Nuclear Expression of Phosphorylated Focal Adhesion Kinase Is Associated with Poor Prognosis in Human Colorectal Cancer

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Abstract. *Aim: To determine whether phosphorylated focal adhesion kinase (P-FAK) has prognostic value in colorectal cancer (CRC) and to test whether it has any association with Tensin 4 (TNS4) expression. Materials and Methods: P-FAK expression was assessed using immunohistochemistry in 462 CRC cases arrayed on a tissue microarray. P-FAK and TNS4 expression were assessed by immunohistochemistry in 40 cases of paired primary colorectal cancer and corresponding hepatic metastases. Results: Nuclear P-FAK expression was observed in 44% of studied cases. Positive nuclear P-FAK expression was associated with shorter disease-specific survival in univariate (p=0.005) and multivariate analysis (p=0.016). P-FAK expression was greater in metastases than the primary tumours (p<0.001) and showed significant association with nuclear TNS4 (p<0.001) in metastases. Conclusion: P-FAK expression is an independent prognostic marker in CRC. The present data suggest that the FAK signalling pathway may interact with TNS4, a known oncogene in CRC.*

Focal adhesion kinase (FAK) is a 125-kDa non-receptor and non-membrane associated protein tyrosine kinase which localizes to focal adhesions. These are sites where integrin molecules mediate attachment between the cell and the extracellular matrix (23-25). FAK has been shown to act as an early modulator in the integrin signalling cascade and facilitates "outside-in" signalling to downstream targets such as extracellular signal-regulated kinase-2 or c-JUN-*N*-terminal kinase (27). FAK has been found to be highly expressed in a variety of tumors, including head and neck, ovarian, thyroid, and colon carcinomas (8, 12-14, 22). Forced FAK expression in ovarian cells has been reported to enhance G1 to S phase transition, suggesting a role for FAK in the promotion of cell proliferation (7, 33). Conversely, knockdown of FAK protein or treatment of cancer cells with antibodies to FAK can induce apoptosis (29, 30). In addition, isolated fibroblasts from *Fak*-knockout mice have been shown to migrate significantly more slowly than their normal counterparts (20) suggesting that FAK also promotes cell migration.

The FAK protein contains numerous domains, and for full activation, FAK requires phosphorylation of the amino acid residue tyrosine 397. The phosphorylated FAK (P-FAK) can complex with SRC-homology 2 (SH2) domain-containing proteins and trigger multiple signalling pathways (21) which alter integrin adhesion dynamics and cause E-cadherin de-regulation with epithelial-mesenchymal transition (3, 5).

The *C*-terminus Tensin-like gene (*TNS4* also known as *CTEN*) is a member of the tensin gene family. Tensin proteins are found in focal adhesions, where they localise to the cytoplasmic tails of integrins. They play a key role in various biological functions, such as cell adhesion, migration, proliferation, differentiation, apoptosis and invasion (17). Human TNS1, TNS2, TNS3 and TNS4 are highly homologous at their *C*-termini (18) but, compared to the other tensins, TNS4 is shorter due to lack of an *N*-terminal actin-binding domain (17). Tensin proteins contain SH2 domains and interact with a variety of other molecules possibly including FAK. Although both TNS4 and FAK

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localise to focal adhesions and both stimulate motility and cause down-regulation of E-cadherin (2, 9), little is known about their interactions.

In the present study, we evaluated P-FAK protein expression in a large series of CRC using immunohistochemistry to test if P-FAK has any prognostic significance in patients with CRC. We hypothesized that FAK may be associated with TNS4 and we tested the expression of TNS4, and P-FAK in a separate series of 40 cases of paired primary CRC and hepatic metastases using immunohistochemistry.

Materials and Methods

Patient selection and characterisation. A tissue microarray (TMA) of the primary tumours was prepared from a series of 462 primary operable CRC from patients who underwent elective surgery between 1st January 1993 and 31st December 2000 at the Nottingham University Hospitals, NHS trust, Nottingham as previously described (28). This is a well-characterised resource in which a prospectively maintained database is used to record relevant clinical and pathological data, including TNM stage, primary tumour site, histological tumour type, histological grade and the presence of extramural vascular invasion. Patients with lymph node metastasis were characteristically-treated with adjuvant chemotherapy, consisting of 5-fluorouracil and folinic acid. The length of follow-up was determined from the date of primary tumour resection, with surviving cases censored for analysis on the 31st December 2003. Disease-specific survival (DSS) was used as the primary end-point. A TMA containing 40 cases of paired primary and metastatic tumour was also prepared as previously described (1). The TMAs used in this study were created from formalin-fixed paraffin-embedded tissue blocks retrieved from the archives of the Pathology department of the Nottingham University Hospitasl Trust. The study was undertaken with full local ethical approval (reference no. 05/Q1605/66).

Immunohistochemistry. TMA sections were stained using the standard streptavidin–biotin complex method, as previously described (2). Hot water bath-assisted retrieval of antigen epitopes was performed in EDTA buffer (Sigma, Gillingham, Dorset, UK) at pH 8.0. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (Dako, Ely, Cambridgeshire, UK) for 10 min and non-specific antibody binding with Normal Swine Serum (NSS) (Dako) for an additional 10 min at room temperature. Primary antibodies and dilutions were as follows: anti-P-FAK (Y397) (Cell Signalling) 1:100 and anti-TNS4 (ab57940; Abcam, Cambridge, Cambridgeshire, UK.) 1:75. These were applied for 45 min at room temperature. After washing with tris-buffered saline (TBS), biotinylated secondary antibody (Dako) (diluted 1/100 in NSS) was applied and incubated for 30 min at room temperature. Slides were then washed with TBS and preformed Strept ABComplex (AB) (Dako; diluted 1/100 in NSS) was applied for 55 min at room temperature. After washing with TBS, slides were incubated using freshly prepared diaminobenzidine (DAB) (Sigma) solution for 10 min at room temperature. Sections were counterstained with haematoxylin (Dako). Negative controls were performed by omitting the primary antibody and substitution with a diluent.

TMA core scoring. The H-score (histochemical score) was used to assess the intensity of staining and the percentage of stained cells following immunohistochemistry (19). The intensity of staining was quantified from 0 to 3 (0=no staining, 1=weak staining, 2=moderate and 3=strong staining) and the percentage of tumour cells in each staining category was estimated. For each intensity category, the two numbers (intensity and percentage of tumour cells at that intensity) were multiplied to produce a score. The scores at all categories were then added together to produce the H-score (thus the maximum H-score possible would be 300 *i.e.* 100% tumour cells showing strong staining). Immunostaining was evalated by two Histopathologists (MI, AA) without prior knowledge of the clinicopathological or patients' outcome data.

Statistical analysis. Statistical analysis was performed using SPSS v. 16.0 software (SPSS Inc., Chicago, IL, USA) (7). Associations between P-FAK and categorical clinical variables were examined using the Pearson chi-square test. Survival was assessed by the Kaplan–Meier method with a log-rank test to assess significance. Multivariate Cox proportional hazards model was used to test the statistical independence and adjust for confounders. Patients whose deaths related to CRC were considered in the disease-specific survival calculations. Deaths as a result of non-CRC-related causes without evidence of recurrence were censored at the time of death. Comparison of expression of nuclear P-FAK in primary tumours and liver was performed on H-score values using the Wilcoxon signed-rank test. Association between nuclear P-FAK and TNS4 was tested using the Fisher's exact test. A p-value of less than 0.05 was considered significant.

Results

Clinicopathological data. The characteristics of the 462 patients enrolled in the current study are summarised in Table I. The patients had a median age of 72 years (range=45-80 years) with a median follow-up of 37 months (range=0-116 months). Fifty seven percent of patients were male. At the time of censoring for data analysis, a total of 221 (49%) patients had died from CRC, while 167 (37%) patients were still alive at the end of follow-up. The majority of tumours 345 (77%) were moderately-differentiated adenocarcinomas. One hundred and twenty-one (27%) tumours were reported to have histological evidence of extramural vascular invasion.

Nuclear P-FAK expression in CRC cases. The number of informative TMA cores for IHC evaluation was 353 cores, while 109 cores were uninterpretable due to loss of tissue from the TMA during the immunohistochemical procedure or no demonstrable viable tumour cells within the core. Expression of P-FAK was predominantly nuclear and the X-tile bio-informatics software (7) (Yale University, New Haven, Connecticut, USA) was used to define optimal cut-off points of the P-FAK H-score values. This program basically divides the total patient cohort randomly into two separate equal training and validation sets ranked by patients' follow-up time. The optimal cut-points were determined by locating the brightest pixel on the X-tile plot

Table I. *Patient and tumour characteristics (n=462).*

Parameter		Category n (%)
Age (years)	Median	72
	Range	57-89
Gender	Male	257 (57%)
	Female	192 (43%)
Status	Alive	167 (37%)
	Dead (cancer-related)	221 (49%)
	Dead (unrelated causes)	60 (13%)
	Unknown	1
Histological type	Adenocarcinoma	382 (85%)
	Mucinous adenocarcinoma	49 (11%)
	Columnar adenocarcinoma	4 (1%)
	Signet ring mucinous adenocarcinoma	6 (1%)
	Unknown	8 (2%)
Histological grade	Welldifferentiated	28 (6%)
	Moderately differentiated	345 (77%)
	Poorly differentiated	67 (15%)
	Unknown	9 (2%)
Tumour site	Colon	230 (52%)
	Rectal	177 (39%)
	Unknown	42 (9%)
Dukes' stage	A	66 (15%)
	B	175 (39%)
	C1	133 (30%)
	C2	20 (4%)
	D	52 (11%)
	Unknown	3 (1%)
TNM stage	0 (T_{is})	3 (1%)
	1	67 (15%)
	2	172 (38%)
	3	149 (33%)
	4	51 (11%)
	Unknown	7 (2%)
Extramural vascular invasion	Negative	219 (49%)
	Positive	121 (27%)
	Unknown	109 (24%)

TNM=Tumour nodes metastasis staging system.

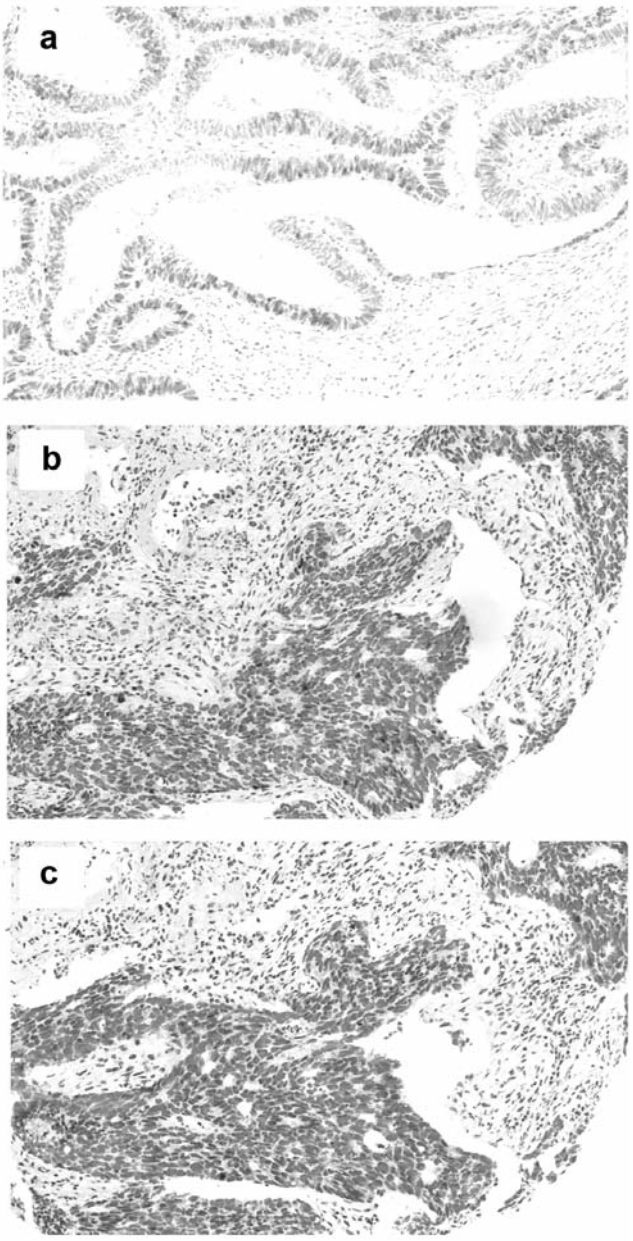

Figure 1. *Nuclear expression of phosphorylated Focal Adhesion Kinase (P-FAK) protein in colorectal cancer (a). Intense nuclear expression of P-FAK in a metastatic tumour deposit (b) and intense nuclear Tensin 4 (TNS4) expression in the same tumour (c). Magnification ×200.*

diagram of the training set and dichotomised the scoring into negative (H-score <30) and positive (H-score >30) groups. Statistical significance was tested by validating the obtained cut-off in the validation set.

Positive nuclear expression was seen in 155 (44%) cases (Figure 1). Analysis of positively-stained P-FAK cases revealed that there was no significant association between the level of nuclear P-FAK expression and known clinicopathological variables, including tumour histological type, tumour grade, tumour site, TNM staging, lymph node metastasis, vascular invasion and distant metastases (Table II).

Positive cytoplasmic P-FAK expression was seen in 22% of cases but this had no correlation with any clinicopathological variables (data not shown).

Association of nuclear P-FAK expression with patient outcome. Univariate survival analyses showed that patients with high nuclear P-FAK expression had shorter DSS than those with low nuclear P-FAK expression (p=0.005, Figure 2). In addition, multivariate Cox proportional hazard analysis revealed that nuclear positivity for P-FAK was a predictor of shorter DSS (p=0.016) (Table III) independent of TNM staging and vascular invasion.

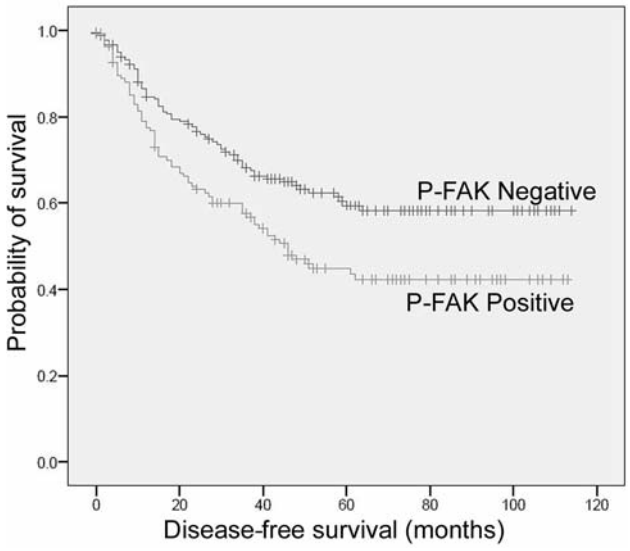

Figure 2. *Kaplan–Meier plots for disease-free survival.*

Table II. *Association of nuclear phosphorylated Focal Adhesion Kinase (P-FAK) expression with clinicopathological features.*

Parameter	Negative P-FAK	Positive P-FAK	χ^2	*p*-Value
Adenocarcinoma	198 (56%)	155 (44%)		
Tumour grade				
Well-differentiated	10 (2.8%)	15 (4.2%)	3.867	0.137
Moderately-differentiated	150 (42.5%)	118 (33.4%)		
Poorly-differentiated	32 (9.1%)	21 (5.9%)		
Tumour site				
Colon	96 (27.2%)	79 (22.4%)	0.806	0.852
Rectum	84 (23.8%)	21 (18.1%)		
TNM stage				
0 (T_{is})	1 (0.3%)	0 (0%)	1.318	0.574
1	34 (9.6%)	23 (6.5%)		
2	80 (22.7%)	54 (15.3%)		
3	61 (17.3%)	54 (15.3%)		
4	19 (5.4%)	22 (6.2%)		
Lymph node stage				
N0	114 (32.3%)	78 (22.1%)	4.536	0.104
N1	39 (11.0%)	37 (10.5%)		
N2	31 (8.8%)	35 (9.9%)		
Vascular invasion				
Negative	127 (35.9%)	101(28.6%)	0.730	0.871
Positive	71 (20.1%)	54 (15.4%)		
Distant metastases				
Negative	178 (50.4%)	130 (36.8%)	2.936	0.235
Positive	18 (5.1%)	23 (6.5%)		

Analysis of TNS4 and P-FAK expression in metastatic CRC. We have previously performed immunohistochemical expression evaluation of TNS4 in a series of 40 matched samples of primary colorectal adenocarcinomas and corresponding liver metastases. Our data showed that high nuclear TNS4 expression is associated with metastatic disease (1). This led us to examine whether the expression levels of these proteins could be correlated to tumour tissue. These samples were stained for P-FAK and, as with the first series of cases, P-FAK staining was nuclear for both primary and metastatic cases. Evaluation of all cases (*i.e.* both primary tumours and metastases) revealed a statistically significant difference between levels of P-FAK expression in the primary and metastatic cases (p=0.001). There was no significant association between TNS4 expression and P-FAK expression in primary tumours (p=0.491), but interestingly, we found a highly significant association betweenTNS4 expression and P-FAK expression in hepatic metastatic cases (p<0.001) (Figure 1, Table IV).

Table III. *Cox proportional hazards analysis for predictors of disease-free survival: effects of TNM, vascular invasion and nuclear phosphorylated Focal Adhesion Kinase (P-FAK) expression in the whole series.*

Parameter	Hazard ratio	95% CI	*p*-Value
Disease-free survival			
TNM stage	1.839	1.582-2.251	<0.001
Vascular invasion	1.431	1.043-2.152	0.002
Nuclear P-FAK expression	1.129	0.763-2.126	0.016

TNM=Tumour nodes metastasis staging system, CI=confidence interval.

Table IV. *Association of nuclear phosphorylated Focal Adhesion Kinase (P-FAK) and nuclear Tensin 4 (TNS4) expression.*

Nuclear P-FAK	Low TNS4	High TNS4
Positive	12	14
Negative	13	1

Discussion

In the present study, we initially sought to investigate the utility of P-FAK as a prognostic marker in a large and well-characterised cohort of CRC cases. Using immunohistochemistry, we were able to detect the expression of P-FAK protein in the nucleus in 44% of tumours. This is similar to published data (26, 31, 32), although ours is the only study to evaluate nuclear staining. In the current study population, nuclear P-FAK expression was not related to any of the clinicopathological parameters examined, although this contradicts other studies showing that P-FAK expression is associated with histological features of poor prognosis (26). However, univariate and multivariate analysis of our

population showed that expression of nuclear P-FAK in CRC had a negative prognostic impact on patient outcome. The mechanistic basis of this is uncertain, although Lim *et al*. found that nuclear P-FAK may physically interact with p53 to suppress transcriptional activation of a number of p53 target genes including *p21*, murine double minute-2 (MDM2) and BCL2-associated X protein (BAX) (16). The latter molecule is an important mediator of p53-induced apoptosis, thereby raising the possibility of an anti-apoptotic effect of nuclear P-FAK.

Both TNS4 and FAK are localized to focal adhesions and can translocate to the nucleus. Tensins contain an SH2 domain which is involved in mediating protein–protein interactions (6, 10, 11) and phosphorylation of tyrosine 397 allows proteins containing SH2 domains to physically interact with P-FAK. Thus, some interaction between TNS4 and P-FAK may be expected and in order to test whether this may occur in tumour tissue, we evaluated a series of paired primary CRC and hepatic metastases. These have been previously examined for TNS4 expression and were therefore tested for P-FAK expression. In contrast to other published studies (4, 15), we found that levels of P-FAK were higher in metastatic deposits than in the primary tumours. The discrepancy between the studies is probably due to the fact that our study evaluated P-FAK (the activated form of FAK), whilst the others studied total FAK (including both activated and non-activated).

Comparison of nuclear TNS4 and nuclear P-FAK immunostaining showed that there was a positive association in the metastases but not in the primary tumours. At this stage, we can only conjecture about the reason for this discrepancy. We have previously shown that localization of TNS4 to the nucleus is associated with metastasis in CRC and it is possible that TNS4 is capable of transporting P-FAK to the nucleus. Conversely, there may be other events occurring during the metastatic process which result in translocation of both TNS4 and P-FAK to the nucleus.

In summary, as far as we are aware of, this is the first study of P-FAK as a prognostic biomarker in CRC. We have shown that nuclear P-FAK is an independent prognostic marker in CRC. In addition, we have shown that Cten and P-FAK expression are positively associated in tumour metastases. Our data suggest that FAK signalling may be important in the biology of CRC and further delineation of this pathway, in particular its interaction with TNS4, is necessary to identify points at which it may be targeted for treatment of CRC.

Conflicts of Interest

There are no conflicts of interest.

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