The Relationship between Oestrogen Receptor-alpha Phosphorylation and the Tumour Microenvironment in Patients with Primary Operable Ductal Breast Cancer

Running title: ER Phosphorylation and the Tumour Microenvironment

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Abstract:

Aims:

Although the role of phosphorylation of oestrogen receptor (ER) at serines 118 (p-S118) and 167 (p-S167) have been studied, the relationship between p-S118, p-S167 and the tumour microenvironment in ER-positive primary operable ductal breast cancers have not been investigated. The aims of this study are to investigate (1) the relationship between p-S118/p-S167 and the tumour microenvironment and (2) the effect of p-S118/167 on survival and recurrence in ER-positive primary operable ductal breast cancers.

Methods and Results:

Patients presenting at 3 Glasgow hospitals between 1995 and 1998 with invasive ductal ER-positive primary breast cancers were studied (n=294). Immunohistochemical staining of p-S118 and p-S167 was performed and their association with clinico-pathological characteristics, cancer-specific survival (CSS) and recurrence-free interval (RFI) were examined.

In the whole cohort, tumour size (P=<0.05) and microvessel density (P=<0.05) were associated with high p-S118 while increased micovessel density (P=<0.05), apoptosis (P=<0.05), general inflammatory infiltrate measured using the Klintrup-Makinen score (P=<0.05) and macrophage infiltrate (P=<0.05) were found to be associated with high p-S167. Only high p-S167 was associated with shorter CSS (P=<0.005) and shorter RFI in the whole cohort (P=0.001) and luminal A (P=<0.05) and B tumours (P=<0.05) separately.

Conclusions:

This study showed that both p-S118 and p-S167 were associated with several microenvironmental factors including increased microvessel density. In particular, p-S167 was associated with reduced RFI and CSS in the whole cohort and RFI in luminal A and B tumours and could possibly be employed to predict response to kinase inhibitors.

(242 words)

Keywords:

Breast cancer, oestrogen receptor, prognosis, tumour microenvironment, serine 118, serine 167

Introduction

Breast cancer, which accounts for 30% of new incidences of cancer in females, is the most common cancer in the UK. With improved treatment modalities, the survival rate has increased significantly with 78.4% patients having 10-year survival [1].

Breast cancer can be categorised according to the expression of immunohistochemical surrogates (oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 1 and 2 and cytokeratin 5 and 6) for molecular classification. ER-positive tumours can be subcategorised into luminal A and B tumours; the former being associated with expression of PR, low proliferation markers (Ki-67), low grade and good outcomes but prone to late recurrences while the latter is associated with high proliferation markers and high grade.

The treatment of ER-positive breast cancers have improved with the introduction of tamoxifen, a competitive inhibitor of ER, and recent reports show that aromatase inhibitors (e.g. letrozole), which inhibit the conversion of androgen to oestrogen, may be more clinically beneficial compared to tamoxifen in post-menopausal women [2]. Patients using either drugs may exhibit *de novo* resistance or acquired resistance, leading to endocrine therapy failure [3]. Studies showed that 90% and 30% of patients with luminal B and luminal A tumours respectively exhibit high recurrence scores [4, 5]. In addition to tamoxifen and aromatase inhibitors, other therapeutic options

including Faslodex and LHRH agonists have been developed and are still under study. Thus, there is a continuing need to identify patients that are more likely to develop resistance and therefore provide more rigorous follow-up.

The ER-Alpha (ER-α) receptor can be phosphorylated at a number of amino acid residues including serines 118 and 167 [6, 7]. There is still not a clear consensus on the role of phosphorylation at serines 118 (p-S118) and 167 (p-S167) in tamoxifen resistance due to conflicting evidence.

The tumour microenvironment has been shown to play an important role in cancer development. Studies have shown that factors including microvessel density, lymphovascular invasion, tumour necrosis, inflammatory infiltrates, tumour stromal percentage and tumour budding are important in determining patient's response to therapy [8-13]. However, the relationship between p-S118/p-S167 and the tumour microenvironment has not been studied. Thus, the aims of the study are to investigate (1) the relationship between p-S118/p-S167 and the tumour microenvironment and (2) the effect of p-S118/167 on survival and recurrence in ER-positive primary operable ductal breast cancers. Considering the importance of the microenvironment and ER phosphorylation status, we hypothesise that these factors may have to be considered jointly in determining recurrence risk.

Materials and Methods:

Patients

Ethical approval for expression studies in human tissue samples was obtained from West of Scotland Research Ethics Service West of Scotland REC4 (REC Ref: Project Number 02/SG007(10), R and D project: RN07PA001). Although patient consent was not obtained, all patient details were anonymised and identifiers were removed. Patients included in this study were diagnosed with operable ER-positive breast cancers at 3 Glasgow hospitals; The Royal Infirmary, Stobhill Hospital, and Western Infirmary between 1995 and 1998 (n=294) and treated with adjuvant tamoxifen. Clinico-pathological characteristics including age, tumour size, invasive grade in histological grade, histological tumour type, nodal status, lymphovascular invasion, type of surgery and adjuvant therapy (chemotherapy and radiotherapy) were retrieved from routine reports. Recurrences and cancer deaths were used as end points. The date and cause of death was confirmed by cross-checks with the Registrar General (Scotland) and the cancer registration system. Recurrences were defined as the date of first recurrence of breast cancer. Recurrence-free interval (RFI) was measured from the time of surgery until the date of first recurrence at any site. Breast cancer-specific survival (CSS) was measured from time of surgery to death due to breast cancer. Patients were followed up regularly after surgery.

Immunohistochemistry (IHC)

One 0.6mm^2 core from each tumour taken during surgery was placed in each of three separate TMA blocks (Beecher Scientific, Silver Spring, MD, USA). 2.5µm-thick paraffin wax sections from each TMA block were mounted on silanised glass slides for IHC. All TMAs were available from previous studies and were designed in triplicates. ER, PR and HER-2 status were performed as described previously [14]. IHC staining on TMAs was also used to assess for Ki-67 using Dako anti-Ki67 (1:100; monoclonal mouse anti-human, Ki-67 antigen, clone MIB1, code M7240, DAKO, Glostrup, Denmark) with a cut-off of 15% [15]. Tumour stromal percentage refers to the area of stroma in a single X10 field with tumour cells at all corners. Tumour budding refers to the detachment of single or cluster of five cancer cells in the stroma at the invasive margins of the tumour [16].

The tissues were first dewax and rehydrated. For antigen retrieval, sections were heated in Sodium Citrate buffer at pH 6 for 1.5 minutes in a pressure cooker until under pressure and then for another 5 minutes once pressure conditions have been achieved. Following this, samples were cooled for 20 minutes. Blocking of endogenous peroxidase was achieved by incubation of tissue in 3% hydrogen peroxidase for 10 minutes. Samples were incubated in 1.5% horse serum for 30 minutes to block non-specific binding. Following this, the samples were incubated in the primary antibodies at 4°C overnight; p-S118 (1:500 dilution; Cell Signalling, #2511) and p-S167 (1:200; Cell Signalling, #5587). The specificity of the antibodies have been shown previously [17, 18]. Phospho-ER epitopes antigenicity have been shown to be

stable [19]. The slides were then washed twice in TBS for 5 minutes before incubation in Dako EnVision™ (K5007, Dako, Copenhagen, Denmark) before washing in TBS buffer again twice for 5 minutes. Diaminobenzidine (SK-4100, Vector Laboratories, Burlingame, CA, USA), which was used as a chromogen, was prepared according to manufacturer's instructions. The slides were counterstained with haematoxylin, dehydrated and mounted with DPX. TMAs of breast cancer patients without linked clinical data were used as positive and negative controls for each antibody.

Slide Scanning and Scoring

The stained tissue microarrays were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK). SlidePath Digital Image Hub, version 4.0.1 (SlidePath's Tissue IA system, Dublin, Ireland) was used for visualisation and automated cell counts. Scoring of tissues was performed by assessors blinded to clinico-pathological characteristics of patients. Samples were scored according to the weighted histoscore/H score method [20]. In brief, staining intensity was graded as negative (0), weak (1), moderate (2) and strong (3) multiplied by the percentage of cells in each category resulting in a range of scores from 0 to 300. Two hundred and forty cores (10% of total tissue cores) were scored for nuclear p-S118 and p-S167 by one observer (KC) and an automated tissue analysis system blinded to patient's details and each other's scores. The Interclass Correlation Coefficient for the observer and the automated results were 0.984 and 0.978 for p-S118 and p-S167 respectively. Subsequently, SlidePath was used to

score the rest of the cores. The use of automated systems have been shown to be an effective alternative to manual scoring of samples [21]. The mean score was taken as the final score for each tumour triplicate.

Statistical Analysis

SPSS version 22 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. The Kaplan-Meier method was used to estimate survival (CSS) and recurrence-free interval (RFI), and the log-rank test was used to assess differences between survival curves. The Cox-proportional hazards model was used for univariate and multivariate survival analysis and the calculation of hazard ratios (HR). Mortality up to March 2010 was included in the analysis and served as a censor date. The X^2 test (or X^2 test for trend where appropriate) was used to determine association between p-S118/p-S167 with clinico-pathological data.

Results:

As shown in Table 1, the majority of patients were above 50 years of age (76.5%), had small (< 20cm in diameter) (66.3%), Grade I or II (80.7%) tumours without lymph node involvement (56.5%). Histologically, most patients were PR-positive (67.3%), HER2-negative (89.8%) and of the Luminal A subtype (64.6%). Proliferative index indicated by Ki-67 was predominantly low (70.1%) and the majority of tumours had significant inflammatory infiltrate (assessed using the Klintrup-Makinen method [22]) (87.8%). The majority of patients were not treated with chemotherapy (71.8%) or radiotherapy (58.2%) but all patients received tamoxifen. Based on the information we have on 293 patients, the patients were on tamoxifen for a median duration of 5 years (IQR – 4.5 to 5.0 years) and a mean duration of 4.69 years. As the median was used to distinguish between low and high expression of p-S118 and p-S167 (92.5 for p-S118 and 14 for p-S167), there were roughly equal numbers of tumours with high or low p-S118 and p-S167. Figure 1 shows the immunohistochemical staining of p-S118 and p-S167 in ER-positive and negative tumours.

Tables 2 and 3 show the relationship between p-S118, p-S167, tumour microenvironmental factors and clinico-pathological characteristics. High p-S118 was associated with tumour size (P=0.011) and microvessel density (P=0.023). Similarly, high p-S167 was associated with increased microvessel density (P=0.009), apoptotic index indicated by TUNEL (P=0.002), general inflammatory infiltrate (P=0.007) and CD68+ macrophage infiltrate (P=0.010).

p-S118 and p-S167 were strongly positively associated with each other (P=0.003).

The patients were followed-up for a median of 70 months (IQR – 59 to 81 months) During the follow-up period, 46 patients experienced recurrence. At the end of follow-up, 110 patients died and of these, 48 deaths could be directly attributed to their disease. Of the other deaths, 15 were due to malignant disease of other organs (including lung and colon cancer), 18 due to vascular diseases (including coronary heart disease and cerebrovascular disease) and 16 due to respiratory diseases (including chronic obstructive pulmonary disease and pneumonia). High p-S167 was associated with significantly shorter CSS (152 vs. 170 months; P=0.003; Figure 2B) and RFI (91 vs. 101 months; P=0.001; Figure 2D) compared to tumours with low p-S167. Mean CSS (158 vs. 160 months; P=0.507; Figure 2A) and RFI (95 vs. 95 months; 0.443; Figure 2B) were not significantly different in tumours with low or high expression of p-S118. When compared together, patients with high p-S167 were more associated with poorer CSS and RFI compared to p-S118 (Figure 3).

Sub-group analyses were performed based on tumour subtypes – luminal A and B. High p-S118 was associated with tumour size (P=0.024), grade (P=0.042), microvessel density (P=0.008), general inflammatory infiltrate (P=0.036) and the use of chemotherapy (P=0.037) or radiotherapy (P=0.046) in luminal A tumours and associated with blood vessel invasion (P=0.042) in luminal B tumours. On the other hand, high p-S167 was associated with PR-

positive status (P=0.032), increased microvessel density (P=0.026), apoptotic index indicated by TUNEL (P=0.013) and CD68+ macrophage infiltrate (P=0.030) in luminal A tumours (Table 4). p-S167 was not significantly associated with any clinico-pathological characteristics in luminal B tumours.

Survival analyses showed that high p-S167 was associated with shorter RFI in both subtypes – Luminal A (92 vs. 105 months; P=0.032; Figure 4A) and Luminal B (128 vs. 153 months: P=0.033; Figure 4B). The same association was seen on multivariate analysis (Table 5) – Luminal A (HR 4.441, 95% CI 1.004-19.638, P=0.049) and B (HR 4.971, 95% CI 1.386-17.834, P=0.014). p-S118 and p-S167 were not associated with CSS in luminal A and B tumours (Table 6).

Discussion

Although there have been studies investigating the role of p-S118, p-S167 and the tumour microenvironment individually in determining survival, the relationship between these factors have not been investigated. The present study observed associations between both p-S118 and p-S167 and several microenvironmental factors. However, only p-S167 had power in stratifying patients according to outcome measures, as it was associated with recurrence-free interval and cancer-specific survival in the full cohort and with recurrence-free interval in the luminal A and B tumours. This may be due to p-S167 associating with more variables associated with the tumour microenvironment.

The weighted histoscore/H score method was used in the present study although the IHC cut-off method and the Allred method is used by some researchers as it is widely accepted that the weighted histoscore/H score method could be more informative than the Allred method. McCarty et al. recommended that this should be the method of choice for assessing ER in breast tumours [23]. Since then, it has been widely adapted in the research field as the method of choice (although Allred is still employed clinically). More recently, Brouckaert et al. discussed reasons why a quantitative assessment of the steroid receptors in breast cancer is the preferred method and that we should employ a weighted histoscore/H score method rather than the quick score or Allred method [24].

In addition, as the weighted histoscore/H score method was employed in the TEAM (adjuvant tamoxifen and exemestane in early breast cancer) clinical trial to assess ER levels and as we plan to take these investigations forward into this cohort, it seemed appropriate to use the same method for assessing phospho-ER as to what was used to assess ER and PR [21, 25]. In addition to the evidence presented by others, we as a group are widely published in the area of biomarker research using the weighted histoscore/H score method and have been employing this method for over 10 years [20]. We have demonstrated that it has high inter-observer reliability, can be easily converted to the Allred score if required and algorithms can be written for automated scoring. The median was used as the cut-off for low and high as it is an unbiased measure and is more informative than 1% or 10% as previously employed by others.

In the present study, ER levels and phospho-ER levels were assess using IHC as ligand binding assays have been demonstrated to provide inaccurate results due to tissues inherently being a heterogenous mix of tumour and stromal cells. Using IHC, we can be sure that the expression status of ER and phospho-ER is unaffected by non-tumour cells. If ER or phospho-ER was assessed using ligand binding assays, results would have varied due to inconsistencies in tumour stromal percentage between specimens [26].

Whilst we recognise the merits of examining tumour samples by a second technique, we believe the IHC strategy allows us to examine ER phosphorylation in multiple cell types (tumour and surrounding

microenvironment) that make up the heterogeneous sample. In addition, the use of IHC allows us to assess expression in different cellular regions. Unfortunately, as we only had archival paraffin-embedded specimens available for this study, immunoblotting of the specimens could not be performed. In addition, it should be recognised that it is standard clinical practice to employ the use of IHC to assess biomarker protein expression in formalin-fixed, paraffin-embedded clinical specimens. IHC is the gold standard method used to assess expression of ER and PR in breast cancer clinical specimens and is utilised to inform appropriate patient treatment strategies. Therefore, we feel that it is appropriate to utilise this technique in the present study, especially as stringent antibody validation using both immunoblotting and IHC was employed.

Due to conflicting evidence, the role of p-S118 and p-S167 in tamoxifen resistance remains unclear. While p-S118 has been reported to be associated with better prognosis, a less malignant phenotype and higher response rate to tamoxifen [17, 27], some studies have also shown its association with poorer response to endocrine therapy [28, 29]. Kirkegaard et al. showed that activated Akt is associated with relapse and death in ER-α positive, tamoxifen-treated patients, thus suggesting that p-S167 may be associated with worse disease outcome while Yamashita et al. showed that p-S167 was predictive of response to endocrine therapy and longer survival after relapse [29, 30]. This study reports that high p-S167 is associated with poor prognosis and higher microvessel density, apoptotic index and macrophage infiltrate, all of which are associated with poorer prognosis [8, 31, 32], thus supporting the association

of p-S167 with poorer outcomes. Given these associations, p-S167 may be involved with signalling pathways associated with inflammatory cytokine release. Future studies should focus on identifying these pathways as they may be useful therapeutic targets.

Svensson et al. showed that high serum oestradiol levels were associated with high levels of extracellular CCL2 and CCL5 in vivo, inducing infiltration of tumour-associated macrophages [33]. Similarly, the role of estradiol in the recruitment and activation of macrophages have also been shown in ovarian cancer [34]. These studies support our findings that ER activation is associated with macrophage infiltration, both of which are associated with poorer prognosis.

p-S167 was found to be a predictor of a shorter recurrence-free interval in both luminal A and B cancers and was also associated with higher microvessel density, apoptotic index and macrophage infiltration in luminal A tumours. This suggests that luminal A tumours can be further subcategorised into 2 groups by p-S167 status. Although patients with luminal A tumours have good prognosis, the difference in recurrence-free interval between patients with high and low expression of p-S167 was about 1 year in this study. Therefore, identification of these patients using p-S167 for more rigorous treatment may be clinically useful. Luminal B tumours have been known to have a more aggressive phenotype, thought to be due to the upregulation of HER2, leading to the upregulation of Akt and MAPK pathways. As p-S167 is associated with these pathways, p-S167 may be important in

stratifying patients with luminal B tumours. Replication of our findings in a larger cohort such as the TEAM trial would establish the utility of p-S167 as an important biomarker for stratifying patients in future clinical trials.

Increasing number of patients undergo ‘switch therapy’ (a sequential switch to aromatase inhibitors after tamoxifen) or have extended treatments of up to 10 years of tamoxifen or aromatase inhibition. It would be interesting to investigate whether these strategies have an effect on levels of p-S118 and p-S167. However, as this was not the case for the present cohort, it was not possible to address this.

Recently, studies have shown that recurrent ESR1 mutations within the ligand binding domain (LBD) in ER-positive endocrine-resistant metastatic breast cancer were identified at higher frequencies in patients who received multiple hormonal treatments, suggesting that the mutations result in increased ER activity and thus increased tumour growth, presenting as a clinical relapse [35, 36]. As p-S167 is associated with a shorter recurrence-free interval, it would be interesting to look at the relationship between p-S167 mutation and LBD mutations of ESR1. As LBD mutations are relatively uncommon in treatment-naïve patients, it would be advisable to examine these mutations in patients who have already received multiple hormonal treatments.

Conclusion

In summary, this study showed the utility of p-S118 and p-S167 in stratifying patients' risk of relapse and the relationship between p-S118 and p-S167 and the tumour microenvironment. As p-S167 was associated with recurrence-free interval and cancer-specific survival in the whole cohort and recurrence-free interval in luminal A and B tumours, p-S167 may be an important biomarker for stratifying patients in the future.

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Tables

Table 1: Clinico-pathological Characteristics of patients with ER-positive operable invasive ductal breast cancers (n=294)

Clinico-pathological characteristics	Patients, n (%)
Age (≤50/ >50 years)	69 (23.5%)/225 (76.5%)
Size (≤20/ 21-50/ > 50mm)	195 (66.3%)/92 (31.3%)/7 (2.4%)
Grade (I/ II/ III)	81 (27.6%)/156 (53.1%)/57 (19.4%)
Molecular subtype (Luminal A/Luminal B/Unknown)	190 (64.6%)/87 (29.6%)/17 (5.8%)
Involved lymph node (Negative/Positive/Unknown)	166 (56.5%)/124 (42.2%)/4 (1.4%)
Progesterone -receptor status (PR-/PR+/Unknown)	95 (32.3%)/198 (67.3%)/1 (0.3%)
HER2 status (HER2-/HER2+/Unknown)	264 (89.8%)/26 (8.8%)/4 (1.4%)
Lymph vessel invasion (Absent/Present)	208 (70.7%)/86 (29.3%)
Blood vessel invasion (Absent/Present)	263 (89.5%)/31 (10.5%)
Microvessel Density (CD34+) (Low/Medium/High/Unknown)	100 (34.0%)/93 (31.6%)/81 (27.6%)/20 (6.8%)
Ki-67 status (Low/High/Unknown)	206 (70.1%)/74 (25.2%)/14 (4.8%)
Tumour necrosis (Absent/Present)	181 (61.6%)/113 (38.4%)
TUNEL (Low/High/Unknown)	134 (45.6%)/124 (42.2%)/36 (12.2%)
General Inflammatory Infiltrate (Low/High)	258 (87.8%)/36 (12.2%)
Chemotherapy (Negative/Positive/Unknown)	211 (71.8%)/81 (27.6%)/2 (0.7%)
Radiotherapy (Negative/Positive/Unknown)	171 (58.2%)/121 (41.2%)/2 (0.7%)
Tumour CD4+ T-lymphocytic infiltrate (Low/Medium/High/Unknown)	145 (49.3%)/60 (20.4%)/81 (27.6%)/8 (2.7%)

Tumour CD8+ T-lymphocytic infiltrate (Low/Medium/High/Unknown)	95 (32.3%)/108 (36.7%)/83 (28.2%)/8 (2.7%)
Tumour CD20+ B-lymphocytic infiltrate (Low/Medium/High/Unknown)	170 (57.8%)/43 (14.6%)/73 (24.8%)/8 (2.7%)
Tumour CD138+ B-Lymphocytic infiltrate (Low/Medium/High/Unknown)	168 (57.1%)/41 (13.9%)/76 (25.9%)/9 (3.1%)
Tumour CD68+ macrophages infiltrate (Low/Medium/High/Unknown)	72 (24.5%)/116 (39.5%)/96 (32.7%)/10 (3.4%)
Tumour Stromal Percentage (Low/High)	194 (66.0%)/100 (34.0%)
Tumour Budding (Low/High)	176 (59.9%)/118 (40.1%)
p-S118 (Low/High)	154 (52.4%)/140 (47.6%)
p-S167 (Low/High)	113 (38.4%)/181 (61.6%)

Table 2: The relationship between p-S118 expression and clinico-pathological characteristics of patients with invasive ER-positive primary operable ductal breast cancers.

Clinico-pathological characteristics	Low p-S118 expression	High p-S118 expression	p-value
Age (≤50/ >50 years)	37/117	32/108	0.922
Size (≤20/ 21-50/ > 50mm)	91/57/6	104/35/1	0.011
Grade (I/ II/ III)	47/84/23	34/72/34	0.107
Molecular subtype (Luminal A/Luminal B)	106/41	84/46	0.226
Involved lymph node (Negative/ Positive)	82/69	84/55	0.350
Progesterone -receptor status (PR-/PR+)	48/106	47/92	0.720
HER2 status (HER2-/HER2+)	142/10	122/16	0.198
Lymph vessel invasion (Absent/Present)	107/47	101/39	0.709
Blood vessel invasion (Absent/Present)	133/21	130/10	0.105
Microvessel Density (CD34+) (Low/Medium/High)	60/48/32	40/45/49	0.023
Ki-67 status (Low/High)	113/36	93/38	0.434
Tumour necrosis (Absent/Present)	90/64	91/49	0.301
TUNEL (Low/High)	69/66	65/58	0.878
General Inflammatory Infiltrate (Low/High)	140/14	118/22	0.121
Chemotherapy (Negative/Positive)	103/50	108/31	0.065
Radiotherapy (Negative/Positive)	97/56	74/65	0.101
Tumour CD4+ T-lymphocytic infiltrate (Low/Medium/High)	80/32/36	65/28/45	0.291
Tumour CD8+ T-lymphocytic infiltrate (Low/Medium/High)	42/60/46	53/48/37	0.198
Tumour CD20+ B-lymphocytic infiltrate (Low/Medium/High)	86/23/39	84/20/34	0.893
Tumour CD138+ B-Lymphocytic infiltrate (Low/Medium/High)	93/19/36	75/22/40	0.380

Tumour CD68+ macrophages infiltrate (Low/Medium/High)	37/64/47	35/52/49	0.659
Tumour Stromal Percentage (Low/High)	94/60	100/40	0.079
Tumour Budding (Low/High)	84/70	92/48	0.067
Cancer-specific survival (months)*	158 (150-165)	160 (153-168)	0.507
Recurrence-free interval (months)*	95 (90-100)	95 (90-100)	0.443
p-S167 (Low/High)	72/82	41/99	0.003

Table 3: The relationship between p-S167 expression and clinico-pathological characteristics of patients with invasive ER-positive primary operable ductal breast cancers.

Clinico-pathological characteristics	Low p-S167 expression	High p-S167 expression	p-value
Age (≤50/ >50 years)	31/82	38/143	0.260
Size (≤20/ 21-50/ > 50mm)	78/33/2	117/59/5	0.689
Grade (I/ II/ III)	37/60/16	44/96/41	0.112
Molecular subtype (Luminal A/Luminal B)	77/27	113/60	0.167
Involved lymph node (Negative/ Positive)	64/49	102/75	0.965
Progesterone -receptor status (PR-/PR+)	39/74	56/124	0.633
HER2 status (HER2-/HER2+)	101/10	163/16	1.000
Lymph vessel invasion (Absent/Present)	83/30	125/56	0.421
Blood vessel invasion (Absent/Present)	100/13	163/18	0.819
Microvessel Density (CD34+) (Low/Medium/High)	50/31/24	50/62/57	0.009
Ki-67 status (Low/High)	83/22	123/52	0.142
Tumour necrosis (Absent/Present)	72/41	109/72	0.634
TUNEL (Low/High)	60/32	74/92	0.002
General Inflammatory Infiltrate (Low/High)	107/6	151/30	0.007
Chemotherapy (Negative/Positive)	74/39	137/42	0.055
Radiotherapy (Negative/Positive)	65/48	106/73	0.869
Tumour CD4+ T-lymphocytic infiltrate (Low/Medium/High)	57/22/29	88/38/52	0.856
Tumour CD8+ T-lymphocytic infiltrate (Low/Medium/High)	37/38/33	58/70/50	0.778
Tumour CD20+ B-lymphocytic infiltrate (Low/Medium/High)	60/18/30	110/25/43	0.579
Tumour CD138+ B-Lymphocytic infiltrate (Low/Medium/High)	72/13/22	96/28/54	0.082

Tumour CD68+ macrophages infiltrate (Low/Medium/High)	37/34/36	35/82/60	0.010
Tumour Stromal Percentage (Low/High)	74/39	120/61	0.987
Tumour Budding (Low/High)	66/47	110/71	0.779
Cancer-specific survival (months)*	170 (165-175)	152 (144-160)	0.003
Recurrence-free interval (months)*	101 (95-107)	91 (86-95)	0.001
p-S118 (Low/High)	72/41	82/99	0.003

Table 4: The relationship between p-S167 expression and clinico-pathological characteristics of patients with invasive Luminal A and B ER-positive primary operable ductal breast cancers.

Clinico-pathological characteristics	Luminal A			Luminal B		
	Low p-S167 expression	High p-S167 expression	p-value	Low p-S167 expression	High p-S167 expression	p-value
Age (≤50/ >50 years)	20/57	23/90	0.464	7/20	13/47	0.872
Size (≤20/ 21-50/ > 50mm)	55/20/2	78/32/3	0.937	15/12/0	31/27/2	0.620
Grade (I/ II/ III)	32/42/3	35/63/15	0.057	2/13/12	5/29/26	0.987
Involved lymph node (Negative/ Positive)	45/32	70/40	0.572	13/14	26/33	0.905
Progesterone -receptor status (PR-/PR+)	32/45	29/84	0.032	5/22	24/36	0.085
HER2 status (HER2-/HER2+)	77/0	113/0		17/10	44/16	0.469
Lymph vessel invasion (Absent/Present)	58/19	88/25	0.815	17/10	29/31	0.302
Blood vessel invasion (Absent/Present)	67/10	105/8	0.266	24/3	50/10	0.728
Microvessel Density (CD34+) (Low/Medium/High)	37/25/12	34/41/32	0.026	9/6/11	14/21/24	0.429
Ki-67 status (Low/High)	77/0	113/0		6/21	8/52	0.466
Tumour necrosis (Absent/Present)	51/26	77/36	0.906	15/12	25/35	0.332
TUNEL (Low/High)	42/24	46/61	0.013	16/8	28/29	0.229
General Inflammatory Infiltrate (Low/High)	75/2	100/13	0.050	24/3	43/17	0.136
Chemotherapy (Negative/Positive)	52/25	90/22	0.067	14/13	41/19	0.217
Radiotherapy (Negative/Positive)	44/33	68/44	0.734	18/9	32/28	0.353
Tumour CD4+ T-lymphocytic infiltrate (Low/Medium/High)	40/18/19	60/23/28	0.909	14/3/9	21/15/24	0.193
Tumour CD8+ T-lymphocytic infiltrate (Low/Medium/High)	25/32/20	36/42/33	0.824	9/6/11	17/27/16	0.141
Tumour CD20+ B-lymphocytic infiltrate (Low/Medium/High)	42/15/20	72/16/23	0.358	15/3/8	31/9/20	0.854

Tumour CD138+ B-Lymphocytic infiltrate (Low/Medium/High)	51/10/15	66/12/33	0.303	17/3/6	24/15/21	0.089
Tumour CD68+ macrophages infiltrate (Low/Medium/High)	26/25/25	22/56/33	0.030	6/9/11	8/25/27	0.518
Tumour Stromal Percentage (Low/High)	51/26	69/44	0.567	16/11	44/16	0.288
Tumour Budding (Low/High)	44/33	70/43	0.608	14/13	36/24	0.633
Cancer-specific survival (months)*	173 (167-178)	163 (155-171)	0.179	158 (143-173)	130 (115-145)	0.064
Recurrence-free interval (months)*	105 (101-108)	92 (88-96)	0.032	153 (138-168)	128 (111-145)	0.033
p-S118 (Low/High)	51/26	55/58	0.025	16/11	25/35	0.198

Table 5: The relationship between the clinico-pathological characteristics of patients with invasive Luminal A and B ER-positive primary operable ductal breast cancers and recurrence-free interval.

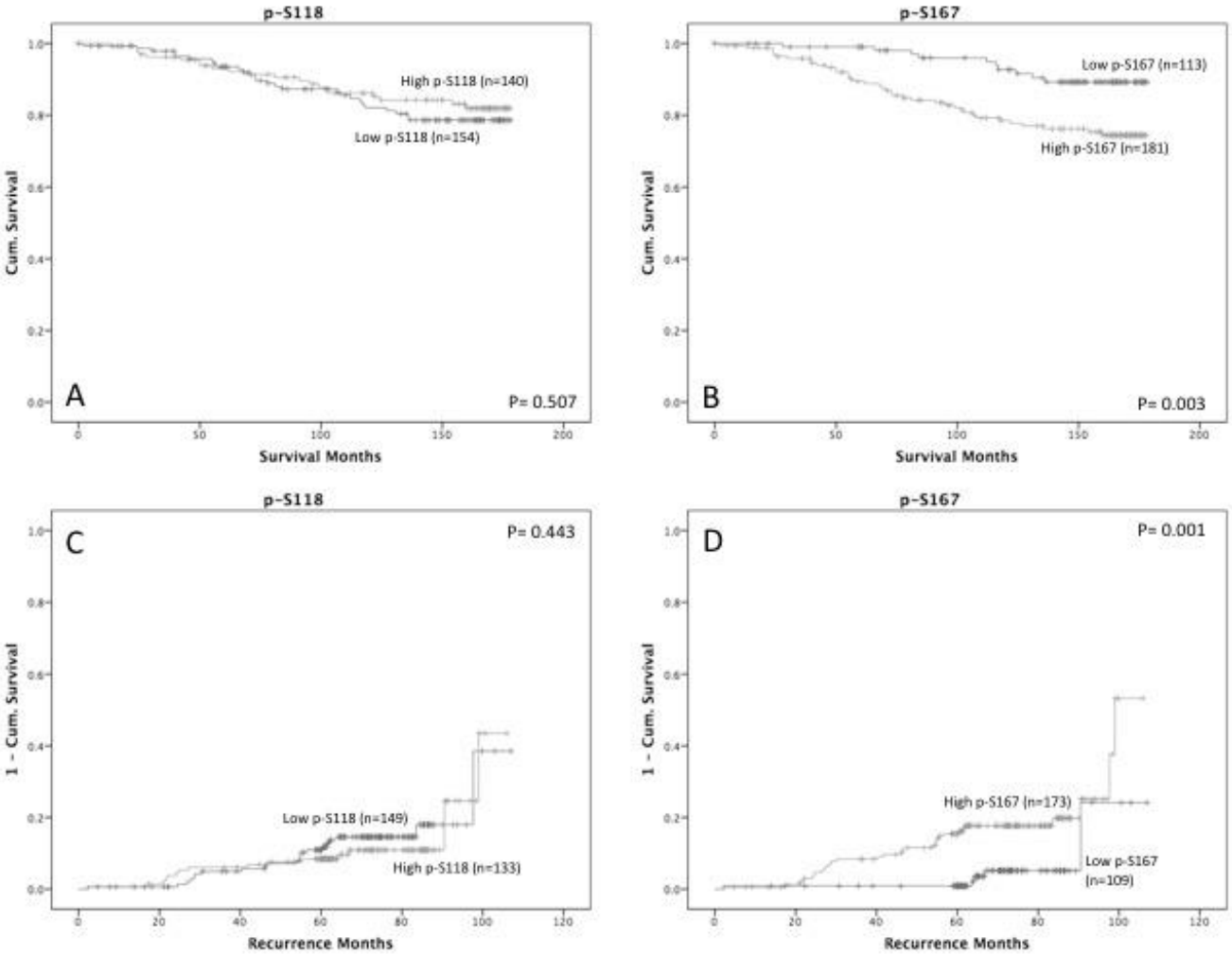
Clinico-pathological characteristics	Luminal A Univariate Hazard ratio (95% CI)	p-value	Multivariate Hazard ratio (95% CI)	p-value	Luminal B Univariate Hazard ratio (95% CI)	p-value	Multivariate Hazard ratio (95% CI)	p-value
Age (≤50/ >50 years)	1.971 (0.446-8.701)	0.371			0.633 (0.243-1.647)	0.349		
Size (≤20/ 21-50/ > 50mm)	1.580 (0.644-3.880)	0.318			1.083 (0.500-2.348)	0.839		
Grade (I/ II/ III)	1.869 (0.820-4.258)	0.137			1.221 (0.578-2.582)	0.600		
Involved lymph node (Negative/ Positive)	1.923 (0.666-5.548)	0.227			5.330 (1.781-15.948)	0.003	4.854 (1.521-15.487)	0.008
Progesterone -receptor status (PR-/PR+)	0.751 (0.272-2.075)	0.581			0.365 (0.151-0.884)	0.026	0.369 (0.151-0.899)	0.028
HER2 status (HER2-/HER2+)	-				1.318 (0.531-3.274)	0.552		
Lymph vessel invasion (Absent/Present)	1.597 (0.571-4.462)	0.372			2.370 (0.979-5.739)	0.056		
Blood vessel invasion (Absent/Present)	2.235 (0.628-7.959)	0.214			1.487 (0.496-4.458)	0.479		
Microvessel Density (CD34+) (Low/Medium/High)	1.777 (0.883-3.577)	0.107			1.586 (0.888-2.832)	0.119		
Ki-67 status (Low/High)	-				1.211 (0.354-4.142)	0.760		
Tumour necrosis (Absent/Present)	1.904 (0.707-5.128)	0.203			2.231 (0.865-5.756)	0.097		
TUNEL (Low/High)	0.385 (0.120-1.231)	0.108			0.659 (0.267-1.626)	0.365		
General Inflammatory Infiltrate (Low/High)	0.043 (0.000-155.046)	0.451			0.483 (0.141-1.653)	0.246		
Chemotherapy (Negative/Positive)	1.234 (0.425-3.584)	0.699			3.606 (1.438-9.044)	0.006	3.837 (1.469-10.023)	0.006
Radiotherapy (Negative/Positive)	0.515 (0.176-1.512)	0.227			0.877 (0.363-2.122)	0.771		
Tumour CD4+ T-lymphocytic infiltrate (Low/Medium/High)	0.662 (0.338-1.297)	0.229			0.877 (0.544-1.415)	0.591		
Tumour CD8+ T-lymphocytic infiltrate (Low/Medium/High)	0.904 (0.492-1.661)	0.745			0.580 (0.333-1.009)	0.054		
Tumour CD20+ B-lymphocytic infiltrate (Low/Medium/High)	1.120 (0.632-1.986)	0.698			0.996 (0.627-1.584)	0.988		
Tumour CD138+ B-Lymphocytic infiltrate (Low/Medium/High)	0.824 (0.452-1.501)	0.526			1.529 (0.927-2.520)	0.096		
Tumour CD68+ macrophages infiltrate (Low/Medium/High)	0.586 (0.289-1.185)	0.137			1.199 (0.644-2.233)	0.566		
Tumour Stromal Percentage	2.396 (0.890-6.449)	0.084			1.607 (0.675-3.828)	0.284		

(Low/High)								
Tumour Budding (Low/High)	1.278 (0.471-3.472)	0.630			1.779 (0.753-4.200)	0.189		
p-S118 (Low/High)	0.678 (0.245-1.876)	0.454			0.790 (0.333-1.869)	0.591		
p-S167 (Low/High)	4.441 (1.004-19.638)	0.049	4.441 (1.004-19.638)	0.049	3.504 (1.029-11.940)	0.045	4.971 (1.386-17.834)	0.014

Table 6: The relationship between the clinico-pathological characteristics of patients with invasive Luminal A and B ER-positive primary operable ductal breast cancers and cancer-specific survival.

Clinico-pathological characteristics	Luminal A Univariate Hazard ratio (95% CI)	p-value	Multivariate Hazard ratio (95% CI)	p-value	Luminal B Univariate Hazard ratio (95% CI)	p-value	Multivariate Hazard ratio (95% CI)	p-value
Age (≤50/ >50 years)	2.741 (0.629-11.932)	0.179			1.375 (0.522-3.619)	0.519		
Size (≤20/ 21-50/ > 50mm)	1.652 (0.728-3.749)	0.230			2.024 (1.046-3.919)	0.036		
Grade (I/ II/ III)	1.480 (0.704-3.111)	0.301			1.305 (0.720-2.365)	0.380		
Involved lymph node (Negative/ Positive)	1.814 (0.700-4.706)	0.221			3.318 (1.457-7.553)	0.004		0.056
Progesterone -receptor status (PR-/PR+)	0.462 (0.183-1.163)	0.101			0.783 (0.361-1.697)	0.535		
HER2 status (HER2-/HER2+)	-				0.917 (0.389-2.161)	0.843		
Lymph vessel invasion (Absent/Present)	1.607 (0.603-4.284)	0.343			3.570 (1.566-8.138)	0.002	2.871 (1.227-6.715)	0.015
Blood vessel invasion (Absent/Present)	3.083 (1.014-9.368)	0.047		0.103	1.628 (0.660-4.017)	0.290		
Microvessel Density (CD34+) (Low/Medium/High)	1.666 (0.887-3.128)	0.112			1.279 (0.808-2.023)	0.294		
Ki-67 status (Low/High)	-				1.754 (0.529-5.816)	0.358		
Tumour necrosis (Absent/Present)	3.586 (1.390-9.255)	0.008	3.704 (1.434-9.568)	0.007	2.241 (1.009-4.977)	0.047		0.466
TUNEL (Low/High)	0.649 (0.236-1.788)	0.403			0.874 (0.409-1.869)	0.728		
General Inflammatory Infiltrate (Low/High)	9.943 (0.000-50.842)	0.383			0.828 (0.336-2.044)	0.683		
Chemotherapy (Negative/Positive)	0.783 (0.258-2.381)	0.667			1.879 (0.895-3.944)	0.096		
Radiotherapy (Negative/Positive)	0.354 (0.116-1.077)	0.067			0.949 (0.449-2.007)	0.892		
Tumour CD4+ T-lymphocytic infiltrate (Low/Medium/High)	0.784 (0.435-1.414)	0.419			0.765 (0.508-1.152)	0.200		
Tumour CD8+ T-lymphocytic infiltrate (Low/Medium/High)	0.811 (0.449-1.464)	0.487			0.551 (0.337-0.899)	0.017	0.580 (0.339-0.991)	0.046
Tumour CD20+ B-lymphocytic infiltrate (Low/Medium/High)	0.824 (0.456-1.490)	0.522			0.731 (0.467-1.142)	0.169		
Tumour CD138+ B-Lymphocytic infiltrate (Low/Medium/High)	1.128 (0.663-1.918)	0.657			1.259 (0.828-1.914)	0.281		

Tumour CD68+ macrophages infiltrate (Low/Medium/High)	0.841 (0.460-1.535)	0.572			1.105 (0.663-1.8430	0.702		
Tumour Stromal Percentage (Low/High)	3.254 (1.258-8.419)	0.015	3.371 (1.301-8.735)	0.012	3.102 (1.458-6.600)	0.003	2.304 (1.051-5.051)	0.037
Tumour Budding (Low/High)	2.468 (0.956-6.365)	0.062			2.592 (1.212-5.545)	0.014		0.481
p-S118 (Low/High)	0.598 (0.224-1.595)	0.304			0.823 (0.392-1.729)	0.607		
p-S167 (Low/High)	2.001 (0.713-5.618)	0.188			2.417 (0.918-6.361)	0.074		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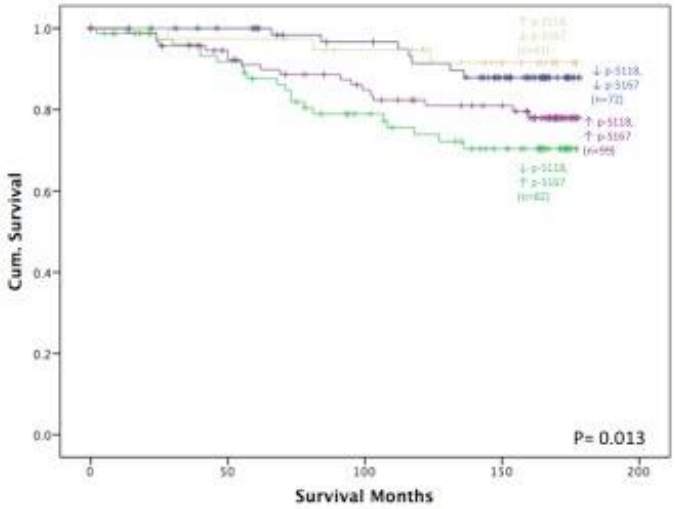
Low p-S118 (n=154)
Cum. Survival
Survival Months
A
P= 0.507
p-S167
Low p-S167 (n=113)
High p-S167 (n=181)
Cum. Survival
Survival Months
B
P= 0.003
p-S118
C
P= 0.443
Low p-S118 (n=149)
High p-S118 (n=133)
1 - Cum. Survival
Recurrence Months
p-S167
D
P= 0.001
High p-S167 (n=173)
Low p-S167
(n=109)
1 - Cum. Survival
Recurrence Months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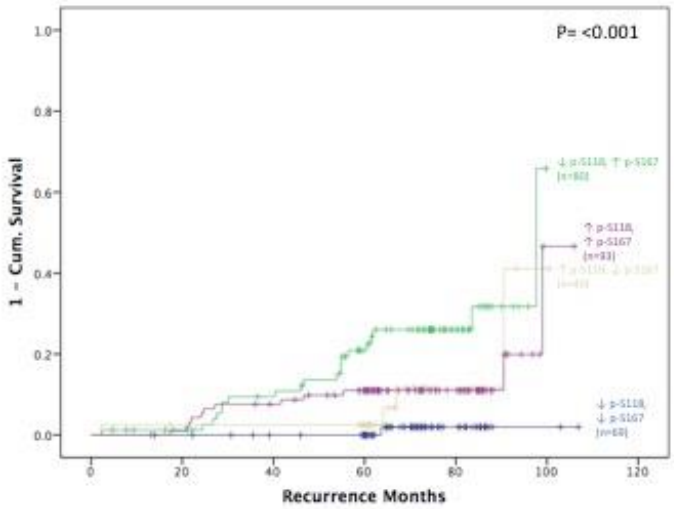
0.4
0.2
0.0
0
50
100
150
200
Survival Months
P= 0.013


1 - Cum. Survival
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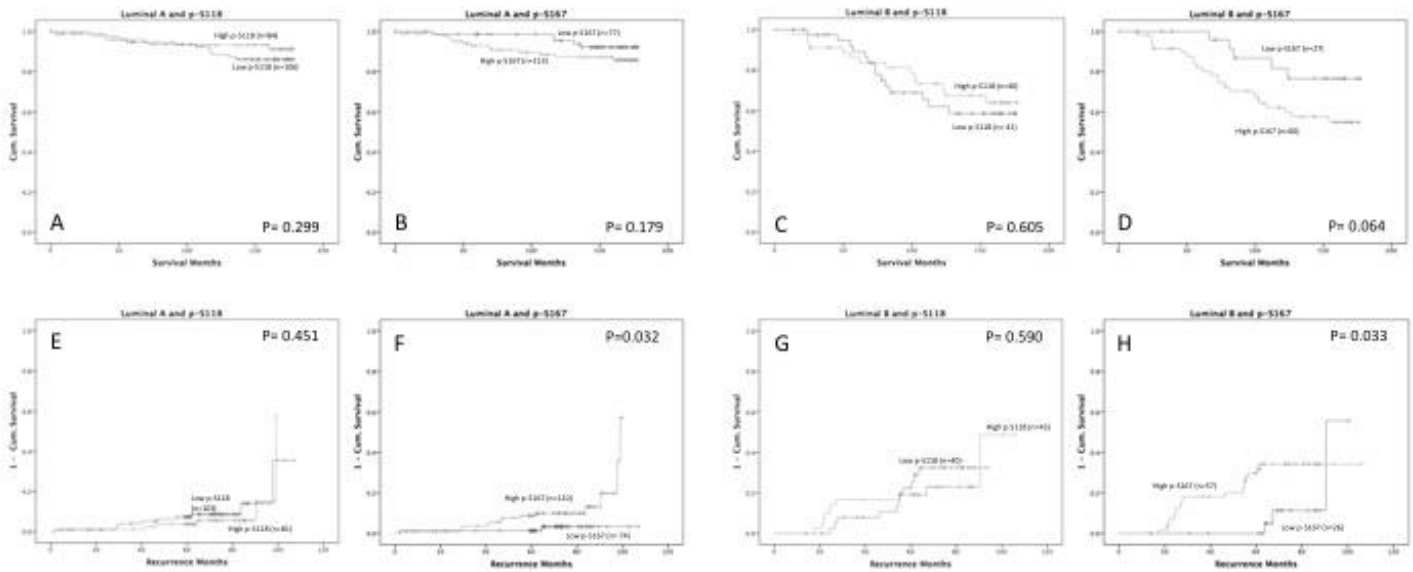
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
Recurrence Months
P= <0.001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E
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P= 0.590
H
P= 0.033