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Comparative Phosphorylation Profiles of Focal Adhesion Kinase among Cancer Colon Cell Lines and Tissues

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ABSTRACT

Focal Adhesion Kinase (FAK) has been indicated in colon cancer development. FAK phosphorylation at Y397 is a critical event in FAK activation. Until recently, FAK phosphorylation in colon cancer has been poorly understood. Therefore, this study conducted western blotting and immunofluorescence analyses to examine the expression and subcellular localization of total FAK and phosphorylated FAK forms in colon cancer cell lines and tumour tissues. This study results showed that differentiated and less aggressive colon cancer cell line; HT-29, expressed lower FAK level compared to non-differentiated and more aggressive colon cancer cell line; HCT 116. Conversely, pY397 was more expressed in HT-29 than in HCT 116, but was more expressed in invasive tissue than in non-invasive and normal tissues. In conclusion, our study suggests a central role of pY397 in the regulation of colon cancer. However, Y397 could be differentially expressed among colon cancer cell lines and tissues.

Key words: Focal adhesion kinase, phosphorylation, differentiation

INTRODUCTION

Focal Adhesion Kinase (FAK) is a 125 KDa non receptor tyrosine kinase which plays an important role in integrin-mediated signalling and performs critical functions in cancer cell adhesion, invasion and migration (Schaller *et al*., 1994; Sawai *et al*., 2005; Watanabe *et al*., 2008) Apart from its catalytic function, FAK acts as a scaffold protein recruiting signalling molecules to the sites of focal adhesion (Schlaepfer *et al*., 1999; Ayaki *et al*., 2001). Tyrosine phosphorylation is an important event required for FAK activation, it is primarily initiated by the autophosphorylation of Y397 residue (Abu-Ghazaleh *et al*., 2001; Matkowskyj *et al*., 2003; McLean *et al*., 2005). Unlike tyrosine phosphorylation, phosphorylation at serine residues has been indicated in cell mitosis and was involved in FAK abrogation (Yamaguchi *et al*., 1997; Yamakita *et al*., 1999; Ma *et al*., 2001).

In spite that several studies have demonstrated higher FAK levels in colon tumour compared to normal one (Owens *et al.*, 1995; Cance *et al.*, 2000; Ayaki *et al.*, 2001) the data available are inconclusive and still need to be further investigated. Owens *et al.* (1995) correlated the development of colon cancer from normal tissue into primary, invasive and metastatic tumour to FAK overactivation. On the other hand, others (Carroll *et al.*, 2000; Matkowskyj *et al.*, 2003) correlated FAK expression and phosphorylation at Y397 to more differentiated colon tumours. Murata *et al.* (2008) demonstrated differential expression patterns of total FAK pY397 between colon cancer cell lines and colon tumour tissues. Moreover, it was thought that FAK overexpression and phosphorylation at Y397 in the well-differentiated colon tumour cells is a transient event required for tumour tissue remodelling and is affected by tumour cells confluence (Glover *et al.*, 2005; Murata *et al.*, 2008).

Here, we commence to investigate the differential expression and phosphorylation patterns of FAK in colon cancer cell lines and tissues. Normal colon and cancer cell lines were examined for FAK expression and phosphorylation (Y397, Y861, Y407, S732 and S910). Additionally, given that colon tumour can exist in variant invasive levels within a distinct tumour region (Park *et al.*, 2000; Jass *et al.*, 2002), we examined pY397 expression in non-invasive and invasive colon tumours in addition to normal tissues. Study results are in agreement with previous reports (Owens *et al.*, 1995; Cance *et al.*, 2000; Ayaki *et al.*, 2001) demonstrated FAK overexpression in more developed colon cancercinomas. However, this study showed that even Y397 appeared to be differentially phosphorylated among colon cancer cell lines and tumour tissues.

MATERIALS AND METHODS

Reagents: Antibodies used were as follows: Anti-FAK (total, pY397, pY407, pY861, pS732, pS910) were all purchased from Upstate (Temecula, USA), HSC 70 and Alexa fluor 488 were purchased from Invitrogen (Paisley, UK). HRP-conjugated IgG goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Thermo Scientific (Rockford, USA). All other reagents were from Sigma (Poole, UK) unless otherwise stated.

Cell culture: Human normal colon cells (CCD-18Co.), colorectal carcinoma cells (HCT 116) and colorectal adenocarcinoma cells (HT-29) were purchased from American Type Culture Collection (ATCC, USA). DMEM (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS) was used to maintain CCD-18Co cells. HCT 116 and HT-29 cells were cultured in McCoy's 5A (Invitrogen, Paisley, UK) supplemented with 10% FBS. Cells were grown in culture flasks and incubated at 37°C, 5% CO_2.

Western blotting: Confluent cells were lysed in ice-cold cell extraction buffer (Biosource, Nivelles, Belgium) containing 1% protease inhibitor cocktail set 1 (Upstate, Temecula, California) and 0.1% phenylmethanesulfonyl fluoride. Protein concentrations were determined using Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, USA). Proteins were resolved by SDS-PAGE using 8% gels. Proteins were immunobilised on nitrocellulose membranes (Amersham Biosciences, UK) and subsequently probed with antibodies that recognise activated forms of FAK. Membranes were subjected to ECL detection reagent (Amersham Biosciences, Germany) and bands were detected with LAS-3000 Luminescent Image Analyzer.

Immunofluorescence: CCD-18Co, HCT 116 and HT-29 cells were grown on Poly-L-Lysine pre-coated coverslips. Cells were fixed with 4% paraformaldehyde and permeabilised using 0.1%

Triton-X, the subsequent processes were carried out at RT. Samples were washed in TBS and blocked with TBS containing 1% BSA, 0.1% sodium azide for 30 min. In a humidified chamber, samples were incubated with 200 μL of primary antibodies of anti-FAK antibodies (total, pY397 and pY861) at 1:250 dilutions for 1 hour. Samples were washed with TBS and probed with 200 μL of Alexa Flour® 488 at a dilution of 1:100 for 30 min. Samples were co-stained with phalloidin (Molecular Probes, Invitrogen) and DAPI (Molecular Probes, Invitrogen) at a dilution of 1:40 and 1:35000 for 20 and 3 min, respectively at RT. Samples were mounted with ProLong® Gold Antifade Reagent (Molecular Probes, Invitrogen).

Tissues immunofluorescence: Matched pairs of human primary tumour and adjacent normal frozen colon tissue section slides were purchased from BioChain Institute (Hayward, USA). Tissues were thawed for 5 min at RT and incubated in blocking solution (10% normal goat serum, 0.075% Triton-X in TBS) for 1 h at RT. In a humidified chamber tissue were probed with 200 μL of anti FAK (pY397) diluted in Normal goat serum (1:50) at 4°C overnight. Tissue sections were washed in TBS and probed with Alexa Fluor 488 (1:1000). Slides were washed in TBS and stained with DAPI for 3 min at RT. Slides were washed and mounted on coverslips with ProLong® Gold Antifade (Molecular probes, Invitrogen).

Microscopy: Cells and tissues were examined by phase contrast-fluorescence microscope (Olympus 1×71, Japan). Images were captured by OSIS XC50 camera (Olympus, Japan). Cell images were captured under 400x magnification, while tissue sections images were captured under and 200x and 600x magnifications.

RESULTS

Focal Adhesion Kinase (total, pY397, pY861 pY407, pS732, pS910) expression in normal and cancer colon cell lines: In order to investigate FAK expression and phosphorylation at some of its major residues, immunoblotting with anti-FAK (total, pY397, pY407, pY861, pS732, pS910) was carried out on a panel of normal and colon cancer cell lines with varying degree of aggressiveness.

FAK was detected in all the cell lines but the level of total FAK appeared to be higher in HCT 116 compared to CCD-18Co and HT-29 ($p<0.05$). The phosphorylated FAK at Y397 residue was undetectable in the normal cell line, CCD-18Co, however, was clearly expressed in both cancer cells; HCT 116 and HT-29. The expression of pY397 appeared to be higher in HT-29 compared to HCT 116 ($p<0.05$) (Fig. 1).

pY407 was undetectable in all colon cell lines analysed in this study. FAK phosphorylation at Y861 was weakly detected in normal colon cells; CCD-18Co, while the protein was undetectable in the colon cancer cells; HCT 116 and HT-29.

Immunoblotting of pS732 showed that FAK phosphorylation at S732 was undetectable in all of cell lines examined. Phosphorylation of S910 residue was detected in CCD-18Co and HT-29, but was undetectable in HCT 116.

The subcellular distribution of FAK and FAK phosphorylated forms (pY397 and pY861) in normal and cancer colon cell lines: Indirect immonofluorescence analysis was performed on a panel of colon cell lines to examine the subcellular localization of total FAK protein, pY397 and

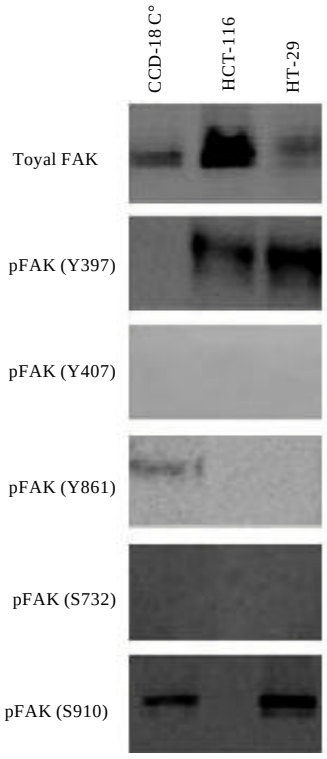

Fig. 1: Western blotting analysis of total FAK and phosphorylated FAK residues in normal and cancer colon cell lines. Equal amounts of lysate samples (30 μg) were analyzed using specific antibodies against FAK (total, pY397, pY407, pY861, pS732, pS910). Results were presented as Mean±standard deviation. $p<0.05$ was considered statistically significant

pY861. Subcellular localization of other forms of phosphorylated FAK protein was not analysed because of the unavailability of specific antibodies for this application.

Cytoplasmic FAK staining was detected in CCD-18Co cells with undetectable staining at cellular protrusions. FAK localization to cellular extensions in the tumourigenic cells; HCT 116 and HT-29, was not observed. FAK Localization to nucleus was observed in CCD-18Co and HT-29 (Fig. 2). pY397 localization was mainly cytoplasmic in CCD-18Co and HCT 116, however, the nuclear localization of the protein was detected in HT-29 (Fig. 3).

pY861 immunostaining analysis showed that the cytoplasmic pY861 was weakly detected in CCD-18Co and was undetectable at cellular peripheries. Cytoplasmic pY861 was clearly observed in HCT 116 and HT-29 with undetectable localization to protrusions (Fig. 4).

pY397 expression in normal and tumour colon tissues: In order to directly examine pY397 expression and distribution in normal and tumour colon tissues immunofluorescence analysis of normal, carcinoma in situ (CIS) and infiltrating carcinoma (IC) was performed. Variant expression levels of pY397 were detected among different invasive stages of colon tumour (Fig. 5).

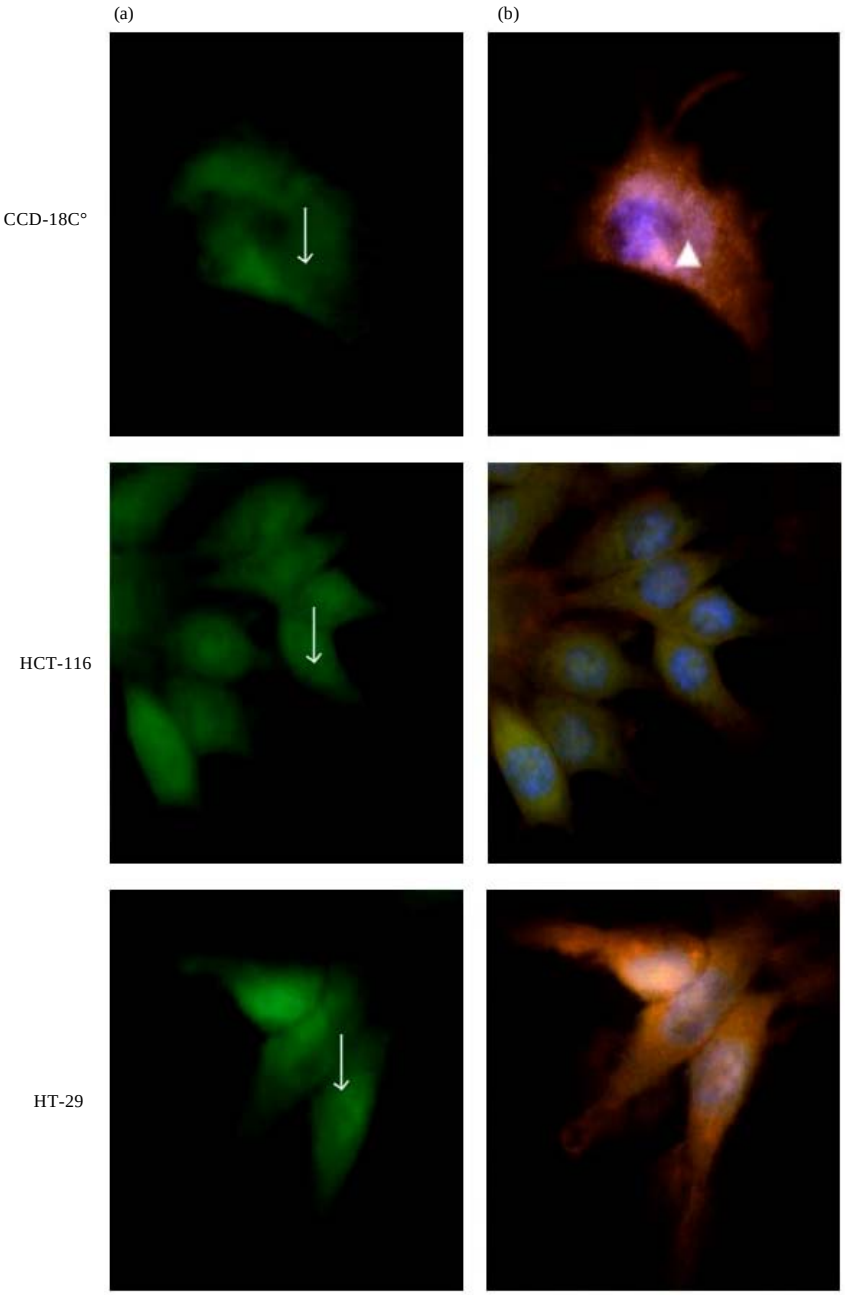

Fig. 2(a-b): FAK subcellular distribution. Colon cells were let to adhere on coverslips, fixed and immunostained with anti-FAK and then with Alexa fluor 488 conjugated antibody (green). Actin cytoskeleton was visualized using phalloidine (red), while nuclei were made visible by staining with DAPI (blue). FAK subcellular localization (a). Composite staining image of FAK, actin cytoskeleton and nuclei (b). Arrow indicates cytoplasmic staining, while arrow head indicates nuclear staining. Magnification: x400

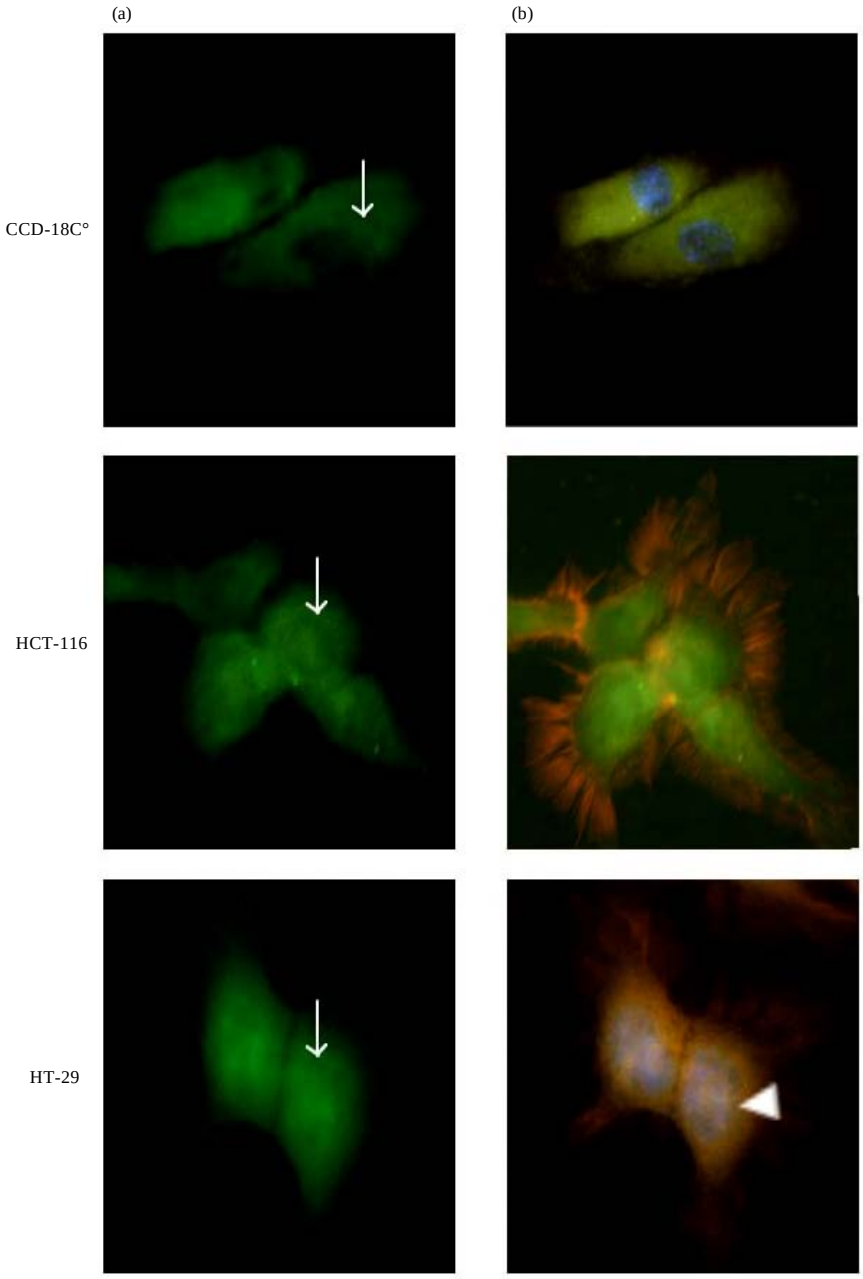

Fig. 3(a-b): Subcellular distribution of pY397 in colon cell lines. Cells were grown on coverslips, fixed and immunostained with anti-pY397 and then with Alexa fluor 488 conjugated antibody (green). Actin cytoskeleton was visualized using phalloidine (red), while nuclei were counter stained blue with DAPI. pY397 subcellular localization (a). Composite staining image of pY397, actin cytoskeleton and nuclei (b). Arrow indicates cytoplasmic staining, while arrow head indicates nuclear staining. Magnification: x4000

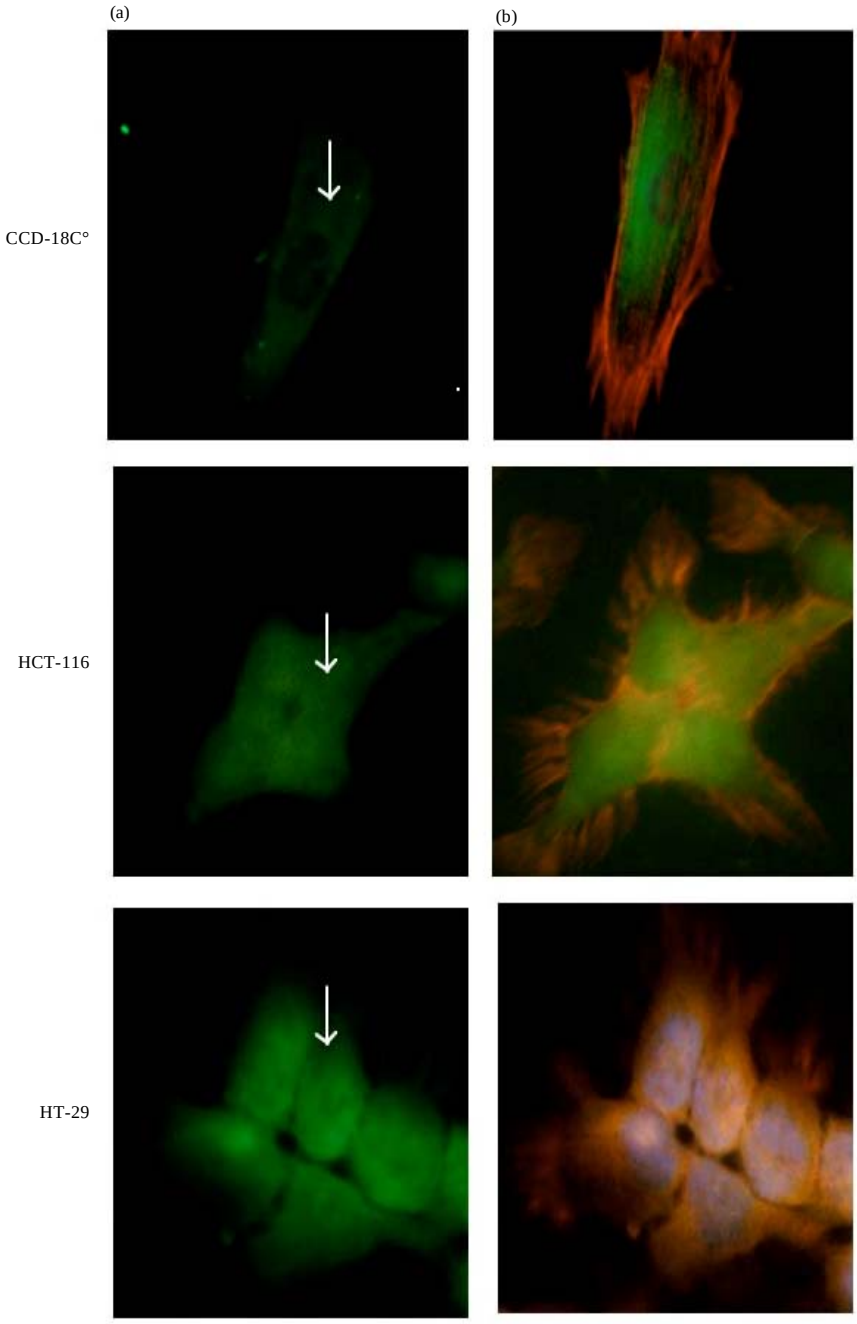

Fig. 4(a-b): Subcellular distribution of pY861 in colon cell lines. Cells were grown on coverslips, fixed and immunostained with anti-pY861 and then with Alexa fluor 488 conjugated antibody (green). Actin cytoskeleton was visualized using phalloidine (red), while nuclei was counter stained blue with DAPI stain. pY861 subcellular localization (a). Composite staining image of the pY861, actin cytoskeleton and nuclei (b). Arrow indicates cytoplasmic staining. Magnification: x400

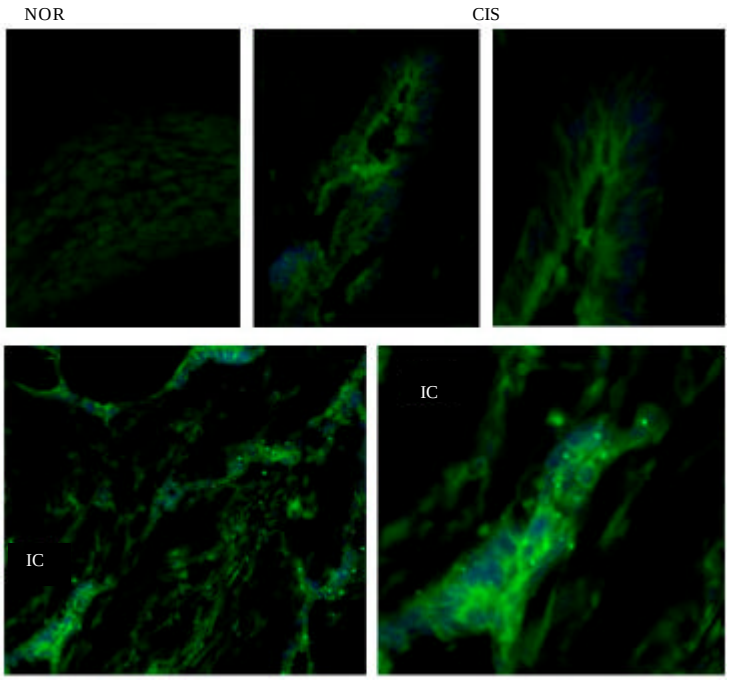

Fig. 5: pY397 staining in human matched pair tissue sections of colon carcinoma. Localization of the site-specific phosphorylated FAK detected with anti-pY397 and Alexa fluor 488 antibodies (green), while nuclei was stained with DAPI (blue). pY397 distribution in normal tissue (NOR). Colon carcinoma in situ (CIS). Infiltrating carcinoma (IC). Arrow indicates staining at basal cell layer, while arrow head indicates staining in the epithelial secretory cells. Magnifications are x 200 (NOR, CIS. left, IC. left) and x600 (CIS. right, IC. right)

Weak pY397 staining was detected in normal colon epithelial cells compared with corresponding tumour tissues. Also, study results showed that CIS differentially expressed pY397 between the basal cell layer (Fig. 5. CIS, arrow) and secretory epithelial cells (Fig. 5. CIS, arrow head) as stronger staining was detected at the basal cell layer.

pY397 analysis of colon tumour tissues showed that pY397 staining was more observable in IC compared to normal and CIS (Fig. 5).

DISCUSSION

FAK has been implicated in the development of colon cancer and higher FAK levels were correlated to more invasive tumours (Owens *et al.*, 1995; Cance *et al.*, 2000). The expression patterns of FAK and pY397 in colon cancer almost indecisive and seemed to differ between cell lines and tissues (Fig. 6).

Our findings demonstrated higher FAK expression with stronger cytoplasmic staining in the more invasive and undifferentiated cells; HCT 116, compared to less invasive and moderately differentiated HT-29 as well as the normal cells; CCD-18Co (Fig. 1 and 2). These findings came in agreement with Owens *et al.* (1995) and Cance *et al.* (2000), who reported higher FAK levels in invasive and metastatic colon tumour compared to non-invasive and normal colon tissues. However,

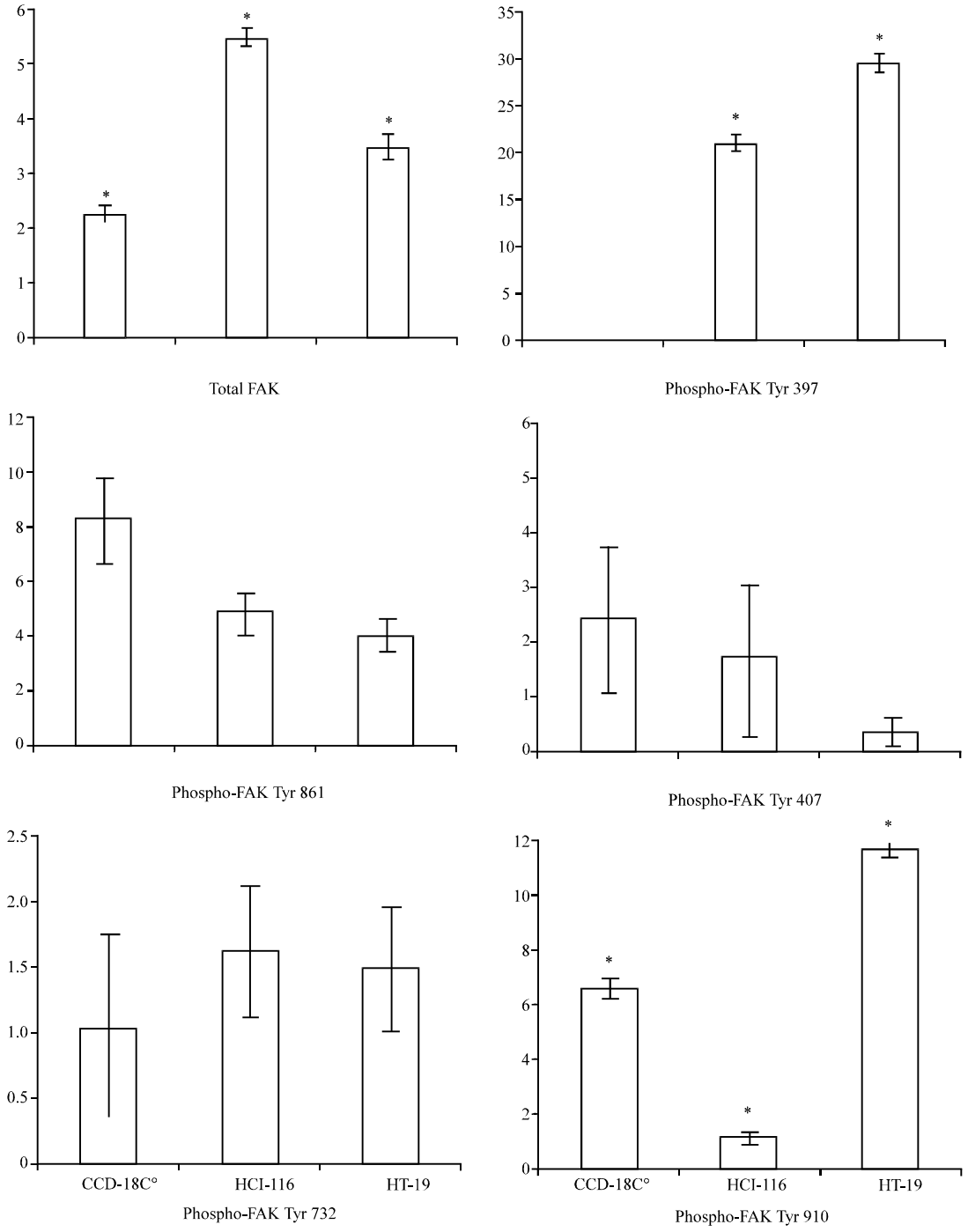

Fig. 6: Supplementary: Densitometric analysis of total and phosphorylated FAK western blot analysis in normal colon and cancer cell lines. Results are presented as Mean±standard deviation (SD). *$p<0.05$ is considered as significant

in view of previous study where the expression patterns of total FAK and pY397 varied between cell lines and tumour tissues, Murata *et al.* (2008) study results came in agreement with the previous mentioned study. That is higher expression of pY397 was demonstrated in HT-29

compared to HCT 116 (Fig. 1 and 3) and higher expression of pY397 was observed in IC compared to CIS (Fig. 5).

Nuclear FAK has been indicated in cellular survival and has been thought to occur subsequent to Y397 phosphorylation (Lim *et al.*, 2008; Murata *et al.*, 2008). Since the current study demonstrated nuclear localizations of total FAK and pY397 in HT-29 (Fig. 2, 3) we suggest a possible involvement of nuclear pY397 in prolonging HT-29 survival. Apart from that, since it was previously demonstrated that FAK phosphorylation at the serine residues; pS843 and pS910 could conversely affect FAK levels (Yamakita *et al.*, 1999; Ma *et al.*, 2001) we suggested that pS910 may act to abrogate total FAK expression in HT-29.

To summarise, in this study, we propose that colon cancer is regulated partially by pY397, however, the protein may exhibit different patterns of expressions between colon cancer cell lines and tissues. We further suggest an inhibitory role of pS910 on total FAK expression, since the absence of pS910 accompanied strong FAK expression in the more aggressive colon cells.

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