreover, it also shows that ERβ can be expressed in aggressive molecular subtypes suggesting that there may be other explanations for this differential expression.

Total ERβ was associated with improved outcome in two studies (Mann *et al*, 2001; Murphy *et al*, 2002) whilst in another Greek study it was not (Stefanou *et al*, 2004). High ERβ1 protein expression has been associated with better outcome (Myers *et al*, 2004; Nakopoulou *et al*, 2004; Omoto *et al*, 2001). One large study looked at ERβ1 in a cohort of 728 patients, in node negative cases ERβ1 expression was found to be associated with better outcome; however, in the node positive group ERβ1 was associated with worse outcome (Novelli *et al*, 2008). There was no correlation with

ERβ1 and outcome in a cohort (ERα+/ERα-) of 167 patients (O'Neill *et al*, 2004) and also no correlation was seen for both ERβ1 and ERβ2 in a ERα negative cohort (255 patients) (Skliris *et al*, 2006).

Since this study has been completed others (Honma *et al*, 2008; Shaaban *et al*, 2008; Sugiura *et al*, 2007) have published concerning the role of ERβ variants (especially ERβ2) in larger cohorts and they are discussed in the later chapters.

The majority of the studies discussed here show that ERβ was associated with good outcome and it was not associated with standard clinicopathological parameters. This contradiction means that ERβ may be valuable in terms of identifying aggressive or indolent cancers not picked up by standard markers.

1.5.5 ERs and tamoxifen in oestrogen signalling

Oestrogen exerts its biological effects through at least four pathways (Figure1.3). In the first pathway (classical ligand dependant) oestrogen (E2) binds to ERs and forms homo or heterodimers depending upon the receptor content. *In vitro* studies have shown that when coexpressed ERα and ERβ preferentially form heterodimers (Cowley *et al*, 1997). Thus it has been proposed that in cells coexpressing both receptors overall oestrogen responsiveness may be determined by the ERα:ERβ ratio (Hall & McDonnell, 1999). ERβ2 also preferentially heterodimerize and inhibit ERα activity suggesting a modulatory role for ERβ (Ogawa *et al*, 1998b). After dimerization, the E2-ER complex recruits coactivator proteins and binds to oestrogen response elements (ERE) in promoters of target genes and activates transcription and subsequent tissue responses. In contrast, tamoxifen recruits corepressors and inhibits transcription (Shang *et al*, 2000). Coregulatory proteins (either coactivators or corepressors) are recruited depending upon the promoter structure, cell type, type of ligand and type of receptor subtype (Klinge, 2000).

In the second pathway (ERE independent) the E2-ER complex can activate transcription by tethering to alternative response elements such as those for Activator Protein 1 (AP-1) through Jun/Fos-proteins (Kushner *et al*, 2000) and can also activate transcription via Specificity Protein 1 (SP-1) (Saville *et al*, 2000). However, ERα and ERβ behave in opposite manners in this pathway; E2 activates

transcription via ERα while ERβ inhibits transcription. Moreover, tamoxifen activates transcription via ERβ (Paech *et al*, 1997).

In the third pathway (ligand independent) growth factors (EGF/IGF-1) or cyclic adenosine monophosphate (c-AMP) activate intra cellular kinase pathways, leading to phosphorylation and activation of ER at ERE containing promoters in a ligand independent manner. This pathway may play a role in mitogenesis within ER positive tissues (Hall *et al*, 2001).

In the fourth pathway [Membrane Initiated Steroid Signalling (MISS) - previously termed non-genomic signalling], E2 activates a putative membrane-associated binding site, possibly a form of ER linked to intracellular growth factor signalling pathways that generate rapid tissue responses (Losel *et al*, 2003). Recent studies have shown membrane ERα and ERβ accounting for 5% of total ER content (Levin, 2001; Razandi *et al*, 2003). However, the studies were mainly done in pituitary and blood vessels, a role for membrane-bound ER in breast has not been fully elucidated (Speirs & Walker, 2007). MISS can be activated by both E2 and tamoxifen (Massarweh & Schiff, 2007). Recently, GRP30, a non-ER protein was identified in the plasma membrane. It is a G protein coupled seven transmembrane receptor and has been thought to play a role in mediating MISS (Thomas *et al*, 2005). However the exact role of MISS is still unclear in breast cancer (Speirs & Walker, 2007).

It is evident from the above signalling pathways that both ERs have overlapping but distinct and sometimes antagonistic functions. Tamoxifen exerts differential effect on target genes depending upon the ER subtype and local context.

1.5.6 Role of ERβ in endocrine responsiveness/resistance

Tamoxifen has been the mainstay of hormonal treatment in ERα+ breast cancer for the past three decades. However, almost all patients with metastatic disease and approximately 40% of patients who receive adjuvant tamoxifen develop tumour recurrence and die from breast cancer (Hurvitz & Pietras, 2008; Normanno *et al*, 2005).

Loss of expression or mutation of ERα, PgR negative status, cross talk between ER and growth factor receptor pathways, metabolism of hormonal agents, oestrogen supersensitivity, oestrogen hypersensitivity and altered expression of coregulators have all been implicated in hormone resistance (Normanno *et al*, 2005). Since its discovery, the role of ERβ in breast hormone responsiveness has also been intensely investigated. ERα is an accepted but imperfect predictive marker for hormone responsiveness. The general assumption is that ERβ and its isoforms with their differential expression pattern and function might play a role in hormone responsiveness. Experimental studies have shown anti-proliferative as well as tumour suppressive effects for ERβ. However, studies investigating the relationship between ERβ expression and hormonal therapy have produced inconsistent results.

Miller *et al.* investigated expression levels of ERα, ERβ1 and ERβ2 proteins in 36 patients in a neo-adjuvant setting with tamoxifen treatment for three months. All tumours were positive for ERα and ERβ1, whereas ERβ2 was positive in 45% cases only. 70% of patients showed a clinical response and 48% showed a pathological response, but there was no significant difference in the expression levels of these receptors between tumours that responded and that did not. Although ERα expression was significantly decreased post treatment, no significant difference was seen between tumours that responded and those that did not (Miller *et al*, 2006). In another study, in the neo-adjuvant setting, again ERβ mRNA level was not predictive of hormone response and there was no significant change in expression level post-treatment (Cappelletti *et al*, 2004). Speirs *et al.* measured ERβ mRNA in 17 breast cancer patients treated with tamoxifen and found ERβ was upregulated in the tamoxifen resistant group and the ERβ upregulation was also seen in tamoxifen resistant cell lines (Speirs *et al*, 1999a). Murphy *et al.* studied cancer samples in 27 ERα+, node negative patients who received tamoxifen. Total ERβ protein as well as ERβ1, ERβ2 and ERβ5 mRNA were measured. High ERβ protein expression correlated with tamoxifen sensitive tumours and no correlation was seen with mRNA levels (Murphy *et al*, 2002). Mann *et al.* performed total ERβ immuno analysis in 118 (ERα+/-) tamoxifen adjuvantly treated patients and showed ERβ+ tumours were significantly associated with increased survival in the whole cohort as well as in the node negative cohort (Mann *et al*, 2001). Hopp *et al.* measured total ERβ by immunoblot analysis in 186 patients (ERα+/-) treated with tamoxifen and found

better survival with high ERβ (Hopp *et al*, 2004). In another study of 52 patients (ERα+/-) high ERβ1 was associated with better relapse free survival (Fleming *et al*, 2004). O'Neill *et al.* showed ERβ1 was associated with a trend for worse survival in 138 (ERα+/-) as well as in 91 ERα+ subgroup. Essilimani-Sahla *et al.* measured total ERβ and ERβ2 protein in 16 tamoxifen-resistant and 34 tamoxifen-sensitive tumours and found that low levels total ERβ, not ERβ2 was associated with tamoxifen resistance (Esslimani-Sahla *et al*, 2004). Saji *et al.* assessed ERβ2 in 18 breast cancer in neo adjuvant tamoxifen setting and found poor response to treatment with ERβ2 expression (Saji *et al*, 2002). Palmieri *et al.* found association between ERβ2 protein and better outcome in a cohort of 23 patients (ERα+) treated with either neo-adjuvant or palliative endocrine treatment (Palmieri *et al*, 2004).

The majority of above studies in relation to hormonal treatment showed good outcome with high ERβ. However, some studies showed no correlation or correlation with poor outcome. Most of the studies were of small numbers, contained both ERα+ and ERα- patients, unselected patients and used varying methods to detect the ERβ or its variants. The role of ERβ in hormonal responsiveness is still unclear.

1.6 Aims and hypothesis

The classical oestrogen receptor (ERα), as a measure of steroid hormone receptor status, is currently an acceptable prognostic marker that predicts the response to hormone therapy. However, it is well known that up to 40% of breast tumours with positive ERα status do not respond to endocrine therapy. Reasons for this lack of response are poorly understood and the role of the more recently identified oestrogen receptor ERβ is unclear. In addition to the full-length "wild-type" ERβ (termed ERβ1) there are a number of variants that are expressed in both normal and cancer tissue and arise from differential splicing (and deletion of exons). A number of studies have employed RT-PCR, *in situ* hybridization or immunohistochemistry to analyse the expression of ERβ in mammary tissue and tumours. The results so far are conflicting, reflecting in part the different case selection or detection techniques used, and because the existence of the variants of ERβ can make it unclear which has

been measured. Nevertheless, it has been suggested that the level of ERβ expression may have prognostic or predictive significance in breast cancer.

The aim of this study is to further investigate the complex expression of the ERβ variants in breast tumours, in order to establish their relative importance in the response of patients to endocrine therapy.

Our hypothesis is that particular variants of ERβ may be more closely associated with outcome following adjuvant tamoxifen treatment of breast cancers. Such variants may be useful as predictive markers.

Table 1.1 Major breast cancer susceptibility genes.

Gene	Associated syndrome	Chromosome site	Gene frequency	Gene penetrance for breast cancer
BRCA1	HBOC	17q21	Rare	Very high
BRCA2	HBOC	13q12-13	Rare	High
p53	Li-Fraumeni	17p13.1	Very rare	High
PTEN	Cowden	10q22-23	Very rare	High
ATM	Ataxia-telangiectasia (heterozygotes)	11q22-23	Common	Low to moderate
STK11	Peutz-Jeghers	19p13.3	Very rare	High

HBOC: Hereditary breast ovarian cancer syndrome.

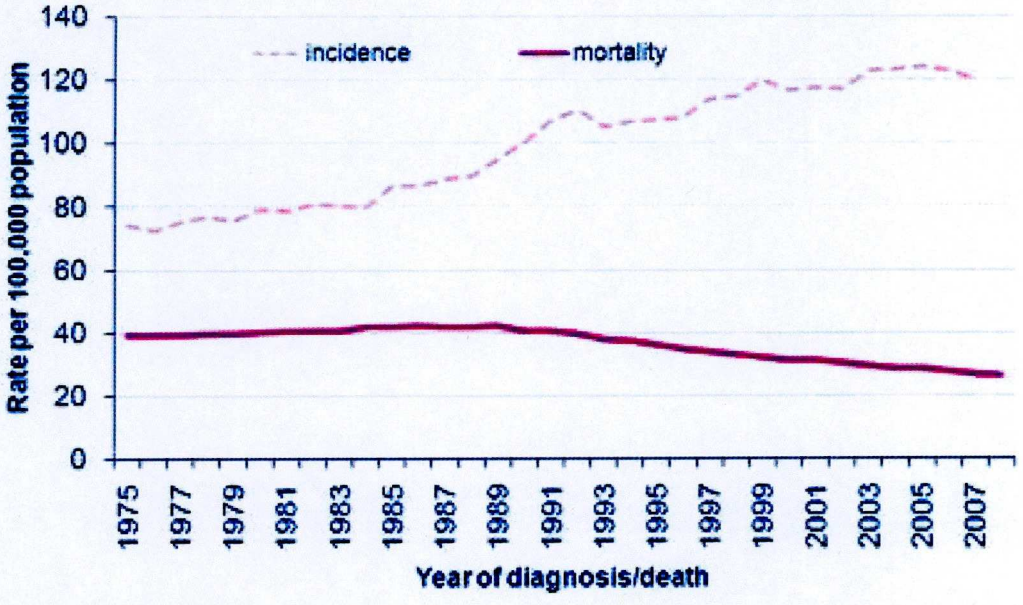


Figure 1.1 Age-standardised incidence and mortality from female breast cancer in Great Britain 1975-2008.
(http://info.cancerresearchuk.org/cancerstats/types/breast/incidence/ 2010, November 27)

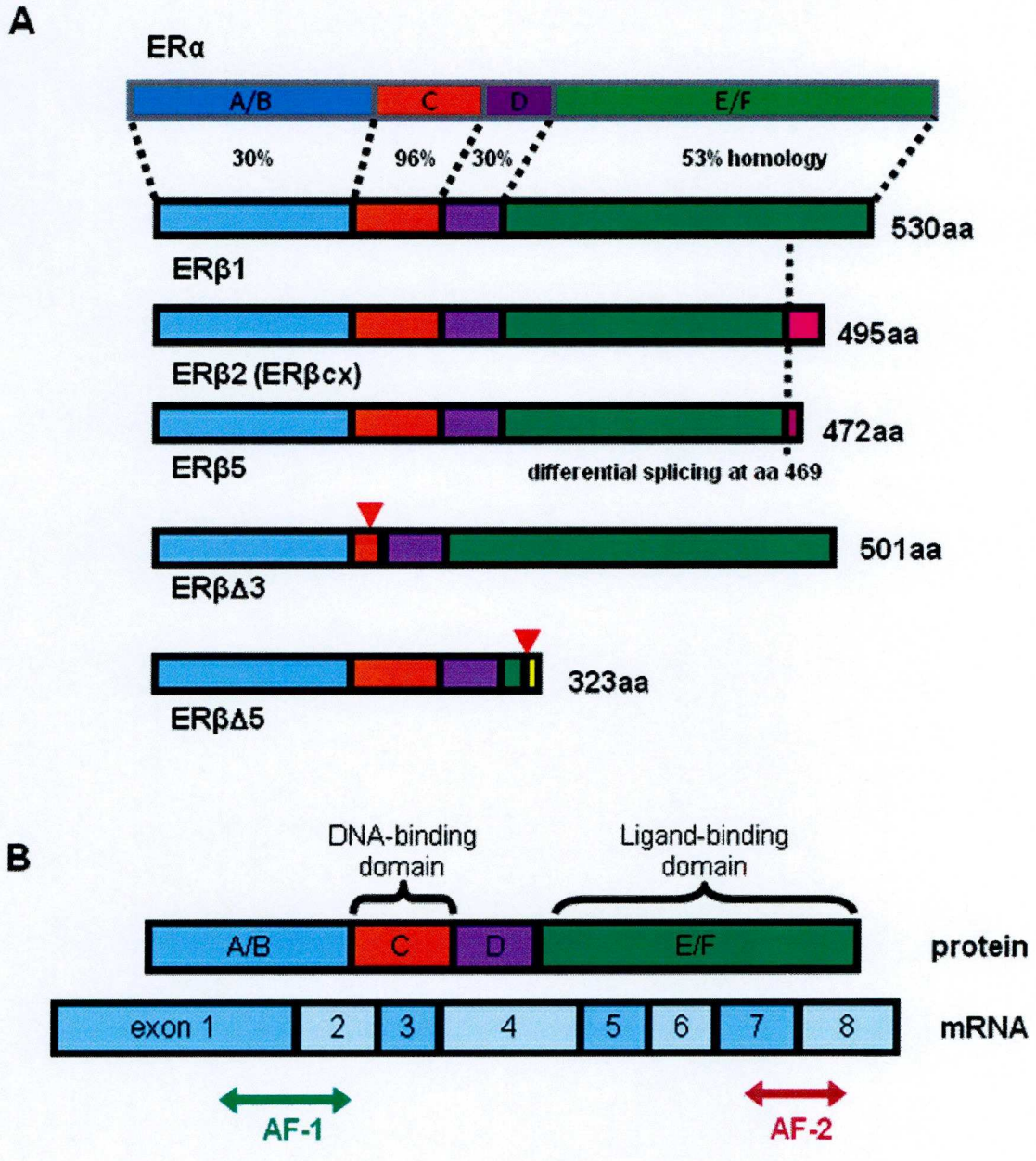


Figure 1.2 Structure of oestrogen receptors.

A. Comparison of ERα, ERβ and ERβ variants protein structure showing homology between ERα and ERβ1.

B. Alignment of ERβ1 protein and mRNA structure showing protein domains A to F, DNA and ligand binding domains, location of Activation Function regions (AF1 and AF2) and mRNA exons 1 to 8.

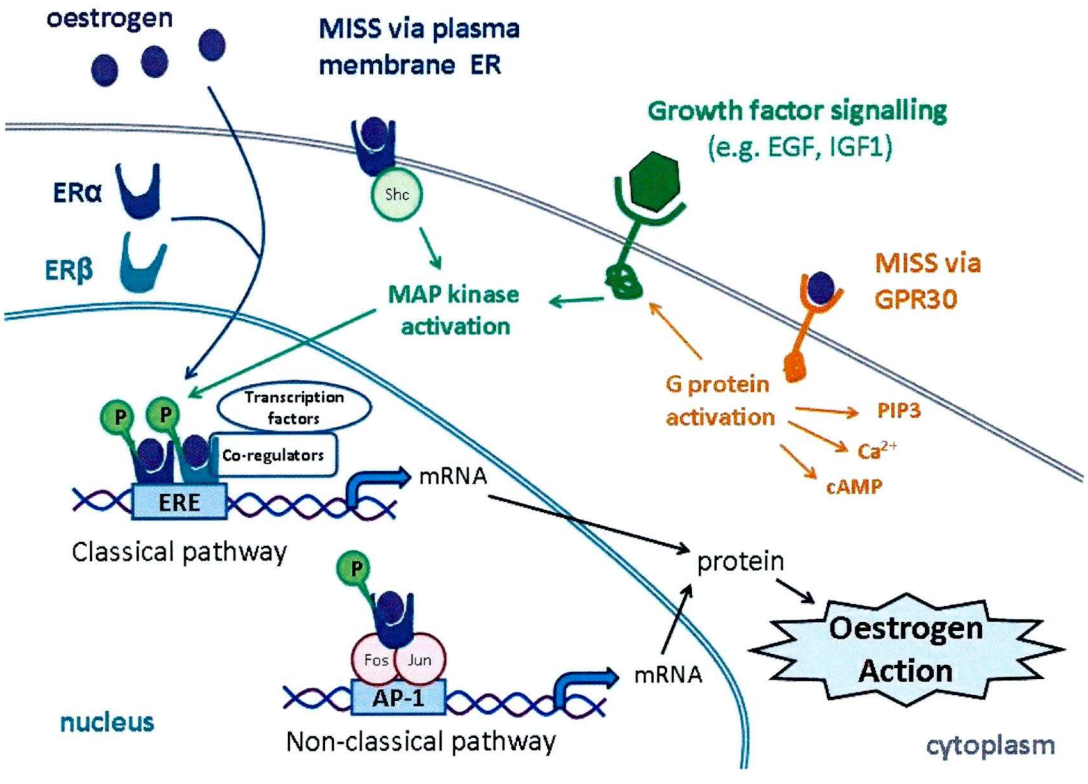


Figure 1.3 The multifaceted mechanisms of oestrogen and oestrogen receptor signalling (adopted from Speirs & Walker, 2007).

In the classical pathway, oestrogen (E2) binds to ERs to form homodimers or heterodimers (as shown here) and recruits co-regulator proteins as part of a complex that binds to EREs in promoters of target genes, regulating transcription and subsequent tissue responses. In the non-classical pathway (which is ERE independent), the E2-ER complex can activate transcription by tethering to alternative response elements such as those for Activator Protein 1 (AP-1) through Jun/Fos-proteins. Growth factors can activate intracellular kinase pathways, leading to phosphorylation and activation of ER in a ligand independent manner, attenuating its activity. In the MISS pathway, E2 binds to plasma membrane ER and activates intra cellular growth factor signalling pathways that generate rapid tissue responses. Recently, GRP30, a non ER protein in the plasma membrane has been also thought to play a role in mediating MISS.

ER-Oestrogen Receptor
P-Phosphorylation
ERE-Oestrogen Response Elements
AP-1 –Activator Protein 1
MISS- Membrane Initiated Steroid Signalling

CHAPTER 2 MATERIALS AND METHODS

2.1 Patient selection

Patients undergoing treatment for invasive breast cancer during the period 1993 and 1999 at the Royal Liverpool University Hospital were identified from a database at the Cancer Tissue Bank Research Centre (CTBRC), University of Liverpool [now the Liverpool Tissue Bank (LTB), http://www.liv.ac.uk/ltb/].

All patients had provided written, informed consent, at the time of their original surgery, for their tissue to be donated to the CTBRC for research purposes and also consented to have their hospital records reviewed in order to provide CTBRC with clinical follow-up data. Ethical approval for the study was obtained from the Liverpool Adult Research Ethics Committee (Reference 01/116), who also approved the collection of samples by the CTBRC with informed consent (Appendix 1).

A total of 141 patients with primary breast cancer were selected from the CTBRC database. They were all post-menopausal women and had received adjuvant hormonal therapy but not systemic chemotherapy. The clinicopathological characteristics of the patients included in this study are detailed in Chapter 3 (Table 3.1).

All cases were subjected to full histopathological review, according to the UK NHSBSP guidelines (National Coordinating Group for Breast Screening, 1997). ERα and Progesterone Receptor (PgR) status was obtained from review of histopathology notes where available or were determined immunohistochemically as described later in this chapter, using a cut-off of 10% positive cells to define the positive and negative groups.

Initial clinical data for these patients was collected by Dr Penny O'Neill (O'Neill *et al*, 2004) by retrospective review of patients' case notes held at the Royal Liverpool University Hospital NHS Trust. Data comprised of patient demographics including age at diagnosis, sex, menopausal status, family history and ethnic origin. The treatment given at diagnosis including details of surgery, radiotherapy and hormonal therapy were also collected. Follow-up data for the patients selected was collected by further retrospective review of patients' case notes held at the Royal Liverpool

University Hospital and at Clatterbridge Centre for Oncology by Dr Helen Innes (Dr H Innes MD thesis, University of Liverpool, 2004). In addition, follow-up data was requested from patients' General Practitioners where this was appropriate. Data recorded included evidence of relapse, including date, site(s), treatment given and outcome of such treatment. Details of last follow-up or date of death, together with cause of death and assessment of whether this was related or unrelated to breast cancer (where this was possible) were also collected. The median follow-up was 71 months for RFS (range 9 to 113) and 79 months for BCS (range 11 to 113).

2.2 Reverse transcription

RNA of suitable quality for 100 cases was obtained from the CTBRC. Testis, uterus, prostate and MCF7 cell line RNAs were obtained from Clontech Laboratories (USA) and ovary RNA was obtained from CTBRC. These were used as controls in the RT reaction and for the initial cloning of control RT-PCR products used to generate standard curves.

Cases were selected for RNA analysis following independent histological review of adjacent sections, so as to avoid high levels of tissue heterogeneity. Samples from all cases consisted of at least 75% tumour cells and 67% of cases had at least 90% tumour cells. Inflammatory infiltrates were present in a minority of cases (at 10% in 15 cases and at 25% in 4 cases).

Total RNA (5μg) from tumour samples were provided in 20μl ethanol. To precipitate the RNA, 2μl of 3M sodium acetate was added and the sample left at -20°C overnight. It was then centrifuged at 13000 g at 4°C for 30 minutes and transferred to ice. The supernatant was removed and 30μl ethanol used to wash the pellet. The pellet was dried at 40°C for 10 minutes to evaporate any remaining ethanol. 5μl Tris EDTA buffer (pH 7.6) was then added to re-suspend the pellet.

RT reactions were carried out in two steps. Initially single and double strand DNA present in the tumour sample were digested by Deoxyribonuclease I (DNase I) (Invitrogen, USA) resulting in purified tumour RNA. In the next step single strand cDNA was prepared from the tumour RNA by a Reverse Transcriptase enzyme reaction. This was performed in duplicate, using Superscript III (Invitrogen), an

RNase H$^-$ Reverse Transcriptase purified to near homogeneity from *E.Coli* containing the *pol* gene of Moloney Murine Leukaemia Virus.

Each 20µl reaction volume initially contained: 3µl total RNA (1µg/1µl), 2µl DNase I (Invitrogen), 2µl DNase I buffer and DEPC-treated water to 20µl. This DNAase digestion reaction was incubated at room temperature for 15 minutes and then terminated by the addition of 2µl EDTA (25mM) and heating to 65°C for 10 minutes. The above reaction volume was split into two 10µl reactions.

In to the each 10µl reaction the following was added: 1µl Oligo $(dT)_{12-18}$ (1mg/ml) and 1µl 10mM dNTP mix (10mM each dATP, dGTP, dCTP, dTTP at neutral pH). The reaction was heated to 65°C for 5 minutes followed by a quick chill on ice. The contents of the tube were collected by brief centrifugation and then added to: 4µl 5X first strand buffer, 2µl 0.1M DTT, 1µl Prime Recombinant Ribonuclease Inhibitor (Eppendorf, Cambridge, UK) and 1µl Superscript III Reverse Transcriptase (Invitrogen). The final reaction mix was heated to 50°C for 60 minutes and the reaction terminated by heating to 70°C for 15 minutes. Parallel reactions were performed in which the RT enzyme was omitted and these acted as controls for genomic DNA contamination. RT reactions (cDNAs) were diluted (1/2 dilutions and 1/50 dilutions) and stored to use in quantitative real-time RT-PCR.

2.3 Quantitative real-time RT-PCR

In real-time quantitative PCR (qRT-PCR), the amount of PCR product is measured at each cycle. This ability to monitor the reaction during its exponential phase enables users to determine the initial amount of target with great precision. The number of cycles and the amount of PCR end-product can theoretically be used to calculate the initial quantity of genetic material (by comparison with a known standard). In real-time PCR, the amount of DNA is measured after each cycle by the use of fluorescent markers. The increase in fluorescent signal is directly proportional to the number of PCR product molecules (amplicons) generated in the exponential phase of the reaction. Fluorescent reporters used include double-stranded DNA-binding dyes (e.g. SYBR® Green) or dye molecules attached to PCR probes (e.g. Taqman® probe). The fluorescence of DNA-binding dyes significantly increases when bound to

double-stranded DNA (dsDNA). The intensity of the fluorescent signal depends on the amount of dsDNA that is present. As dsDNA accumulates, the dye generates a signal that is proportional to the DNA concentration and can be detected using real-time PCR instruments. Taqman® probes require a pair of PCR primers in addition to a probe with both a reporter and a quencher dye attached. The probe is designed to bind to the sequence amplified by the primers. During qPCR, the probe is cleaved by the 5′-nuclease activity of the Taq DNA polymerase; this releases the reporter dye and generates a fluorescent signal that increases with each cycle.

Quantitative real-time reverse transcriptase polymerase chain reactions (qRT-PCR) were performed in a Bio-Rad Icycler PCR Machine (Bio-Rad Laboratories Ltd., Hertfordshire, UK). ERβ1 (full-length), C-terminal truncations (ERβ2/ERβcx, ERβ5) and exon deletions (ERβΔ5, ERβΔ3) were quantified with appropriate primers and Taqman probe (Table 2.1). For ERβΔ5 and ERβΔ3 PCR, one primer crossed the deleted exon boundary (Figure 2.1). ERα, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and HPRT (hypoxanthine ribosyltransferase) were quantified with IQ SYBR Green Supermix (Bio-Rad). GAPDH and HPRT were used to determine RNA integrity and RT efficiency and also to validate the quantities of candidate genes in PCR.

For candidate gene PCR, 4μl of a 1/2 dilution of cDNA was used per reaction (equivalent to cDNA from approximately 150ng of total RNA). For control gene PCR (HPRT and GAPDH) and ERα, 4μl of a 1/50 dilution of cDNA was used (equivalent to cDNA from approximately 6ng of total RNA). Oligonucleotide primers used for qRT-PCR are shown in Table 2.1 and have been previously validated (Critchley *et al*, 2002; Poola, 2003). Reactions included 1x IQ Supermix (Bio-Rad) and 0.5μM or 1 μM of each PCR primer and 0.2μM of the appropriate Taqman probe (Table 2.1). The PCR reactions consisted of a hot-start *Taq* polymerase activation step of 95°C for 3 minutes, followed by conditions shown to produce unique, specific bands for each mRNA (Table 2.1 and Figure 2.2). All amplicons crossed introns to avoid amplification of genomic DNA (Figure 2.1).

Absolute quantitation of mRNA for each gene was calculated using standard curves produced with the relevant gene's cloned cDNA dilutions (Figure 2.3). Briefly, within the BioRad Icycler software, threshold cycle values are calculated for control samples (i.e. the point in the PCR reaction at which the amplification curves crosses

a specific fluorescent value). These are plotted against the log of the starting amounts (in this case attomole amounts of cloned PCR products), which should give a straight line. Data at either end of the graph which do not fit on the straight line are removed; the remaining data are re-plotted and define the dynamic range for the assay (within which quantitation is reliable). The software calculates an efficiency measure (100% efficiency being equivalent to a doubling in DNA amount with each cycle) and a regression equation for the line. Using this regression equation, starting quantities (in attomoles) for test samples are calculated from their threshold cycle values. This is the amount of cDNA that has been put in the PCR reaction; from the proportion of the Reverse Transcriptase (RT) reaction used in the PCR reaction and the amount (in μg) of RNA in the RT reaction, this is converted to attomoles per μg RNA.

The mRNA levels of ERα, ERβ1, ERβ2, ERβ5, ERβΔ5 and ERβΔ3 are corrected by a factor calculated by referencing both house-keeping control genes against their mean values in the cohort. This process was performed for all PCR reactions including housekeeping control genes. To calculate a correction factor from these control genes (for differences in cDNA quality arising from RNA quality, RNA amount and RT efficiency) the quantities of control gene cDNA for each RT reaction was divided by the mean value of the control gene for the whole cohort. This was done individually for each control gene (HPRT and GAPDH) and a mean correction factor calculated (reducing any abnormal effects of either control gene if used separately). Dividing each test gene quantity by this correction factor ratio (for each RT reaction individually as this is where the main variances in cDNA quality arise) normalises all quantities to the control genes without changing the units (attomoles per μg RNA). Replicate corrected absolute quantities for each test gene were then averaged for each sample. These values were used in all subsequent analysis.

2.4 Agarose gel electrophoresis

Polymerase chain reaction products were separated and identified by gel electrophoresis. A 3% agarose gel was prepared by disolving an appropriate quantity of Seakem agarose (Flowgen) in TAE buffer (40mM Tris acetate, 1mM EDTA, pH 7.6) by boiling in a microwave oven. After cooling to 65°C, ethidium bromide

(0.5μgm/ml) was added for visualisation of the DNA and the gel was poured into a prepared tray and allowed to cool. 5μl of PCR product was mixed with 2μl loading dye [0.25% (w/v) Orange G (sigma), 0.025% (w/v) Xylene Cyanol (sigma) and 40% (w/v) Sucrose] and 3μl of water, and this 10μl sample was wet-loaded into the gel immersed in TAE electrophoresis buffer. Molecular weight markers (PhiX174/HaeIII, Abgene) were included on each gel. The gels were run at 120-150V, scanned on a Typhoon 9400 fluorescent imager (Amersham) and analysed with ImageQuant version 4.1 software (Molecular Dynamics). This fluorescent scanner provides an image of the gel, with DNA visualised by chelation of ethidium bromide. Examples of gel electrophoresis are shown in Figure 2.2.

2.5 Cloning and DNA sequence analysis

PCR products were cloned into PCR2.1 TOPO plasmid vectors using TOPO TA cloning® kit (Invitrogen) according to manufacturers guidelines. The cloned cDNA inserts and flanking vector sequences were then amplified by colony PCR with M13 primers. The PCR reactions were treated with ExoSAP (Amersham Pharmacia Biotech, to remove unused PCR primers) and sequenced individually with both M13 primers using DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Pharmacia Biotech). Sequencing products were purified (AutoSeq plate, Amersham Pharmacia Biotech) and analysed on a MegaBACE 1000 DNA analyser (Molecular Dynamics). The identities of the cloned cDNAs were confirmed by performing web-based database searching via BLAST at the NCBI (www.ncbi.nlm.nih.gov). Example DNA sequence and BLAST alignments are given for ERβΔ5 (Figure 2.4) and ERβΔ3 (Figure 2.5).

2.6 Immunohistochemistry (IHC)

Histological sections (4μm) were cut from archival formalin-fixed, paraffin-embedded specimens and provided by CTBRC on 2% 3-aminopropyltriethoxysilane (APES) coated slides. All sections were cut from formalin-fixed, paraffin-embedded archival specimens in September 2001 and stored in room temperature until September 2005, when this experiment was performed.

Mouse anti-human ERβ2 monoclonal antibody MCA2279S (clone no 57/3; Serotec Ltd, Oxford, UK) was employed to recognise the ERβ2 isoform. Optimisation of the immunohistochemistry was initially performed by antibody kindly donated by Dr P Saunders (Edinburgh) and further validated with MCA2279S purchased from Serotec. Specificity of the antibody was confirmed by incubation with pre-immune serum. For detection of ERα, a mouse anti-human ERα monoclonal antibody was used (Clone 1D5, Dakocytomation Ltd, Ely, Cambridge, UK). Progesterone receptor (PgR) status was measured using a mouse monoclonal anti-PgR antibody (Clone 636, Dakocytomation Ltd, Ely, Cambridge, UK).

ERβ2 immunostaining

Formalin-fixed and paraffin wax-embedded sections of normal and malignant breast tissues were used for immunostaining. After de-waxing and rehydration, the slides were immersed in 3% hydrogen peroxide (30ml 30% hydrogen peroxide in 270ml of alcohol) for 15 minutes to block the endogenous peroxidases. Antigen retrieval was by microwaving the slides for 10 minutes in Antigen Unmasking Solution (H3300, Vector Laboratories Ltd., Peterborough, UK). Then slides were incubated in Protein Block Serum-Free (DakoCytomation, California, USA) for 10 minutes. Slides were incubated overnight at 4°C (Saunders *et al*, 2002) with ERβ2 antibody diluted (1:25) in 0.1% (w/v) BSA in phosphate buffered saline. Antibody binding was detected by applying biotinylated link antibody solution, followed by the streptavidin-HRP solution (LSABP2® System-HRP, DakoCytomation, California, USA), both for 30 minutes at room temperature. Slides were then incubated in 3,3'-diaminobenzidine (Sigma, St Louis, USA) for five minutes before counterstaining in Harris Haemotoxylin (Sigma, St Louis, USA) for 1.5 minutes. Slides were agitated in acid alcohol [1% (v/v) HCL in 70% (v/v) alcohol] for 15 seconds and then quickly dipped in Scott's tap water substitute for 15 seconds. Sections were dehydrated and mounted with DPX (Fisher Scientific UK Ltd, Loughborough, UK). Positive (control tissue) and negative (no primary antibody) controls were included in each batch of staining. In some negative controls the ERβ2 antibody was pre-incubated with a molar excess of immunising synthetic peptide CMKMETLLPEATMEQ (MCA928, clone W3/25, Serotec Ltd, Oxford, UK) (Saji *et al*, 2002; Saunders *et al*, 2002) prior to application to sections from specimens previously shown to stain positively. Nuclear staining

was abolished in these blocked controls, but some cytoplasmic staining remained. Scoring of tumour sections was performed for nuclear staining only.

ERα and PgR immunostaining

The immunostaining technique was similar to the above except the following steps. Slides were not incubated in Protein Block Serum-Free (DakoCytomation, California, USA) after antigen retrieval step. The ERα antibody (1:30 dilution) and PgR antibody (1:50 dilution) were applied to slides and incubated for 40 minutes at room temperature.

Assessment of immunostaining

Analysis was restricted to the epithelial component of all tissues. Stained slides were analysed independently by two observers (myself and Dr Vijay Aachi, Consultant Breast Pathologist, Royal Liverpool and Broadgreen University Hospital) using light microscopy. The percentage of positively stained malignant cells was estimated as was the staining intensity (weak, moderate and strong), and an immuno-score calculated according to the Allred system as shown in Table 2.2 (Harvey *et al*, 1999). The Allred scoring system is the currently validated and accepted scoring system across the UK and many other countries for ERα and PgR.

2.7 Statistical methods

Power calculations were performed using the PS program (Dupont & Plummer, 1998) with survival analysis implementation of Schoenfeld and Richter (Schoenfeld & Richter, 1982). Based on estimates of proportions of ERβ2 positive cases from the previous study from our laboratory (Davies *et al*, 2004) and available outcome data, we determined that this study would have 80% power with an α value of 0.05 to detect a hazard ratio below 0.73 or above 1.40 in the whole cohort (below 0.63 or above 1.74 in the ERα + cohort), which we considered appropriate to give an indication of clinical utility.

All other statistical analyses were performed using the SPSS® package (Windows, v.13). The degree of agreement between observers was assessed using the Kappa statistic; a value of >0.61 was taken to be a satisfactory agreement (Altman, 1991). Pearson correlation and Spearman's rank correlation were used as a measure of association between abundance of mRNA and the degree of immunohistochemical staining. The Student's t-test and the Mann-Whitney U test (MW) were used to compare the levels of mRNA or protein between cases defined by other parameters. For paired data, paired T-tests and Wilcoxon signed ranks tests were used.

Relapse Free Survival (RFS) was defined as any recurrence or metastasis to local, contralateral, regional, and distant locations. Breast Cancer Survival (BCS) was calculated from any breast cancer related mortality. Optimal cut-points (e.g. for the continuous variables given by qRT-PCR analysis) were determined using Receiver Operating Characteristic (ROC) plots for relapse free survival (RFS) and breast cancer survival (BCS) at 5 years after surgery. Individualized ROC plots were determined for various subgroup analyses. Curves for outcome were generated using the Kaplan-Meier method for censored data, with surviving patients' data being censored at the date of their last clinic visit. Curves from different groups of patients were compared using the log rank test. Unadjusted hazard ratios (HR) ± 95% confidence intervals (CI) were obtained using Cox's univariate analysis. Cox's regression model was used for multivariate survival analysis (Altman, 1991) and stratified Kaplan Meier plots with log rank tests were used to further investigate the interaction between two outcome related variables.

Table 2.1 qRT-PCR conditions

Gene	Primers/Probes	Conc. (µM)	Amplicon	Cycling Conditions	PCR product size
ERβ1 [a]	For- TTTGGGTGATTGCCAAGAGC Rev- AGCACGTGGGCATTCAGC Probe- CCTCCCAGCAGCAATCCATGCG	0.5 0.5 0.2	bases 1731-1917 in NM_001437	50 cycles: 94°C 20 sec., 68°C 60 sec.	186 bp
ERβ2 [b]	For- ATCCATGCGCCTGCTAAC Rev- GAGTGTTTGAGAGGCCTTTTCTG TCCTGATGCTCCTGTCCCACGTCA	0.5 0.5 0.2	bases 1265-1343 in AF124790	50 cycles: 95°C 20 sec., 52°C 20 sec.	78 bp
ERβ5 [a]	For- TTTGGGTGATTGCCAAGAGC Rev- CACTTTTCCCAAATCACTTCACC CCTCCCAGCAGCAATCCATGCG	0.5 0.5 0.2	bases 89-265 in AF061055	50 cycles: 94°C 20 sec., 65°C 60 sec..	177 bp
ERβΔ5 [a]	For- CGGCAAGGCCAAGAGAAG [d] Rev- GCATTTCCCCTCATCC TAGTGCTCACCCTCCTGGAGGCTGA	0.5 0.5 0.2	bases 1144- 1525 in NM_001437	50 cycles: 94°C 20 sec., 61°C 60 sec.	241 bp
ERβΔ3 [a]	[d] For- TTTAAAAGAAGCATTCAAGGCTC Rev- AACTCCTTGTCGGCCAACTT TAGTGCTCACCCTCCTGGAGGCTGA	0.5 0.5 0.2	bases 935-1335 in NM_001437	50 cycles: 94°C 20 sec., 61°C 60 sec.	283 bp
ERα [c]	For- CCACCAACCAGTGCACCATT Rev- GGTCTTTTCGTATCCCACCTTTC	1.0 1.0	bases 1030-1137 in NM_000125	40 cycles: 95°C 20 sec. 60°C 20 sec., 72°C 30 sec.	128 bp
HPRT [c]	For- GTGTTGGATATAAGCCAGACTTTGTT Rev- AACTCAACTTGAACTCTCATCTTAGGC	1.0 1.0	bases 597-763 in NM_000194	40 cycles: 94°C 30 sec., 64°C 60 sec.	167 bp
GAPDH [c]	For- GCATCCTGGGCTACACTGAG Rev- TCCACCACCCTGTTGCTGTA	0.5 0.5	Bases 917-1079 in NM_002046	40 cycles: 94°C 30sec., 65°C 90 sec.	163 bp

a primers from Poola (Poola 2003), b primers from Critchley *et al.* (Critchley, Henderson et al. 2002), c primers designed in-house, d primer crosses exon boundary

Table 2.2 Allred scoring system used for scoring immunohistochemistry.

Percentage score for proportion of staining	**Score for staining intensity**
0 = No nuclear staining	0 = No staining
1 = <1% nuclei staining	1 = Weak staining
2 = 1-10% nuclei staining	2 = Moderate staining
3 = 11-33% nuclei staining	3 = Strong staining
4 = 34-66% nuclei staining	
5 = 67-100% nuclei staining	

Allred score = Percentage staining score + Intensity score

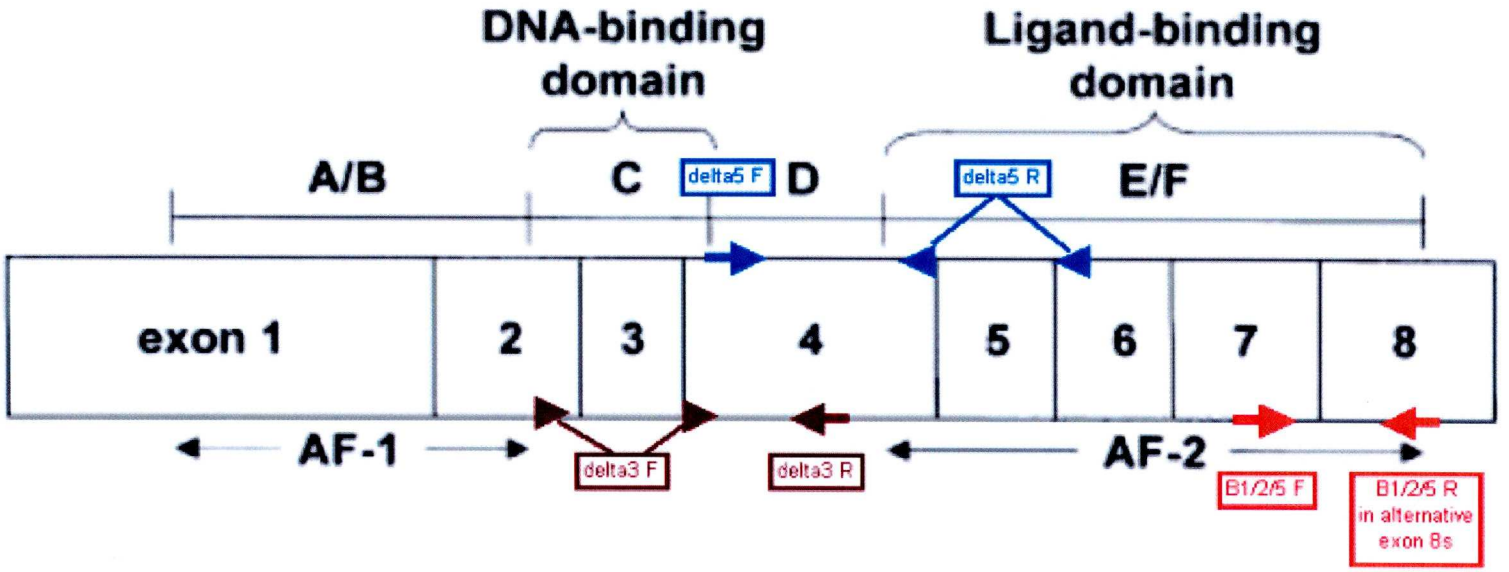


Figure 2.1 ERβ structure with location of primers for the ERβ variant specific qRT-PCR. Primers were designed in such a way to maximise the specificity.
F-Forward primer; R-Reverse primer.
For deletion variants, at least one primer crossed the exon boundary.
For C-terminal variants ERβ1 and ERβ5 the same forward primers were used, but different specific reverse primers. For ERβ2, separate forward and reverse primers were used.

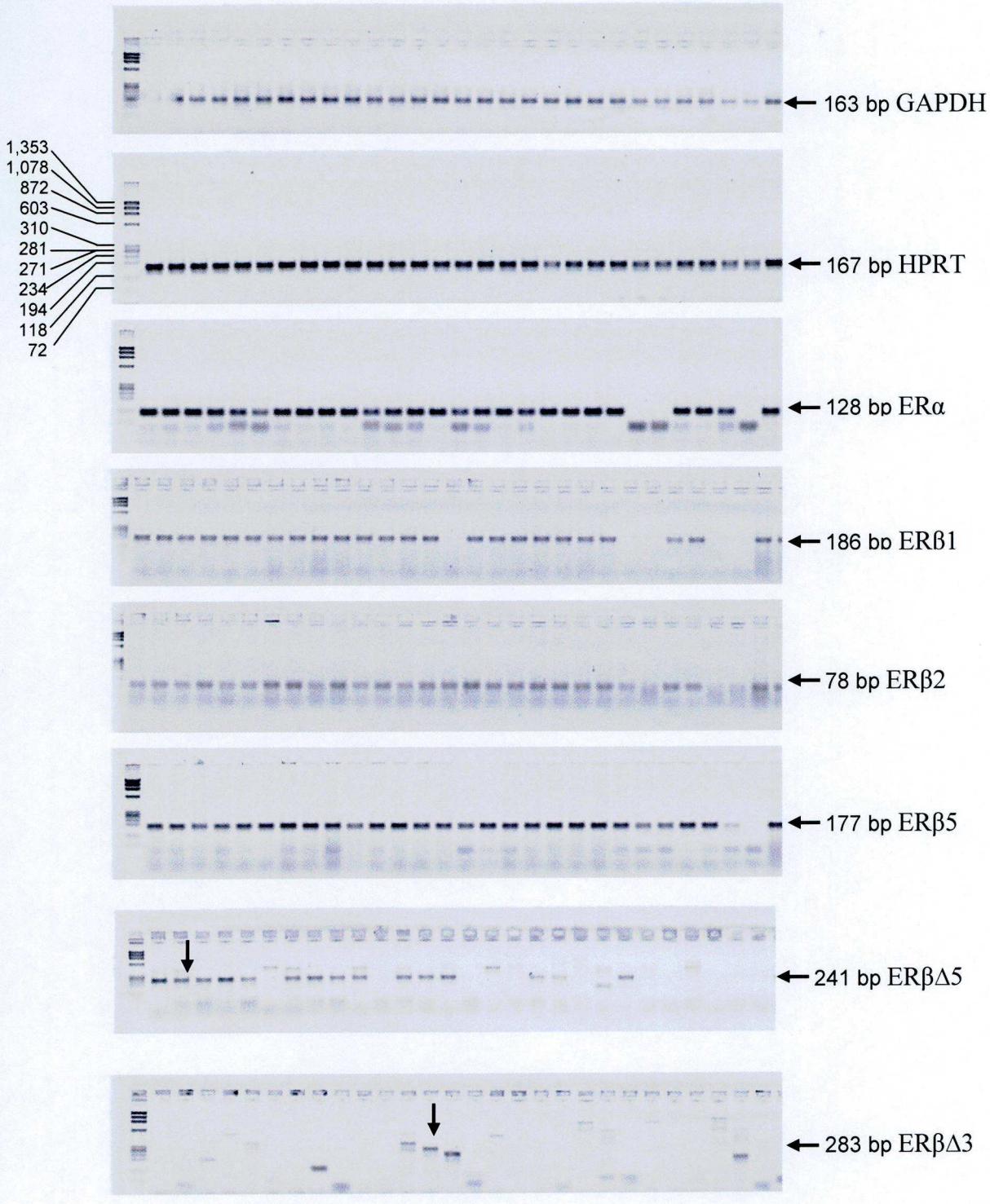
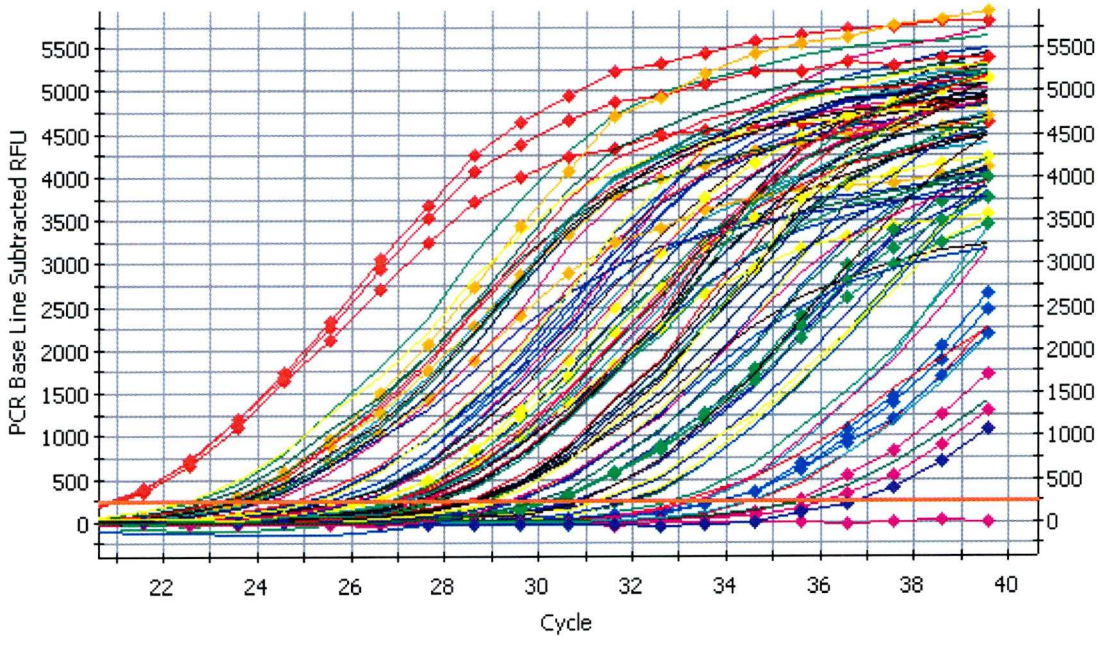


Figure 2.2 Examples of agarose gel electrophoresis for qRT-PCR products. On the left hand side of the gel, standard sizes of the molecular weight markers are shown and on the right hand side the PCR products identity and their expected sizes are shown.

A



B

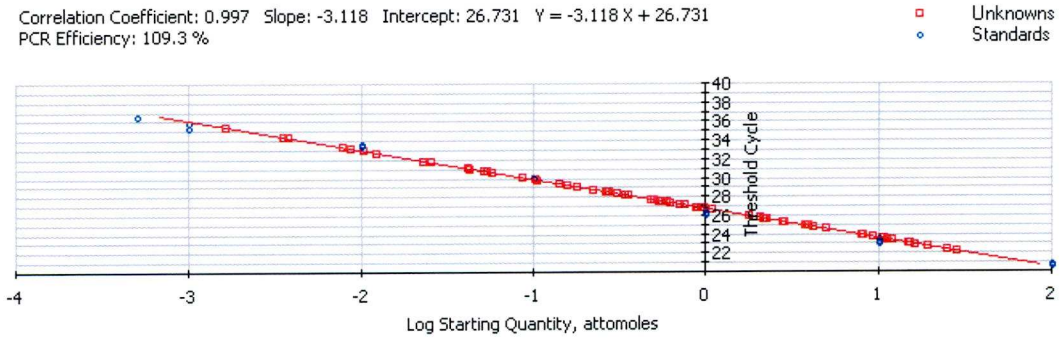
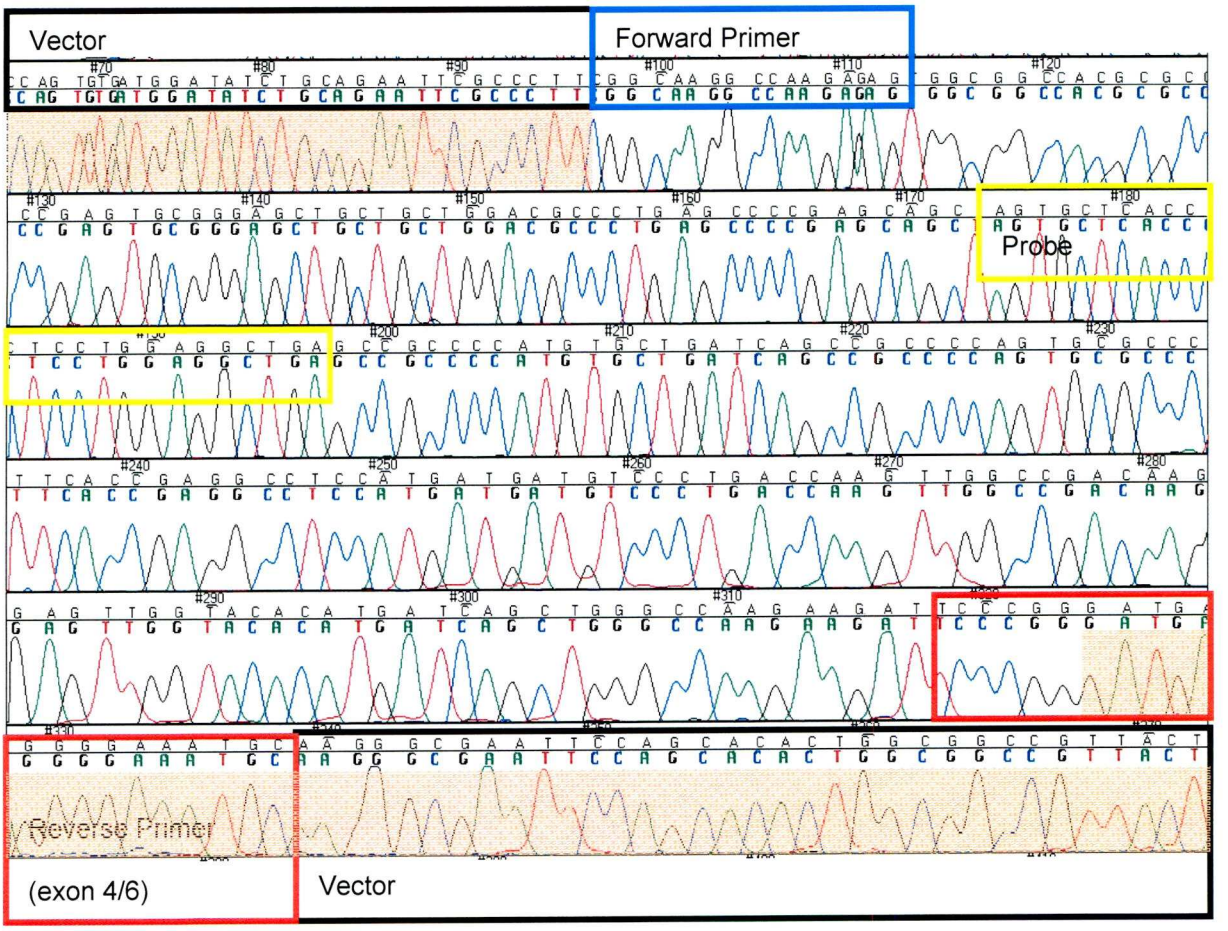


Figure 2.3 Real time PCR quantitation (A) and standard curve (B).

In A, Relative Fluorescent Units (RFU) for base line subtracted data are plotted against cycle number. Rainbow coloured curves with squares were from serially diluted cloned PCR standards (blue coloured circles in plot B). Various coloured lines without square markers represent unknown breast cancer samples (red squares in standard curve in plot B). The orange horizontal line in A is the threshold at which Ct values are calculated.

In B, Ct values for standards were plotted against log of known starting quantities to provide a linear plot from which unknown tumour sample values can be extrapolated.

A. ERβΔ5 DNA sequence analysis



B. ERβΔ5 BLAST result

>ref|NM_001437.2| Homo sapiens estrogen receptor 2 (ER beta) (ESR2), transcript variant a, mRNA
Length=2169

GENE ID: 2100 ESR2 | estrogen receptor 2 (ER beta) [Homo sapiens]
(Over 100 PubMed links)

Score = 403 bits (446), Expect = 8e-110
Identities = 227/228 (99%), Gaps = 1/228 (0%)
Strand=Plus/Plus

```
Query  97    CGGCAAGGCCAAGAGA-GTGGCGGCCACGCGCCCCGAGTGCGGGAGCTGCTGCTGGACGC  155
             ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1194  CGGCAAGGCCAAGAGAAGTGGCGGCCACGCGCCCCGAGTGCGGGAGCTGCTGCTGGACGC  1253

Query  156   CCTGAGCCCCGAGCAGCTAGTGCTCACCCTCCTGGAGGCTGAGCCGCCCCATGTGCTGAT  215
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1254  CCTGAGCCCCGAGCAGCTAGTGCTCACCCTCCTGGAGGCTGAGCCGCCCCATGTGCTGAT  1313

Query  216   CAGCCGCCCCAGTGCGCCCTTCACCGAGGCCTCCATGATGATGTCCCTGACCAAGTTGGC  275
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1314  CAGCCGCCCCAGTGCGCCCTTCACCGAGGCCTCCATGATGATGTCCCTGACCAAGTTGGC  1373

Query  276   CGACAAGGAGTTGGTACACATGATCAGCTGGGCCAAGAAGATTCCCGG  323
             ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1374  CGACAAGGAGTTGGTACACATGATCAGCTGGGCCAAGAAGATTCCCGG  1421
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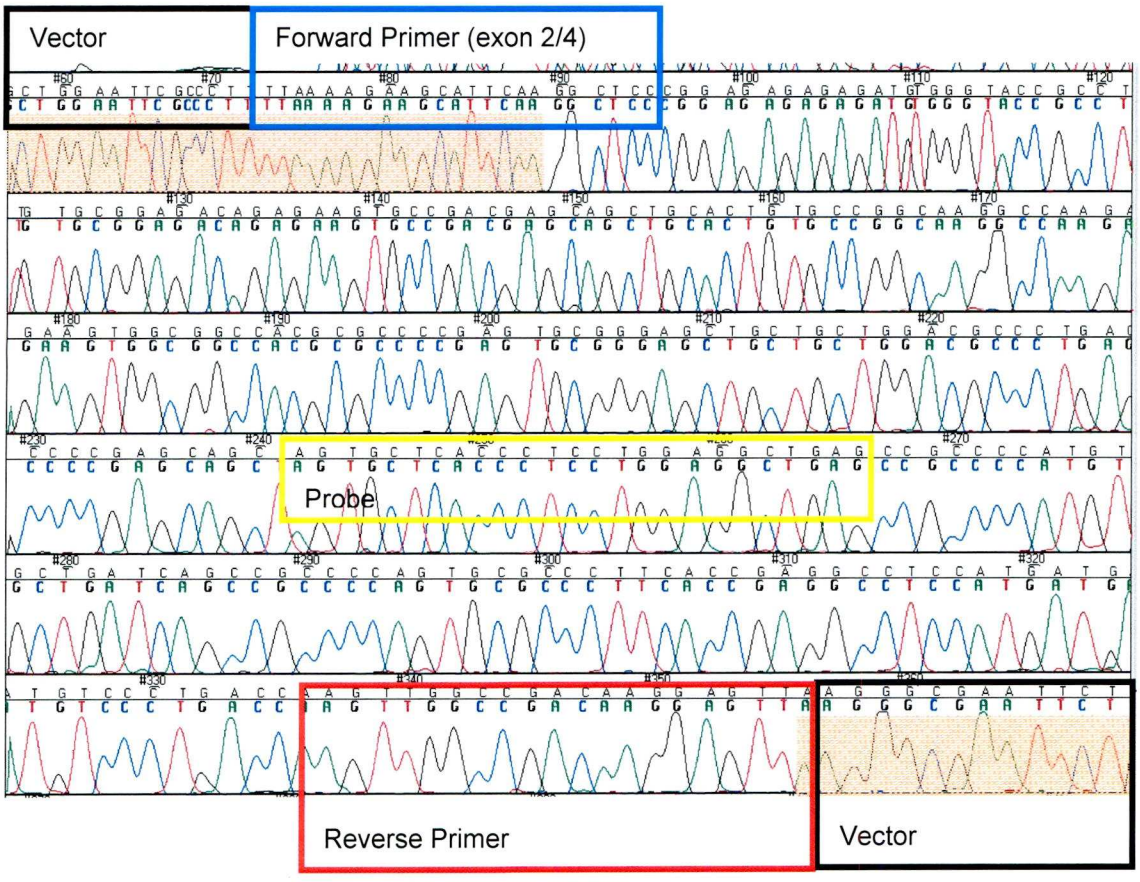
Figure 2.4 ERβΔ5 sequence analysis (A) and BLAST result (B) confirming the identity of PCR product; alignment match indicated by white background in A.

A. ERβΔ3 DNA sequence analysis



B. ERβΔ3 BLAST result

>ref|NM_001437.2| Homo sapiens estrogen receptor 2 (ER beta) (ESR2), transcript variant a, mRNA
Length=2169

GENE ID: 2100 ESR2 | estrogen receptor 2 (ER beta) [Homo sapiens]
(Over 100 PubMed links)

Score = 480 bits (532), Expect = 4e-133
Identities = 266/266 (100%), Gaps = 0/266 (0%)
Strand=Plus/Plus

```
Query  90    GGCTCCCGGAGAGAGAGATGTGGGTACCGCCTTGTGCGGAGACAGAGAAGTGCCGACGAG  149
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Sbjct  1120  GGCTCCCGGAGAGAGAGATGTGGGTACCGCCTTGTGCGGAGACAGAGAAGTGCCGACGAG  1179

Query  150   CAGCTGCACTGTGCCGGCAAGGCCAAGAGAAGTGGCGGCCACGCGCCCCGAGTGCGGGAG  209
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1180  CAGCTGCACTGTGCCGGCAAGGCCAAGAGAAGTGGCGGCCACGCGCCCCGAGTGCGGGAG  1239

Query  210   CTGCTGCTGGACGCCCTGAGCCCCGAGCAGCTAGTGCTCACCCTCCTGGAGGCTGAGCCG  269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1240  CTGCTGCTGGACGCCCTGAGCCCCGAGCAGCTAGTGCTCACCCTCCTGGAGGCTGAGCCG  1299

Query  270   CCCCATGTGCTGATCAGCCGCCCCAGTGCGCCCTTCACCGAGGCCTCCATGATGATGTCC  329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1300  CCCCATGTGCTGATCAGCCGCCCCAGTGCGCCCTTCACCGAGGCCTCCATGATGATGTCC  1359

Query  330   CTGACCAAGTTGGCCGACAAGGAGTT  355
             |||||||||||||||||||||||||
Sbjct  1360  CTGACCAAGTTGGCCGACAAGGAGTT  1385
```

Figure 2.5 ERβΔ3 sequence analysis (A) and BLAST result (B) confirming the identity of PCR product; alignment match indicated by white background in A.

CHAPTER 3 RESULTS 1

CLINICOPATHOLOGICAL DATA

3.1. Introduction

Patient selection is a very important process when evaluating biomarkers in a treatment specific population. There is a need to avoid factors that might unduly influence apparent expression of the biomarkers and to carefully select patients who received uniform treatment. Nevertheless, it is also useful if the cohort is representative of the general population. Every effort should be made to avoid selection bias and the cohort should have enough numbers to provide valid results. Therefore, having selected the cohort, here we analyse standard clinical markers in order to verify that they behaved as one might expect. This validates the cohort for further investigation of novel markers.

The Patient's clinical and pathological characteristics were obtained from Candis Cancer Tissue Bank Research Centre (CTBRC), University of Liverpool, Liverpool. Patient's treatment and follow-up data, which were collected retrospectively and stored in CTBRC were available for outcome analysis. All the patients who underwent operations for primary breast cancer between 1993 and 1999, and kindly donated tissue for research were initially analysed. From this large patient group, a specific cohort of patients who were postmenopausal women and received adjuvant endocrine therapy but not chemotherapy was then selected; patient had either mastectomy or wide local excision with or without axillary lymph node surgery and radiotherapy. Patients who had neo-adjuvant therapy were also excluded from the study. This gave us 141 cases for the study. Paraffin tissue sections for immunohistochemistry (IHC) were available for all the cases and suitable quantity of tumour RNA for qRT-PCR was available for 100 cases.

3.2 Summary of patient characteristics – IHC cohort

All the patients (Table 3.1) were postmenopausal women (n=141) and median age was 68 years (range 47 to 87). Postmenopausal status was obtained from patients notes. Staging investigations to exclude metastatic disease varied but generally

included chest radiograph and liver function tests and none of the patients had distant metastasis at the time of diagnosis.

They had been treated by surgery (47 mastectomy, 94 wide local excision). The overwhelming majority of cancers were less than 5cm in size (63 cancers were less than 2 cm in size and 74 were 2-5 cm in size) and in the remaining 4 cases, 3 cases were more than 5 cm in size (5.5, 6.5 & 8 cm) and it was not available for 1 case. Some form of axillary staging was carried out in118 patients. 51 cases had positive nodes in the axilla. Further analysis of nodal status showed that 36 cases were positive for 1-3 nodes, 10 cases were positive for 4-9 nodes and the remaining 5 cases had more than 10 positive nodes.

70 cases had adjuvant radiotherapy either to breast (n=40), breast and axilla (n=25) or breast and supra clavicular fossa (n=2) or chest wall (n=1) and, chest wall and supra clavicular fossa (n=2). All patients did receive adjuvant endocrine therapy; either tamoxifen (n= 133) or as part of the ATAC trial (n=8, blind to regimen at the time of study), but no one received systemic chemotherapy or primary endocrine therapy.

The majority of the cancers were invasive ductal carcinoma (n=121) and 11 cases were invasive lobular carcinoma and the remaining were rare type of tumours (Table3.2). The majority of the tumours were grade 3 (n=61) followed by grade 2 (n=58), and the remaining were grade 1 (n=22). Vascular invasion was present in 60 cases and was absent in 81 cases.

ERα status was obtained from CTBRC where available (n=136) and for the remaining cases (n=5) ERα staining was done as part of the current study using standard protocol. Progesterone receptor (PgR) status was obtained from CTBRC for 121 cases and for the rest of the cases (n=20), PgR staining was carried out as part of the current study. For ERα and PgR status a cut-off of 10% positive cells was used to define the positive and negative groups (O'Neill *et al*, 2004). In this cohort, 98 cases were positive and 43 were negative for ERα and, 69 cases were positive for PgR and 72 were negative for PgR. All patients received adjuvant endocrine therapy irrespective of ERα status (ERα status was not routinely measured for breast cancer patients before 1996).

3.3 Association between clinicopathological characteristics – IHC cohort

ERα+ status was associated with low-grade tumours (Chi-square P<0.001) (Figure 3.1), invasive ductal carcinoma (Chi-square P=0.032) and PgR positivity (Chi-square P<0.001), but not related to tumour size, nodal status and vascular invasion. PgR positivity was associated with low-grade tumours (Chi-square P<0.001), invasive ductal carcinoma (Chi-square P=0.030) and negative nodal status (Chi-square P=0.024). Positive nodal status was associated with large tumour size (Chi-square P=0.021, T-test P=0.0047) (Figure 3.2), high-grade tumours (Chi-square P=0.020), and lymphovascular invasion (Chi-square P<0.001). Invasive ductal carcinoma was associated with high-grade tumours (Chi-square P=0.006) and lymphovascular invasion (Chi-square P=0.001).

3.4 Survival analysis for clinicopathological characteristics – IHC cohort

Survival analysis [Relapse free survival (RFS) and Overall survival (BCS)] was performed for the established markers using Kaplan Meier method. Survival Curves for outcome were generated using the Kaplan-Meier method for censored data, with surviving patients' data being censored at the date of their last clinic visit; curves from different groups of patients were compared using the log rank test.

In Kaplan-Meier survival analysis, ERα+ status, PgR positivity, low grade (grade 1 *vs.* 2 *vs.* 3 and grade 1&2 *vs.* 3), negative nodal status, small primary tumour and cancers other than IDC were associated with good outcome (Table 3.3). Vascular invasion was not associated with outcome. Kaplan Meier survival plots for ERα status, tumour grade, nodal status and PgR status were shown in Figure 3.3 for RFS and Figure 3.4 for BCS.

Cox univariate analysis showed that ERα positive status, PgR positive status, low grade of the tumour (1&2 *vs.* 3), negative nodal status and cancers other than invasive ductal carcinoma (IDC *vs.* other types) were associated with better relapse free (RFS) and overall survival (BCS). Small tumours were associated with better

overall survival and also showed a trend towards better relapse free survival (Table 3.3). Lymphovascular invasion was not associated with survival.

Cox multivariate analysis was carried out including ERα status, PgR status, grade of the tumours (1&2 *vs.* 3), nodal status, tumour size (less than 2cm *vs.* more than 2cm) and tumour histology (IDC *vs.* Other types). For RFS, only negative nodal status (HR 3.08 CI 1.56 to 6.06; P=0.001) and low grade (HR 1.54 CI 1.10 to 2.16; P=0.012) were associated with good outcome. There was a trend for better RFS for PgR positive status (HR 1.54 CI 0.28 to 1.09; P=0.086).

For BCS, negative nodal status (HR 2.85 CI 1.33 to 6.11; P=0.007) and PgR positive status (HR 0.40 CI 0.18 to 0.91; P=0.029) were associated with good outcome. There was a trend for better BCS for low tumour grade (HR 1.42 CI 0.97 to 2.08; P=0.069).

In the ERα+ tamoxifen treated cohort (n=91), PgR positivity, low grade-(grade1 *vs.* 2*vs.* 3 and grade1&2 *vs.* 3), negative nodal status and small primary tumour were associated with good outcome in Kaplan-Meier survival analysis and Cox univariate analysis (Table 3.3). In multivariate analysis for RFS only negative nodal status (HR 3.91 CI 1.48 to 10.3; P=0.006) was associated with good outcome. There was a trend for better RFS for low grade (HR 1.52 CI 0.97 to 2.39; P=0.070). For BCS, similar to the whole cohort, negative nodal status (HR 4.39 CI 1.53 to 12.56; P=0.006) and PgR positive status (HR 0.33 CI 0.12 to 0.91; P=0.033) were associated with good outcome.

3.5 Summary of patient characteristics – qRT-PCR cohort

All the patients were postmenopausal women (n=100) and median age was 68 years (range 48 to 87). They had been treated by surgery (33 mastectomy, 67 wide local excision) and 44 cancers were less than 2 cm in size and 53 were 2-5 cm in size and in the remaining 3 cases, 2 cases were more than 5 cm in size (5.5 & 8 cm) and it was not available for 1 case. Some form of axillary staging was carried out in 88 patients. 39 cases had positive nodes in the axilla. Further analysis of nodal status showed that 31 cases were positive for 1-3 nodes, 5 cases were positive for 4-9 nodes and the remaining 3 cases had more than 10 positive nodes.

49 cases had adjuvant radiotherapy either to breast (n=28), breast and axilla (n=17) or breast and supra clavicular fossa (n=2) or chest wall (n=1) and, chest wall and supra clavicular fossa (n=1). All patients did receive adjuvant endocrine therapy; either tamoxifen (n= 93) or as part of the ATAC trial (n=7, blind to regimen during study period), but no one received systemic chemotherapy or primary endocrine therapy.

The majority of the cancers were invasive ductal carcinoma (n=85) and 8 cases were invasive lobular carcinoma and the remaining were rare type of tumours. The majority of the tumours were grade 3 (n=42) and grade 2 (n=44), and the remaining were grade 1 (n=14). Vascular invasion was present in 41 cases and was absent in 59 cases (Table 3.1).

ERα and PgR status were obtained from CTBRC for the majority of the cases, but as described previously additional staining was performed where status was unknown; 70 cases were positive and 30 were negative for ERα. In this cohort, 53 cases were positive and 47 were negative for PgR.

3.6 Association between clinicopathological characteristics – qRT-PCR cohort

Most of the correlations between clinicopathological characteristics seen in IHC cohort were maintained in the 100 patient cohort, but there were some differences.

ERα+ status was associated with low-grade tumours (Chi-square P<0.001) and PgR positivity (Chi-square P<0.001), but not related to tumour size, nodal status, tumour histology and lymphovascular invasion. Positive nodal status was associated with PgR positivity (Chi-square P=0.021), lymphovascular invasion (Chi-square P=0.001) and high-grade tumours (Chi-square P=0.045), but was no longer associated with tumour size (Chi-square P=0.109) or grade (Chi-square P=0.126) in this 100 patient cohort. Invasive ductal carcinoma type was associated with lymphovascular invasion (Chi-square P=0.003) and high-grade tumours (Chi-square P=0.014). Lymphovascular invasion was also associated with higher TNM stage (Chi-square P=0.017).

3.7 Survival analysis for clinicopathological characteristics – qRT-PCR cohort

Survival analysis was performed for this cohort similar to the immuno cohort using Kaplan Meier method. In Kaplan Meier survival analysis, ERα+ status, PgR positivity, low grade (grade1 *vs.* 2*vs.* 3 OR grade1&2 *vs.* 3), negative nodal status, small primary tumour and cancers other than IDC were associated with good outcome (Table 3.4). Vascular invasion was not associated with outcome.

Cox univariate analysis showed that ERα positive status, PgR positive status, low grade of the tumour (1&2 *vs.* 3), negative nodal status and invasive ductal carcinoma (IDC *vs.* Other types) were associated better relapse free (RFS) and overall survival (BCS). Small tumours were associated with better overall survival, but not with relapse free survival (Table 3.4). Lymphovascular invasion did not show correlation with survival.

Cox multivariate analysis was carried out including ERα status, PgR status, grade of the tumours (1-2 *vs.* 3), nodal status and tumour size (less than 2cm Vs more than 2cm) tumour histology (IDC *vs.* other types). For RFS, negative nodal status (HR 3.05 CI 1.36 to 6.84; P=0.007) and low tumour grade (HR 1.55 CI 1.05 to 2.27; P=0.026) were associated with good outcome. For BCS, once again negative nodal status (HR 3.46 CI 1.33 to 8.99; P=0.001) and low tumour grade (HR 1.75 CI 1.11 to 2.77; P=0.017) were associated with good outcome.

In the ERα+ tamoxifen treated cohort (n=64), low grade (grade1 *vs.* 2 *vs.* 3 and grade1&2 *vs.* 3) and negative nodal status were associated with good outcome in Kaplan-Meier survival analysis and Cox univariate analysis. There was a trend for PgR positivity for BCS (Table 3.4). In multivariate analysis, for RFS, only negative nodal status (HR 3.14 CI 1.09 to 9.01; P=0.03) was associated with good outcome; for BCS, only grade (HR 3.08 CI 1.21 to 7.81; P=0.018) was associated with good outcome.

3.8 Discussion

When undertaking a study of predictive markers, or a survey of expression in relation to clinicopathological features, it is important to assess the extent to which the cohort reflects the wider population, or the clinically relevant treatment group.

Patients selected in this study were from the CTBRC (Cancer Tissue Bank Research Centre) database from a single institute. The patient selection was done with the aim of the study in mind, to assess the role of ERβ in adjuvant endocrine treated postmenopausal primary breast cancer. Comparison of this database and the cohort of patients selected for this study were made with the Surveillance, Epidemiology, and End Results (SEER) data of the National Cancer Institute (Carter *et al*, 1989) (Table 3.5). The majority of breast cancers were invasive ductal carcinoma followed by lobular carcinoma and there were more node-negative cancers than node-positive cancers in both studies. The majority of tumours were 2-5 cm in size in both studies (55% *vs.* 53 %); however, tumours measuring less than 2 cm were significantly higher in this study (45% *vs.* 34%). This is despite a somewhat lower proportion of smaller tumours (30%) in the CTBRC as a whole, presumably as there was a tendency to bank predominantly large tumours. Hence this cohort may not be fully representative of a random sample of breast cancers. However, the patients included in the study were highly selective, as they are all postmenopausal women and received adjuvant endocrine therapy and no neo-adjuvant therapy. Possible reasons for this discordance with unselected series include that this cohort being postmenopausal, is likely to contain more screen detected tumours which are usually smaller; and being selected for adjuvant endocrine therapy only, is likely to contain fewer large tumours that required chemotherapy.

Significant benefits of careful patient selection include more consistent assessment of hormonal factors and a better measure of treatment specific outcomes. Measurement of oestrogen receptor expression in postmenopausal women is more consistent as expression may vary with menstrual status in premenopausal women. Patients who received neo-adjuvant chemotherapy or endocrine therapy were excluded, so that any effect on ERβ levels from previous treatment was avoided. Patients receiving adjuvant chemotherapy were excluded, to focus on the endocrine treatment effect alone.

This retrospective cohort included 43 patients who received endocrine therapy, but later found to be ERα negative. Inclusion of such patients allowed the investigation of relationships between ERβ and clinicopathological characteristics in primary tumours in relation to both ERα positive and negative status. Whilst this allows one to address the biology of breast cancer, relation to outcome is best studied in the more restricted ERα positive subgroup receiving endocrine treatment.

In this study, the majority of the tumours were invasive ductal carcinoma, and this histological type was associated with worse outcome compared to other types. Ellis *et al.* analysed a series of 1621 women, who underwent primary breast cancer operation, but did not receive any adjuvant systemic treatment. Invasive lobular carcinoma and special types like tubular, invasive cribriform and mucinous carcinoma were associated with better prognosis than invasive ductal carcinoma of no special type (Ellis *et al*, 1992). Hence the prognostic impact of invasive ductal carcinoma is apparently maintained in this postmenopausal endocrine treated cohort.

ERα positive patients had better survival compared to ERα negative patients, as expected from the prognostic and predictive value of ERα. Association between ERα positive status and better relapse free survival was first noted in 1977 (Knight *et al*, 1977). Further studies showed that ER positivity was associated with both relapse free as well as overall survival (Hawkins *et al*, 1987; Parl *et al*, 1984). Barnes *et al.* showed in a study of 831 patients (659 ERα+ and 172 ERα-) treated with adjuvant tamoxifen, with 6.5 years of median follow-up, ERα+ patients had better survival (Barnes *et al*, 2004). Association between ERα status and outcome in the present study were entirely consistent with these previous findings and the known biology of tamoxifen action.

Low grade tumours were associated with better relapse free and overall survival. Again this was well recognised in previous studies (Elston & Ellis, 1991; Fisher *et al*, 1993). Positive nodal status was associated with high-grade tumours, large tumours and lymphovascular invasion. Many studies have shown a direct relationship between tumour size and the probability of lymph nodal involvement (Carter *et al*, 1989; Nemoto *et al*, 1980). Positive nodal status and large tumour were associated with poor prognosis, as seen in previous studies (Carter *et al*, 1989; Donegan, 1992; Gebauer *et al*, 2002).

Therefore, even though the study was highly selective and relatively small, the association between the clinicopathological characters were similar to most of the published literature from unselected patient cohorts. In spite of the relatively small number of cases studied, the outcome analysis of the standard markers (e.g. nodal status, grade, size and ERα) was again consistent with previous larger studies. Hence this cohort provides a good basis to further explore novel associations between outcome and alternative markers, such as those provided by ERβ and its splice variants.

Table 3.1 Patient characteristics

Characteristics		**Immunohistochemistry Cohort n=141 (%)**	**qRT-PCR Cohort n=100**
Surgery	Mastectomy	47 (33.3)	33
	WLE	94 (66.7)	67
Radiotherapy	Yes	70 (49.6)	49
	No	71 (51.4)	51
Histology	Invasive ductal	121 (85.8)	85
	Others	20 (14.2)	15
Grade	G1	22 (15.6)	14
	G2	58 (41.1)	44
	G3	61 (43.3)	42
Size	T1	63 (44.7)	44
	T2	74 (52.5)	53
	T3	3 (2.1)	2
	Unknown	1 (0.7)	1
Nodal status	+	51 (36.2)	39
	-	67 (47.5)	49
	Unknown	23 (16.3)	12
Vascular invasion	Present	60 (42.6)	41
	Absent	81 (57.4)	59
ERα	+	98 (69.5)	70
	-	43 (30.5)	30
PgR	+	69 (47.5)	53
	-	72 (52.5)	47

Table 3.2 Histological types

Histological Type	**No of cases-whole cohort n=141 (%)**	**No of cases-qRT-PCR cohort (n=100)**
Invasive ductal carcinoma	121 (85.8)	85
Invasive lobular carcinoma	11 (7.8)	8
Mucinous carcinoma	5 (3.5)	3
Papillary	1 (0.7)	1
Tubular	1 (0.7)	1
Medullary	1 (0.7)	1
Mixed mucinous and papillary	1 (0.7)	1

Table 3.3 IHC cohort -Univariate and Kaplan Meier (KM) survival analyses.

	Survival	Whole cohort (n=141)			ERα+ and tamoxifen adjuvant cohort (n=91)		
		Cox Hazard Ratio	Cox P value	KM Log rank P value	Cox Hazard Ratio	Cox P value	KM Log rank P value
ERα+	RFS	2.83 (1.67 to 4.81)	0.0001	<0.001	N/A	N/A	N/A
	BCS	2.32 (1.28 to 4.21)	0.005	0.004	N/A	N/A	N/A
PgR+	RFS	2.74(1.53 to 4.91)	0.001	<0.001	0.40 (0.19 to 0.87)	0.020	0.016
	BCS	3.52 (1.74 to 7.14)	0.0005	<0.001	0.25 (0.10 to 0.61)	0.002	0.001
Grade (1&2 *vs.* 3)/(1*vs*2*vs*3)	RFS	1.90(1.43 to 2.51)	0.00001	<0.001	1.92 (1.30 to 2.82)	0.001	<0.001
	BCS	2.07 (1.49 to 2.87)	0.00001	<0.001	2.28 (1.46 to 3.55)	<0.001	<0.001
Positive nodal status	RFS	4.26(2.21 to 8.25)	0.0002	<0.001	5.07 (2.02 to12.7)	0.001	<0.001
	BCS	4.01 (1.92 to 8.37)	0.0002	<0.001	5.34 (1.90 to 15.06)	0.002	<0.001
Tumour size	RFS	1.67 (0.96 to 2.87)	0.070	0.07	-	-	-
	BCS	2.09 (1.10 to 3.95)	0.024	0.02	2.61 (1.06 to 6.46)	0.04	0.031
Cancers other than IDC	RFS	3.50 (1.09 to 11.23)	0.035	0.02	-	-	-
	BCS	8.41 (1.16 t0 61.14)	0.035	0.01	-	-	-

N/A – Not applicable

Table 3.4 qRT-PCR cohort-Univariate and Kaplan Meier (KM) survival analyses.

	Survival	**Whole cohort (n=100)**			**ERα+ and tamoxifen adjuvant cohort (n=64)**		
		Cox Hazard Ratio	**Cox P value**	**KM Log rank**	**Cox Hazard Ratio**	**Cox P value**	**KM Log rank**
ERα+	RFS	2.30 (1.21 to 4.37)	0.011	0.009	N/A	N/A	N/A
	BCS	2.14 (1.02 to 4.51)	0.046	0.04	N/A	N/A	N/A
PgR+	RFS	2.03(1.05 to 3.92)	0.036	0.032	-	-	-
	BCS	2.38 (1.08 to 5.23)	0.031	0.026		0.08	0.07
Grade (1&2 *vs*	RFS	1.69(1.22 to 2.34)	0.002	<0.005	2.57 (1.3 to 5.06)	0.007	0.006
3)/(1*vs*2*vs*3)	BCS	2.03 (1.35 to 3.04)	0.001	<0.001	4.85 (1.93 to 12.18)	0.001	0.001
Positive nodal	RFS	3.84(1.75 to 8.40)	0.001	<0.001	4.14 (1.53 t0 11.23)	0.005	0.003
status	BCS	4.52 (1.79 to 11.43)	0.001	<0.001	4.31 (1.35 to 13.77)	0.014	0.007
Tumour size	RFS	1.55 (0.81 to 3.0)	0.188	-	-	-	-
	BCS	2.23 (1.01 to 4.93)	0.048	0.042	-	-	-
Cancers other than IDC	RFS	4.20(1.01 to 17.46)	0.049	0.02	-	-	-
	BCS	27.66 (0.43 to 1777)	0.118	0.01	-	-	0.04

Table 3.5 Comparison of CTBRC cases with SEER data.

		SEER data	**CTBRC data**	**IHC cohort**	**qRT-PCR cohort**
Number of cases		24 740	379	141	100
Histological type	Ductal (NST)	83.4%	88.2%	85.8%	85%
	Lobular	7.4%	6.3%	7.8%	8%
	Others	9.3%	5.5%	6.4%	7%
Size	<2cm	33.6%	29.5%	45.0%	44.0%
	2-5cm	55.4%	63.0%	52.9%	53.0%
	>5cm	10.9%	7.5%	2.8%	2.0%
Nodal Status	Negative	54.4%	46.0%	56.8%	55.7%
	Positive	45.5%	54.0%	43.2%	44.3%

CTBRC: Cancer Tissue Bank Research Centre

SEER: Surveillance, Epidemiology, and End Results (SEER) data of the National Cancer Institute

NST: No Specific Type

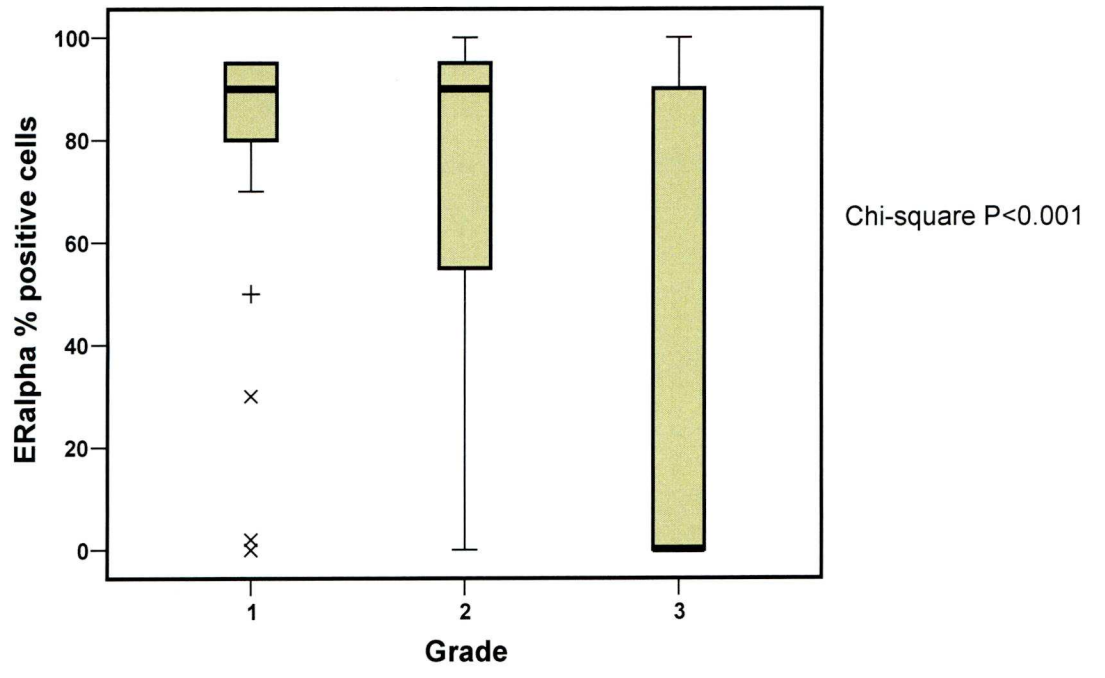


Figure 3.1 Association between ERα positivity and low grade tumours.

ERα+ status was associated with low-grade tumours.

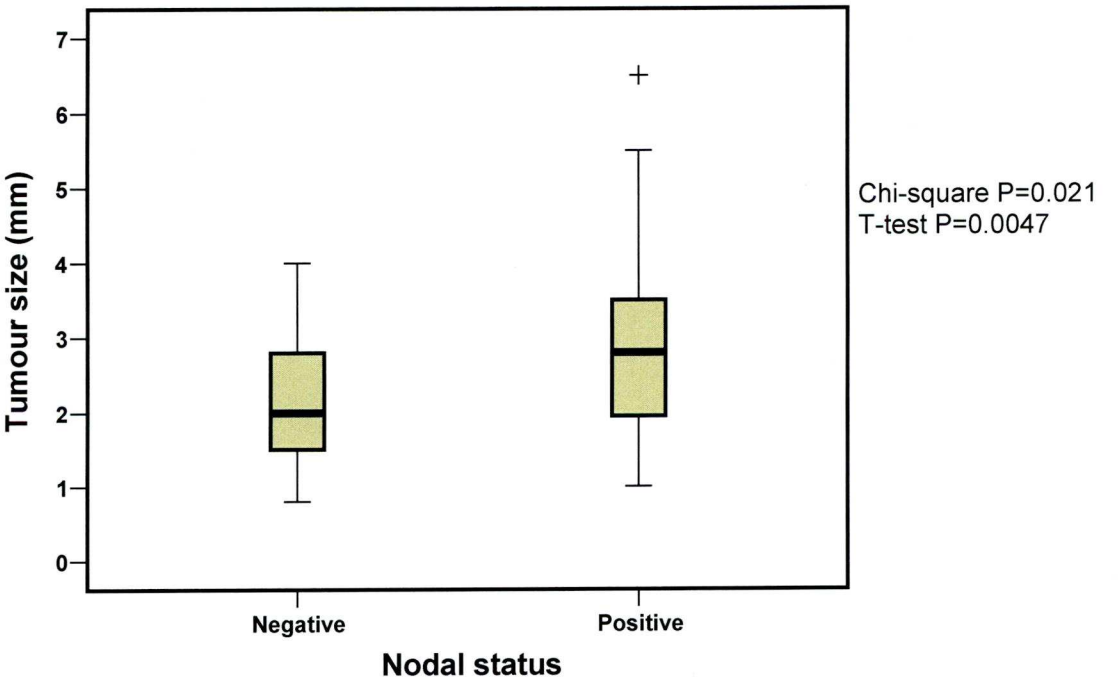


Figure 3.2 Association between positive nodal status and large tumours.

Positive nodal status was associated with large tumour size.

The box represents the interquartile range, the line across the box indicates the median. The whiskers extend from the box to the highest and lowest values [excluding outliers (+) and extremes (x)]

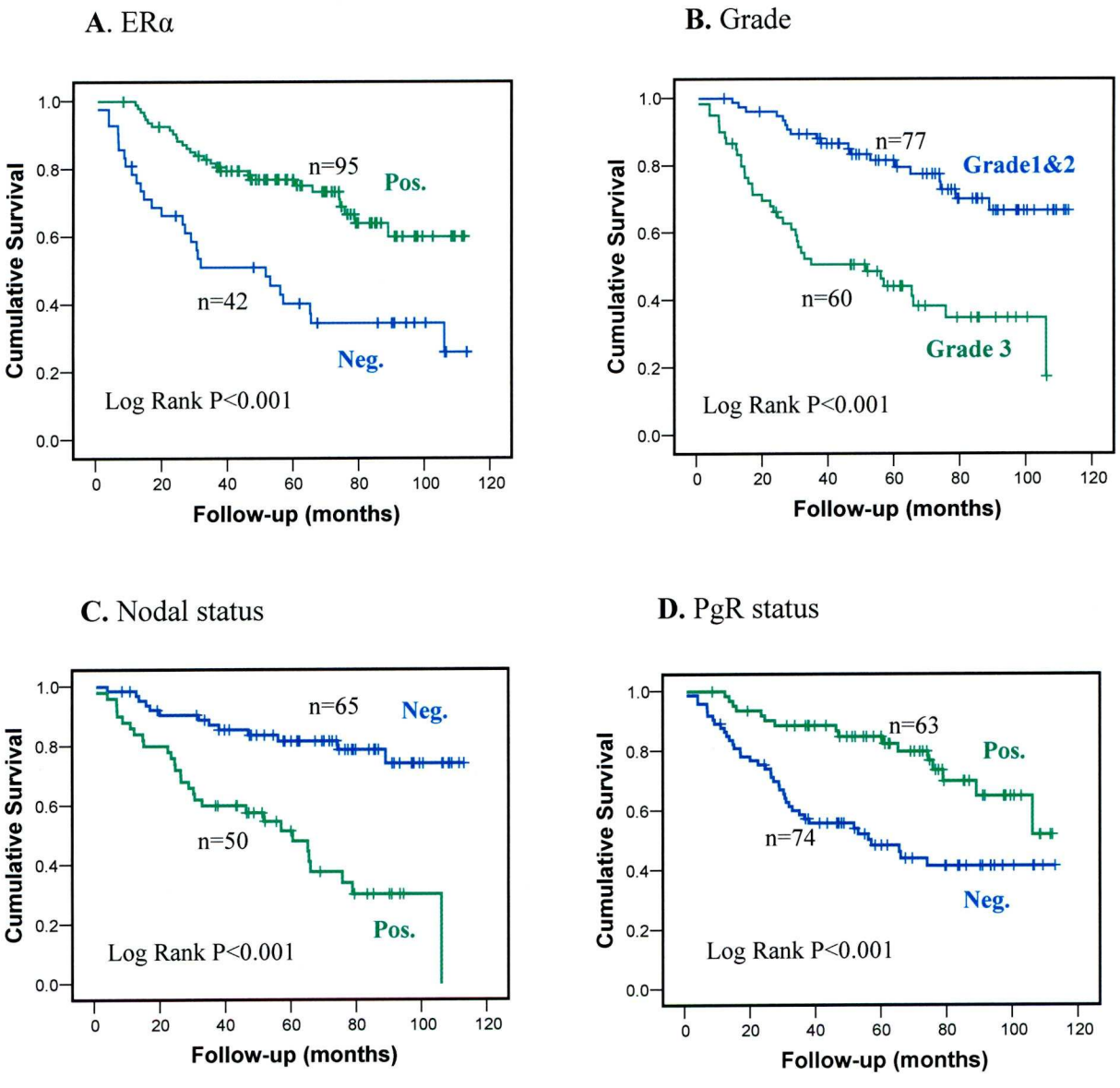


Figure 3.3 Kaplan Meier survival plots for relapse free survival (RFS) for standard markers in IHC cohort. ERα+ status, PgR+ status, lower tumour grade and negative nodal status were associated with better RFS.

Crosses on the lines represent censored data.

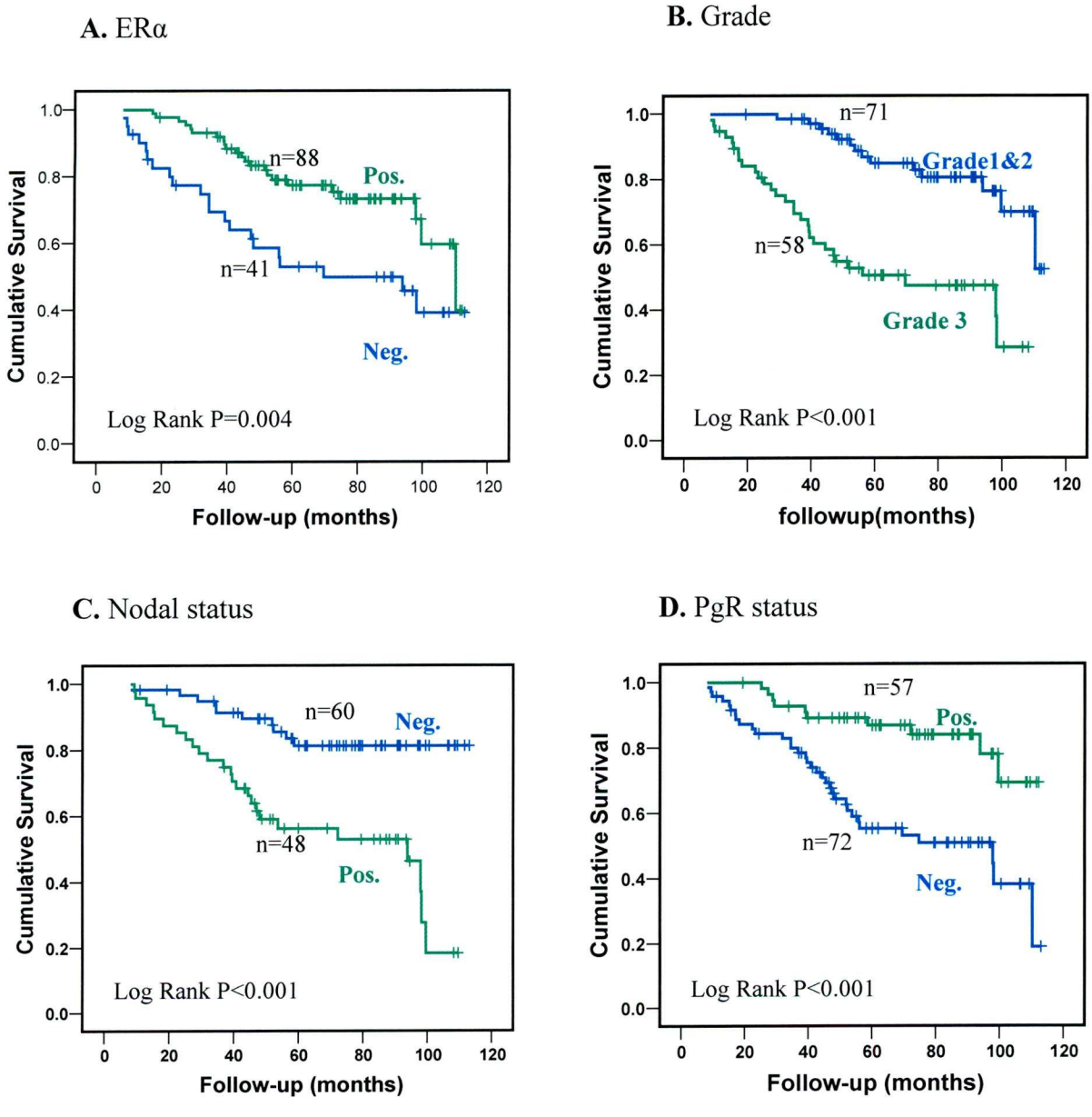


Figure 3.4 Kaplan Meier survival plots for breast cancer survival (BCS) for standard markers in IHC cohort. ERα+ status, PgR+ status, lower tumour grade and negative nodal status were associated with better BCS.

Crosses on the lines represent censored data.

CHAPTER 4 RESULTS 2

mRNA QUANTITATION AND ANALYSIS FOR ERα, ERβ1, ERβ2, ERβ5, ERβΔ5 AND ERβΔ3

4.1 Introduction

The hypothesis being tested relies on measurement of individual splice variants of ERβ. This can be achieved using validated, variant specific, quantitative RT-PCR assays. Relationship to clinicopathological parameters and outcomes can then be tested using appropriate statistical methods.

Oestrogen receptors were measured at the mRNA level, fully quantitatively, in 100 cases where suitable quantity of tumour RNA was available, in samples where the proportion of the tumour cells exceeded 75%. As mentioned earlier, 70 cases were ERα+ and the remaining 30 were ERα- by immunohistochemistry. House-keeping genes, hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured to assess the quality of the tumour sample cDNA. Oestrogen receptor alpha (ERα), Oestrogen receptor beta (ERβ) isoforms ERβ1, ERβ2 and ERβ5, and deletion variants ERβΔ5 and ERβΔ3 were fully quantitated by real-time, polymerase chain reaction (PCR). Their expression levels were compared with other standard parameters and used in survival analysis for better understanding of these molecular markers.

4.2 Optimization of mRNA assays

Standard protocols and primers were available to perform quantitative PCR for ERα, HPRT and GAPDH genes (Dr Michael Davies, personal communication). Quantitative PCR was performed in duplicate on a Bio-Rad Icycler Real-Time PCR machine (Bio-Rad Laboratories Ltd., Hertfordshire, U.K.). Expression levels of mRNA for each gene were calculated using standard curves produced with the relevant cloned cDNAs by trying various dilutions and temperature settings. Optimal conditions were selected on the basis of sensitivity and reproducibility of these standard curves. The PCR mix and the protocol are detailed in the Methods section.

For ERβ1, ERβ2, ERβ5, ERβΔ5 and ERβΔ3 primers were previously validated (Critchley *et al*, 2002; Poola, 2003). All the primers were initially evaluated using different temperature conditions and PCR mixes (e.g. varying primer concentration and $MgCl_2$ concentration). The optimal conditions and primers which gave unique bands were then used to evaluate these variants in the breast tumour samples (Table 2.2). Testis, prostate and uterus RNAs were used as controls in each PCR experiment as a quality control reference. Each tumour was amplified at least in duplicate and the mRNA level was taken as the average from any positive sample. If there was no amplification in both duplicates, the mRNA expression was inferred as absent. Identity of PCR products were confirmed by agarose gel electrophoresis (Figure 2.2), cloning, DNA sequencing and BLAST searching via the NCBI website (Figures 2.4 and 2.5).

4.3 Measurement of ERα mRNA

ERα mRNA expression was noted in all except three samples irrespective of ERα immuno status. The mRNA level ranged between 0.03 attomoles per μg total RNA and 257 attomoles per μg total RNA. There was good correlation between ERα immunostaining and mRNA expression (%+ cells, Pearson=0.308 P=0.002; Spearman=0.480 P<0.001) across the whole cohort (Figure 4.1); but the significance was lost when the correlation was limited to 70 ERα+ cases (Pearson=0.151 P=0.23; Spearman=0.145 P=0.25). Mean ERα mRNA level was significantly high (P=0.006 T-test, P<0.001 MW) in ERα+ cases (mean 33 attomoles per μg total RNA) than ERα- cases (mean 5.1 attomoles per μg total RNA) as one would expect. The three cases which did not express mRNA were all ERα- by immunohistochemistry.

4.3.1 Correlation with clinicopathological characteristics

There was no correlation of ERα mRNA expression with tumour size, nodal status, vascular invasion and invasive ductal carcinoma, but there was a correlation with grade (Spearman P=0.001) and ERα mRNA expression was significantly high in low-grade tumours (MW P=0.001) (Figure 4.2.A).

ERα mRNA levels correlated with PgR immunostaining (%+ve cells, Spearman P<0.001) and were higher in PgR+ tumours (MW P<0.001), although mean levels were not significantly different between in PgR+ and PgR- cases (T test P=0.29).

When the analysis was limited to 70 ERα+ cases, there was no correlation between ERα mRNA expression and other standard parameters. The differential expression noted in low grade and PgR+ tumours was also no longer evident.

4.3.2 Correlation with survival status

First a ROC curve was obtained to identify the optimal cut-point for ERα mRNA values using breast cancer 5-year relapse. The area under the curve was 0.67 (CI 0.56-0.78) and the P value was 0.007 (Figure 4.12A). The optimum mRNA cut-off point was 8 attomoles per μg of total RNA. In Kaplan Meier survival analysis, using this cut-off value, the ERα mRNA values above the cut-off was significantly associated with good outcome in the 100 cohort group [RFS Log Rank P=0.029, BCS Log Rank P=0.01 (Figure 4.3)]. Similar good outcome was also noted in Cox univariate analysis (RFS HR=2.08 CI 1.06 to 4.06 P=0.03, BCS HR=2.78 CI 1.23 to 6.29 P=0.01). In multivariate analysis of ERα protein and mRNA, only ERα immuno status retained its significant association with outcome in RFS (Cox P=0.011) whereas ERα mRNA was the strongest in BCS (Cox P=0.014). In the 70 ER+ cohort, there was no relation between ERα mRNA and outcome as one would expect.

4.4 ERβ1 quantitative assay

ERβ1 was detected in 84 tumour samples and in the remaining 16 cases it was below the limit of detection. The mean ERβ1 mRNA expression was 0.00084 attomoles per μg total RNA. ERβ1 mRNA level was significantly higher in ERα+ cases than ERα- cases (T test P=0.002; MW P<0.001). The mean ERβ1 level in ERα+ case was 0.0011 attomoles per μg total RNA and in ERα- case was 0.00015 attomoles per μg total RNA, a fold difference of 7.3. The ERα mRNA level is approximately 1000 times higher than ERβ1 mRNA level across the whole cohort (Paired T test P<0.001).

4.4.1 Correlation with clinicopathological characteristics

ERβ1 mRNA level correlates with ERα+ staining (%+ cells, Pearson P=0.008) (Figure 4.4) and there was a trend for PgR staining (%+ cells, Pearson P=0.057), with ERβ1 mRNA level significantly higher in PgR positive tumours (T test P=0.041; MW P=0.042). ERβ1 mRNA correlates inversely with grade of the tumour (Spearman P=0.001), being significantly higher in low grade tumours (T test P=0.016; MW P=0.005) (Figure 4.2.B).

There was no significant relationship between ERβ1mRNA and tumour size, histological type, vascular invasion or nodal status.

4.5 ERβ2 quantitative assay

ERβ2 mRNA was detected in all tumour samples. The range of mRNA level was between 0.00018 and 0.025 attomoles per μg total RNA. There was no significant difference in expression levels between ERα+ and ERα- tumours (%+ cells, Pearson P=0.386) (Figure 4.4). The ERβ2 mRNA level was significantly lower than ERα mRNA levels (Paired T test P<0.001).

4.5.1 Correlation with clinicopathological characteristics

There was significantly higher expression of ERβ2 mRNA in histological types other than invasive ductal carcinoma (MW P=0.044) and a trend for low expression in tumours with lymphovascular invasion (MW P=0.061). There was no significant relationship between ERβ2mRNA and ERα status, PgR status, tumour size, grade and nodal status.

4.6 ERβ5 quantitative assay

ERβ5 mRNA was expressed in all 100 cases. The range is between 0.000092 attomoles per μg total RNA and 0.040 attomoles per μg total RNA. The mean ERβ5 mRNA level was 0.0050 attomoles per μg total RNA. ERβ5 mRNA level correlates inversely with ERα staining (%+ cells, Pearson P=0.001; Spearman P<0.001) (Figure 4.4) and PgR staining (%+ cells Pearson P=0.013; Spearman P=0.030). The mean

ERβ5 mRNA level was significantly higher in ERα- cases compared to ERα+ cases (T test P<0.001; MW P=0.001; Paired T test P<0.001) and in PgR- cases compared to PgR+ cases (T test P=0.029; Paired T test P<0.001).

4.6.1 Correlation with clinicopathological characteristics

There was no significant correlation between ERβ5 mRNA and tumour size, grade, invasive ductal carcinoma, lymphovascular invasion and nodal status.

4.7 ERβΔ5 and ERβΔ3 quantitative assay

ERβΔ5 mRNA was only detected in 20 cancers, 17 of which were ERα- and 14 of them were high grade tumours. The mean value was 0.000028 attomoles per μg total RNA. ERβΔ3 was only detected in 2 cases, one was ERα- and the other was ERα+, and both were positive for ERβΔ5.

4.7.1 Correlation with clinicopathological characteristics

ERβΔ5 positive status was associated with ERα negative status ($P< 10^{-8}$ Chi-square), levels were higher in ERα- cases ($P=10^{-9}$ MW) and there was a significant inverse correlation with ERα staining (P< 0.0002 Pearson). Similar associations were seen between high ERβΔ5 and PgR negative status (P=0.009 Chi-square, P=0.007 MW) and there was a significant inverse correlation with PgR immunostaining (P=0.023 Pearson). ERβΔ5 positive status was also associated with high grade (P=0.005 Chi-square, P=0.002 MW) and there was a significant positive correlation with grade (P=0.006 Spearman).

Further investigation indicated an association with *p53* mutation (Chi-square 7.71 P=0.006) and proliferation, as measured by Ki67 (Chi-square 3.99 P=0.046). ERβΔ5 positive samples had significantly high expression of Ki67 (% + cells P=0.002 MW) with mean number of Ki67 positive cells (49%) being higher than in cases negative for ERβΔ5 (21%, P=0.000004 T test). [Ki67 expression data and *p*53 mutation data were obtained from CTBRC; Ki67 data (n=19) was kindly provided by O'Neill *et al.*

and has been published previously (O'Neill *et a*, 2004).); *p*53 mutation status (n= 20) was kindly provided by Innes *et al.* (Innes, MD Thesis, University of Liverpool, 2004) and is based on sequencing of PCR products covering the coding region of the *TP*53 gene.] There was no association with histology, size and vascular invasion. There was no association with histology, size and vascular invasion.

4.8 Comparison of expression of ERβ1, ERβ2, ERβ5, ERβΔ5 and ERβΔ3 mRNA

Mean levels of ERβ1 (0.0008 attomoles per μg total RNA) were significantly lower ($P< 10^{-10}$ paired T-test) than either ERβ2 (0.006 attomoles per μg total RNA) or ERβ5 (0.005 attomoles per μg total RNA), but there was no significant difference in mean ERβ2 and ERβ5 mRNA levels (Figure 4.5).

ERβ variant mRNAs were differentially expressed in the ERα+ and ERα- tumours; in ERα+ cases ERβ2 was expressed at the highest level followed by ERβ5 then ERβ1, but in ERα- cases ERβ5 was the highest followed by ERβ2 then ERβ1 (Figure 4.5). Mean ERβ1 levels were significantly higher in ERα+ cases than ERα- cases (P=0.002 T-test, P=0.0001 MW), but mean ERβ5 levels were significantly lower in ERα+ cases (P=0.0004 T-test, P=0.001 MW). In the full cohort (n=100), ERβ2 mRNA levels correlated positively with ERβ1 mRNA (Pearson 0.36, P=0.0003) and to a lesser extent with ERβ5 mRNA (Pearson 0.22, P=0.025), but there was no significant correlation between ERβ1 and ERβ5 (Pearson 0.08, P=0.42) (Figure 4.6). However, within the ERα+ cohort (n=70), levels of mRNA for all C-terminal variants correlated with each other (P< 0.005 Pearson).

There was a significant positive correlation of ERβΔ5 mRNA with ERβ2 (P=0.02 Pearson) and ERβ5 (P< 0.0005 Pearson) mRNA, and positive correlations with grade (P=0.006 Spearman), but no significant correlation with ERβ1 mRNA. ERβΔ5 expression could not be accounted for by deletion within ERβ1 alone. In a significant proportion of cases the measured amounts of ERβΔ5 was greater than that of ERβ1 (Figure 4.7), but could be accounted for if a proportion of ERβ2 and/or ERβ5

mRNAs also contained deletions of exon 5. Preliminary work to detect such complex variants (data not shown) indicates that they do exist.

4.9 Association of ERβ1, ERβ2, ERβ5, ERβΔ5 and ERβΔ3 mRNA with patient survival

Using ROC-derived optimal cut points for the 100 case (ERα+ and ERα-) qRT-PCR cohort in Kaplan Meier Log Rank analysis, there were relationships between higher ERβ isoform expression and good outcome for ERβ1 (P=0.065 RFS, P=0.047 BCS; Figure 4.8), ERβ2 (P=0.046 RFS, P=0.042 BCS; Figure 4.9) and ERβ5 (P=0.028 RFS, P=0.023 BCS; Figure 4.10).

In the ERα- cases, high ERβ5 mRNA was associated with better outcome (RFS P=0.008 Log Rank, HR 0.25 CI 0.08-0.75 P=0.014; BCS P=0.066 Log Rank, HR 0.33 CI 0.097-1.14 P=0.080; Figure 4.11). The 5 year cumulative relapse-free population was 59% in the ERβ5-high group (n=23) compared to 0% in the ERβ5-low group (n=6); the 5 year cumulative BCS was 67% in the ERβ5-high group (n=23), compared to 40% (n=5) in the ERβ5-low group. No associations were found between other ERβ isoform mRNA levels and outcome in this (admittedly small) cohort. Only nodal status was also significantly associated with poor outcome in these ERα- cases (RFS P=0.024 Log Rank, HR 2.9 CI 1.1-7.7 P=0.031). There was no association between ERβ5 and nodal status (P=0.77 Chi-square) and in multivariate analysis high ERβ5 mRNA was associated with better outcome (RFS HR 0.21 CI 0.06-0.82 P=0.024) independent of nodal status.

Further outcome analysis for ERβ splice variant mRNA was limited to ERα positive women who received adjuvant tamoxifen and had a defined breast cancer related outcome (n=62 RFS, n=58 BCS). High grade (RFS P=0.006, BCS P=0.0008) and positive nodal status (RFS P=0.003, BCS P=0.007) maintained their association with worse outcome (Log Rank). ROC plots indicated a significant relationship between good outcome and high qRT-PCR values for ERβ2 (RFS area under curve 0.68 CI 0.52-0.84, P=0.036) (Figure 4.12) and ERβ5 (RFS area under curve 0.68 CI 0.54-0.83, P=0.033). Optimal cut-points for qRT-PCR data determined by ROC analysis for RFS and BCS at 5 years were 0.00068 (ERβ1), 0.00393 (ERβ2) and 0.00224

(ERβ5) attomoles per μg total RNA. Significant relationships were found between the subsequent dichotomized qRT-PCR categorizations and good outcome for both ERβ2 and ERβ5, but not ERβ1 (Figure 4.13).

High ERβ2 mRNA was associated with better outcome (RFS P=0.0095 Log Rank, HR 0.32 CI 0.13-0.79; BCS P=0.011 Log Rank, HR 0.25 CI 0.08-0.79) and a similar relationship was seen with ERβ5 (RFS P=0.06 Log Rank, HR 0.44 CI 0.19-1.06; BCS P=0.018 Log Rank, HR 0.28 CI 0.09-0.85). These variables exhibited a significant association with each other ($P=10^{-6}$ Chi square, P=0.001 Pearson) and were highly concordant (81% of cases), but in multivariate analysis ERβ2 emerged as the strongest marker for both RFS and BCS. The 5 year cumulative relapse-free population was 81% in the ERβ2-high group (n=30) compared to 55% in the ERβ2-low group (n=32); the 5 year cumulative BCS was 89% in the ERβ2-high group (n=29), compared to 62% in the ERβ2-low group (n=29).

When grade, size, nodal status and PgR status were included in Cox multivariate analysis of the ERα+ tamoxifen-treated cohort, high ERβ2 had independent significance for good outcome: for RFS, ERβ2 (HR 0.31 CI 0.11-0.86, P=0.024) and nodal status (HR 3.7 CI 1.2-11.5, P=0.022) were independently significant; for BCS, ERβ2 (HR 0.17 CI 0.05-0.65, P=0.0095) and grade (HR 1.8 CI 1.03-3.3, P=0.041) were independently significant. Notably there was no significant association between ERβ2 and grade, size, nodal status or PgR status in this treatment-specific cohort (all P> 0.35 Chi-square).

In ERα+, node negative cases (n=33), using a lower cut-off (0.00185 attomoles per μg total RNA) defined by ROC analysis of this subgroup, ERβ2 was significantly associated with better outcome (RFS P=0.0005, BCS P=0.00002 Log Rank) (Figure 4.14); the 5 year cumulative relapse-free population was 96% in the ERβ2-high group (n=26), compared to 39% in the ERβ2-low group (n=7) and the 5 year cumulative BCS was 95% in the ERβ2-high group (n=25), compared to 27% (n=6) in the ERβ2-low group.

ERβΔ5 positive status was not associated with RFS or BCS.

4.10 Discussion

4.10.1 Optimisation and choice of assay

ERα, ERβ1, ERβ2, ERβ5, ERβΔ5 and ERβΔ3 mRNAs were measured fully quantitatively with standard curves prepared from known quantities of respective cloned cDNAs. This technique allows us to measure the absolute starting quantity of mRNAs in attomoles and also shows objective evidence of PCR efficiency and the range over which the assays are valid. This makes comparison between assays or different genes easier and more reproducible. In most of the previous studies mRNAs were evaluated either relatively using the ΔΔct method (which can introduce bias as it usually assumes equal PCR efficiencies) or semi-quantitatively by measuring the intensity of fluorescence of gels of PCR products (which has a limited dynamic range).

The limitation of measuring genes by qRT-PCR is that it may measure RNA molecules from epithelial cells as well as stromal cells, fibroblasts and endothelial cells. It has been well proved that ERβ is expressed in comparatively larger amount than ERα in stromal cells, fibroblasts and endothelial cells (Speirs *et al*, 2002). Hence part of the expression level seen may be due to non tumour cells.

HPRT and GAPDH were measured to validate the quantity and quality of the RNA, and account for differences in cDNA synthesis. Their levels were used to normalize the candidate gene expression by a factor relative to the mean of expression over the cohort. Use of 2 control genes minimizes bias due to either one. Positive controls (RNA from testis, ovary and prostate and MCF7 cells) and negative controls (no cDNA in the PCR or no reverse transcriptase in cDNA synthesis reactions) were used in each PCR for quality control and to rule out genomic DNA contamination.

Variant specific primers were used for candidate genes to measure the mRNA. To maximize specificity and sensitivity of detection, and make correlation with other markers more relevant, PCR conditions were optimised and PCR product identity was confirmed by gel electrophoresis and DNA sequencing. For ERβ2 this was particularly important as primer design was limited by the sequence overlap between ERβ2 and ERβ5; it was achieved by shortening the extension time in the PCR to favour the shorter PCR product.

4.10.2 ERα mRNA qRT-PCR

ERα mRNA was quantifiable in 97 cases out of 100 patients, which includes 27 ERα negative patients. The 3 cases which were negative were ERα negative by immunohistochemistry. There was a 10,000 fold difference between low and high ERα mRNA levels, which is a wider range than seen for most genes, including the ERβ variants. There was good correlation with ERα immunostaining, which confirms utility of qRT-PCR, suggests that the RNA samples were broadly representative of the tumour cell populations scored by immunohistochemistry and indicates the importance of transcriptional control mechanism in determining ERα protein levels. Of note, the expression of the ERα immunostaining is usually bimodal (Collins *et al*, 2005) compared to the broader distribution of ERα mRNA expression seen in our study. It is possible that this bimodal distribution may due to some post transcriptional control, combined with a narrower dynamic range for immunostaining compared to qRT-PCR analysis.

The correlation of ERα mRNA with PgR status and low grade tumour across the whole cohort mirrors that of ERα protein expression. This again suggests that total RNA samples were representative of the tumour sections for immuno. That these correlations with grade and PgR were lost when considering the ERα positive cohort is most likely due to exclusions of the more extreme cases with lowest levels of ERα mRNA, which are also lowest for PgR and of generally higher grade, hence narrowing the range over which correlations were assessed.

The dichotomized ERα mRNA level showed good correlation with survival, as expected from its association with ERα immuno status. In multivariate analysis, compared to immuno status ERα mRNA was a better marker for BCS (although not for RFS). Such results are unlikely to influence the current clinical practice of measuring ERα by immunohistochemistry.

4.10.3 ERβ1, ERβ2 and ERβ5 mRNA assay

Comparison of the mRNA results presented with those of others is difficult as not all studies have been as thorough in terms of specificity of their primer design or their quantification methodology. It is also possible that others may have underestimated ERβ mRNA levels by using assays of insufficient sensitivity. Extra care needs to be

taken when detecting ERβ mRNA, which is shown by their greatly reduced range and level of expression compared to ERα, as demonstrated quantitatively here.

Many early studies used PCR primers designed to differentiate between ERα and ERβ, which did not take into account ERβ C-terminal variants or deletion variants, as they were unknown at that time. Our results indicate that the mRNA levels of ERβ2 and ERβ5 often exceed that of ERβ1; hence, many early studies could have overestimated "ERβ" expression by measuring total ERβ. The level of expression of exon deletion variants measured here indicate that they are unlikely to impact on measurement of ERβ, being expressed at relatively low levels and detected in a minority of cases.

In studies of ERβ C-terminal variants, many studies utilized variations of the "triple primer" assays (Iwao *et al*, 2000; Omoto *et al*, 2002) to detect ERβ1, ERβ2 and ERβ5 by means of agarose gel electrophoresis. Despite careful optimization and validation by some, this assay has limited dynamic range and suffers from primer competition (where multiple variants compete for primer usage in the PCR reaction). Perhaps more importantly, for assessing the importance of ERβ variants with outcome, the semi-quantitative approaches do not lend themselves to assessment of optimal cut-points for use in grouping, as required for survival analysis when limited numbers of cases are available for study.

In this study, ERβ variants were expressed much lower than ERα (1000 times lower for ERβ1 and 100 times lower for ERβ2 and ERβ5) as previously reported (de Cremoux *et al*, 2002; Speirs *et al*, 1999a). This low expression of ERβ mRNA was also noted *in situ* hybridisation studies (Sasano *et al*, 1999). ERβ2 and ERβ5 were expressed at detectable levels in all tumours, and wild-type ERβ1was expressed in 84% of cases. More interestingly ERβ2 and ERβ5 mRNA levels were significantly higher than ERβ1 in individual samples, as previously reported (Leygue *et al*, 1999; Omoto *et al*, 2002). This may be due to ERβ1 down regulation in tumours compared to normal tissue (Shaaban *et al*, 2003), although we have not measured ERβ1 in matched normal tissues to support this hypothesis. Omoto *et al.* compared the mRNA of ERβ1 and ERβ2 by RT-PCR in breast cancers and adjacent normal breast tissues in 22 cases and found that ERβ1 level is lower and ERβ2 level is higher in cancers compared to the normal breast tissues (Omoto *et al*, 2002). Iwao *et al.* found both

ERβ1 and ERβ2 higher and ERβ5 lower in normal tissues compared to breast cancers by triple-primer PCR assay in 112 cancers and 11 adjacent normal tissues (Iwao *et al*, 2000). ERβ5 was more abundantly expressed in ERα- cases and this was seen previously in a cohort containing both Caucasians and African American patients (Poola *et al*, 2005b). These results indicate that rather than the expression of total ERβ being altered, individual splice variants may be downregulated or upregulated in tumourigenesis.

In this study, ERβ2 did not show any association with ERα. However, ERβ1 positively correlated with ERα and to a less extent with PgR, with significantly high ERβ1 in ERα+ and PgR+ cancers. On the contrary ERβ5 inversely correlated with ERα and PgR, with significantly high ERβ5 in ERα- cancers. This again shows that ERβ1 and ERβ5 showing different association with ERα status and this may be due to different transcriptional/epigenetic control. These findings were not consistent with the study of Poola *et al.* (Poola *et al*, 2005b), who quantitatively measured ERβ1 and ERβ2 in 60 ERα- (20 Caucasians and 40 African Americans) and 74 ERα+ (34 Caucasians and 40 African Americans) breast cancers. There was no significant difference in mRNA levels of ERβ1 and ERβ5 in ERα positive or negative patients in their study. This can partly be explained by the case selection, as the study did not comment on the menopausal status of the patients and, also ERα positivity was estimated by immunohistochemistry in some patients with cut off for positivity at >5% and in the remaining patients ERα+ status was determined by ligand binding assay. In our study, all patients were postmenopausal and ERα status was determined by immunohistochemistry with 10% cut off value, as universally accepted. Another factor to consider is the ethnicity of the patients investigated. Their cohort is made up of Caucasians (Americans) and African Americans, ours was mainly Caucasians of British origin. ERβ expression profile differs in various ethnic groups as seen in their study as well as other studies (Poola *et al*, 2002c). These differences may explain the discrepancies in the expression levels of ERβ1 and ERβ5 in the above study. Another study (Iwao *et al*, 2000) used triple-primer PCR assay to measure ERβ1, ERβ2 and ERβ5 in 112 cancers (both premenopausal and post menopausal) and found no significant difference in the proportion of ERβ variant expression between ERα+ and ERα- patients. However when they measured the ERβ mRNA using q-RTPCR method using primers located in exon 5 and 7 in the same cohort of patients, ERβ

mRNA levels were significantly higher in ERα- breast cancers than ERα+ (Iwao *et al*, 2000). Again this Japanese study had different case selection and different PCR techniques and different ethnic population, which may have contributed to the difference in expression levels.

The variants were differentially expressed; in ERα+ cases, ERβ2 was highly expressed followed by ERβ5 and ERβ1. In ERα- cases, ERβ5 is highly expressed followed by ERβ2 and ERβ1. ERβ2 correlated with the others, but ERβ1 and ERβ5 did not correlate with each other in the whole cohort, although they correlated with each other in the ERα+ cohort. Hence it would seem that ERβ C-terminal splice variants are differentially regulated and in ERα- cases in particular there is a clear difference in the transcriptional control of ERβ1 and ERβ5.

ERβ1 correlated positively and ERβ5 inversely with ERα status and PgR in our study. This is interesting as studies by semi-quantitative PCR for ERβ have shown inverse correlation with PgR (Cullen *et al*, 2001; Dotzlaw *et al*, 1999) or no correlation (de Cremoux *et al*, 2002). Similarly for ERα there was positive or negative correlation (Bieche *et al*, 2001; Knowlden *et al*, 2000; Kurebayashi *et al*, 2000) and no correlation (de Cremoux *et al*, 2002; Dotzlaw *et al*, 1997) with ERβ mRNA. From our study, investigating individual variants in a fully quantitative manner has shown differential expression of the individual variants in tumours depending upon their ER/PgR status (ERβ1 positive correlation, ERβ2 no correlation and ERβ5 negative correlation). The above studies measured the total ERβ and, depending upon the location of their primers, this may have influenced the proportional amount of these variants and may have resulted in spurious values resulting in this discordant association with ER/PgR status.

ERβ1 is the only variant showing association with low grade tumours similar to ERα. Sugiura *et al.* evaluated ERβ1 and ERβ2 mRNA in fully quantitative manner in 150 cases (ERα+ and ERα-) by qRT-PCR and found both correlated with each other similar to our study. ERβ1 also positively correlated with PgR status and low tumour grade while ERβ2 did not correlate with any standard clinicopathological parameters similar to our study. Interestingly, the ERβ1 and ERβ2 mRNA expression correlated with respective protein expression as well (Sugiura *et al*, 2007).

Leygue *et al.* evaluated ERβ1, ERβ2 and ERβ5 by the Triple primer-PCR technique in 53 breast cancers. ERβ1 correlated inversely with tumour grade while ERβ2 and ERβ5 correlated positively (Leygue *et al*, 1999).

However most of the ERβ RNA studies using quantitative or semi-quantitative, specific or non-specific primers have not shown any association with tumour grade, tumour size and nodal status (Bieche *et al*, 2001; Cullen *et al*, 2001; de Cremoux *et al*, 2002; Dotzlaw *et al*, 1999; Iwao *et al*, 2000). One RT-PCR study evaluated ERβ with primers in the N terminal region in 60 breast cancers and found association between ERα+/ERβ+ status and, node positivity and high grade tumours (Speirs *et al*, 1999a). Again this observation was seen in a relatively small cohort using non-specific primers measuring all the variants and this may explain the association with poor prognostic markers not seen by others.

4.10.4 ERβΔ5 and ERβΔ3 mRNA assay

Deletion variant ERβΔ5 positivity was associated with high grade tumours and this was previously noted in a study of 43 breast cancer patients of both premenopausal and post menopausal status (Poola *et al*, 2002b).

Another significant finding in this study is the disparity in the amounts of ERβΔ5 and ERβ1 (10 out 20 cases expressing higher levels of ERβΔ5 than ERβ1) which supports the notion that ERβ2 or ERβ5 variants can also harbour exon deletions. As exon 5 deletion leads to a functionally distinct truncated protein not detectable with the reagents used, in theory this could explain some of the discrepancy between ERβ2 protein and mRNA. This would only be relevant to the small number of cases in which mRNA levels were high when protein levels were low, and in all cases ERβΔ5 was only a minor component compared to the ERβ2 mRNA level (Figure 4.7).

ERβΔ3 was detected in 2 cancers and both were positive for ERβΔ5. ERβΔ3 expression has not previously been reported in breast cancer (Herynk & Fuqua, 2004). Our study shows that this variant may be expressed in breast cancer, but rarely.

4.10.5 ERβ splice variants and outcome

All C-terminal variants (high expression as defined by ROC curve) showed association with better outcome in the whole cohort, but in the ERα+ tamoxifen treated cohort, ERβ2 and ERβ5 were associated with better outcome in KM survival analysis. In Cox multivariate analysis, ERβ2 was the only variant independently associated with better outcome (RFS and BCS). ERβΔ5 positive status was not associated with RFS or BCS, even though there was association with ERα and PgR negative status, high grade tumours, high Ki67 expression and *p*53 mutation.

As with the investigation of clinical and biological correlations, many comparisons between the present study and previous work are difficult due to the different methods of assessments of ERβ, most of which were either not variant specific or not fully quantitative. Furthermore, it is in determining the relationship with outcome that most studies fall short in terms of numbers studied and case selection. These factors are closely linked, in that smaller studies of cases carefully matched for treatment are more likely to provide relevant insight than the same size, or even larger, studies in which treatment has not been taken into consideration. It is the intention of treatment to affect an improvement in patient outcome, and the treatment regime is often determined by the perception of risk of recurrence. Despite this, many studies do not take in to account these clearly confounding factors and try to relate ERβ expression in groups of patients receiving a variety of different treatments. This is understandable given that clinical samples are often drawn from limited retrospective cohorts, but requires that comparison between studies take into account the possible selection biases.

Davies *et al.* (Davies *et al*, 2004) studied ERβ1, ERβ2 and ERβ5 mRNA in semi-quantitative manner in a overlapping cohort of 105 patients (both ERα+ and ERα-) and found association with better outcome for ERβ2 and ERβ5 in whole cohort as well as in the ERα+ subgroup. ERβ1 was not associated with outcome. The present study done in fully quantitative manner showed outcome results similar to the semi-quantitative data for ERβ2 and ERβ5. However, ERβ1 showed association with better outcome in the whole cohort (ERα+ and ERα-) and this association was lost in the ERα+ cohort. By fully quantitating, and assigning cut-points with ROC curve analysis, association with outcome is better demonstrated in this study.

The advantage of our study is that all patients received adjuvant endocrine treatment and 93 patients (63 ERα+ and 30 ERα-) received tamoxifen only. Our outcome result can also be taken as a surrogate marker for tamoxifen responsiveness/resistance in our cohort. Murphy *et al.* measured ERβ1, ERβ2 and ERβ5 mRNA in semi-quantitative manner in a cohort of 27 node negative ERα+ patients who were either sensitive or resistant to tamoxifen and found no significant difference in their levels in these two groups (Murphy *et al*, 2002). Chang *et al.* estimated ERβ mRNA by RT-PCR in 30 cancers (21 sensitive to tamoxifen and 9 resistant) and the ERβ mRNA level were high in the resistant group with marginal significance (Fisher's exact test P=0.11) (Chang *et al*, 2005). Speirs *et al.* semi-quantitatively measured ERβ mRNA in a small cohort of 17 patients who either responded or were resistant to tamoxifen, and found higher ERβ mRNA in tamoxifen resistant group (Speirs *et al*, 1999a).

Cappelletti *et al.* evaluated ERα and ERβ mRNA with RT-PCR, pre and post treatment, in a cohort of 47 patients who received neo-adjuvant toremifene for three months. There was no significant correlation with endocrine response to tumour (shrinkage) or pre and post ERβ mRNA level. In contrast ERα mRNA was high in tumours which responded to treatment and their level was down regulated after treatment (Cappelletti *et al*, 2004). These four mRNA studies either showed no relation or poor correlation with outcome in endocrine treatment setting. However the cohorts were small and different endocrine regime and different PCR assessment methods were used, making it difficult to come to a meaningful consensus.

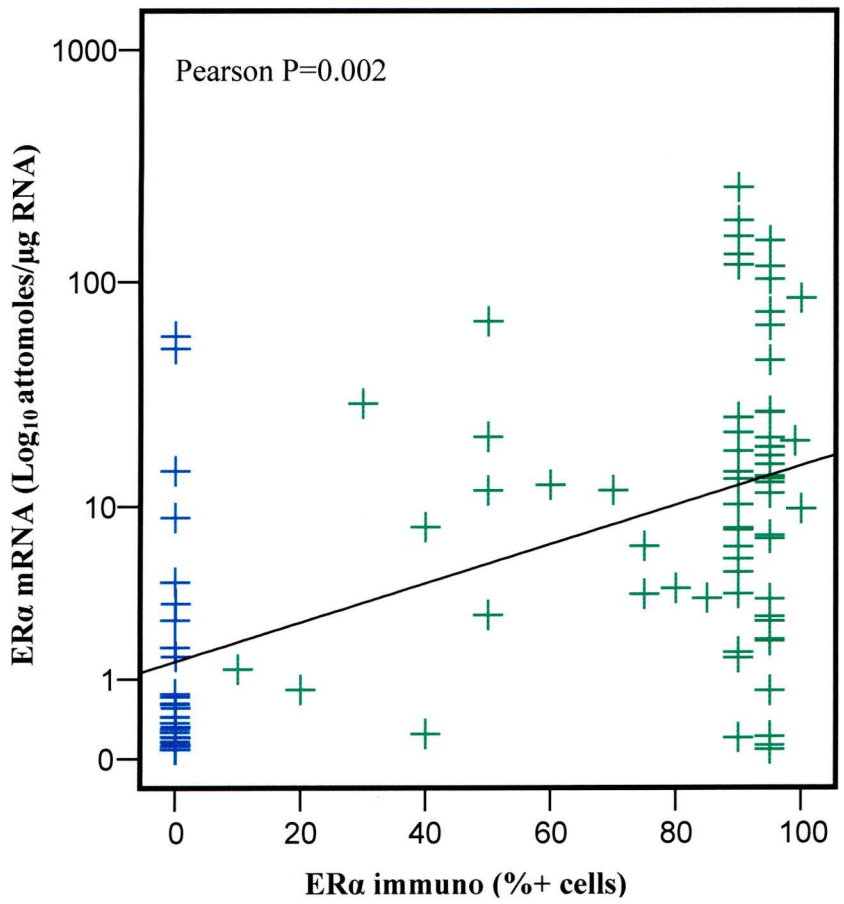


Figure 4.1 Scatter plot of ERα mRNA expression in relation to ERα protein expression.

Markers are coloured according to ERα immune status.

Green = ERα positive cases.

Blue = ERα negative cases.

Line represents linear regression for all cases.

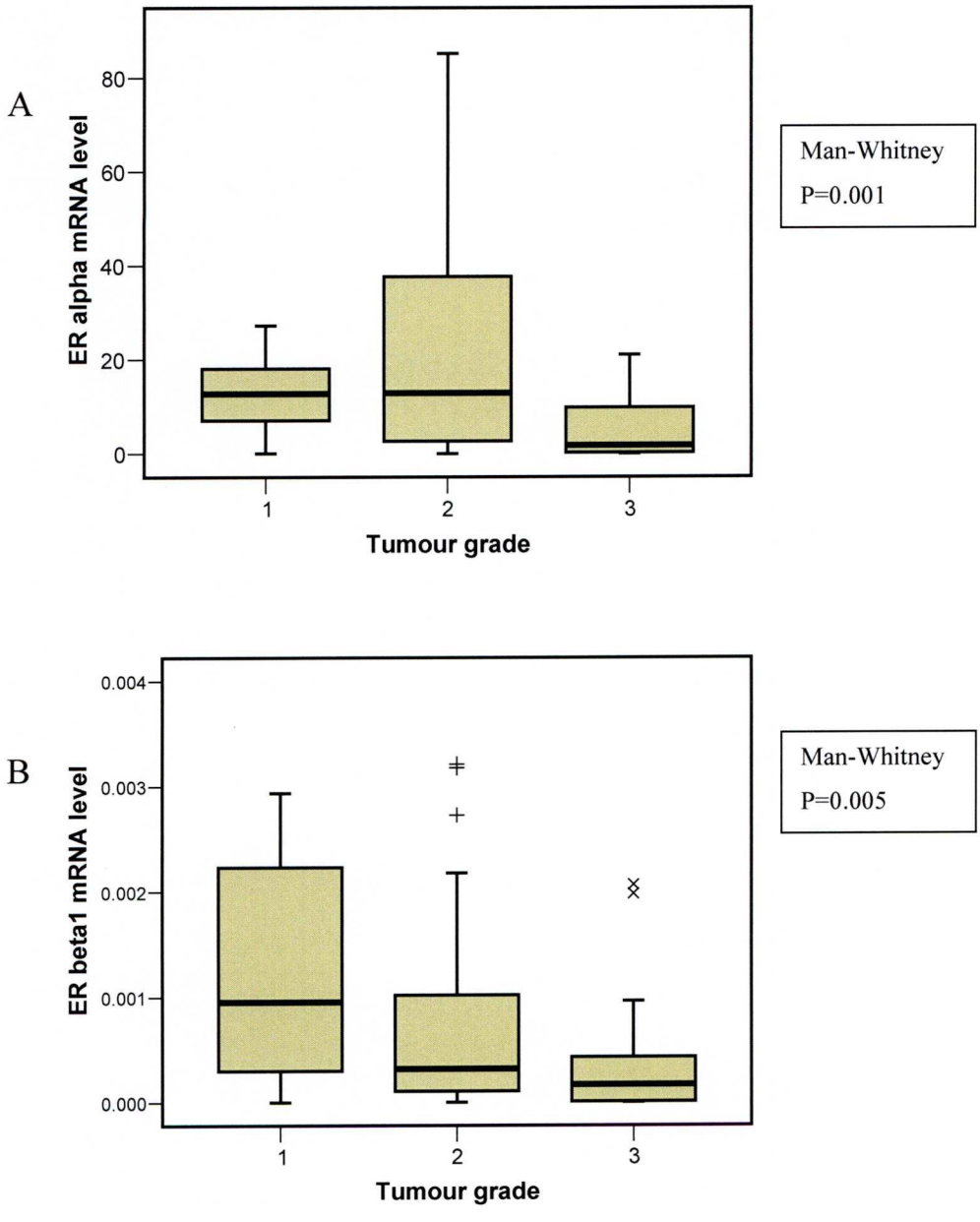
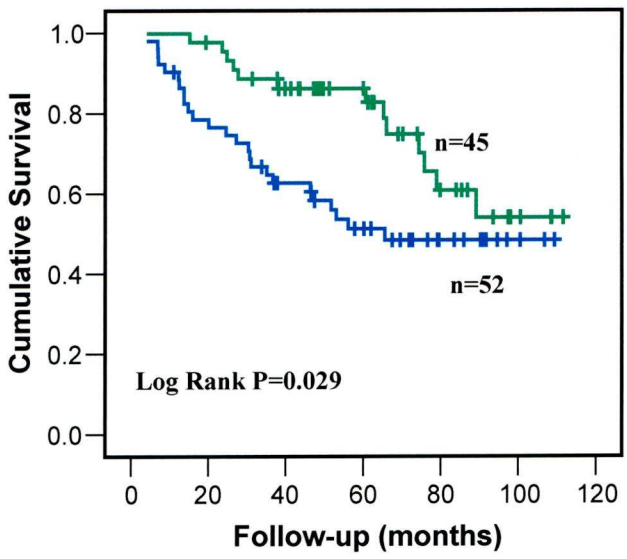


Figure 4.2 Correlation between mRNA level and tumour grade. ERα mRNA level (A) and ERβ1 mRNA level (B) were high in low grade tumours.

The box represents the inter-quartile range; the line across the box indicates the median. The whiskers extend from the box to the highest and lowest values [excluding outliers (+) and extremes (**x**)]

A



B

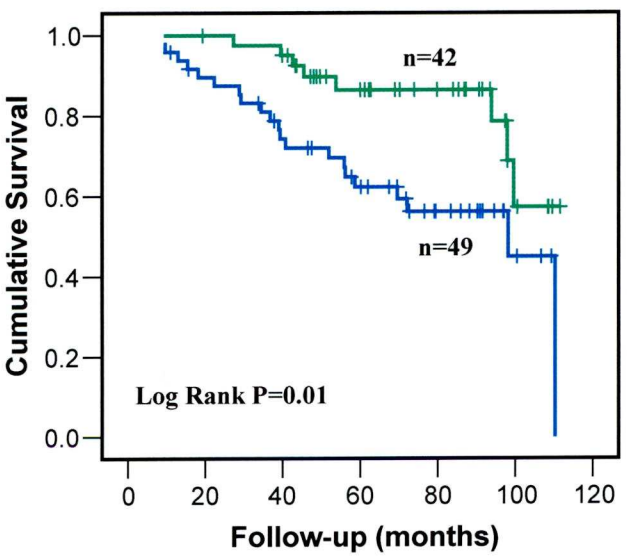


Figure 4.3 Kaplan Meier survival plots RFS (A) and BCS (B) for ERα mRNA dichotomized by ROC cut-point as high and low. High ERα mRNA levels were associated with better outcome.

Green line – high mRNA; Blue line – low mRNA

Crosses on the lines represent censored data.

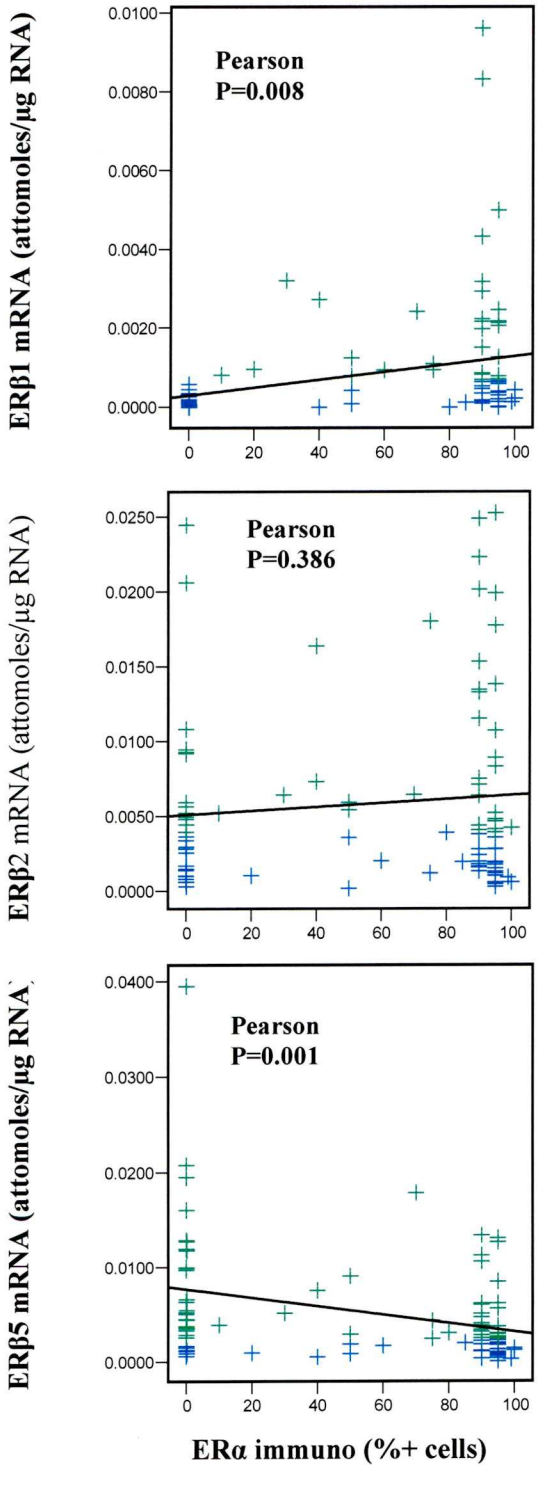


Figure 4.4 Scatter plots of ERβ variant mRNA expression in relation to ERα protein expression. ERβ1 showed a positive association with ERα protein expression, whereas ERβ5 showed a negative association and ERβ2 showed no association.

Markers are coloured according to mRNA level (dichotomized by respective ROC cut points used in survival analysis); green = high and blue = low. Lines represent linear regression for all cases.

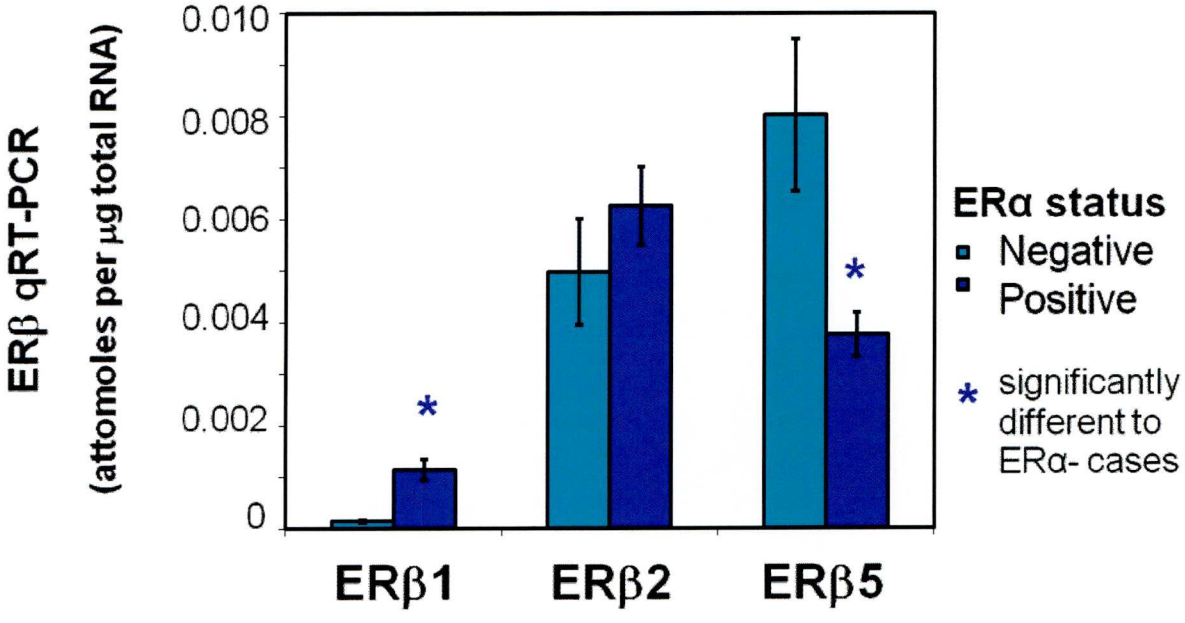


Figure 4.5 ERβ mRNA expression levels categorised by ERα status.

Mean ERβ1 levels were significantly higher in ERα+ cases than ERα- cases, but mean ERβ5 levels were significantly lower in ERα+ cases. Mean ERβ2 levels were not significantly different in ERα+ or ERα- cases.

Bars represent mean qRT-PCR values and error-bars are standard error of means.

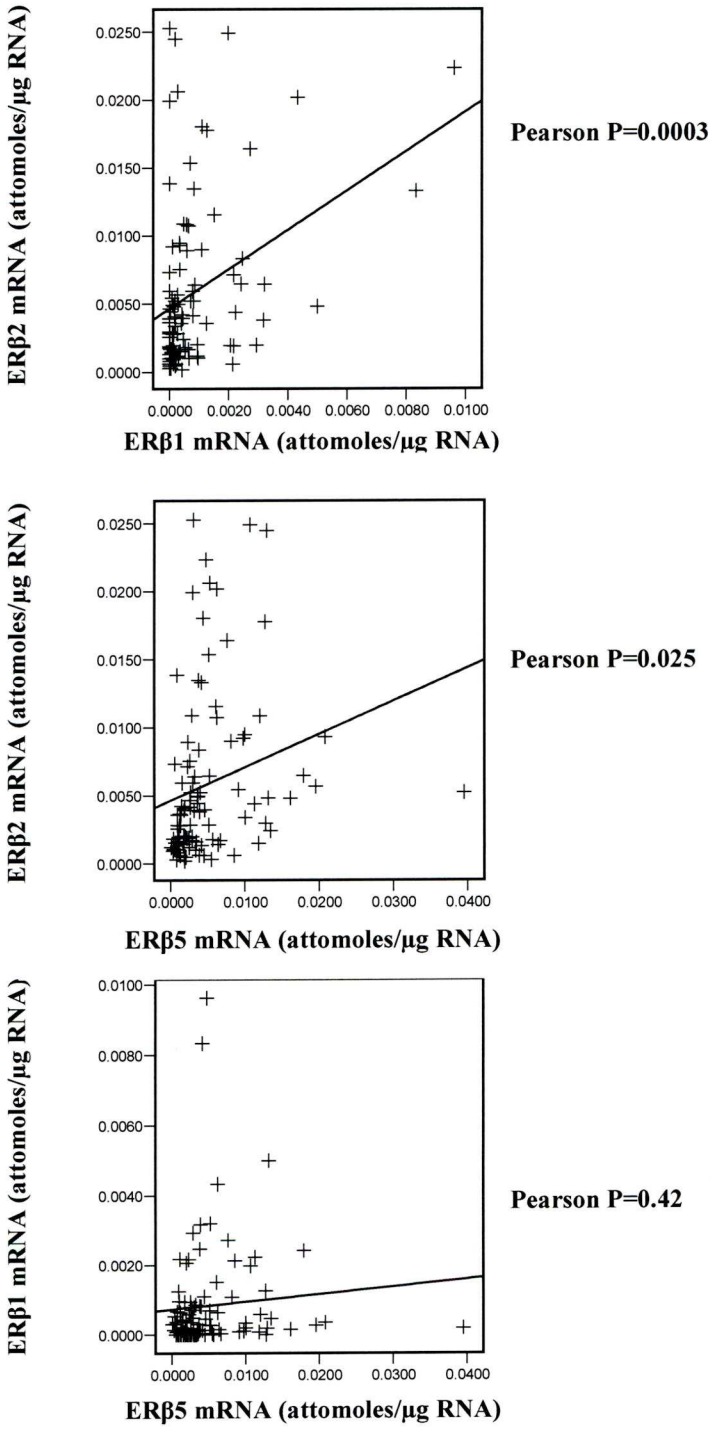


Figure 4.6 Scatter plots of ERβ variant mRNA expression in whole cohort showing correlation with each other. ERβ2 mRNA levels correlated positively with ERβ1 mRNA and to a lesser extent with ERβ5 mRNA, but there was no significant correlation between ERβ1 and ERβ5.

Lines represent linear regression for all cases.

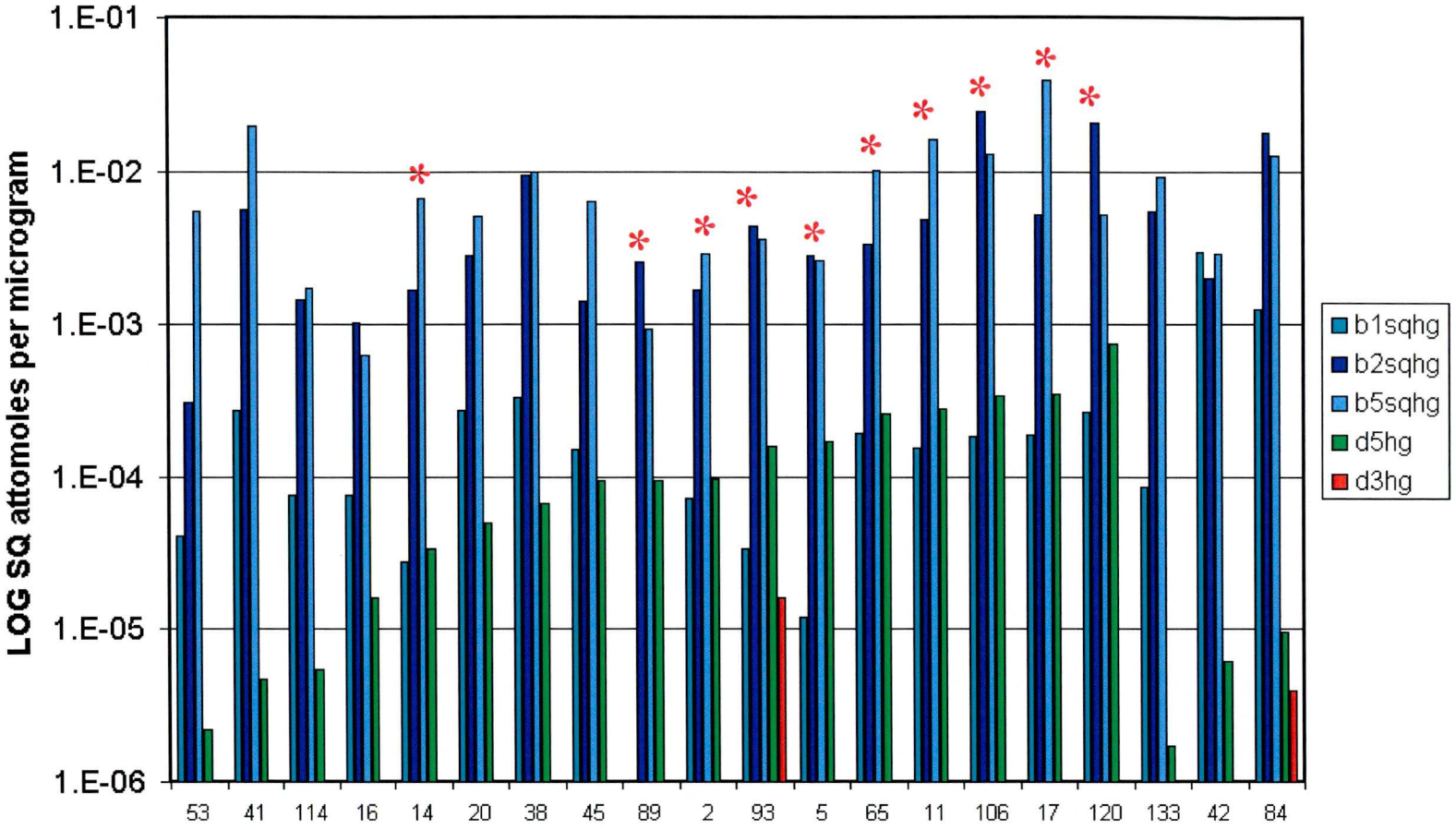
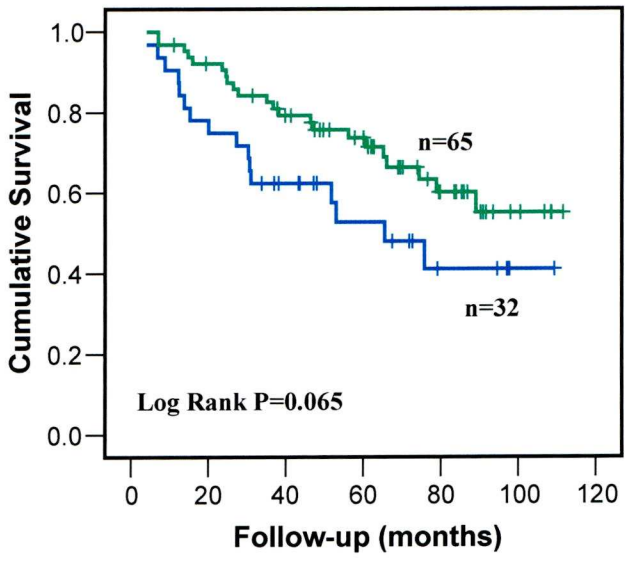


Figure 4.7 Relative expressions of ERβ splice variants in cases with detectable level of ERβΔ5. ERβΔ5 mRNA levels were lower than ERβ2 and ERβ5 in all 20 cases. However, in 10 cases ERβΔ5 levels were higher than ERβ1.

* indicates cases where ERβΔ5 mRNA > ERβ1mRNA

A



B

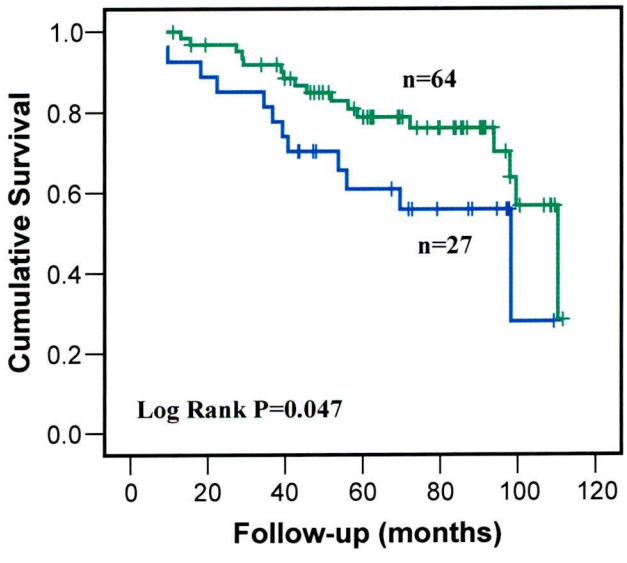
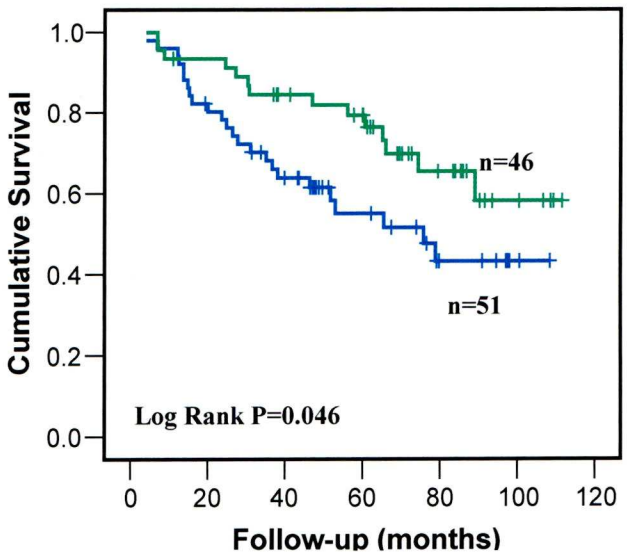


Figure 4.8 Kaplan Meier survival plots for RFS (A) and BCS (B) for ERβ1 mRNA dichotomized by ROC cut-point as high and low in the whole cohort. High ERβ1 mRNA levels were associated with better outcome.

Green line – high mRNA; Blue line – low mRNA

Crosses on the lines represent censored data in the whole cohort

A



B

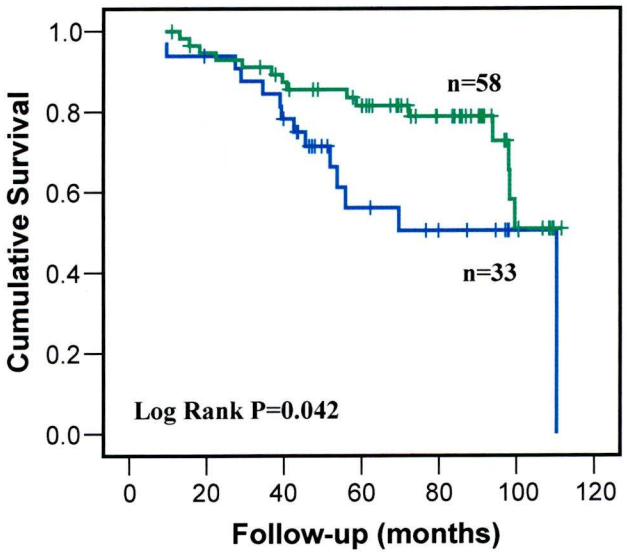
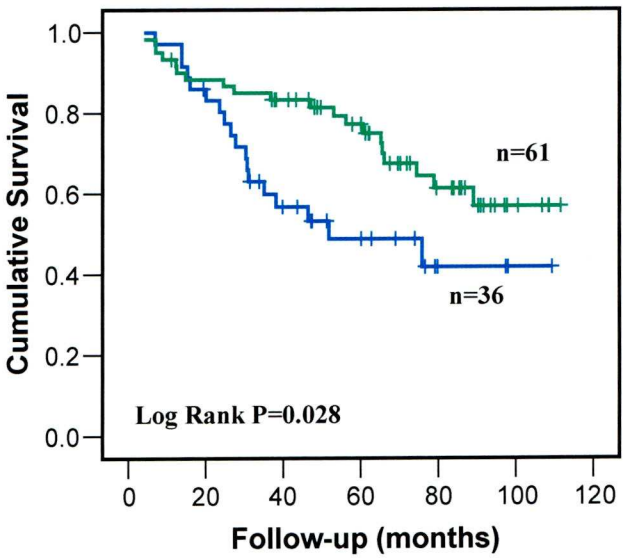


Figure 4.9 Kaplan Meier survival plots for RFS (A) and BCS (B) for ERβ2 mRNA dichotomized by ROC cut-point as high and low in the whole cohort. High ERβ2 mRNA levels were associated with better outcome.

Green line – high mRNA; Blue line – low mRNA
Crosses on the lines represent censored data

A



B

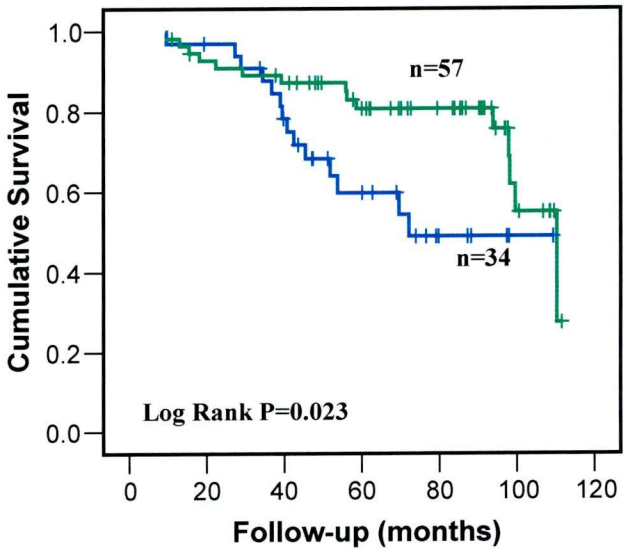
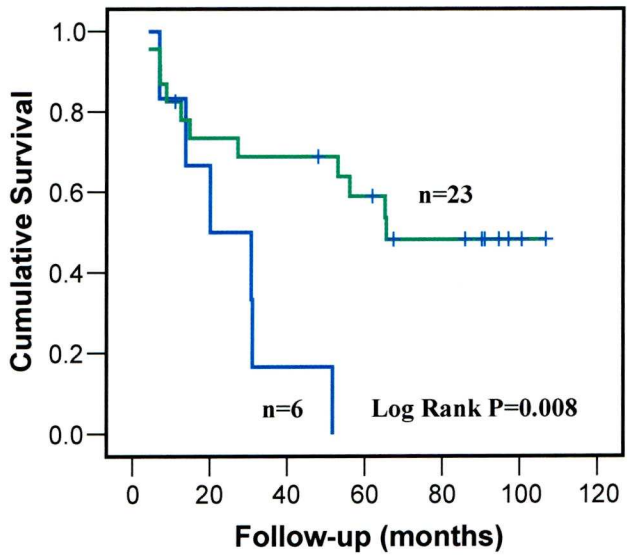


Figure 4.10 Kaplan Meier survival plots for RFS (A) and BCS (B) for ERβ5 mRNA dichotomized by ROC cut-point as high and low in the whole cohort. High ERβ5 mRNA levels were associated with better outcome.

Green line – high mRNA; Blue line – low mRNA
Crosses on the lines represent censored data

A



B

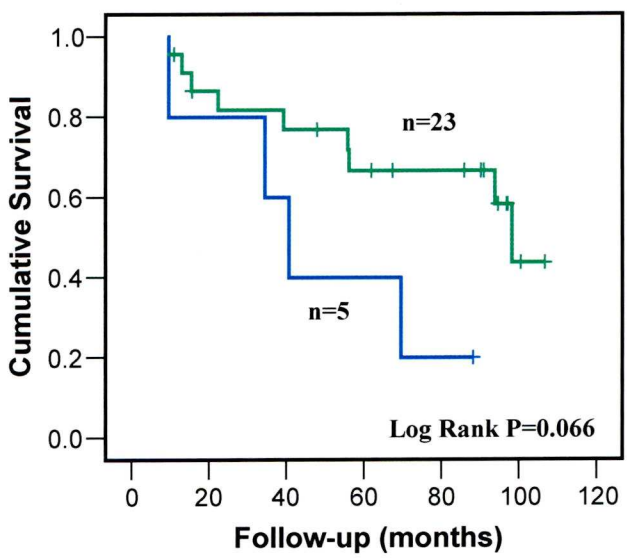
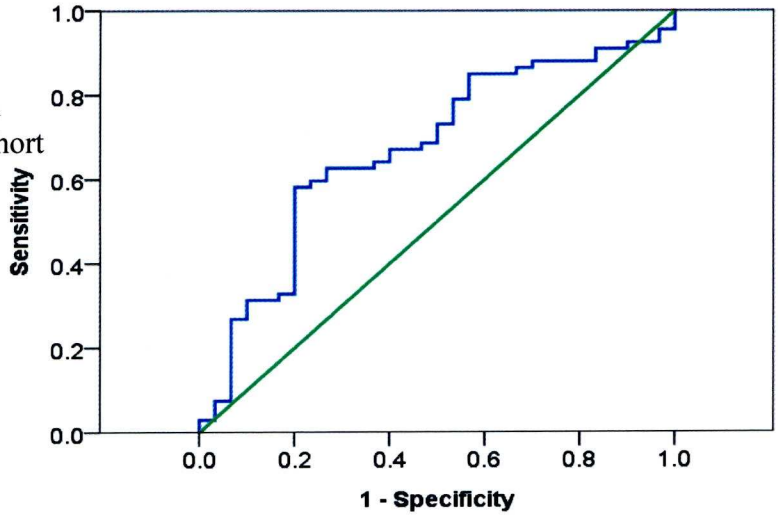


Figure 4.11 Kaplan Meier survival plots for RFS (A) and BCS (B) for ERβ5 mRNA dichotomized by ROC cut-point as high and low in the ERα- tamoxifen treated cohort. High ERβ5 mRNA levels were associated with better outcome.

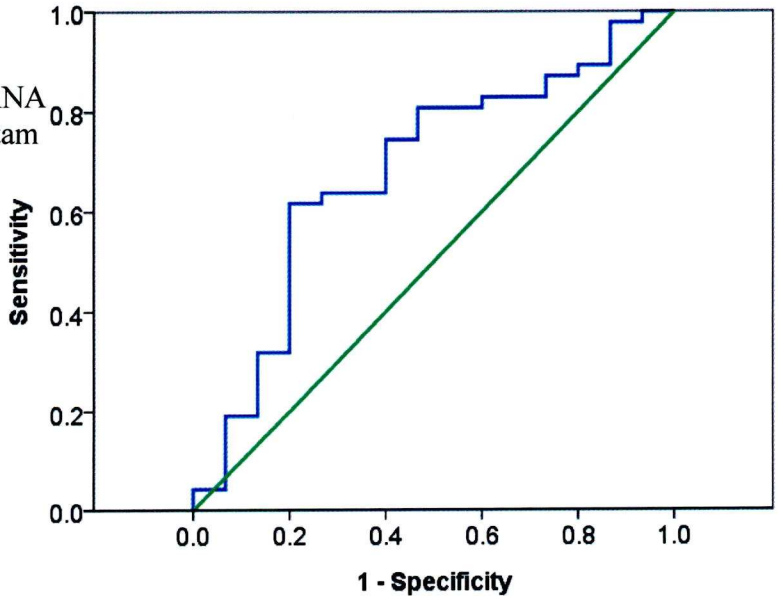
Green line – high mRNA; Blue line – low mRNA
Crosses on the lines represent censored data

A
ERα mRNA
In whole cohort



Area under Curve 0.67
95%CI 0.56-0.79
P=0.007

B
ERβ2 mRNA
In ERα+ tam
cohort



Area under Curve 0.68
95%CI 0.52-0.84
P=0.036

Figure 4.12 ROC curves to calculate the optimal cutpoint for ERα (A) and ERβ2 (B) mRNA level using relapse free survival at 5 years as the state variable.

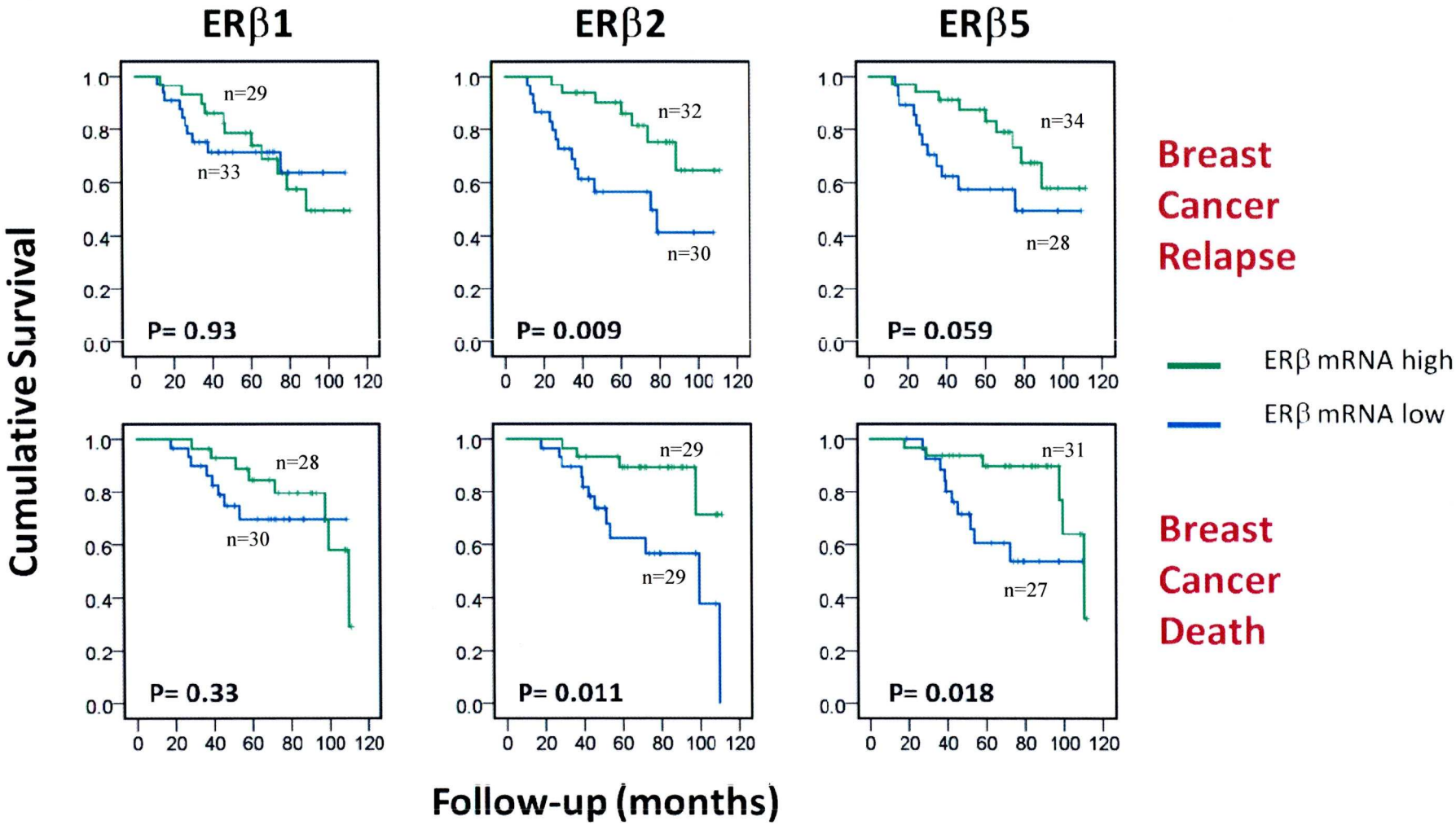


Figure 4.13 Kaplan Meier plots of breast cancer outcomes using optimized cut-points for each ERβ mRNA isoforms in ERα+ tamoxifen treated cohort. High ERβ2 and ERβ5 mRNA levels were associated with better outcome, while ERβ1 levels were not.

Green line – high mRNA; Blue line – low mRNA. Crosses on the lines represent censored data. P values are given for Log Rank test.

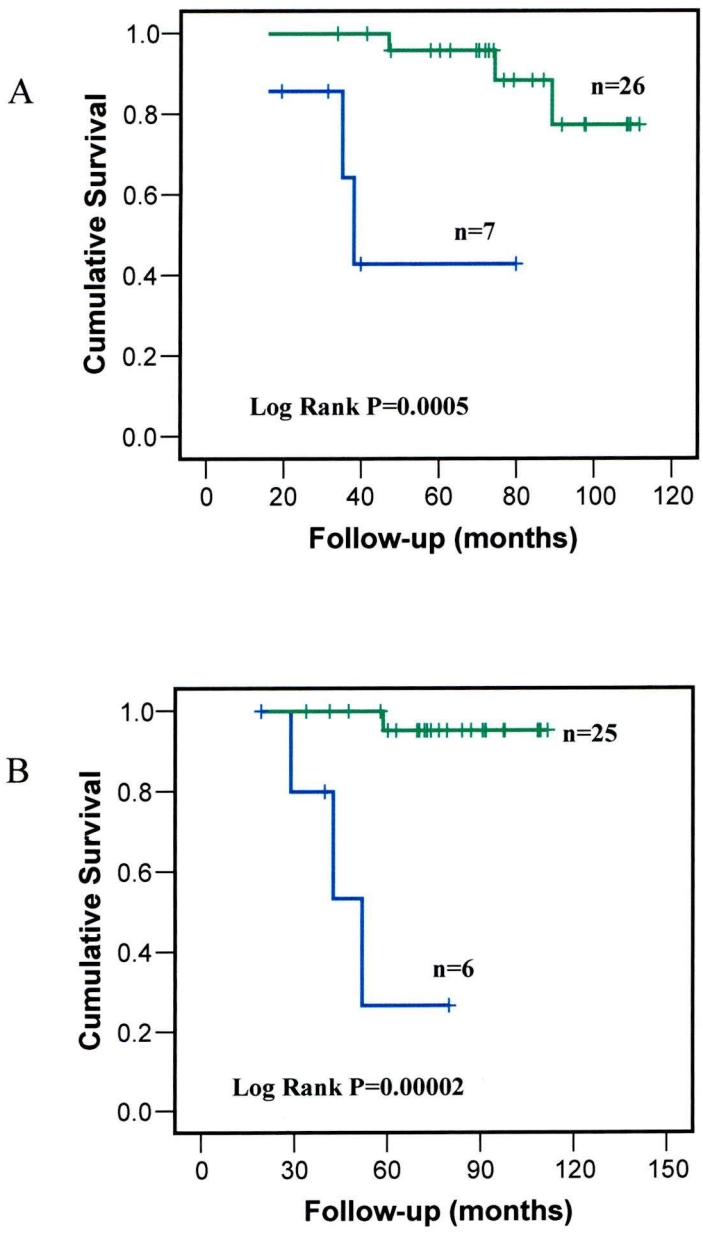


Figure 4.14 Kaplan Meier survival plots for RFS (A) and BCS (B) for ERβ2 mRNA dichotomized by ROC cut-point as high and low in the ERα+ node negative tamoxifen treated cohort. High ERβ2 mRNA levels were associated with better outcome.

Green line – high mRNA; Blue line – low mRNA

Crosses on the lines represent censored data

CHAPTER 5 RESULTS 3

ERβ2 IMMUNOHISTOCHEMISTRY ESTIMATION AND ANALYSIS

5.1 Introduction

Biomarkers can make use of measurements of mRNA levels, but a functional role for ERβ variants in breast cancer is more heavily suggested by expression of the corresponding protein. Furthermore, immunohistochemical assessment is more commonly used in the clinical setting for prognostic and predictive purposes (e.g. ER and PR). Therefore, as ERβ2 mRNA emerged as the most significant marker for outcome and suitable reagents became available, expression of ERβ2 protein was assessed in order to address its relationship to mRNA expression as well as to treatment outcome.

5.2 ERβ2 Immunohistochemistry

A cohort of 141 cases were analysed by immunohistochemistry for ERβ2 (Table 3.1) including 98 ERα+ cases. ERβ2 staining was assessed by 2 observers (R.V., V.A.) using the Allred scoring system and also as percentage positive cells (%+), with good agreement between observers (Allred Spearman 0.91 $P = 1.0 \times 10^{6}$, %+ Pearson 0.92 $P = 3.4 \times 10^{59}$). At the cut-point used for outcome analysis the Kappa score was 0.87. A consensus score was produced and used herein. The frequencies of each score were detailed in Table 5.1 and representative examples of immunostaining are shown in Figure 5.1.

5.3 Correlation with clinicopathological characteristics

ERβ2 immunostaining significantly correlated with that for ERα both for %+ cells (Pearson 0.42 $P=7.8 \times 10^{7}$) and for Allred score (Spearman 0.40 $P=4.1 \times 10^{6}$). ERβ2 immunostaining was greater in ERα+ cases (mean %+=69) than in ERα- cases (mean %+=52) and this difference was statistically significant (P=0.00001 T-test). ERβ2

immunostaining also correlated, to a lesser extent, with PgR (%+ Pearson 0.18 P=0.035) and ERβ2 Allred score was greater in PgR+ cases than PgR- cases (P=0.033 MW). The percentage of ERβ2 positive cells were somewhat lower in grade 3 cases (P=0.042 MW), in keeping with the association with ERα status. There was no association with vascular invasion, nodal status, age or size, or with ERβ1-specific immunostaining (ERβ1 immunohistochemistry data was available for 82 cases from CTBRC).

5.4 Association of ERβ2 protein with patient survival

Using the Allred scoring system, two groups of patients were designated as ERβ2 low (score 5 or lower, n=41, 29%) and ERβ2 high (score 6 or higher, n=100, 71%). The cut-point used was that indicated by ROC curve analysis and confirmed by testing of the limited number of possible groupings provided by the Allred scoring method. Similar dichotomization was provided by a cut-point of 65% for proportion of immuno-positive cells, but in keeping with published recommendations (Carder *et al*, 2005), the Allred score was used predominantly for further analysis as it also takes staining intensity into account.

The high/low ERβ2 Allred score groups were significantly associated with ERα status (P=0.001 Chi square) and within the subgroup of ERα positive women who received adjuvant tamoxifen (n=85) there were 18 ERβ2 low cases and 67 ERβ2 high cases (79%).

Within the group as a whole (ERα+ and ERα- cases), high ERβ2 protein levels were significantly related to a better relapse free survival (RFS P=0.049 Log Rank), but not for breast cancer survival (BCS P=0.16 Log Rank). However, in both cases the survival curves converge at later time-points (Figure 5.2). With shorter follow-up time a stronger relationship with outcome was seen: e.g. at 5 years for relapse (RFS P=0.018 Log Rank, HR 0.50 CI 0.27-0.90 P=0.020) and 7 years for survival (BCS P=0.048 Log Rank, HR 0.50 CI 0.27-0.90 P=0.020). If ERα status and ERβ2 immunoscore were combined in multivariate analysis of 5 year RFS, only ERα status was independently significant (HR 0.38 CI 0.22-0.66 P=0.001) whereas ERβ2 did not retain independent significance (HR 0.76 CI 0.43-1.33 P=0.33). With the

addition of further parameters of grade, size and nodal status, only nodal status (HR 3.1 CI 1.5-6.2 P=0.001) and grade (HR 1.5 CI 1.1-2.2 P=0.026) were independently significant.

When considering only ERα+, tamoxifen-treated cases there was no relationship between ERβ2 immunostaining and outcome (RFS P=0.95, BCS P=0.65 Log Rank) (Figure 5.3).

5.5 Association between ERβ2 mRNA and protein

When assessing the relationship between ERβ2 immunostaining and mRNA level for paired samples from each case, no correlation was seen between levels of protein and mRNA for ERβ2, by using %+ cells (Pearson 0.12 P=0.24) or Allred score (Spearman 0.08 P=0.40). A similar lack of correlation was seen previously for ERβ1 using semi-quantitative analysis of ERβ1 mRNA in an overlapping cohort (O'Neill *et al*, 2004); this was re-confirmed using quantitative ERβ1 mRNA measurement in 82 patients from this study [Pearson (%+) 0.02 P=0.87; Spearman (Allred) 0.03 P=0.83].

However when ERβ2 protein expression were dichotomized into high and low with the cut off used for survival, patients with an Allred score of 5 or below (n=21) had higher mean ERβ2 mRNA than cases with an Allred score of 6 or more (n=79, P=0.045 T-test), indicating a possible inverse relationship.

Using the cut-points optimized for outcome analysis, the majority of cases with high ERβ2 mRNA levels also had high levels of ERβ2 protein, but only a minority of those cases with high ERβ2 protein were also classified as having high ERβ2 mRNA (Table 5.2). Hence, ERβ2 mRNA expression is frequently associated with expression of significant levels of ERβ2 protein, but ERβ2 protein expression is often dissociated from mRNA expression (being high in many cases with low mRNA levels). Overall there is a subset of cases (35%) with concomitant high ERβ2 mRNA and protein, and another subset of cases (44%) in which high protein levels are accompanied by low mRNA levels.

5.6 Association of ERβ2 protein and mRNA with patient survival

As discussed in the previous chapter ERβ2 mRNA levels were associated with outcome in univariate analysis in the whole cohort. Since high ERβ2 protein levels also showed better RFS in the whole cohort, they were entered into a Cox multivariate model and they were independently associated with better RFS in the whole cohort (ERβ2 protein HR 0.40 CI 0.20–0.80 P=0.010, ERβ2 mRNA HR 0.43 CI 0.22–0.83 P=0.013).

However, in the ERα+ tamoxifen-treated cohort, in multivariate analysis of mRNA and protein, only high ERβ2 mRNA levels were significantly associated with lower RFS (HR 0.28 CI 0.212–0.72 P=0.008), but a trend remained for protein (HR 0.42 CI 0.15–1.19 P=0.10). Similar results were obtained for analysis of BCS.

Further outcome analysis was carried out in the 35% of cases with both high ERβ2 protein and mRNA level; this group had a significantly better outcome than those with low levels of either mRNA or protein, and those cases with low levels of both mRNA and protein had the worst outcome (RFS P = 0.009; BCS P=0.021 Log Rank, Figure5.4). When cases with both high ERβ2 protein and RNA were compared with rest of the group consisting of all other cases, they had significantly better outcome [(RFS P=0.002 Log Rank, HR 0.67 CI 0.51–0.88 P=0.004) (BCS P=0.003 Log Rank, HR 0.61 CI 0.43–0.87 P=0.006)] (Figure 5.5). This association was also seen in the ERα+ tamoxifen treated cohort [(RFS P=0.004 Log Rank, HR 0.61 CI 0.43–0.88 P=0.009) (BCS P = 0.009 Log Rank, HR 0.56 CI 0.34–0.91 P=0.020)] (figure 5.6). The outcome benefit of concomitant high ERβ2 mRNA and protein levels was particularly marked at a shorter follow-up in the ERα+ tamoxifen treated cohort. This measure was the only independent marker of improved outcome using Cox multivariate analysis including grade, size, PgR status and nodal status at 5 years for RFS (HR 0.48 CI 0.24–0.95 P=0.036) and at 7 years for BCS (HR 0.46 CI 0.23–0.92 P=0.029). In the ERα+ tamoxifen-treated, node negative cases, having both high ERβ2 mRNA and protein was significantly related to an improved BCS (P=0.028 Log Rank).

5.7 Discussion

As with mRNA measurement, many studies of ERβ protein used antibodies that targeted either N terminal epitopes which will potentially detect all the variants or C-terminal ERβ1-specific epitopes. So there were not many studies specifically looking for ERβ2 protein. In considering the possible role of ERβ2 protein, this discussion will exclude ERβ2 mRNA studies, since mRNA cannot be taken as a surrogate marker for protein expression.

5.7.1 ERβ2 immunohistochemistry

Immunostaining is a technique which often varies between studies, due to the use of different protocols, different antibodies and because some parameters (e.g. tissue fixation) are beyond the control of investigators using retrospective cohorts.

Here ERβ2 immunohistochemistry was done with monoclonal antibody MCA2279S (clone no 57/3; Serotec) which has been previously used by other studies (Saunders *et al*, 2002) and was the reagent of choice in a subsequent larger study (Shaaban *et al*, 2008). The method used was similar to other studies in terms of antigen heat retrieval and overnight incubation of primary antibody at 4° centigrade, although the former is sometimes difficult to replicate between laboratories as centres used different heating methods: autoclave treatment for 7 minutes (Honma *et al*, 2008), pressure cooking for 15 minutes (Esslimani-Sahla *et al*, 2005) and microwaving for 15 minutes (Palmieri *et al*, 2004).

A blocking step was used to block cytoplasmic staining that was at the time considered as non-specific, but some cytoplasmic staining remained as previously noted with ERβ (O'Neill *et al*, 2004; Shaaban *et al*, 2003; Skliris *et al*, 2003). Only nuclear staining was included in scoring, although subsequent studies (Shaaban *et al*, 2008) have indicated that cytoplasmic ERβ2 staining might not only be real, but might have biological significance. It would be interesting to see whether cases with high mRNA and low protein in our study had significant cytoplasmic staining. Additionally, it would be interesting to explore if the presence of cytoplasmic staining is in any way related to the excess protein staining seen in a proportion of cases with low mRNA. If this ERβ2 protein that is not transcriptionally regulated is stabilized in some way, it may be that it is detected in cytoplasm due to the cells

having reduced ability to degrade it (which normally occurs in the cytoplasm). Alternatively it may represent non-liganded ERs in cytoplasm, as ERs normally reside in cytoplasm and undergo nuclear translocation after ligand binding (Leung *et al*, 2006).

ERβ2 staining was also noted in stromal and endothelial cells, as previously seen (Esslimani-Sahla *et al*, 2005) and excluded from scoring. At present it is not clear whether this non-epithelial ERβ2 expression contributes to carcinogenesis or hormone responsiveness. Interplay between epithelial and stromal cells is important in breast development and it has been proposed that ERβ2 may play a role in these paracrine pathways.

Compared to ERα staining, which is usually bimodal in distribution, ERβ2 staining seems to be more varied and widely expressed by many cells. This makes assigning a cut-point for ERβ protein expression challenging, but is probably related to differing biology of the two oestrogen receptors. ERα is usually highly expressed in only a specific subset of normal breast epithelial cells, but is aberrantly expressed at these same high levels in a larger proportion of cancerous breast cells in some cases of breast cancer. In contrast ERβ1 is more widely expressed in normal breast epithelia (Speirs *et al*, 2002), most likely at lower levels, and is lost in a more variable proportion of cancer cells (Shaaban *et al*, 2003). However ERβ2 appears to be less expressed in normal breast compared to DCIS and invasive cancer (Esslimani-Sahla *et al*, 2005), although no correlation between these two protein variants was evident.

Allred score and percentage of immunostaining were measured for this study. Allred score has been accepted as a standard measure for ERα (Leake *et al*, 2000), as this standardized approach also helps with correlation with other studies it has been recommended for ERβ (Carder *et al*, 2005). Some studies have used percentage positivity alone (Esslimani-Sahla *et al*, 2004; Honma *et al*, 2008) with a variety of cut-off values.

In our study the cut-off was Allred score 5 or lower and 6 or higher. Using this cut-off, 71% of cases had high ERβ2 protein and 29% had low ERβ2 protein. The antibody dilution was 1 in 25 in this study. When higher dilutions were tried, different positive values were observed and the consistency of staining was lost. Other studies with the same antibody used dilutions of 1 in 10 (Shaaban *et al*, 2008)

or 1 in 20 (Honma *et al*, 2008). Shaaban *et al.* reported 83% positive cases using a 20% cut-off and Honma *et al.* reported 85% positive cases using a 10% cut-off. Using these same cut-offs our positive cases would be 94% and 96% respectively, indicating that our staining was stronger than reported elsewhere. Although these studies used the same antibody, the antigen retrieval methods and incubation times varied. These factors may have influenced the extent of staining reported here and this is reflected in the higher cut-point chosen for outcome analysis. It is difficult to assign a universal cut-off for ERβ2 at this stage until the cut-off levels are validated in large prospective studies and uniform protocols are accepted world-wide (Carder *et al*, 2005).

5.7.2 Correlation with other clinicopathological characteristics

ERβ2 protein expression correlates with ERα and PgR positivity, and ERβ2 protein was significantly higher in ERα and PgR positive cases. Similar to ERα there was correlation with grade, as low levels of ERβ2 were seen in high grade tumours. In keeping with most previous studies there were no clear links to many clinical and pathological parameters.

Sugiura *et al.* showed significant association in 150 patients between ERβ2 protein and ERα positivity and low tumour grade similar to the current study (Sugiura *et al*, 2007). In a large study by Shaaban *et al.* (757 patients), ERβ2 protein expression correlated positively with ERα, PgR and grade 2 tumours, and inversely with vascular invasion and distant metastasis. Positive association was also seen with androgen receptor and BRCA1 (Shaaban *et al*, 2008). In another large study of 442 patients, ERβ2 protein did not show any correlation with standard clinicopathological markers except for ERα positivity (Honma *et al*, 2008).

Saji *et al.* showed correlation between ERβ2 and PgR negative status in 115 ERα+ tumours (Saji *et al*, 2002). However, no correlation with standard clinicopathological markers were seen with ERβ2 protein in a number of other studies (Esslimani-Sahla *et al*, 2004; Palmieri *et al*, 2004; Skliris *et al*, 2006).

This study was specifically designed to address the potential role of ERβ variants in endocrine-treated breast cancer, with further selection towards post-menopausal cases (as discussed previously). Although such selection is valuable in studies of

outcome, it does introduce some bias in comparison to unselected/consecutive cohorts. Whilst mitigated by the inclusion of a representative proportion of ERα- cases, it is possible that some differences in association with clinical or biological parameters is influenced by the case selection.

5.7.3 ERβ2 protein and outcome

Using the Allred cut-off score (selected by using ROC curve), high ERβ2 was associated with better relapse free survival in the whole cohort, and at shorter follow-up high ERβ2 patients had significantly better RFS and BCS. These outcome associations were not maintained in the ERα positive cohort. It seems the correlation between ERα and ERβ2 may be partly responsible for the outcome associations in the whole cohort.

In comparing with other studies, as previously discussed, it is important to consider the case selection and treatment received. This is sometimes difficult as, although different treatment sub-groups are noted, treatment-specific outcome results are not always given. Other studies which evaluated ERβ2 protein only are discussed here.

Sugiura *et al.* evaluated both ERβ2 mRNA and protein with outcome in a cohort of 150 patients (ER+ and ER-). High ERβ2 mRNA was associated with better RFS and OS in both univariate and multivariate analysis. However, high ERβ2 protein was only associated with better RFS in univariate analysis and not for OS (Sugiura *et al*, 2007). Though Sugiura *et al.* study results are concordant with our findings, there are some subtle differences between this study and ours in terms of patient selection, treatment received by patients, antibody used and ERβ2 positivity rate and so on.

Shaaban *et al.* evaluated ERβ1, ERβ2 and ERβ5 protein expression in a cohort of 757 patients (ERα+ and ERα-) and 250 patients received endocrine therapy in this cohort.

High ERβ2 significantly correlated with disease free survival and overall survival in the whole cohort, and also predicted response to endocrine therapy. Patients who were ERα+/ERβ2+ had significantly better overall survival than others. Considering ERβ2 immunostaining alone in our whole cohort, the results presented here are superficially similar to those seen by Shaaban *et al.* (Shaaban *et al*, 2008) in their larger cohort. For these results to have clinical significance in endocrine treated

breast cancer we would hope that the associations with outcome would hold true for the ERα+ tamoxifen-treated cohort, but it was not clear whether this was the case.

Honma *et al.* examined ERβ1 and ERβ2 protein in 442 breast cancer patients (377 ERα+ and 65 unknown hormone status) who received adjuvant tamoxifen monotherapy and found ERβ1 expression was the strongest marker for disease free and overall survival. However, high ERβ2 expression also showed better disease free survival (P=0.03) and overall survival (P=0.038) in Kaplan Meier survival analysis (Honma *et al*, 2008).

5.7.4 ERβ2 protein and mRNA correlation

Associations between high levels of ERβ2 protein (immunoscore) or mRNA (qRT-PCR) and improved outcome have been seen, but only the qRT-PCR results are statistically significant in the clinically relevant ERα+ cohort. It is therefore important to establish the relationship between mRNA and protein levels in clinical samples. Notably, many previous mRNA studies made conclusions regarding biological or clinical relevance based on the presumption that level of protein correlates with that of mRNA.

Some studies compared the ERβ mRNA expression levels with the ERβ protein. Shaw *et al.* found no correlation in 37 out of 61 cancers for total ERβ (Shaw *et al*, 2002). Omoto *et al.* also found that ERβ1 protein expression was not directly proportional to mRNA level (Omoto *et al*, 2002). A similar lack of correlation was seen previously for ERβ1 using semi-quantitative analysis of ERβ1 mRNA in an overlapping cohort (O'Neill *et al*, 2004); this was re-confirmed in 82 patients using quantitative ERβ1 mRNA measurement from this study [Pearson (%+) 0.02 P = 0.87; Spearman (Allred) 0.03 P = 0.83]. This lack of correlation again raises the role of translational control, protein stabilisation and heterogeneous sample for RNA extraction skewing the mRNA level.

In this cohort of breast cancers there was no evidence of a significant relationship between ERβ2 mRNA and protein as a whole. The disparity between protein and RNA expression for ERβ2 is even suggestive of an inverse relationship in a

proportion of cases in the current study. However, in a Japanese study, in a cohort of 150 patients (ERα+ and ERα-cases) ERβ1 and ERβ2 qRT-PCR significantly correlated with ERβ1and ERβ2 protein, respectively (Sugiura *et al*, 2007). A rabbit polyclonal antibody raised against ERβ2 specific epitope was used in this study and Allred score above 3 was used as a cut off for positivity (Sugiura *et al*, 2007). The study by Sugiura *et al.* contradicts all the other studies published so far, at the same time it is encouraging, as we know breast cancer phenotype seems to be different in various ethnic and geographical regions and whether these phenotypic differences play a role in translational control is an interesting thought.

Due to tissue heterogeneity, any mRNA analysis of tissue homogenates without selection can contribute to discordance with immunostaining results that are scored on specific cell types. In contrast to mRNA estimation, protein evaluation by immunohistochemistry looks into epithelial cells only. Tissue heterogeneity in terms of amount of tumour and connective tissue in the specimen used to prepare may influence the amount of specific mRNAs in each sample. To reduce this bias, the cases were selected for RNA analysis following independent histological review of adjacent sections, so as to avoid high levels of tissue heterogeneity. Samples from all cases consisted of at least 75% tumour cells and 67% of cases had at least 90% tumour cells. Inflammatory infiltrates were present in a minority of cases (at 10% in 15 cases and at 25% in 4 cases). During mRNA and protein correlation, whether or not these 19 cases were excluded, they did not unduly influence the correlation.

The recent study by Cummings *et al.* comparing the expression of ERβ1, ERβ2 and ERβ5 mRNAs in whole tumour tissue, and in micro-dissected epithelial cells and stromal cells obtained from 25 breast tumours have shown greater gene expression in whole tissue compared to micro-dissected material. Interestingly variants were differentially expressed in different cell population with ERβ1 was significantly more in stromal cells and ERβ5 was significantly highly expressed in the epithelium and ERβ2 was low in both cell types. Again in three individual tumours depending upon the cellular composition the variants were differentially expressed suggesting phenotypic differences in tissue composition (Cummings *et al*, 2009).

A further consideration when comparing RNA and protein level is the possibility of discordance arising from sampling of different areas of the tumour. In the same way

that protein expression is not homogeneous, mRNA levels may vary between the sample used for RNA analysis and that used for protein analysis. Hence, use of different areas of the tumour for mRNA and protein measurement can also effect correlations seen. That not all mRNAs are translated into protein and different proteins can have different stabilities and this makes like to like comparison between mRNA and protein expression difficult. However, good correlation between ERα mRNA and protein was seen in this study.

A major factor in the discordance is that many cases express high levels of protein, but low mRNA levels; a situation that is not likely to arise from expression of mRNA in non-tumour cells. It is however possible that heterogeneity of expression in the different parts of the tumour specimen used for mRNA and protein analysis contributes to the lack of correlation and *in situ* analysis of mRNA and protein in adjacent tissue slices might address this.

That correlation between ERα mRNA and protein was seen in this cohort does support the notion that sample selection for RNA has been effective, but it must be noted that the situation for ERα is somewhat different as it is generally not expressed in the types of cells that might contribute to the samples used for RNA extraction.

Although ERβ2 protein levels are apparently not directly related to mRNA levels, that does not mean that ERβ2 protein expression is not related to mRNA levels in some cases, or that expression of ERβ2 protein is not important in cases with high ERβ2 mRNA (associated with better outcome). A significant proportion of cancers (35%) had both high protein and high mRNA levels and these had a significantly better outcome than the remaining cases, with cases where both mRNA and protein were low having the worst outcome. This good outcome observed for those cases assessed as having both high mRNA and protein levels was independent in multivariate analysis.

This suggests that transcription of ERβ2 mRNA drives ERβ2 protein levels in some cases, and these cases do particularly well on tamoxifen treatment. It is possible therefore that the relatively poor utility of ERβ2 protein assessment by immunostaining as a measure of outcome prediction may be due to high levels of ERβ2 protein in some cases (with lower levels of ERβ2 mRNA) being related to

some form of protein stabilization, or detection of inactive ERβ2. It is therefore perhaps unsurprising that previous studies of ERβ2 protein expression did not find significant associations between ERβ2 and outcome in ERα+ tamoxifen treated cases as these did not include concomitant mRNA measurement. They were thus unable to distinguish between ERB2 protein associated with increased transcription and that possibly present due to some form of post-transcriptional control (or perhaps the breakdown of normal control).

Table 5.1 Frequency of Allred score in breast cancer samples.

Allred score	Whole cohort n=141		qRT-PCR cohort n=100	
	No of cases	Percentage of cases (%)	No of cases	Percentage of cases (%)
0	2	1.4	1	1
3	3	2.1	0	0
4	9	6.4	5	5
5	27	19.1	15	15
6	39	27.7	30	30
7	61	43.3	49	49

Table 5.2 Association between ERβ2 mRNA and protein using optimal cut-points from survival analysis.

ERβ2	mRNA high	mRNA low	Total
Protein high	35	44	79
Protein low	14	7	21
Total	49	51	100

Pearson Chi-square 3.32 P=0.068

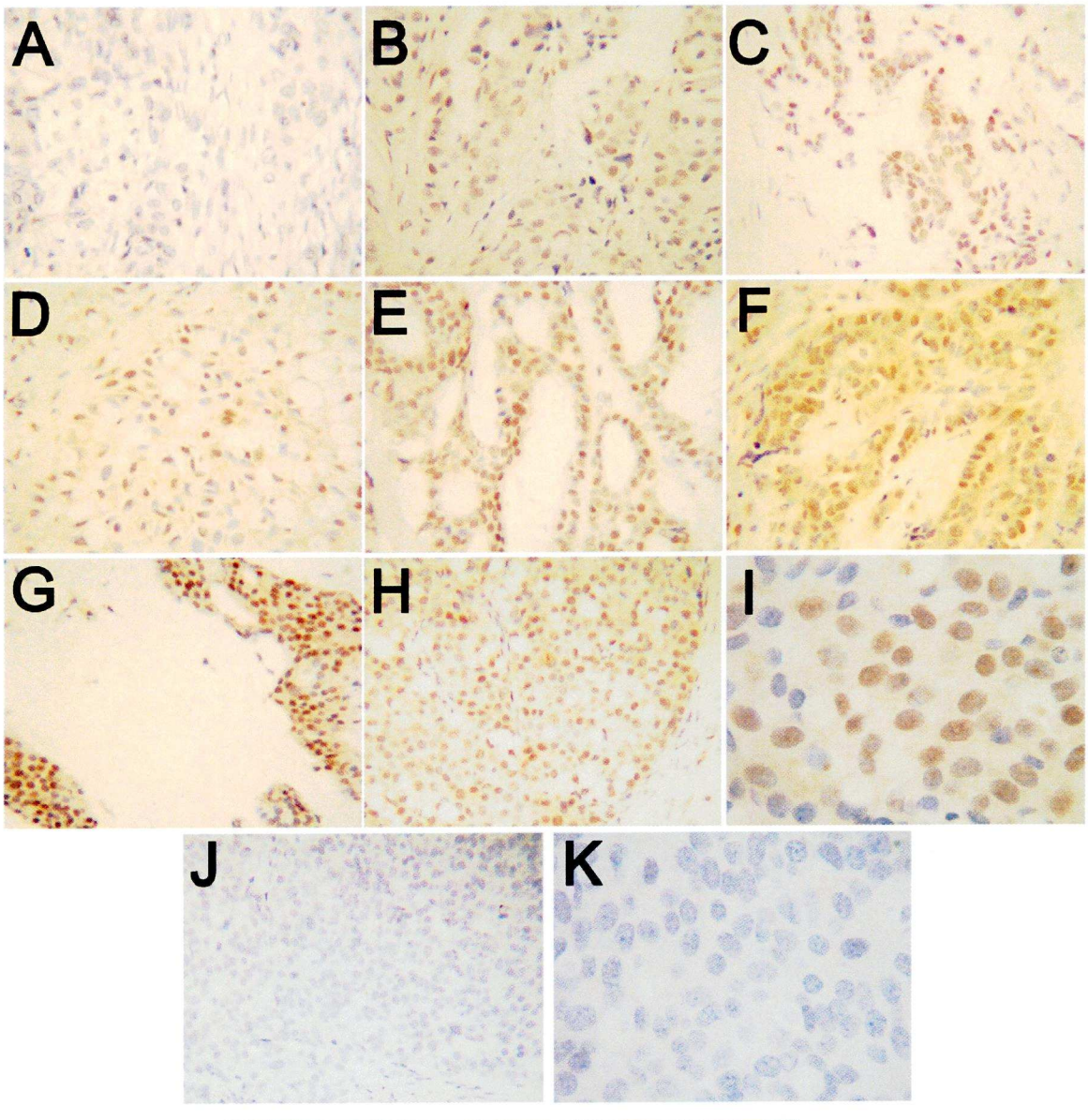
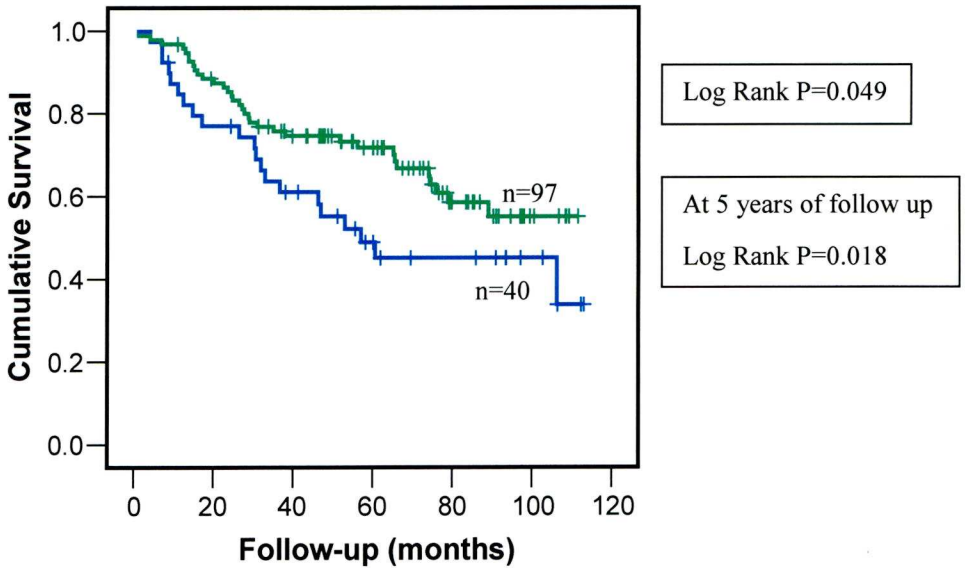


Figure 5.1 Immunohistochemical staining for ERβ2. Breast carcinomas showing different levels of staining; examples of Allred score 0 (A), 3 (B), 4 (C), 5 (D), 6 (E), 7 (F) and 8 (G).

H-K are low (H, J) and high (I, K) magnification images of the same tumour stained normally (H, I) and following blocking with synthetic peptide (J, K).

A



B

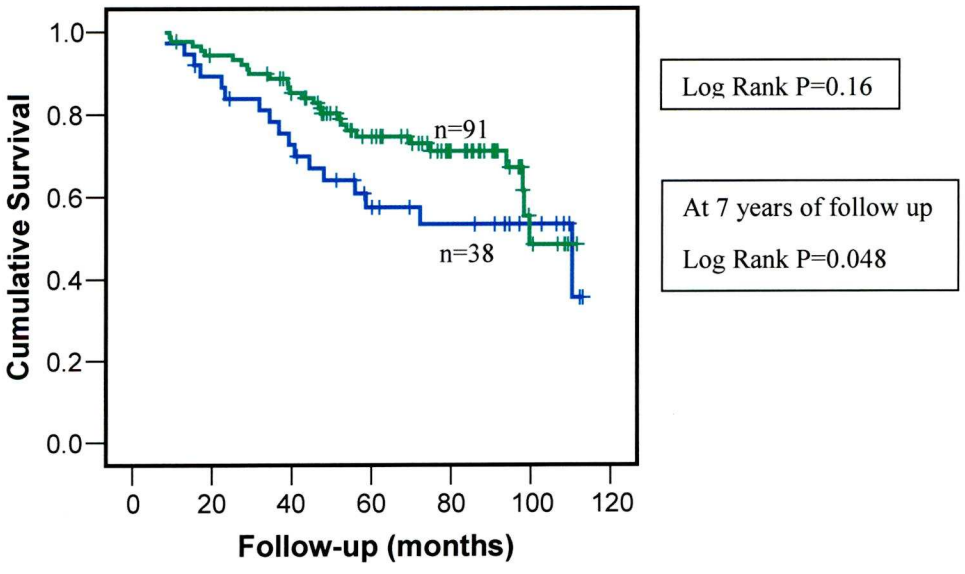
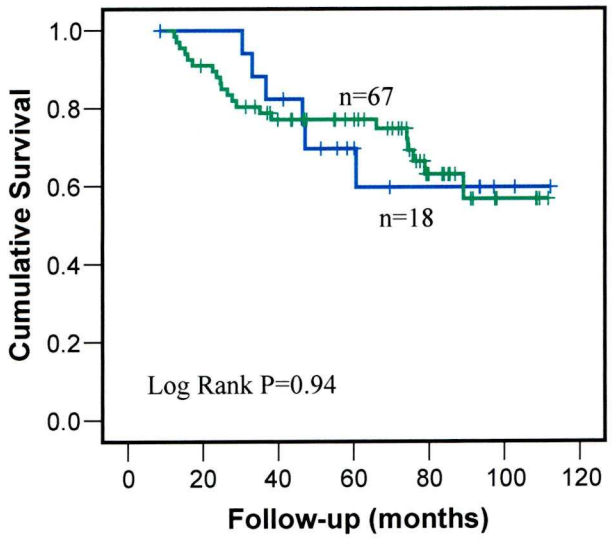


Figure 5.2 Kaplan Meier survival plots RFS (A) and BCS (B) for ERβ2 protein dichotomized by high and low Allred score in the whole cohort. High ERβ2 protein level was associated with good outcome in the whole cohort.

Green line – high protein; Blue line – low protein

Crosses on the lines represent censored data.

A



B

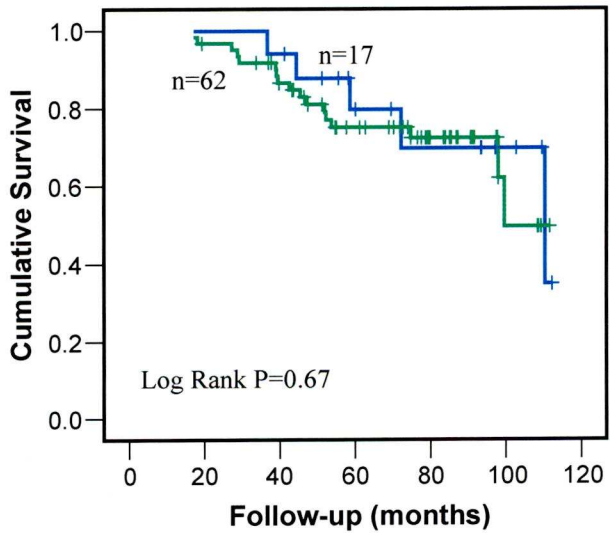
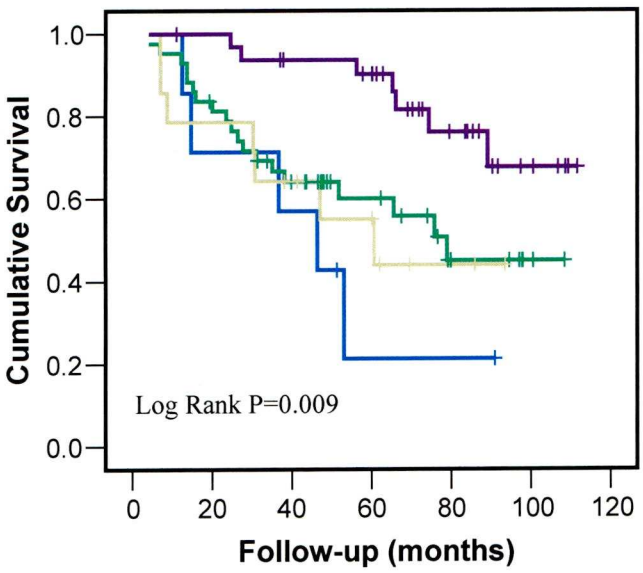


Figure 5.3 Kaplan Meier survival plots RFS (A) and BCS (B) for ERβ2 protein dichotomized by high and low Allred score in the ERα+ tamoxifen cohort. ERβ2 protein level was not associated with outcome in the ERα+ tamoxifen cohort.

Green line – high protein; Blue line – low protein

Crosses on the lines represent censored data.

A



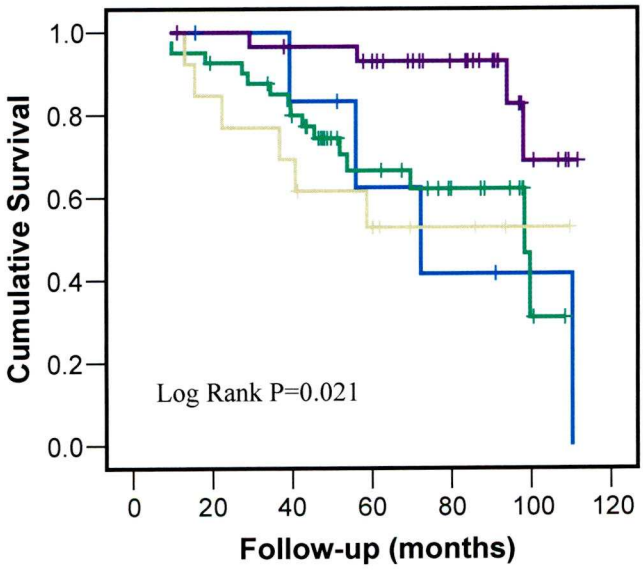
Violet - high protein and high mRNA (n=33)

Green - high protein and low mRNA (n=43)

Grey - low protein and high mRNA (n=14)

Blue - low protein and low mRNA (n=7)

B



Violet - high protein and high mRNA (n=30)

Green - high protein and low mRNA (n=41)

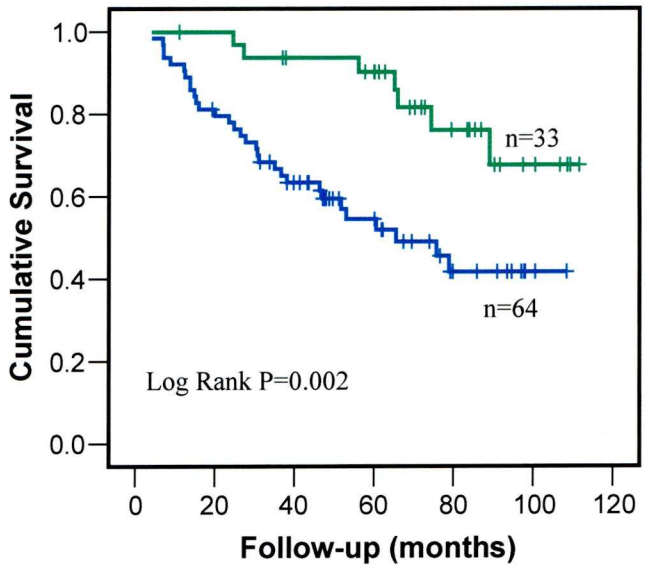
Grey - low protein and high mRNA (n=13)

Blue - low protein and low mRNA (n=7)

Figure 5.4 Kaplan Meier survival plots RFS (A) and BCS (B) for ERβ2 by categorizing the whole cohort into four categories. Cases expressing high levels of both mRNA and protein had better outcome followed by cases expressing higher levels of either.

Crosses on the lines represent censored data.

A



B

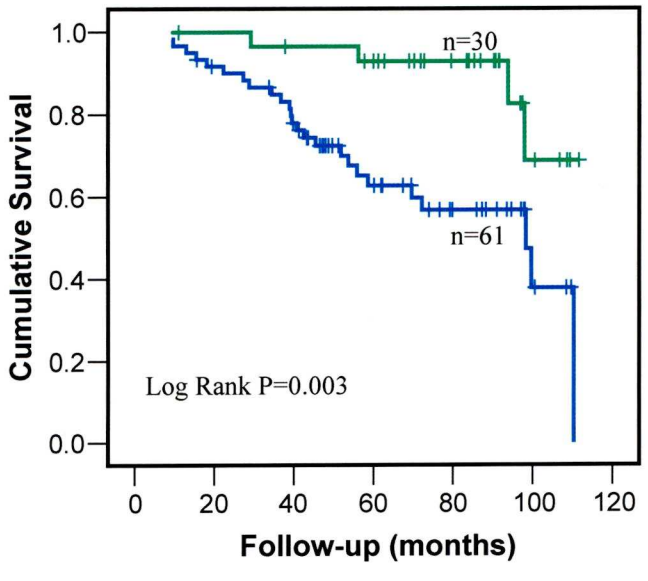
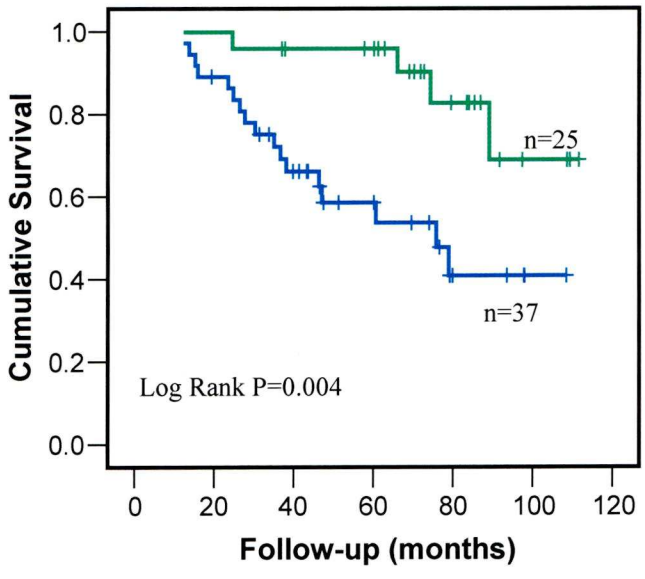


Figure 5.5 Kaplan Meier survival plots RFS (A) and BCS (B) for cases with both high ERβ2 protein and mRNA *Vs* other cases in the whole cohort. Cases expressing high levels of both were associated with better outcome.

Green line – high protein and mRNA; Blue line – high protein and low mRNA/low protein and high mRNA/both low

Crosses on the lines represent censored data.

A



B

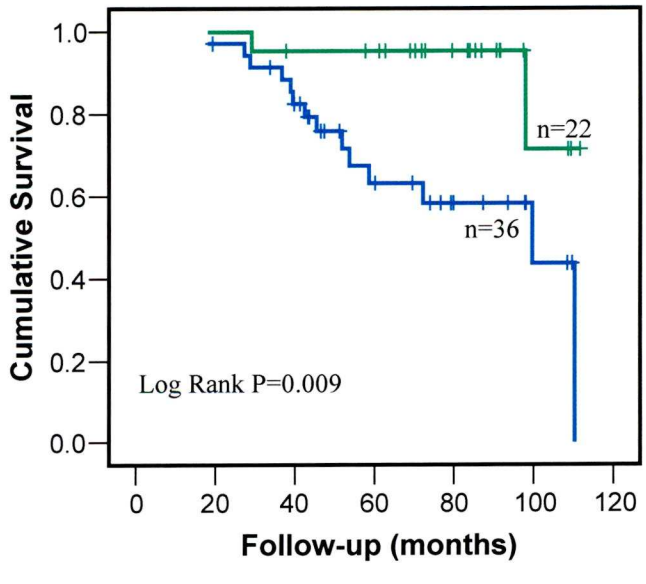


Figure 5.6 Kaplan Meier survival plots RFS (A) and BCS (B) for cases with both high ERβ2 protein and mRNA *Vs* other cases in the ERα+ tamoxifen cohort. Cases expressing high levels of both were associated with better outcome.

Green line – high protein and high mRNA; Blue line – high protein and low mRNA/low protein and high mRNA/both low

Crosses on the lines represent censored data.

CHAPTER 6 FINAL DISCUSSION AND CONCLUSIONS

6.1 Final discussion

There have been numerous studies to date that attempt to address the potential importance of ERβ assessment in breast cancer management. Many of these studies make use of mRNA levels as a surrogate marker for ERβ expression, despite the fact that few have attempted to relate mRNA to protein levels. Other studies do not address the expression of ERβ isoforms, but use techniques that rely on detection of N-terminal protein or mRNA sequences that are shared by most variants. A good proportion of studies also fail to take into account menopausal status, stage of the disease or the treatment given. This study therefore set out to quantitate the expression levels of ERβ wild-type (ERβ1) and variant (ERβ2, ERβ5, ERβΔ3, ERβΔ5) mRNA together with protein expression for ERβ2 and address their relationship to patient survival in a treatment-specific cohort of postmenopausal women receiving adjuvant endocrine treatment but not chemotherapy.

That this retrospective cohort includes a proportion of ERα- cases (30%), similar to the general breast cancer population, allows reasonable assessment of ERβ isoform expression in relation to other clinical and histological parameters. ERα- cases afforded some insight into expression of ERβ5 and exon-deleted variants, but were excluded in the important assessment of outcome for endocrine treatment, which was limited to ERα+ cases, reflecting current breast cancer management. Despite this subgroup analysis, the cohort studied was of significant power to detect outcome-related associations for previously validated markers (e.g. grade, nodal status, PgR status) and ERβ variants.

ERβ5 is similar to ERβ2 in structure and the alternative C-terminal exons for these proteins overlap. ERβ5 mRNA levels are similar to and correlate with ERβ2 in ERα+ cases and this is reflected in similar associations with improved outcome. There has been some recent interest in the potential role of ERβ1 and ERβ5 isoforms in ERα- breast cancers to act as targets for chemopreventative drugs (Poola *et al*, 2005b). We find that protein levels of ERβ1 and ERβ2 are significantly expressed in ERα- cases, but with no clear relationship to outcome. Both ERβ2 and ERβ5

mRNAs were present in greater amounts than ERβ1 in ERα- tumors, but, unlike ERβ2 and ERβ1, ERβ5 mRNA levels were higher in ERα- cases than ERα+ cases. These results indicate some differences in regulation of these isoforms that might have some consequence for breast cancer. We found an association of high ERβ5 mRNA levels with better outcome in our cohort of endocrine-treated ERα- cases, with no such relationship for ERβ1 or ERβ2 mRNA. Despite the relatively small number of cases, we were also able to demonstrate that ERβ5 was related to better outcome independent of nodal status in the ERα- subgroup. This potentially indicates that cases with high ERβ5 are more likely to benefit from endocrine treatment. However, whether the better outcome of ERα- cases with high ERβ5 is related to their treatment with tamoxifen is unclear and further studies will be required. Given that protein and mRNA levels of ERβ isoforms do not seem to be directly related, it remains to be seen if ERβ5 protein plays a significant role in ERα- breast disease. In the recent study by Shaaban *et al.*, ERβ5 protein expression significantly correlated with outcome in cohort of 757 patients obtained from consecutive cases enrolled to the Nottingham Tenovus primary breast carcinoma series over a period of 12 years (Shaaban *et al*, 2008).

ERβΔ5 can act as a dominant-negative isoform of both ERα and ERβ (Inoue *et al*, 2000). As ERα expression is an indicator of good prognostic outcome and predictive of tamoxifen benefit, the naturally occurring dominant-negative ERβΔ5 could suppress the beneficial effects of ERα expression and contribute to tumor progression. Detectable levels of ERβΔ5 were predominantly found in cancers which were ERα-, of high grade and highly proliferative; ERβΔ5 was also associated with *p*53 mutation. Although the low levels and restricted incidence of such variants may preclude any clear role in behavior of breast cancer (especially in ERα+ tamoxifen-treated cancers), it would be interesting to clarify if splicing leading to exon deletion is more prevalent in more aggressive forms of cancer. This may be a gene-specific effect, which would point towards a functional role for these ERβ splice variants, or a consequence of more widespread error-prone splicing. Of note both cases harbouring exon 3 deletion also expressed the exon 5 deleted ERβ, suggesting that the latter may be the case in some cancers. Any functional role for ERβ exon

deletion variants would presumably rely on expression of the equivalent protein variant, which has yet to be demonstrated.

Previous assessments of the role of ERβ2 (also known as ERβcx) in breast cancer outcome have been limited. One previous study of 50 ERα positive cases using immunostaining with a different antibody raised to the same ERβ2-specific epitope (Esslimani-Sahla *et al*, 2004) failed to show any predictive association with adjuvant tamoxifen treatment. However this analysis was based on detecting differences in staining between "sensitive" and "resistant" cases using the crude measure of relapse within 5 years of tamoxifen therapy. Unpublished observations (Saji *et al*, 2005) similarly failed to show any predictive value in an adjuvant setting. However, an association of ERβ2 protein with a favorable outcome has been seen in a metastatic and locally advanced setting (Palmieri *et al*, 2004). Our own previous data (Davies *et al*, 2004) was based on a semi-quantitative RTPCR analysis using an assay in which ERβ5 is co-amplified with ERβ2 and distinguished based on size of the PCR product, similar to the triple-primer assay used elsewhere (Iwao *et al*, 2000; Leygue *et al*, 1999). Here the results have been confirmed using independent cDNA synthesis reactions and different splice variant specific PCR conditions. The fully quantitative nature of the qRT-PCR results allows comparison of mRNA levels between different variants or of variant levels between tumors, but necessitated selection of optimal cut points (in this case using ROC analysis) for the dichotomization required for standard outcome analysis with Kaplan Meier plots. It should be noted that, whilst such dichotomization is useful in demonstrating associations with outcome, true utility of ERβ variant mRNA measurement will only be demonstrated with larger patient cohorts and may be better achieved by treating mRNA quantitation as a continuous variable, as in other RT-PCR based outcome predictors (Paik *et al*, 2004).

Our findings indicate that determination of ERβ isoform mRNAs (in particular ERβ2) may be useful in delineating ERα+ cases that respond well to adjuvant tamoxifen treatment. In node negative cases, where the need for additional markers of response is greatest, our study shows that low ERβ2 mRNA levels are significantly related to worse outcome. However, ERβ2 protein levels are apparently not directly related to mRNA levels and ERβ2 protein staining of breast cancer

sections does not relate to outcome for ERα+ cases. Rather there is some association of ERβ2 immunostaining with better outcome in broader cohorts of patients (including ERα- cases), due in part to a correlation between ERα and ERβ2 protein levels. A similar lack of association between ERβ2 immunostaining and outcome has been demonstrated in the neo-adjuvant setting (Miller *et al*, 2006). In this regard our result is concordant with Sugiura *et al.* who examined ERβ1 and ERβ2 in 150 patients (both ER+ and ER-) who had either no adjuvant treatment or some form of adjuvant treatment. They found correlation between ERβ1 mRNA and protein as well as ERβ2 mRNA and protein. In univariate Kaplan Meier survival analysis, higher levels of either protein or mRNA of ERβ1 and ERβ2 were associated with better outcome. However, in multivariate analysis only ERβ2 mRNA was an independent marker of RFS or OS (Sugiura *et al*, 2007). There are some differences between this study and our own. The antibody used in their study was even though raised against the same epitope as ours it was rabbit polyclonal antibody and the ERβ2 positivity rate in the cut off used for survival analysis was only 51% compared to 71% in our cohort. There were also differences in the ethnicity of the population studied and the treatment received by the patients.

As previously discussed, Shaaban *et al.* (Shaaban *et al*, 2008) evaluated ERβ1, ERβ2 and ERβ5 protein expression in a large retrospective cohort (757 patients) containing both ERα+ and ERα- patients, treated with various adjuvant therapies. High ERβ2 significantly correlated with better outcome in the whole cohort, and also predicted response to endocrine therapy. This was somewhat similar to our outcome with ERβ2 in the whole cohort of 141 patients, but this association with outcome was lost when the analysis was limited to ERα positive tamoxifen treated patients. It was not clear whether this subgroup analysis was performed in Shaaban *et al.* cohort. More recently, Honma *et al.* examined ERβ1 and ERβ2 protein in 442 breast cancer patients (364 ER+ & 78 ER-) who received adjuvant tamoxifen monotherapy. Both ERβ1 and ERβ2 protein expression showed better disease free survival and overall survival in KM univariate analysis. However, ERβ1 expression was the strongest marker for both disease free and overall survival in further analysis (Honma *et al*, 2008).

That expression of ERβ2 protein may be important is demonstrated by the good outcome in those cases assessed as having both high mRNA and protein levels and

the independent status of these markers in multivariate analysis. It would seem that at least in those cases where transcription of ERβ2 mRNA drives ERβ2 protein, these cases do particularly well on tamoxifen treatment. Conversely, where ERβ2 levels are depleted both in terms of mRNA and protein, this is associated with worse outcome. The complication arises in the apparent expression of relatively high levels of ERβ2 protein in some cases that cannot be explained by transcriptional control. It is possible therefore that the relatively poor utility of ERβ2 protein assessment by immunostaining as a measure of outcome prediction in post-menopausal, ERα+, tamoxifen-treated breast cancer may be due to high levels of ERβ2 protein in some cases being related to some form of protein stabilization. One explanation for these cases having a poorer outcome, than those in which mRNA an protein are both high, would be that the protein is stabilised and does not fulfil the apparently protective role of ERβ2. Shaaban *et al.* has shown cytoplasmic expression of ERβ2 protein is related to poor outcome. Given that the cytoplasm is the normal route for degradation; might this cytoplasmic staining be related to a failure to degrade abnormal ERβ2? It is currently unclear if those cases with cytoplasmic staining are the same ones that have high levels of ERβ2 nuclear protein but low levels of ERβ2 mRNA.

Whilst our data would suggest that ERβ2 could contribute to an improved outcome in a subgroup of patients, it provides further evidence that determination of ERβ2 protein by immunostaining is unlikely to provide the predictive test that is needed for better targeting of additional therapy in those women for whom tamoxifen is not likely to be sufficient. The failure to link protein expression to outcome measures does not preclude the use of ERβ2 mRNA in a clinical setting. That mRNA measurement (e.g. by qRT-PCR) remains largely outside the remit of clinical laboratories has historically been due largely to technical constraints, although with the advent of tests such as Oncotype DX this is now being addressed (Paik *et al*, 2004). More important is the need for larger trials to validate any such markers (be they RNA or protein based). Such trials should, as here, be based on specific treatments. With the increased use of alternative adjuvant endocrine therapies such as aromatase inhibitors, it will be worthwhile re-investigating the predictive power of ERβ2 and ERβ5 in other treatment-based cohorts.

6.2 Conclusions

This study was unique in that mRNAs of individual splice variants were measured in a clinically-relevant, treatment-specific cohort with concomitant ERβ2 protein expression. Irrespective of the clinical utility of any ERβ based measurement, the data presented here clearly implicates different roles for ERβ splice variants in the behaviour of breast cancer, both in terms of associations with clinicopathological characteristics and with treatment-specific outcome. Hence the data support the hypothesis that particular variants of ERβ may be more closely associated with outcome following adjuvant tamoxifen treatment of breast cancers. It remains unclear if such variants are useful as predictive markers. Nevertheless they offer further insight in to the complex molecular landscape of breast cancer.

Interventional studies will be required to confirm if this role is important, or if ERβ variant levels merely act as surrogate markers for other control pathways.

The differential expression pattern seen for the alternatively spliced variants indicates a complex level of control at the transcriptional and splicing level in breast cancers. Differential expression of C-terminal variants was also seen in various normal tissues, together with our results, splicing control is most likely functionally important. However, the low levels of deletion variants and association of ERβΔ5 with more aggressive, largely ERα- cancers are potentially related to aberrant splicing.

Disassociations between protein and mRNA levels can be partly explained by tissue heterogeneity (Cummings *et al*, 2009), however it supports an important modulating role for translational control (Smith *et al*, 2010) and hint at subset of tumours in which ERβ2 protein may be stabilised. Associations of ERβ2 protein with better outcome were found, but only in the wider cohort and may in part be due to a correlation with ERα expression.

6.3 Future directions

Our study and recent larger studies (Shaaban *et al*, 2008; Honma *et al*, 2008; Sugiura *et al*, 2007) have shown association between wild-type ERβ1 and ERβ2 and breast cancer outcome. The general consensus is that high ERβ2 expression is associated

with better outcome. However, further larger studies with standardized protocols are needed before these results can be translated to tests to be used in clinical practice.

It would be interesting to see whether ERβ1 and ERβ2 protein can be measured retrospectively from previous trials like ATAC and BIG 1-98. Although conclusions would be limited by the case selection used for these trials, they provide large clinically relevant cohorts, at least some of which are available as tissue microarrays through the *Trans*ATAC study. This will also provide an opportunity to evaluate the role of ERβ in relation to aromatase inhibitors.

There is also a need to include ERβ immunostaining in future prospective studies with other potential markers, which will eventually pave a way towards clinical utility in breast cancer management. As for drug treatments, prospective trials are widely considered to be important to validate biomarkers. The practical aspects of prospective studies (such as the case-by-case assessment of markers alongside clinical care) more closely mirror intended use (compared to processing and assessment of large retrospective cohorts).

ERβ2 mRNA could be added to other mRNA based studies like Oncotype DX to see whether it will add to the value to their results. This may not be very feasible in the short term, as this test is centralized and subject to a number of patent and licensing issues as well as regulatory approval, but could be considered if a reassessment of markers was conducted. There are also more practical issues, such as validating the measurement of ERβ2 qRTPCR from paraffin embedded tissue and positioning this additional marker in the risk score formula.

Further functional studies are needed to examine the role of ERβ in breast carcinogenesis. With the discovery of co-regulator proteins which play an important role in ER signalling it will be interesting to see how they can be utilized in clinical practice to increase the efficacy of the selective oestrogen receptor modulators. The importance of the binding of these co-regulators to ERβ will need to be considered when assessing ERα function.

It might be useful to study the association between ERβ and other established markers like PgR and HER2 to understand the interaction between these pathways. For example, there is interplay between ERα and growth factor pathways through

phosphorylation of this ER at different sites. Further work along similar lines with ERβ isoforms might help explain some aspects of their biology.

On a more general point, the work here has shown that measurement of ERβ splicing variants (be they isoforms or aberrant mRNA products) has some utility. More recent technologies, such as microarrays that measure multiple splicing events and extremely high throughput RNA sequencing on "next generation sequencing" platforms, allow us to study such events on a wider scale. These could be used to address if differences in splicing events seen are specific to these pathways or are part of a wider deregulation of splicing associated with cancer phenotype.

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APPENDIX

Liverpool NHS
Health Authority

LIVERPOOL RESEARCH ETHICS COMMITTEE

Hamilton House
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Liverpool
L3 6AL

Tel: (voice) 0151 285 2000
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Direct Dial:

5 July 2001

Our Ref: 01/116

Dr M Davies
Clatterbridge Cancer Research Trusts
J K Douglas Laboratories
Clatterbridge Hospital
Bebington
CH63 4JY

Dear Dr Davies

RE: A RETROSPECTIVE STUDY OF OESTROGEN RECEPTOR BETA AND ITS RELATIONSHIP WITH OTHER HISTOPATHOLOGICAL PARAMETERS AND PATIENT OUTCOME INCLUDING RESPONSE TO HORMONE THERAPY IN INVASIVE BREAST CANCER

The above study was discussed at a meeting of the Liverpool Research Ethics Committee on 4 July 2001. I am pleased to inform you that no ethical objection and approval has now been granted.

The Trust or appropriate Health Service Authority must be asked for permission for the study to proceed. Please contact Angela Ball, Clinical Trials Centre, Linda McCartney Building, Fourth Floor, Royal Liverpool University Hospital, Prescot Street, Liverpool L7 8XP.

Conditions of Approval

(a) The Liverpool Research Ethics Committee shall grant ethical approval for a fixed period of three years, renewable annually thereafter.

(b) Where it is felt appropriate, the Liverpool Research Ethics Committee may suspend or withdraw ethical approval (if, for example, the procedures being performed differ significantly from the agreed protocol) and notify the appropriate NHS body of this.

(c) Applications deemed to fall under the following category may also be dealt with by Chairman's Action:

"Investigations that pose no ethical problems, and are without risk of distress or injury, psychological or physical, to the subject, e.g. some epidemiology, some surveys of the public's eating or smoking habits, assessment of patient information and education, should nevertheless be the subject of an application but may be expeditiously handled by Chairman's Action "

Cont/d ....

(d) In accordance with ICH GCP Guidelines, an annual study update must be provided to the Committee. Failure to file the annual report by the due date will result in automatic suspension of the study without further notice.

(e) A copy of the final report must be submitted on completion of the study.

(f) All serious adverse events must be reported promptly to the Committee.

Any proposed amendments to the protocols must be notified to the Liverpool Research Ethics Committee for approval before implementation.

The LREC is fully compliant with the International Conference on Harmonisation/Good Clinical Practice (ICH) Guidelines.

May I please remind you to state the LREC reference number (<u>at the top of this letter</u>) on all your correspondence.

Yours sincerely

pp Dr E J Tunn
Chairman
Liverpool Research Ethics Committee

LIVERPOOL RESEARCH ETHICS COMMITTEE
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