# The relationship between phosphorylation status of focal adhesion kinases, molecular subtypes, tumour microenvironment and survival in patients with primary operable ductal breast cancer

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**ABSTRACT**

**Background:** Despite advances in therapies to treat breast cancer, over 100,000 patients die in the UK of this disease per year, highlighting the need to develop effect predictive and prognostic markers for patients with primary operable ductal breast cancer. Therefore, the aim of the present study was to examine the relationship between membranous, cytoplasmic and nuclear expression of focal adhesion kinase (phosphorylated at Y 397, Y 861 and Y 925), molecular subtypes, tumour microenvironment and survival in patients with primary operable ductal breast cancer.

**Methods:** 474 patients presenting between 1995 and 1998 with primary operable ductal breast cancer were included in this study. Using tissue microarrays expression of membranous, cytoplasmic and nuclear tumour cell phosphorylation of FAK at $Y^{397}$, $Y^{861}$ and $Y^{925}$ was assessed, and associations with clinicopathological characteristics, tumour microenvironment and cancer-specific survival (CSS) were examined.

**Results:** No significant association was observed for ph-FAK $Y^{861}$ with survival at all sites. However, high expression of membranous ph-FAK $Y^{397}$ was associated with increased tumour grade (P<0.001), molecular subtypes (P<0.001), increased tumour necrosis (P<0.001), high Klintrup–Mäkinen grade (P<0.001), increased CD138+ plasma cells (P=0.031), endocrine therapy (P=0.001) and poor cancer specific survival (P=0.040). Similarly, high expression of nuclear ph-FAK $Y^{397}$ was associated with decreased age (P=0.042), increased CD138+ plasma cells (P=0.001) and poor cancer specific survival (P=0.003). Furthermore, high expression of cytoplasmic ph-FAK $Y^{925}$ was associated with decreased tumour grade (P<0.001), less involved lymph node (P=0.020), molecular subtypes (P<0.001), decreased tumour necrosis (P<0.001), low Klintrup–Mäkinen grade (P<0.001), decreased CD4+ T-cells (P=0.006), decreased CD138+ plasma cells (P=0.034), endocrine therapy (P<0.001), chemotherapy (P=0.048), and improved cancer specific survival

(P=0.044). On multivariate analysis, high expression of nuclear ph-FAK $Y^{397}$ was independently associated with reduced cancer specific survival (P=0.017).

**Conclusion:** The results of the present study show that membranous and nuclear ph-FAK $Y^{397}$ and cytoplasmic ph-FAK $Y^{925}$ were associated with prognosis in patients with primary operable ductal breast cancer. In addition, high expression of nuclear ph-FAK $Y^{397}$ was an independent prognostic factor in patients with primary operable ductal breast cancer and could be incorporated into clinical practice.

## INTRODUCTION

Breast cancer is a heterogeneous disease and is the most common cancer in females worldwide. In the UK, breast cancer is the second most common cancer representing 30% of all female cancers. Despite the routine use of hormone receptor status [1], TNM staging system [2], and the recent introduction of molecular subtypes to identify likely outcomes and plan treatment, it is clear such staging approaches are suboptimal and require refinement.

Recently, there has been an increasing interest in studying the tumour microenvironment and how it affects disease outcome. Indeed, it is clear that the interaction between the tumour and its microenvironment are crucial for long-term survival. In breast cancer, many studies have highlighted the association between components of the tumour microenvironment and patient survival [3, 4, 5]. In particular, some phenotypic features such as the local inflammatory infiltrate and stromal infiltrate have recently been reported to have independent prognostic value in patients with primary operable ductal breast cancer [6, 7, 8]. Therefore, there is now increasing interest in the signalling transduction pathways that may be important in regulating such important phenotypic features.

Focal adhesion kinase (FAK) is a cytoplasmic non-receptor protein tyrosine kinase that plays a critical role in cell motility and undergoes intracellular activation by multiple factors, such as Src Kinase, phospholipase and growth-factor-receptor-bound protein-7 [9]. In human cancers, increased tumour expression of FAK has been shown in several cancer types including lung, cervical and colon cancer when compared to normal tissue [10, 11, 12]. Phosphorylation of FAK at $Y^{397}$ although not a measure of full activation itself, is a surrogate measure of activation and is associated with enhanced tumour growth, migration, invasion, adhesion and spreading, as well as tumour angiogenesis through regulation of both cancer cells and their microenvironment [13, 14, 15]. In two studies of more than 1800 patients with

breast cancer, total FAK expression was significantly associated with neo-angiogenesis and reduced cancer specific survival [16; 17]. Indeed, it has been reported that nuclear FAK regulates the anti-tumour immune response [18]. Moreover, auto-phosphorylation of FAK at $Y^{397}$ allows binding of Src Kinase or other substrates such as ERK and PI3K to promote tumorigenesis [15; 19]. Once auto-phosphorylated, phosphorylation of FAK $Y^{861}$ can occur via Src [20] and phosphorylation of FAK $Y^{925}$ can occur via ERK [21] to mediate distinct downstream effects.

In the last decade, a number of studies have documented the role of total FAK in many types of cancer including breast cancer [10, 11, 12]. However, there is less information about the role of phosphorylated form at each site (membranous, cytoplasmic and nuclear). Therefore, it was of interest to examine in more detail expression of ph-FAK $Y^{397}$ (auto-phosphorylation) and ph-FAK $Y^{861}$ (Src dependent phosphorylation) at different sites and highlight the relationship of each phosphorylation site individually with clinicopathological characteristic to provide novelty to our work.

Therefore, the aim of the present study was to examine relationship between phosphorylated membrane, cytoplasmic and nuclear FAK at $Y^{397}$ (ph-FAK $Y^{397}$), $Y^{861}$ (ph-FAK $Y^{861}$), and $Y^{925}$ (ph-FAK $Y^{925}$), molecular subtypes, the tumour microenvironment and cancer specific survival in patients with primary operable ductal breast cancer.

## PATIENTS AND METHODS

### Patient cohort

Patients presenting with primary operable ductal breast cancer at Glasgow Royal and Western Infirmaries and Stobhill Hospital, in the West of Scotland, between 1995 and 1998 that had formalin-fixed paraffin embedded blocks of the primary tumour available for evaluation were studied (n=474). The tissue was obtained from the Glasgow Biorepository and was approved by the Research Ethics Committee of the West Glasgow University Hospitals NHS Trust (REC reference 07/s0704/61). Individual patient consent was not required for this study.

### Clinical and pathological characteristics

Clinicopathological data included age, tumour size, tumour grade, lymph node status, type of surgery, and type of adjuvant therapy (chemotherapy, hormonal therapy and/or radiotherapy) was retrieved from the routine reports. Tumour grade was assigned according to Nottingham Grading System. Specific information on DCIS and on the resection margin status in this patient cohort was not available. ER and PR status were assessed on tissue microarrays (TMAs) using immunohistochemistry (IHC) and scored according to the American Society of Clinical Oncology and College of American Pathologists guidelines with cut-off value of 1% positive tumour nuclei [22]. HER-2 status were assessed visually using TMA sections as previously described i.e. a score 3+ is regarded as positive; 2+ is regarded as equivocal, leading to referral for HER-2 FISH; and 0 and 1+ are regarded as negative [23].

Proliferation index was assessed by Ki-67 IHC using established protocols in the Department of Pathology, Glasgow Royal Infirmary with appropriate positive and negative controls [24]. Blood (BVI) and lymph (LVI) vessel invasion were assessed, on 2.5 μm thick

sections, using IHC staining with the lymphatic endothelial marker D2-40 (SIG-3730, Covance, USA) diluted 1:100 and vascular endothelial marker Factor VIII (Leica, UK) diluted 1:100 as previously described [25]. Patients were routinely followed up after surgery. Date and cause of death was cross-checked with the cancer registration system and the Registrar General (Scotland). Death records were complete until 31st of May 2013 and that served as the censor date. Cancer-specific survival was measured from the date of primary surgery until the date of death from breast cancer.

**Assessment of the tumour microenvironment**

Full-section haematoxylin and eosin (H&E) slides taken at the deepest point of invasion were used to score local inflammatory infiltrate according to Klintrup Mäkinen (KM) grade as previously described [6]. Briefly, the inflammatory cell infiltrate at the invasive margin was classified as either low-grade (no increase or mild/patchy increase in inflammatory cells) or high-grade (prominent inflammatory reaction forming a band at the invasive margin, or florid cup-like infiltrate**)**. Individual immune cells type was assessed using IHC staining on TMA sections for helper and cytotoxic T-lymphocytes, macrophages and plasma cells using CD4+, CD8+, CD68+ and CD138+ staining as previously described [26]. Full-section H&E slides were also used to score the tumour stroma percentage (TSP) and tumour budding as previously reported [8; 27]. Briefly, a single field was examined for TSP and graded as either low (≤50%) or high (>50%) and 5 fields were examined for budding and the highest bud count per field was used.

**Immunohistochemistry**

Immunohistochemical expression of ph-FAK $Y^{397}$, ph-FAK $Y^{861}$ and ph-FAK $Y^{925}$ was carried out using previously constructed tissue microarrays as previously reported [23]. TMAs contained 0.6-mm cores with three cores per patient and were sectioned at 2.5um

thickness. TMA sections were dewaxed in histoclear before being rehydrated using graded alcohols. Antigen retrieval for ph-FAK $Y^{397}$, ph-FAK $Y^{861}$ and ph-FAK $Y^{925}$ was performed using Tris-EDETA buffer (pH 9) at 96$^{o}$C for 20 minutes before cooling. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 15 minutes for ph-FAK $Y^{397}$; 20 minutes for ph-FAK $Y^{861}$ and 10 minutes for ph-FAK $Y^{925}$. Normal horse serum was applied for 60 minutes at 25$^{0}$C as a blocking solution. TMA sections were then incubated for 60 minutes at 25$^{0}$C with primary antibody for ph-FAK $Y^{397}$ (ab39967, Abcam, UK) and ph-FAK $Y^{861}$ (44-626G, Invitrogen, UK) at a concentration of 1:200. For ph-FAK $Y^{925}$, TMA sections were incubated with primary antibody (PA5-21148, SAB, UK) overnight at 4$^{0}$C at a 1:6000 dilution. Envision (Dako) secondary antibody was added for 30 minutes at room temperature. Proteins were visualised using DAB substrate then counterstained in haematoxylin and blued with Scotts' tap water before being dehydrated through a series of graded alcohols. Cover slips were applied using distrene, plasticizer, xylene (DPX).

**Scoring method**

Stained TMA sections were scanned using a Hamamatsu NanoZoomer (Welwyn Garden City, Hertfordshire, UK) at x20 magnification and visualized using Slidepath Digital Image Hub, version 4.0.1 (Leica Biosystems, UK). Assessment of tumour-specific membrane, cytoplasmic and nuclear ph-FAK $Y^{397}$, ph-FAK $Y^{861}$ and ph-FAK $Y^{925}$ expression was performed by two examiners (NMA and FJG) blinded to clinical data at x20 magnification (total magnification x400) using the weighted histoscore. The weighted histoscore provides an assessment of the percentage and density of staining and is calculated as follows: 0x% not stained + 1x% weakly stained + 2x % moderately stained + 3x % strongly stained as shown in figure 3 (A, B, C and D). This gives a range of scores from 0 to 300. To ensure reproducibility, 20% of tumours were co-scored by a second investigator; the intra-class correlation coefficient for all markers was > 0.70.

## Statistical analysis

Patients were split into low and high expression using the median for all phosphorylation sites. Survival analysis was examined using Kaplan-Meier curves and the log-rank test. The relationships between FAK phosphorylation, molecular subtypes, and the tumour microenvironment were examined using the Chi-square test for linear trend. Univariate survival analysis was performed using Cox regression survival analysis to assess hazard ratios and 95% confidence intervals. Multivariate survival analysis was performed using cox regression survival analysis using a backwards conditional method assessed independent prognostic factors along with common clinical and pathological factors. A *P*-value $<0.05$ was considered statistically significant and the study conformed to the remark criteria. All analysis was performed using SPSS version 24.0 (IBM SPSS IL, USA).

## RESULTS

The majority of patients were older than 50 years (71%), had tumours size ≤2 cm (58%), had grade II carcinoma (41%), no axillary lymph node involvement (53%), and had low Klintrup–Mäkinen grade (73%).

The correlation between tumour-specific ph-FAK $Y^{397}$, ph-FAK $Y^{861}$, and ph-FAK $Y^{925}$ expression in patients with primary operable ductal breast cancer is presented in Table 1. Membranous ph-FAK $Y^{397}$ expression correlated with cytoplasmic ph-FAK $Y^{397}$ expression ($P<0.001$), nuclear ph-FAK $Y^{397}$ expression ($P=0.001$), membranous ph-FAK $Y^{861}$ expression ($P<0.001$), cytoplasmic ph-FAK $Y^{861}$ expression ($P<0.001$), nuclear ph-FAK $Y^{861}$ expression ($P=0.002$), and membranous ph-FAK $Y^{925}$ expression ($P<0.001$). Cytoplasmic ph-FAK $Y^{397}$ expression correlated with nuclear ph-FAK $Y^{397}$ expression ($P<0.001$), membranous ph-FAK $Y^{861}$ expression ($P=0.002$), cytoplasmic ph-FAK $Y^{861}$ expression ($P<0.001$), and nuclear ph-FAK $Y^{861}$ expression ($P<0.001$). Nuclear ph-FAK $Y^{397}$ expression correlated with cytoplasmic ph-FAK $Y^{861}$ expression ($P=0.002$), nuclear ph-FAK $Y^{861}$ expression ($P<0.001$), membranous ph-FAK $Y^{925}$ expression ($P=0.003$), and nuclear ph-FAK $Y^{925}$ expression ($P=0.019$). Membranous ph-FAK $Y^{861}$ expression correlated with cytoplasmic ph-FAK $Y^{861}$ expression ($P<0.001$), nuclear ph-FAK $Y^{861}$ expression ($P=0.001$), and membranous ph-FAK $Y^{925}$ expression ($P<0.001$). Cytoplasmic ph-FAK $Y^{861}$ expression correlated with nuclear ph-FAK $Y^{861}$ expression ($P<0.001$). Nuclear ph-FAK $Y^{861}$ expression correlated with membranous ph-FAK $Y^{925}$ expression ($P=0.001$). Cytoplasmic ph-FAK $Y^{925}$ expression correlated with nuclear ph-FAK $Y^{925}$ expression ($P=0.001$). When assessing the correlation coefficients (CC), correlations between cytoplasmic ph-FAK $Y^{397}$ and both membranous ph-FAK $Y^{397}$ expression ($CC=0.513$) and cytoplasmic ph-FAK $Y^{861}$ ($CC=0.404$), and between nuclear ph-FAK $Y^{397}$ and nuclear ph-FAK $Y^{861}$ ($CC=0.439$) were considered as significant ($CC>0.4$).

The median follow-up for alive patients (n=275) was 150 months with 96 cancer-specific deaths and 90 non-cancer deaths. The relationship between tumour-specific ph-FAK $Y^{397}$, ph-FAK $Y^{861}$, ph-FAK $Y^{925}$ expression and cancer specific survival (CSS) in the full cohort was examined as shown in Table 2 (n=474). High expression of membranous ph-FAK $Y^{397}$ was associated with reduced CSS (P=0.040) (Figure 1A) with 10-year survival reduced from 80% (low expression) to 71% (high expression). Similarly, high expression of nuclear ph-FAK $Y^{397}$ was associated with reduced CSS (P=0.003) (Figure 1B) with 10-year survival reduced from 84% (low expression) to 72% (high expression). However, high expression of cytoplasmic ph-FAK $Y^{925}$ was associated with increased CSS (P=0.044) (Figure 1C) with 10-year survival increased from 75% (low expression) to 83% (high expression). No other phosphorylation sites at any cellular location were associated with CSS.

The relationship between tumour-specific phosphorylation, molecular subtypes, and the tumour microenvironment was assessed. High expression of membranous ph-FAK $Y^{397}$ (n=419; Table 3) was significantly associated with increased tumour grade (P<0.001), molecular subtypes (P<0.001), increased tumour necrosis (P<0.001), high Klintrup–Mäkinen grade (P<0.001), increased CD138+ plasma cells (P=0.031), and endocrine therapy (P=0.001). Whereas, high expression of nuclear ph-FAK $Y^{397}$ (n=419, Table 4) was associated with decreased age (P=0.042), and increased CD138+ plasma cells (P=0.001). However, high expression of cytoplasmic ph-FAK $Y^{925}$ (n=443; Table 5) was significantly associated with decreased tumour grade (P<0.001), no involved lymph node (P=0.020), molecular subtypes (P<0.001), decreased tumour necrosis (P<0.001), low Klintrup–Mäkinen grade (P<0.001), decreased CD4+ T-cells, decreased CD138+ plasma cells (P=0.034), endocrine therapy (P<0.001), and chemotherapy (P=0.048).

The relationship between FAK phosphorylation, clinicopathological characteristics and CSS is presented in Table 6 (n=474). In univariate analysis, tumour size (P<0.001),

tumour grade (P<0.001), involved lymph node (P<0.001), molecular subtypes (P<0.001), tumour necrosis (P<0.001), tumour budding (P<0.001), tumour stroma percentage (P<0.001), Klintrup–Mäkinen grade (P=0.095), CD8+ T-cells (P=0.004), CD138+ plasma cells (P=0.003), blood vessel invasion (P<0.001), lymph vessel invasion (P<0.001), membranous ph-FAK $Y^{397}$ (P=0.042), nuclear ph-FAK $Y^{397}$ (P=0.003), and cytoplasmic ph-FAK $Y^{925}$ (P=0.046) were associated with CSS. On multivariate analysis, involved lymph node (P=0.019), molecular subtypes (P=0.009), tumour necrosis (P<0.001), tumour budding (P=0.005), tumour stroma percentage (P=0.007), CD8+ T-cells (P=<0.001), CD138+ plasma cells (P=0.019), blood vessel invasion (P=0.002), lymph vessel invasion (P=0.023), and nuclear ph-FAK $Y^{397}$ (P=0.017) were independent prognostic factors.

## DISCUSSION

The present study examined the level of phosphorylation of FAK at (tyrosine residue) $Y^{397}$, $Y^{861}$, and $Y^{925}$ and assessed their relationship with clinicopathological characteristics, the tumour microenvironment and survival in patients with primary operable ductal breast cancer. The study showed a significant correlation between membranous, cytoplasmic and nuclear ph-FAK $Y^{397}$ , membranous, cytoplasmic and nuclear ph-FAK $Y^{861}$, and membranous ph-FAK $Y^{925}$, suggesting these sites may work together to regulated downstream pathways in these patients. Furthermore, membrane and nuclear ph-FAK $Y^{397}$ associated with decreased survival whereas nuclear ph-FAK $Y^{925}$ associated with improved survival. This suggests that FAK can differentially effect survival depending on the site of phosphorylation and highlights that each site needs to be investigated individually in patients with primary operable ductal breast cancer.

It has previously been shown that phosphorylation of FAK at tyrosine residue 861 ($Y^{861}$) is reliant on the auto phosphorylation of FAK at $Y^{397}$. Indeed, it has been reported that the auto-phosphorylation of FAK at Y 397 allows a conformational change that unmasks the binding site for Src kinase which, in turn, facilitates further phosphorylation of FAK at Y 861 [9]. Similarly, this auto phosphorylation may also lead to the unmasking of the $Y^{925}$ site allowing phosphorylation at this site by ERK. To confirm this, it would be of interest to examine the association between ph-FAK $Y^{397}$, ph-FAK $Y^{861}$, and FAK $Y^{925}$.

In the present study, a correlation was observed from the scatter plots between cytoplasmic ph-FAK Y397 and both membranous ph-FAK Y397 expression (CC=0.513) and cytoplasmic ph-FAK Y861 (CC=0.404), and between nuclear ph-FAK Y397 and nuclear ph-FAK Y861 (CC=0.439). A correlation coefficient (CC) between (0.40-0.59) may be considered to have a moderate correlation as reported in the bmj (Correlation and regression) [28].

In the current study, it was observed that high expression of membrane and nuclear ph-FAK $Y^{397}$ were associated with outcome in early stage breast cancer. In addition, high expression of membrane ph-FAK $Y^{397}$ was associated with increased tumour grade, molecular subtypes, increased tumour necrosis, low Klintrup–Mäkinen grade, increased CD138+ plasma cells, and endocrine therapy, suggesting it is associated with aggressive disease. In addition, high expression of nuclear ph-FAK $Y^{397}$ was associated with decreased age and increased CD138+ plasma cells. Recently, Dwyer and co-workers have confirmed a role for FAK in NF-κB signalling [30] and is associated with severe drop in the immune response [31],suggesting that auto-phosphorylation of FAK at Y397 can potentially modulate inflammation within the tumour microenvironment in patients with primary operable ductal breast cancer[30]. Based on the previous information, this may suggest that both canonical and non-canonical pathways are activated and this activation was significantly associated with inflammatory characteristics. However further work is required to substantiate this hypothesis.

In the present study, there was a significant association between membranous and nuclear ph-FAKY397which may suggest that FAK moves to the nucleus once activated at this site to modulate the pro-tumorigenic inflammatory response. Interestingly, Lachowski and co-workers suggested that Yes-associated protein (YAP) translocates to the nucleus and become active under control of FAK [31]. Therefore, inhibiting phosphorylation at this site may improve survival in patients with primary operable ductal breast cancer. However further work is required to substantiate this hypothesis.

Compared with low, high phosphorylation of nuclear ph-FAK $Y^{397}$ was significantly associated with poorer cancer specific survival. That may be due to nuclear ph-FAK $Y^{397}$ promoting P53 degradation in the nucleus [32]. Therefore, overexpression of FAK in human tumour cells might contribute to malignancy by promoting survival under conditions that

would normally lead to cell death. Indeed, Golubovskaya and co-workers have reported in a cell line study that inhibition FAK phosphorylation at $Y^{397}$ and $Y^{577}$ has been shown to have synergistic effects with inhibition of EGF-receptor signalling, leading to apoptosis in breast cancer cells [33, 34]. More recently, it was reported that in 600 patients with breast cancer, the FAK promoter region contains p53 binding sites, and that p53 inhibits FAK transcription and regulates its expression in tumour samples. Furthermore, it was shown that FAK overexpression and p53 mutations are directly associated [35]. Therefore, from the present work, it is suggested that ph-FAK $Y^{397}$ would present a rational therapeutic target in patients with primary operable ductal breast cancer, which is of particular interest now with many inhibitors entering clinical trials [36].

The results of the present study also show that high expression of cytoplasmic ph-FAK $Y^{925}$ was significantly associated with decreased tumour grade, molecular subtypes, no involved lymph node, decreased tumour necrosis, low Klintrup–Mäkinen grade, decreased CD138+ plasma cells, endocrine therapy, chemotherapy, and better cancer specific survival. This suggests that cytoplasmic ph-FAK $Y^{925}$ is not regulating the same downstream pathways as ph-FAK $Y^{397}$. This is not surprising as this site resides in the C terminal domain and provides binding sites for SH2 (Src homology-2) domain-containing proteins, whereas $Y^{397}$ resides in the kinase domain [37, 38, 39]. Furthermore, this site is activated by ERK signalling pathway, which has previously been shown to be associated with improved survival and decreased inflammation suggesting that FAK can differentially regulate the inflammatory response depending on the sites phosphorylated [40, 41, 42].

The main limitation of the present study was that although there was extensive characterization of tumour and host characteristics these observations were cross-sectional in nature. Therefore, the associations observed may not be causal in effect. In addition, although there was 10-year follow-up of the patients, contemporary management of ductal breast cancer has changed over this period. Nevertheless, the present results provide

important information pertaining to the importance of the FAK signal transduction pathways in the development of aggressive phenotypic characteristics in patients with primary operable ductal breast cancer.

In summary, the results of the present study showed that membranous and nuclear ph-FAK $Y^{397}$, and cytoplasmic ph-FAK $Y^{925}$ were differentially associated with clinicopathological characteristic and survival but only nuclear ph-FAK $Y^{397}$ was an independently prognostic factor in patients with primary operable ductal breast cancer. Therefore, nuclear ph-FAK $Y^{397}$ could be a potential target in treatment of patients with primary operable ductal breast cancer. Furthermore, the results suggest it is important to know which site is phosphorylated and not just to look at a surrogate of FAK activation when assessing patient survival.

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**Table 1. The relationship between tumour ph-FAK $Y^{397}$, ph-FAK $Y^{861}$, and ph-FAK $Y^{925}$ expression in patients with primary operable ductal breast cancer.**

| | **Ph-FAK $Y^{397}$ cytoplasmic expression** | **Ph-FAK $Y^{397}$ nuclear expression** | **Ph-FAK $Y^{861}$ membranous expression** | **Ph-FAK $Y^{861}$ cytoplasmic expression** | **Ph-FAK $Y^{861}$ nuclear expression** | **Ph-FAK $Y^{925}$ membranous expression** | **Ph-FAK $Y^{925}$ cytoplasmic expression** | **Ph-FAK $Y^{925}$ nuclear expression** |
|---|---|---|---|---|---|---|---|---|
| **Ph-FAK $Y^{397}$ membranous expression** | **0.513**** **<0.001** | 0.156** **0.001** | 0.337** **<0.001** | 0.308** **<0.001** | 0.152** **0.002** | 0.232** **<0.001** | -0.077 0.126 | -0.007 0.883 |
| **Ph-FAK $Y^{397}$ cytoplasmic expression** | | 0.249** **<0.001** | 0.157** **0.002** | **0.404**** **<0.001** | 0.215** **<0.001** | 0.098 0.051 | -0.014 0.781 | 0.037 0.459 |
| **Ph-FAK $Y^{397}$ nuclear expression** | | | 0.047 0.344 | 0.151** **0.002** | **0.439**** **<0.001** | 0.149** **0.003** | 0.005 0.914 | 0.118* **0.019** |
| **Ph-FAK $Y^{861}$ membranous expression** | | | | 0.392** **<0.001** | 0.163** **0.001** | 0.249** **<0.001** | 0.055 0.271 | -0.043 0.386 |
| **Ph-FAK $Y^{861}$ cytoplasmic expression** | | | | | 0.380** **<0.001** | 0.076 0.127 | 0.051 0.305 | -0.080 0.108 |
| **Ph-FAK $Y^{861}$ nuclear expression** | | | | | | 0.171** **0.001** | 0.092 0.069 | 0.075 0.136 |
| **Ph-FAK $Y^{925}$ membranous expression** | | | | | | | -0.036 0.448 | 0.010 0.827 |
| **Ph-FAK $Y^{925}$ cytoplasmic expression** | | | | | | | | 0.158** **0.001** |

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

*. Correlation is significant at the 0.05 level (2-tailed)

**Table 2. The relationship between FAKs phosphorylation and survival in patients with primary operable ductal breast cancer (n=474).**

| | | **Membrane** | | | **Cytoplasmic** | | | **Nuclear** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | ***No*** | ***10 yrs. CCS*** | ***P-value*** | ***No*** | ***10 yrs. CCS*** | ***P-value*** | ***No*** | ***10 yrs. CCS*** | ***P-value*** |
| **ph-FAK $Y^{397}$** | | | | **0.040** | | | 0.448 | | | **0.003** |
| | Low expression | 322 | 80% | | 213 | 77% | | 206 | 84% | |
| | High expression | 97 | 71% | | 206 | 79% | | 12 | 72% | |
| **ph-FAK $Y^{861}$** | | | | 0.151 | | | 0.623 | | | 0.255 |
| | Low expression | 323 | 80% | | 214 | 80% | | 208 | 81% | |
| | High expression | 103 | 71% | | 212 | 76% | | 208 | 74% | |
| **ph-FAK $Y^{925}$** | | | | 0.139 | | | **0.044** | | | 0.109 |
| | Low expression | 359 | 81% | | 239 | 75% | | 415 | 78% | |
| | High expression | 85 | 70% | | 204 | 83% | | 29 | 93% | |

**Table 3. The relationship between membranous ph-FAK $Y^{397}$ expression, molecular subtypes, tumour microenvironment and survival in patients with primary operable ductal breast cancer (n=419).**

| All patients (n=419) | Patients | Low phosphorylation (n=322) | High phosphorylation (n=97) | P-value |
|---|---|---|---|---|
| **Age ≤50/>50 years/ missed** | 128/291/0 | 99/223 | 29/68 | 0.874 |
| **Tumour size (≤2, 2.1-5, >5)/ / missed** | 240/168/11/0 | 196/115/11 | 44/53/0 | 0.057 |
| **Tumour grade (I/ II/ III)/ missed** | 75/164/180/0 | 67/133/122 | 8/31/58 | **<0.001** |
| **Involved lymph node (negative/positive)**/ missed | 223/191/5 | 170/147 | 53/44 | 0.861 |
| **Molecular subtypes (A, B, TN, HER-2)/ missed** | 194/102/73/30/12 | 161/80/49/13 | 33/22/24/17 | **<0.001** |
| **Tumour necrosis (low/high)/ missed** | 188/231/0 | 164/158 | 24/73 | **<0.001** |
| **Tumour budding (low/high)/ missed** | 268/151/0 | 205/117 | 63/34 | 0.818 |
| **Tumour stroma percentage (low/high)/ missed** | 277/142/0 | 211/111 | 66/31 | 0.647 |
| **Klintrup–Mäkinen grade (low/high)/ missed** | 298/121/0 | 244/78 | 54/43 | **<0.001** |
| **CD4+ (low/moderate/high)/ missed** | 177/86/148/8 | 136/67/112 | 41/19/36 | 0.816 |
| **CD8+ (low/moderate/high)/ missed** | 126/141/144/8 | 96/114/105 | 30/27/39 | 0.490 |
| **CD68+ (low/moderate/high)/ missed** | 118/145/147/9 | 85/114/116 | 33/31/31 | 0.203 |
| **CD138+ (low/moderate/high)/ missed** | 220/55/135/9 | 175/47/93 | 45/8/42 | **0.031** |
| **Blood vessel invasion (yes/no)/ missed** | 368/51/0 | 284/38 | 84/13 | 0.673 |
| **Lymph vessel invasion (no/yes)/ missed** | 280/139/0 | 217/105 | 63/34 | 0.655 |
| **Endocrine therapy (no/yes)/ missed** | 119/294/6 | 78/238 | 41/56 | **0.001** |
| **Chemotherapy (no/yes)/ missed** | 230/186/3 | 184/135 | 46/51 | 0.076 |
| **Alive/cancer death/non cancer death/ missed** | 233/89/85/12 | 182/62/68 | 51/27/17 | 0.866 |
| **Cancer specific survival (months)[a]/ missed** | 419/0 | 152 (146-158) | 138 (125-151) | **0.040** |

A means 95% CI

**Table 4. The relationship between nuclear ph-FAK $Y^{397}$ expression, molecular subtypes, tumour microenvironment and survival in patients with primary operable ductal breast cancer (n=419).**

| All patients (n=419) | Patients | Low phosphorylation (n=206) | High phosphorylation (n=213) | P-value |
|---|---|---|---|---|
| **Age (≤50/>50 years)/ missed** | 127/291/1 | 53/153 | 74/138 | **0.042** |
| **Tumour size (≤2, 2.1-5, >5)/ missed** | 231/164/11/13 | 112\86\8 | 127\82\3 | 0.136 |
| **Tumour grade (I/ II/ III)/ missed** | 75/164/179/1 | 33/89/84 | 42/75/95 | 0.973 |
| **Involved lymph node (negative/positive)**/ missed | 223/190/6 | 111/93 | 112/97 | 0.867 |
| **Molecular subtypes (A, B, TN, HER-2)/ missed** | 194/102/72/30/21 | 98/51/31/15 | 96/51/41/15 | 0.495 |
| **Tumour necrosis (low/high)/ missed** | 188/230/1 | 98/108 | 90/122 | 0.293 |
| **Tumour budding (low/high)/ missed** | 267/151/1 | 136/70 | 131/81 | 0.369 |
| **Tumour stroma percentage (low/high)/ missed** | 277/141/1 | 143/63 | 134/78 | 0.180 |
| **Klintrup–Mäkinen grade (low/high)/ missed** | 298/120/1 | 154/52 | 144/68 | 0.123 |
| **CD4+ (low/moderate/high)/ missed** | 176/86/148/9 | 85/52/64 | 91/34/84 | 0.418 |
| **CD8+ (low/moderate/high)/ missed** | 125/141/144/9 | 57/70/74 | 68/71/70 | 0.348 |
| **CD68+ (low/moderate/high)/ missed** | 117/145/147/10 | 58/67/76 | 59/78/71 | 0.688 |
| **CD138+ (low/moderate/high)/ missed** | 219/55/135/10 | 119/34/48 | 100/21/87 | **0.001** |
| **Blood vessel invasion (yes/no)/ missed** | 367/51/1 | 181/25 | 186/26 | 0.968 |
| **Lymph vessel invasion (no/yes)/ missed** | 279/139/1 | 135/71 | 144/68 | 0.604 |
| **Endocrine therapy (no/yes)/ missed** | 118/294/7 | 55/147 | 63/147 | 0.534 |
| **Chemotherapy (no/yes)/ missed** | 230/185/4 | 113/90 | 117/95 | 0.922 |
| **Alive/cancer death/non cancer death/ missed** | 232/89/85/13 | 126/32/39 | 106/57/46 | 0.356 |
| **Cancer specific survival (months)[a]/missed** | 419/0 | 157 (150-164) | 141 (133-149) | **0.003** |

A means 95% CI

**Table 5. The relationship between cytoplasmic FAK $Y^{925}$ expression, molecular subtypes, tumour microenvironment and survival in patients with primary operable ductal breast cancer (n=443).**

| All patients (n=443) | Patients | Low phosphorylation (n=239) | High phosphorylation (n=204) | P-value |
|---|---|---|---|---|
| **Age (≤50/>50 years)/ missed** | 126/317/0 | 68/171 | 58\146 | 0.996 |
| **Tumour size (≤2, 2.1-5, >5)/ missed** | 258/172/13/0 | 134/98/7 | 124\74\6 | 0.373 |
| **Tumour grade (I/ II/ III)/ missed** | 85/180/178/0 | 37/83/119 | 48/97/59 | **<0.001** |
| **Involved lymph node (negative/positive)/ missed** | 235/203/5 | 114/121 | 121/82 | **0.020** |
| **Molecular subtypes (A,B, TN, HER-2)/ missed** | 213/109/71/28/22 | 101/48/57/20 | 112/61/14/8 | **<0.001** |
| **Tumour necrosis (low/high)/ missed** | 209/234/0 | 91/148 | 118/86 | **<0.001** |
| **Tumour budding (low/high)/ missed** | 287/156/0 | 160/79 | 127/77 | 0.303 |
| **Tumour stroma percentage (low/high)/ missed** | 295/148/0 | 161/78 | 134/70 | 0.709 |
| **Klintrup–Mäkinen grade (low/high)/ missed** | 322/121/0 | 155/84 | 176/37 | **<0.001** |
| **CD4+ (low/moderate/high)/ missed** | 195/89/151/8 | 93/44/95 | 102/45/56 | **0.006** |
| **CD8+ (low/moderate/high)/ missed** | 139/144/152/8 | 71/72/89 | 68/72/63 | 0.194 |
| **CD68+ (low/moderate/high)/ missed** | 131/150/152/10 | 72/76/82 | 59/74/70 | 0.891 |
| **CD138+ (low/moderate/high)/ missed** | 237/55/141/10 | 115/31/84 | 122/24/57 | **0.034** |
| **Blood vessel invasion (yes/no)/ missed** | 390/53/0 | 208/31 | 182/22 | 0.824 |
| **Lymph vessel invasion (no/yes)/ missed** | 302/141/0 | 163/76 | 139/65 | 0.989 |
| **Endocrine therapy (no/yes)/ missed** | 117/318/8 | 85/150 | 32/168 | **<0.001** |
| **Chemotherapy (no/yes)/ missed** | 250/190/3 | 125/113 | 125/77 | **0.048** |
| **Alive/cancer death/non cancer death/ missed** | 251/93/86/13 | 128/59/46 | 123/34/40 | 0.590 |
| **Cancer specific survival (months)[a] /missed** | 443/0 | 145(138-153) | 156 (149-163) | **0.044** |

A means 95% CI

**Table 6. Multivariate analysis of FAK phosphorylation, clinicopathological characteristics and cancer specific survival in patients with primary operable ductal breast cancer (n=474).**

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| **All patients (n=474)** | **Hazard ratio (95% CI)** | ***P*-value** | **Hazard ratio (95% CI)** | ***P*-value** |
| **Age (≤50/>50 years)** | 1.22 (0.77-1.91) | 0.397 | | |
| **Tumour size (≤2, 2.1-5, >5)** | 2.11 (1.49-2.97) | **<0.001** | | 0.108 |
| **Tumour grade (I/ II/ III)** | 1.87 (1.38-2.53) | **<0.001** | | 0.458 |
| **Involved lymph node (negative/positive)** | 2.76 (1.80-4.23) | **<0.001** | 1.85 (1.11-3.10) | **0.019** |
| **Molecular subtypes (A, B, TN, HER-2)** | 1.49 (1.23-1.80) | <0.001 | 1.35 (1.08-1.69) | **0.009** |
| **Tumour necrosis (low/high)** | 4.38 (2.65-8.24) | **<0.001** | 3.34 (1.77-2.30) | **<0.001** |
| **Tumour budding (low/high)** | 2.53 (1.69-3.79) | **<0.001** | 2.00 (1.23-3.24) | **0.005** |
| **Tumour-stroma percentage (low/high)** | 2. 19 (1.46-3.27) | **<0.001** | 1.86 (1.18-2.92) | **0.007** |
| **Klintrup–Mäkinen grade (low/high)** | 1.44 (0.94-2.19) | **0.095** | | 0.789 |
| **CD4+ (low/moderate/high)** | 0.96 (0.80-1.25) | 0.983 | | |
| **CD8+ (low/moderate/high)** | 0.70 (0.54-0.89) | **0.004** | 0.55 (0.40-0.74) | **<0.001** |
| **CD68+ (low/moderate/high)** | 0.86 (0.67-1.10) | 0.222 | | |
| **CD138+ (low/moderate/high)** | 1.38 (1.11-1.72) | **0.003** | 1.37 (1.05-1.79) | **0.019** |
| **Blood vessel invasion (no/yes)** | 3.39 (2.14-5.39) | **<0.001** | 2.28 (1.35-3.86) | **0.002** |
| **Lymph vessel invasion (no/yes)** | 4.14 (2.75-6.25) | **<0.001** | 1.85 (1.09-3.15) | **0.023** |
| **Membranous Ph-FAK Y $^{397}$** | 1.60 (1.02-2.51) | **0.042** | | 0.495 |
| **Nuclear Ph-FAK Y$^{397}$** | 1.92 (1.25-2.97) | **0.003** | 1.76 (1.11-2.81) | **0.017** |
| **Cytoplasmic Ph-FAK Y$^{925}$** | 0.65 (0.43-0.99) | **0.046** | | 0.281 |

## LEGENDS

**Figure 1.** The association between FAK phosphorylation and cancer specific survival in patients with primary operable ductal breast cancer.

(A) Kaplan-Meier survival curve showing association between cancer specific survival and membranous ph-FAK $Y^{397}$.

(B)

Kaplan-Meier survival curve showing association between cancer specific survival and cytoplasmic ph-FAK $Y^{925}$.

**Figure 2.** Flow chart showing patient inclusion and exclusion criteria for study.

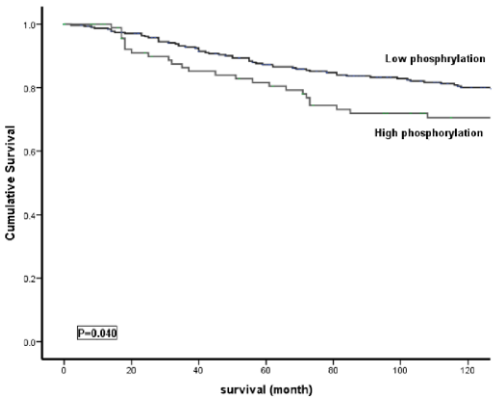


Figure 1A

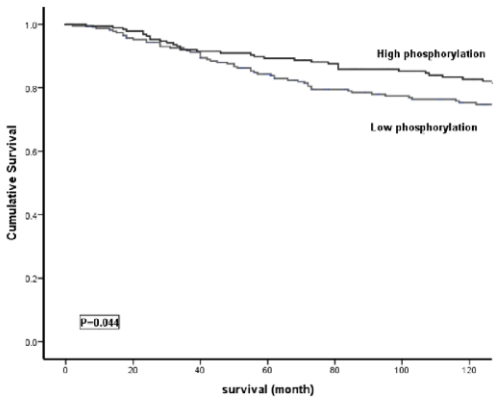


Figure 1B

**Figure 2** Flow chart showing patient inclusion and exclusion criteria for study.