Lung Cancer (2006) **53**, 263–271

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/lungcan

Up-regulation of focal adhesion kinase in non-small cell lung cancer

Stephana Carelli[a,b], Giorgia Zadra[a], Valentina Vaira[c,d], Monica Falleni[c,d], Luca Bottiglieri[c,d], Mario Nosotti[e], Anna Maria Di Giulio[a], Alfredo Gorio[a,b,*], Silvano Bosari[c,d,**]

[a] *Laboratory of Pharmacology, Department of Medicine, Surgery and Dentistry, University of Milan, Polo H. San Paolo, Milan, Italy*
[b] *Clinical Pharmacology, IRCCS Humanitas, Rozzano, Milan, Italy*
[c] *Division of Pathology, Department of Medicine, Surgery and Dentistry, University of Milan, AO San Paolo, Milan, Italy*
[d] *Fondazione Ospedale Maggiore Policlinico, Regina Elena e Mangiagalli, Milan, Italy*
[e] *Division of Thoracic Surgery, Fondazione Ospedale Maggiore Policlinico, Regina Elena e Mangiagalli, Milan, Italy*

Received 6 February 2006; received in revised form 19 April 2006; accepted 1 June 2006

KEYWORDS
FAK;
NSCLC;
Tumourigenesis;
Cell migration;
Cancer progression;
Immunohistochemistry;
Real-time RT-PCR

Summary Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase linked to the integrin and growth factor receptor-signalling pathways that regulates a number of the biological processes involved in neoplastic transformation, invasion and metastases, such as cell adhesion, migration and apoptosis. Its up-regulation might play a role in the tumourigenesis of invasive tumours, but its involvement in human lung cancer tissues has not yet been determined.

We immunohistochemically compared FAK expression and localisation in 60 formalin-fixed and paraffin-embedded non-small cell lung cancer (NSCLC) tissues with that in the surrounding non-neoplastic tissue and in a further five microscopically normal lungs. FAK mRNA levels were quantitatively determined by real-time RT-PCR in frozen tissue specimens of all of the tumours and 21 matched non-neoplastic lung parenchymas, and protein expression in 16 homogenates of the matched neoplastic/non-neoplastic specimens was evaluated by Western blotting.

The three different techniques showed that FAK is weakly expressed in non-neoplastic lung parenchyma and up-regulated in NSCLCs. Moreover, Western blotting and real-time RT-PCR indicated a statistically significant correlation between FAK up-regulation and higher disease stages (I + II versus III + IV, $p = 0.019$ and 0.028, respectively). Our results provide evidence that FAK is up-regulated in NSCLCs, and suggest its potential involvement in lung cancer progression.

* Corresponding author at: Laboratory of Pharmacology, Department of Medicine, Surgery and Dentistry, University of Milan, Polo H. San Paolo, Via A. Di Rudinì 8, 20142, Milano, Italy. Tel.: +39 0250323032; fax: +39 0250323033.
** Corresponding author at: Division of Pathology, Department of Medicine, Surgery and Dentistry, University of Milan, AO San Paolo, and Fondazione Ospedale Maggiore Policlinico, Regina Elena e Mangiagalli, Via A. Di Rudinì 8 20142, Milano, Italy.
E-mail addresses: alfredo.gorio@unimi.it (A. Gorio), silvano.bosari@unimi.it (S. Bosari).

0169-5002/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.lungcan.2006.06.001

1. Introduction

A number of factors regulate tumour cell growth, migration and proliferation, and a major role is played by interactions with the extracellular matrix (ECM) [1]. Integrins are cell membrane-spanning proteins that bind to the ECM and contribute to the assembly of the cellular focal adhesion sites in which focal adhesion kinase (FAK) is located. FAK is a non-receptor tyrosine kinase protein that was originally detected in Rous sarcoma virus (v-src)-transformed chicken embryo fibroblasts [2]. A primary autophosphorylation site (Tyr397) is located in the N-terminal region of FAK and directs the interaction with the Src homology-2 domain [3]. Two highly conserved tyrosine residues (Tyr576 and Tyr577) are part of the central catalytic domain that is involved in signalling events to downstream effectors [4], and multiple protein—protein interaction sites are present in the C-terminal region [5]. FAK is a point of convergence for the signalling events mediated by both integrins and growth factor receptors [6] that regulate processes, such as cell adhesion [5], motility [7], migration [8], and apoptosis [9].

FAK phosphorylation leads to the activation of various downstream intracellular signalling molecules such as Src family kinases [10], phosphatidylinositol 3-kinase [11], phospholipase C [12] and Grb7 [13]. As all of these effectors activate pathways that are intimately involved in neoplastic transformation, invasion and metastases, FAK may play an important role in tumourigenesis [14], a hypothesis that is supported by the original isolation of the human FAK gene from a primary human sarcoma [15] and the high FAK levels found in several tumour cell lines of different origin [16]. FAK is also up-regulated in different primary human tumours including breast, colorectal [17], prostate [18], thyroid [19], ovarian [20], and brain tumours [21]. The extent of its mRNA expression has been linked to the progression of epithelial and mesenchymal tumours to invasive and metastatic phenotypes [22], and to poor prognoses in patients with hepatocellular carcinoma [23] and acute myeloid leukemia [24]. Finally, at cell level, FAK regulates matrix metalloproteinase expression and secretion, and EGF-stimulated chemotaxis motility [14].

The FAK signal transduction pathway may be a promising target for cancer therapy [14]. It has recently been shown that inhibiting FAK expression and activation by inducing FAK-related non-kinase (FRNK) increases the sensitivity of transformed human kidney epithelial cells to 5-fluorouracil, which suggests that FAK may play a role in tumour chemoresistance [25]. The same has been found in pancreatic adenocarcinoma cells, in which the suppression of FAK expression by means of mRNA interference enhances gemcitabine-induced *in vitro* and *in vivo* cytotoxicity, and increases tumour susceptibility to chemotherapy [26]. Moreover, the activation of FAK and its molecular partners improves cell resistance to ionizing radiation in lung adenocarcinoma cells [27]. Lung cancer is the leading cause of cancer death in Western countries: its incidence continues to increase [28] and it has a poor prognosis as only 15% of affected subjects survive for 5 years or more after diagnosis [29]. However, despite the increasing importance of FAK in human cancer, there is still a lack of data concerning FAK expression in primary human NSCLCs. The aim of this study was to investigate the expression and clinical significance of FAK in 60 surgically resected stage I—IV NSCLCs with known clinicopathological features.

2. Materials and methods

2.1. Patients and samples

The study population consisted of 60 NSCLC patients who underwent curative surgical resection, without having previously received chemotherapy or radiation therapy; all of the enrolled patients have their informed consent to the study.

Tissue samples representative of the tumour, non-neoplastic lung parenchyma (normal tissue sections and reactive lung parenchyma adjacent to carcinomas) and five microscopically normal lung specimens were routinely fixed in 10% buffered neutral formalin, and processed for conventional histopathological examination. Immediately adjacent neoplastic specimens and 21 macroscopically non-neoplastic tissue samples (taken as far as possible from the neoplastic area) were snap frozen in liquid nitrogen within 10 min of surgical resection, and standard 5 μm thick sections from paraffin-embedded blocks were stained with hematoxylin and eosin, and examined by light microscopy. Tumour histotype and stage were, respectively, determined according to the WHO classification of lung and pleural tumours (1999) and the TNM staging system (1997). All of the non-neoplastic samples were microscopically judged to be benign.

The clinical follow-up of 43 patients was available for a mean post-surgical period of 28 months (range: 12—45); the follow-up data were obtained by means of direct patient contact at 2-month intervals for the first 2 years and 4-month intervals thereafter. At the time of the last follow-up, 13 patients had died for cancer-related causes. The clinicopathological characteristics of the study patients are shown in Table 1.

2.2. Tissue homogenisation and protein quantification

Fresh frozen lung tissues were homogenised under isotonic condition (25 mM Tris—HCl, pH 7.4, 250 mM sucrose, 1% Triton X-100 (v/v), 1% NP-40 (v/v), 5 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulphonylfluoride, 1 mM aprotinin, 1 mM leupeptin, 2 mM sodium orthovanadate, 2 mM sodium floride) using an ULTRA-TURRAX T25 homogeniser (IKA-labortechnik GmbH, Staufen, Germany). The homogenates were centrifuged at 2800 rpm for 20 min at 4 °C, after which the supernatants were collected and their protein concentration determined by means of Lowry Ciocalteau's method using bovine serum albumin as standard.

2.3. Western blotting

Equal amounts of solubilised protein (75 μg) were heated in Laemmli sample buffer (Biorad, Hercules, CA) containing 2-β mercaptoethanol (70 mM), separated by one-dimensional SDS-PAGE gel under reducing conditions, and electroblotted onto nitrocellulose membrane. The membrane was blocked in TBS with 0.05% Tween-20 (v/v), containing 5% slim milk

Table 1 Clinicopathological characteristics of NSCLC patients and tumours

Clinicopathological characteristics	No. of cases (%)
Age (years)	
Mean	65
Range	22—79
Gender	
Man	48 (80)
Woman	12 (20)
Tumour histotype	
ADK	42 (70)
SQ	11 (18)
Others[a]	7 (12)
Tumour grade	
G1	2 (3)
G2	26 (44)
G3	32 (53)
Pathological tumour stage	
T1	8 (13)
T2	38 (63)
T3	3 (5)
T4	11 (18)
N0	29 (48)
N1, N2	31 (52)
Stage grouping	
I	24 (40)
II	16 (27)
III	18 (30)
IV	2 (3)

ADK: adenocarcinoma; SQ: squamous cell carcinoma.
[a] Two adenosquamous carcinomas; three large-cell carcinomas; two pleomorphic carcinomas.

(w/v), and probed with anti-FAK polyclonal antibody specific for the C-terminal portion of the protein (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. The membrane was then incubated with goat anti-rabbit IgG-horseradish peroxidase (1:10,000 dilution; Chemicon, Temecula, CA). p53 and β_1-Integrin expression was assessed using specific polyclonal antibodies (1:200 dilution, Santa Cruz Biotechnology). Membranes were then incubated using anti-β-actin (1:200 dilution; Santa Cruz Biotechnology) as internal control. FAK activation was investigated using the FAK pY Sampler Pack (Biosource, Camarillo, CA), which contains polyclonal antibodies recognising phosphorylated tyrosines (Tyr397, Tyr407, Tyr576, Tyr577, Tyr861); FAK phosphorylation was normalised to its expression levels. The proteins were visualised by means of an enhanced chemiluminescence detection system (ECL™, Amersham, Arlington Heights, IL). After being acquired using a GelDoc™ image capture system (Biorad), the autoradiograms were quantified using Quantity One™ software.

2.4. Immunohistochemistry

Five micrometer thick sections representative of the tumour and non-neoplastic lung parenchyma were immunostained using a standard avidin—biotin—peroxidase complex (ABC) technique and the Super Sensitive Non-Biotin HRP Detection System (Menarini, Florence, Italy), with the anti-FAK antibody that recognises the N-terminal portion of the protein (Santa Cruz Biotechnology). Briefly, the slides were dewaxed, rehydrated and processed using an Optimax Plus automated immunostainer (BioGenex, San Ramon, CA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in distilled water for 10 min and, after extensive washing in TBS/Triton X-100, the slides were incubated with the primary antibody (1:500 dilution) overnight at 4 °C; 3,3′-diaminobenzidine was used as the chromogenic substrate. Positive and negative controls were included. Each tissue section was separately evaluated by two pathologists (MF and SB) who were unaware of the clinicopathological characteristics.

The intensity of FAK immunostaining (1 = weak, 2 = moderate, and 3 = intense) and the percentage of positive tumour cells (0% = negative, 1—40% = 1, 41—70% = 2, and >70% = 3) were assessed in at least five areas at ×400 magnification; in the case of heterogeneous immunostaining, the predominant pattern was used for scoring purposes. The scores of each case were multiplied to give a final score of 0, 1, 2, 3, 4, 6 or 9, and the tumours were finally classified as negative: score 0; low expressors (LE): score ≤ 3; intermediate expressors (IE): score 4; or high expressors (HE): score ≥ 6. The staining of the adjacent non-neoplastic parenchyma in each section was also evaluated. The intensity of FAK immunostaining (1 = weak, 2 = moderate, and 3 = intense) and the percentage of positive tumour cells (0% = negative, 1—40% = 1, 41—70% = 2, and >70% = 3) were assessed in at least five areas at ×400 magnification; in the case of heterogeneous immunostaining, the predominant pattern was used for scoring purposes. The scores of each case were multiplied to give a final score of 0, 1, 2, 3, 4, 6 or 9, and the tumours were finally classified as negative: score 0; low expressors (LE): score ≤ 3; intermediate expressors (IE): score 4; or high expressors (HE): score ≥ 6. The staining of the adjacent non-neoplastic parenchyma in each section was also evaluated.

2.5. Real-time RT-PCR

FAK mRNA levels were quantified by real-time quantitative RT-PCR based on TaqMan™ technology, using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described [30]. The amount of FAK mRNA was normalised to an endogenous reference (β-actin, ACTB) and expressed as *n*-fold FAK mRNA levels in relation to a calibrator or 1× sample (MCF7 cell line). The amount of target was determined by applying the $\Delta\Delta C_t$ method (Applied Biosystems Sequence Detector User Bulletin #2).

2.5.1. RNA extraction, cDNA synthesis and real-time RT-PCR conditions

Total RNA was isolated from the frozen and homogenised neoplastic and non-neoplastic tissues using a commercial guanidinium isothiocyanate-based kit (RNeasy®, Qiagen, Valencia, CA) according to the manufacturer's instruc-

tions. The amount of RNA in the sample was quantified spectrophotometrically, and 100 ng/sample was used for cDNA synthesis.

The reaction mixtures for the reverse transcription and real-time RT-PCR were prepared using Applied Biosystems reagents according to the manufacturer's instructions; the reactions for real-time quantitative amplification were performed in duplicate.

2.5.2. Primers and probes

A FAK-specific gene expression assay, including cDNA-specific primers and a TaqMan™ MGB probe, was performed using Assay-on-Demand chemistry (Hs00178587_m1, Applied Biosystems). The primers and TaqMan™ probe for β-actin mRNA were designed to be intron-spanning using Primer Express Software (Applied Biosystems) in order to avoid amplifying genomic DNA. The nucleotide sequences of the primers and probe were ACTB forward: 5′-CCTTCCTGGGCATGGAG-3′; ACTB reverse: 5′-AAGGAGGAGCATGATCTTGATCTT-3′; ACTB probe: 5′-(6-Fam)-CCTGTGGC ATCCACGAAACTACCTTC-(Tamra)-3′.

2.6. Statistical analyses

The Western blotting results were statistically evaluated using Fisher's exact test and the *t*-test; the immunohistochemical data were analysed using the χ^2-test.

An independent *t*-test and Wilcoxon's two-group test were used for the real-time RT-PCR analysis; the results of the matched NSCLC and non-neoplastic lung tissues were compared using an adjusted *t*-test and Wilcoxon's test, and the graphical representation was based on a quartile distribution.

All of the analyses were performed using Ministat 2000 (2.1 version, Pubblicazioni Medico Scientifiche, Udine, Italy) and GraphPad Prism 4.0 software; *p*-values of <0.05 were considered statistically significant.

3. Results

3.1. FAK Western blotting

Western blotting was used to evaluate p125^{FAK} protein expression in 16 NSCLCs (six stage I, six stage II, four stage III) (Table 1) and paired non-neoplastic lung parenchymas. FAK expression was significantly increased in 13 NSCLC samples in comparison with their non-neoplastic counterparts. The Western blotting of seven samples is shown in Fig. 1(panel A); the ratio between the optical density of the neoplastic (T) and non-neoplastic (N) tissues (OD T/N) of the same patients was calculated and expressed graphically (Fig. 1, panel B). The increased expression of FAK in the NSCLCs is documented by the mean ratio of 2.98 (range 0—7.3), and comparable results were obtained using a polyclonal antibody specific for the FAK N-terminal portion (data not shown). FAK activity is regulated by tyrosine phosphorylation [32], and so the phosphorylation status of FAK tyrosine residues measured by specific antibodies can be considered an index of protein activation. All of the evaluated tyrosine residues (see Section 2) were phosphorylated in the neoplastic and non-neoplastic specimens, with no quan-

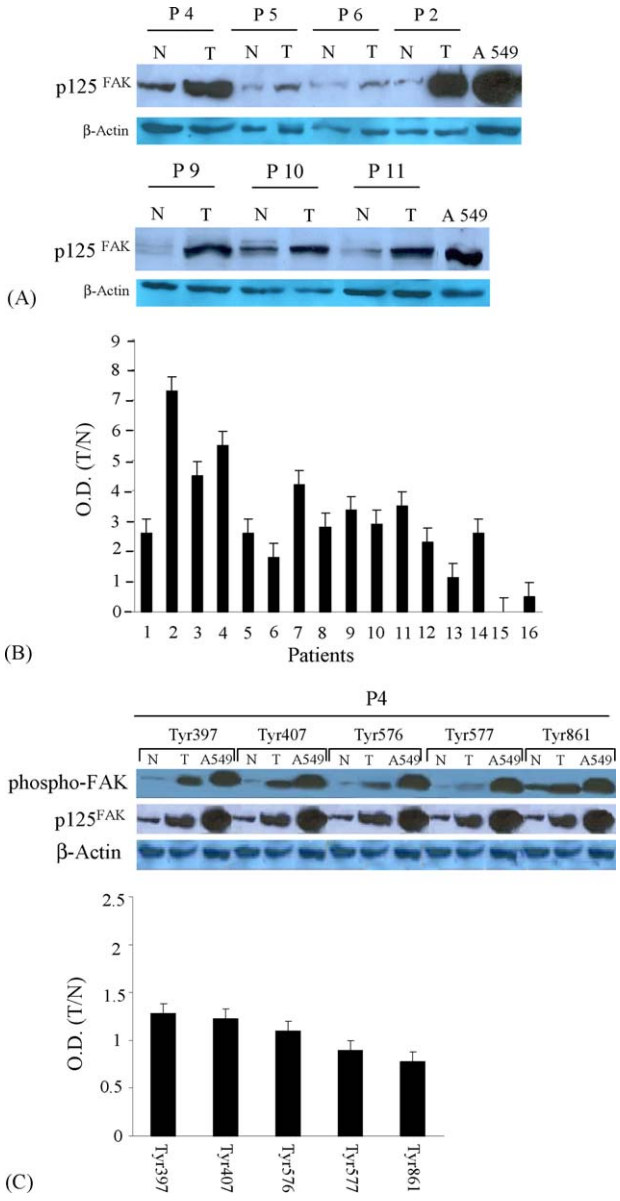

Fig. 1 Expression of p125^{FAK}. (A) Western blot of p125^{FAK} expression in matched neoplastic (T) and non-neoplastic (N) homogenised tissues from 7 of 16 NSCLC patients; as a positive control, the expression of FAK was determined in the A549 cell line. Band intensities clearly indicate significant FAK up-regulation in neoplastic tissue in comparison with the non-neoplastic parenchyma of the same patient. (B) Plot of quantitative densitometric ratio between neoplastic and non-neoplastic tissue (OD T/N) from all 16 patients. FAK immunolabelling is greater in the neoplastic tissue (mean 2.98). (C) Tyrosine phosphorylation of FAK studied by means of polyclonal antibodies recognising specific tyrosines (Tyr397, Tyr407, Tyr576, Tyr577, Tyr861; see Section 2). FAK phosphorylation was densitometrically quantified and normalised to protein expression levels. The FAK phosphorylation ratio (OD T/N) between neoplastic and non-neoplastic tissue in one patient (P4) is reported; the same analysis was made in all other 15 patients with similar results (data not shown). Mean values ± S.E. of two independent experiments with similar results.

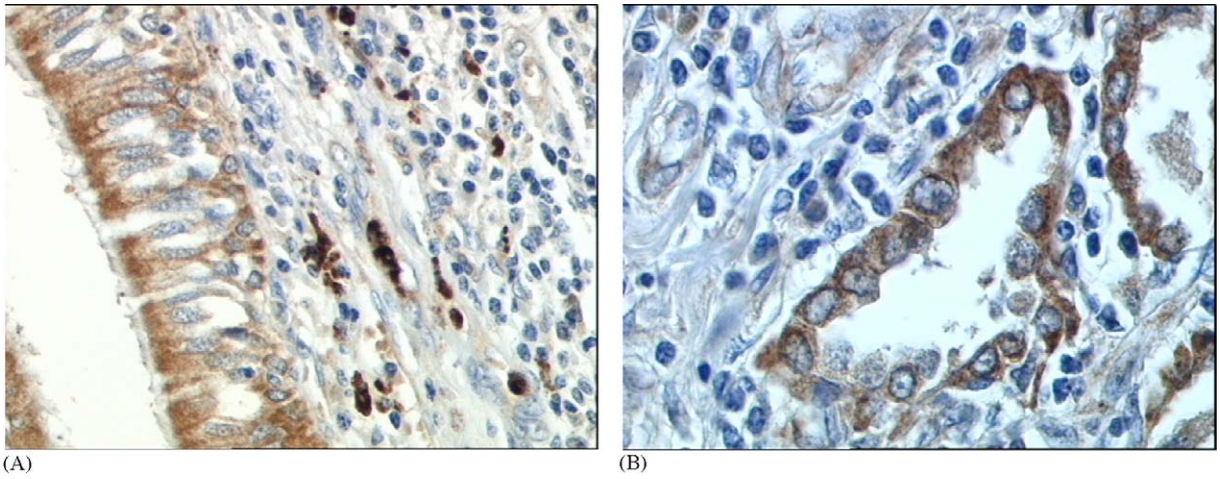

Fig. 2 FAK protein expression in non-neoplastic lung parenchyma. (A) FAK immunostaining in ciliated and basal epithelial cells of the bronchial mucosa. (B) Intense FAK protein accumulation in the cytoplasm and perinuclear cisterns of hyperplastic alveolar cells. In both images, FAK immunostaining can also be seen in some inflammatory elements of the subepithelial stromal tissue. Original magnification: (A) ×400 and (B) ×600.

titative difference between the two tissue types (Fig. 1, panel C). These results suggest that the up-regulation of FAK expression does not correlate with increased activation in NSCLCs.

3.2. Tissue localisation of FAK by immunohistochemistry

FAK immunoreactivity was detected in both normal and neoplastic lung cells. In normal bronchus, FAK protein expression was detected in epithelial ciliated and basal cells, chondrocytes, and the ductal and serous cells of the submucosal glands; FAK immunostaining was also detected in the endothelial cells of the vessels within the lung parenchyma. Immunoreactivity was greater in reactive lesions, such as squamous metaplastic bronchial epithelium, and in the hyperplastic/reactive pneumocytes surrounding the neoplasia. Fig. 2 shows FAK protein immunodetection in non-neoplastic lung parenchyma.

In comparison with the non-neoplastic lung parenchymas, FAK was overexpressed in every neoplastic tissue section. Furthermore, FAK immunoreactivity was stronger in the NSCLCs than in previously described reactive lung lesions, with moderate-strong immunoreactivity in between 40% and more than 90% of tumour cells. Forty-six of the NSCLCs (77%) were HE (moderate-intense immunoreactivity in more than 70% neoplastic cells; scores 6 and 9), 10 (17%) were IE (moderate immunoreactivity in 40—70% of neoplastic cells; score 4), and 4 (7%) were LE (moderate-intense immunoreactivity in less than 40% neoplastic cells; scores 2 and 3). FAK was also observed in the endothelial cells of the vessels within the neoplastic stroma.

Furthermore, as shown in Table 2, there was no significant difference in FAK immunostaining between the two most represented tumour histotypes (ADK versus SQ, $p = 0.947$, χ^2-test). Examples of FAK immunoreactivity in adenocarcinoma and squamous cell carcinoma are shown in Fig. 3.

3.3. Quantitative evaluation of FAK mRNA by real-time RT-PCR

FAK mRNA levels were investigated in 60 NSCLCs and 21 matched non-neoplastic lung tissues (16 of these matched samples were also studied by Western blotting, see Fig. 1). The non-neoplastic lung samples were used to establish the basal levels of FAK mRNA, and FAK transcripts ranging from 0.5*n* to 3.3*n* (mean 1.3*n*; median 1.1*n*) were determined in all of them. Three tumour samples were discarded because of RNA degradation, but FAK mRNA was assayed in all of the remaining 57 and ranged from 0.5*n* to 4.5*n* (mean 1.8*n*; median 1.5*n*). Both Wilcoxon's test and the *t*-test showed that FAK mRNA levels were significantly higher in the NSCLCs whether all of the samples were considered ($p = 0.016$ and 0.013) (Fig. 4), or just the 21 matched neoplastic and non-neoplastic lung samples ($p = 0.038$ and 0.04).

3.4. Comparative analyses and clinicopathological correlation

The Western blotting and immunohistochemical evaluations of FAK expression were comparable: the NSCLC cases with a high level of FAK protein content also showed intense

Table 2 FAK protein expression in NSCLCs

Tumour histotype	FAK immunostaining		
	HE[a]	IE[a]	LE[a]
ADK, $n = 42$ (70%)	34 (81)	6 (14)	2 (5)
SQ, $n = 11$ (18%)	9 (82)	1 (9)	1 (9)
Others, $n = 7$ (12%)	3 (43)	3 (43)	1 (14)

Values in parentheses are in percent.
[a] HE: high expressors; IE: intermediate expressors; LE: low expressors. See Section 2 for tumour classification of FAK immunoreactivity.

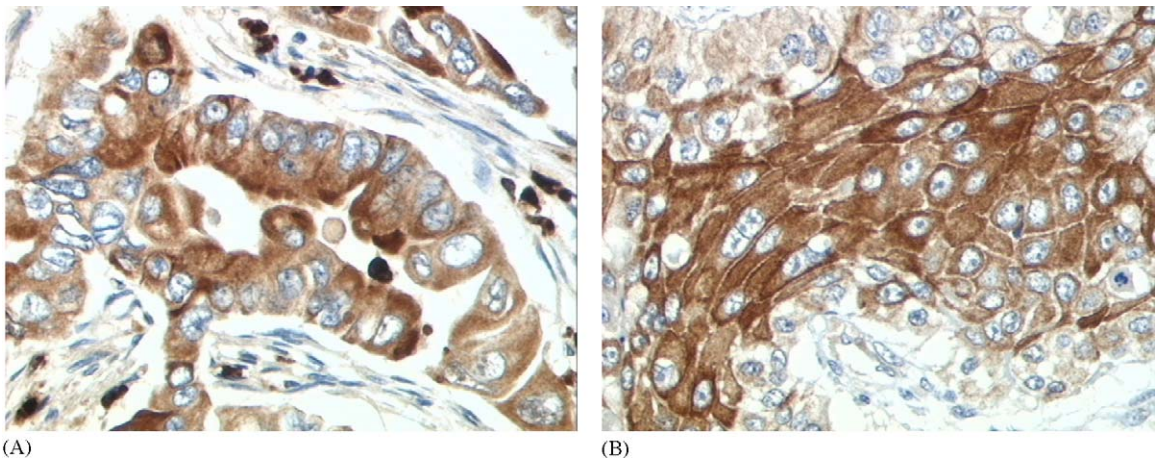

Fig. 3 FAK protein overexpression in NSCLCs as detected by immunohistochemistry. (A) Lung adenocarcinoma and (B) lung squamous cell carcinoma. Original magnification: (A) ×600 and (B) ×400.

immunostaining. Considering all of the FAK protein expression values, Western blotting showed a statistically significant correlation between higher protein levels and a high tumour stage (stage I + II versus stage III + IV, $p = 0.019$). The FAK mRNA overexpression revealed by real-time RT-PCR not only correlated with a high tumour stage (stage I + II versus stage III + IV, $p = 0.028$), but there was also a statistically significant association between FAK mRNA level and tumour size (T1 versus T2, T3 and T4, $p = 0.046$).

The association between FAK mRNA measured by real-time RT-PCR and the presence of node metastases at the time of resection was also observed. The 21 matched specimens were analysed separately and sub-classified on the basis of the expression ratio between the neoplastic and non-neoplastic sample (a two-fold greater expression of FAK mRNA in tumours was set as threshold). There was a trend toward a correlation between a two-fold increase in mRNA levels and the presence of positive nodes (N1 or N2), as the majority of these overexpressing tumours (5/7) were N-positive; among the neoplastic specimens with mRNA levels that were ≤2-fold higher, there was a casual distribution (8 N-negative and 6 N-positive) (data not shown).

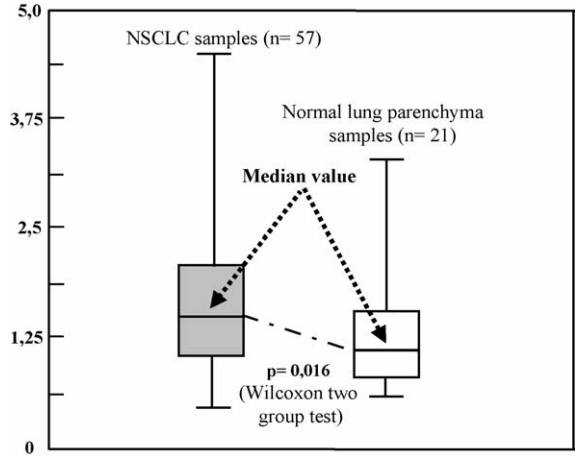

Fig. 4 Box plot diagram of FAK mRNA expression analysed by real-time RT-PCR. FAK mRNA levels were evaluated in 57 neoplastic and 21 non-neoplastic lung tissues (21 matched samples). FAK mRNA level (expressed as *n*-fold the calibrator sample MCF7 cell line) was significantly higher in the neoplastic than the non-neoplastic lung parenchyma ($p = 0.016$, Wilcoxon's two-group test). Median values indicated by arrows.

3.5. Expression of β_1-integrin and p53 in NSCLCs

The correlations between FAK up-regulation and changes in other adhesion- and apoptosis-related proteins were evaluated by Western blotting in 12 of the above samples. The expression of β_1-integrin was comparable in the neoplastic and non-neoplastic lung tissues ($p = 0.13$, *t*-test), as can be seen in the example of Western blotting and densitometric analysis of all of the examined cases in Fig. 5 (panel A). The up-regulation of FAK therefore seems to be specific among adhesion-related molecules. We also assessed the pro-apoptotic p53 protein, which is capable of suppressing FAK expression [31], and found that the increased FAK expression in NSCLCs did not correspond to changes in p53 expression in any of the 12 cases ($p = 0.3$, *t*-test) (Fig. 5, panel B).

4. Discussion

FAK is a non-receptor tyrosine kinase involved in cell motility [7], migration [8], apoptosis [9] and cell matrix interactions [6]. All of these processes are crucial for neoplastic transformation, invasion and dissemination, and suggest a potential role of FAK in the development and progression of cancer [14]. FAK is overexpressed in a variety of human tumours including breast [17], colorectal [17,33], prostate [18,34], thyroid [19], ovarian [20], endometrial [35], and brain cancers [21]. Agents aimed at inhibiting the FAK signal transduction pathway may be useful in the development of effective new anticancer therapies [14,36]. Inhibited FAK activity or modulated FAK expression have recently been associated with the decreased resistance of human pancreatic adenocarcinoma cells to acquired apoptosis with detachment from the ECM [37], the increased sensitivity of transformed human kidney epithelial cells to 5-fluorouracil

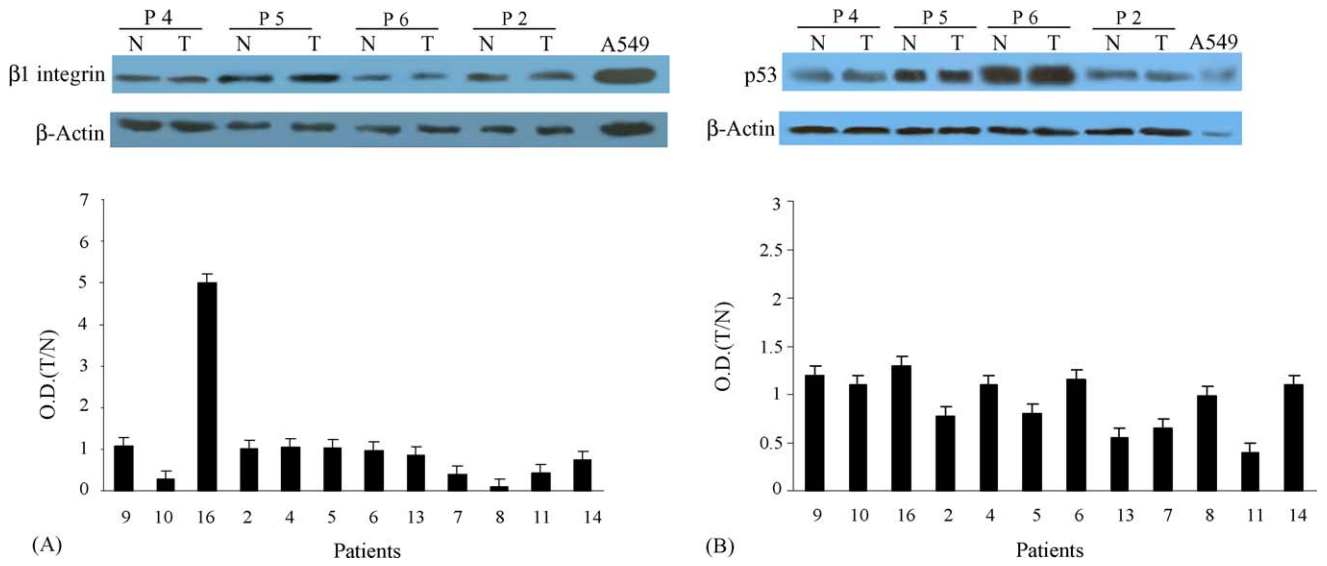

Fig. 5 FAK regulation in NSCLCs. β_1-integrin (A) and p53 (B) expression in matched neoplastic (T) and non-neoplastic homogenates (N) of 12 NSCLC patients. Western blotting of four patients is shown; the quantitative densitometric ratio between the neoplastic and non-neoplastic tissue (OD T/N) of all 12 analysed patients are plotted under the gel images. As a gel loading control, the membranes were exposed to β-actin antibody. Mean values ± S.E. of two independent experiments with similar results.

[30], and enhanced gemcitabine-induced *in vitro* and *in vivo* cytotoxicity [26].

This paper describes FAK expression in NSCLCs and non-neoplastic lung parenchyma for the first time. We analysed 60 tumours by means of immunohistochemistry and quantitative real-time RT-PCR, 16 of which were also analysed by Western blotting, and provide immunohistochemical and molecular evidence of FAK up-regulation in NSCLCs. FAK distribution within neoplastic tissue was quite diffuse and homogeneous, thus supporting its potential role in conferring an advantage on growth and survival, and giving tumour cells more invasive behaviour [16]. We also found that FAK up-regulation is common in NSCLCs, regardless of tumour histotype and grade: weak-moderate FAK immunostaining was detected in ciliated basal epithelial cells, ductal cells of the submucosal glands of the bronchus, as well as in chondrocytes and vessel endothelial cells of non-neoplastic lung parenchyma. Interestingly, more intense FAK protein immunostaining was observed in bronchial squamous metaplastic epithelium and hyperplastic alveolar cells, which are considered reactive changes that may evolve into dysplastic and neoplastic transformations.

These immunohistochemical observations may partially explain our quantitative Western blotting and real-time RT-PCR evaluations of FAK protein and mRNA in non-neoplastic lung tissues. FAK mRNA levels were significantly higher in the neoplastic than in the non-neoplastic lung samples, which is consistent with previous observations of FAK overexpression in human malignancies [16]. Moreover, all of the neoplastic samples whose FAK overexpression was determined by Western blotting and real-time RT-PCR came from patients with a high tumour stage at the time of resection. Taken together, these findings suggest that FAK up-regulation may be an early event in lung neoplastic transformation, and further support previously published data favouring its crucial role in the development and maintenance of human cancers [17,32,33].

We also found that the expression of β_1-integrin does not change in NSCLCs, and so it is possible that FAK up-regulation may be specific to NSCLCs. Furthermore, the up-regulation is not accompanied by any changes in FAK activity, as assessed by evaluating the extent of protein phosphorylation [32]: the five tyrosine residues analysed (Tyr397, Tyr407, Tyr576, Tyr577 and Tyr861) proved to be equally phosphorylated in the neoplastic and non-neoplastic tissues. It is known that one of the possible mechanisms involved in FAK regulation is the action of p53, which inhibits FAK transcription [31], but we found that the expression of p53 in our tumour samples overexpressing FAK was comparable with that in the control non-tumoural lung tissues. It is possible that the up-regulation of FAK may be due to the amplification of its gene or p53 gene mutations.

FAK overexpression in human malignancies has been associated with cancer cell migration and invasiveness, and more aggressive tumour behaviour including distant and/or nodal metastases [17,23,24,38,39]. The lack of FAK signalling in the endothelial cells of FAK−/− mice embryos has shown that the function of this protein is crucial for morphogenesis and the formation of blood vessels [40]. In addition, FAK activation in mouse microvascular endothelial cells is necessary to regulate *in vitro* tube formation [41]. We found FAK immunoreactivity in the endothelial cells of both neoplastic and adjacent non-neoplastic lung parenchyma, similarly to previously described findings in the vascular network of oral squamous cancers [41], and the microvascular endothelial cells of malignant astrocytic tumours [42]. These characteristics may explain the invasive and metastatic malignant phenotype of tumours overexpressing FAK.

In conclusion, our study provides the first immunohistochemical and molecular evidence that increased FAK transcript levels and protein expression are common events in NSCLC. By immunohistochemistry, FAK protein overexpression was also detectable in reactive changes, such as the squamous cell metaplasia of bronchial epithelial cells

and alveolar hyperplasia. The immunohistochemical detection of FAK overexpression in all of our cases regardless of tumour stage supports the hypothesis that FAK may play a role in maintaining the malignancy of the morphological and biological characteristics of transformed lung cells, rather than a crucial role in the later stages of NSCLC progression. The detection of FAK overexpression, particularly at protein level, may represent a novel target for inhibitory therapies in lung cancer [43]. However, the up-regulation of FAK could not be statistically related to disease-free or overall survival in our patients probably because of the short follow-up period. The prognostic significance of FAK in NSCLCs therefore requires further evaluation.

Acknowledgement

This work was supported in part by a grant from *Associazione Italiana Ricerca sul Cancro* (AIRC).

References

[1] Aplin AE, Howe AK, Juliano RL. Cell adhesion molecules, signal transduction and cell growth. Curr Opin Cell Biol 1999;11: 737—44.
[2] Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT. pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA 1992;89:5192—6.
[3] Cobb BS, Schaller MD, Leu TH, Parsons JT. Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. Mol Cell Biol 1994;14:147—55.
[4] Owen JD, Ruest PJ, Fry DW, Hanks SK. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. Mol Cell Biol 1999;19:4806—18.
[5] Schaller MD, Otey CA, Hildebrand JD, Parsons JT. Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J Cell Biol 1995;130:1181—7.
[6] Schlaepfer DD, Hanks SK, Hunter T, van der Geer P. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature 1994;372:786—91.
[7] Cary LA, Chang JF, Guan JL. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J Cell Sci 1996;109:1787—94.
[8] Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, et al. FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol 2000;2:249—56.
[9] Xu LH, Owens LV, Sturge GC, Yang X, Liu ET, Craven RJ, et al. Attenuation of the expression of the focal adhesion kinase induces apoptosis in tumor cells. Cell Growth Differ 1996;7:413—8.
[10] Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. Mol Cell Biol 1994;14:1680—8.
[11] Chen HC, Guan JL. Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. Proc Natl Acad Sci USA 1994;91:10148—52.
[12] Zhang X, Chattopadhyay A, Ji QS, Owen JD, Ruest PJ, Carpenter G, et al. Focal adhesion kinase promotes phospholipase C-gamma1 activity. Proc Natl Acad Sci USA 1999;96:9021—6.
[13] Han DC, Guan JL. Association of focal adhesion kinase with Grb7 and its role in cell migration. J Biol Chem 1999;274: 24425—30.
[14] Hauck CR, Hsia DA, Punte XS, Cheresh DA, Schlaepfer DD. FRNK blocks v-Src stimulated invasion and experimental metastases without effects on cell motility or growth. EMBO J 2002;21:6289—302.
[15] Weiner TM, Liu ET, Craven RJ, Cance WG. Expression of growth factor receptors, the focal adhesion kinase, and other tyrosine kinases in human soft tissue tumors. Ann Surg Oncol 1994;1:18—27.
[16] Gabarra-Niecko V, Schaller MD, Dunty JM. FAK regulates biological processes important for the pathogenesis of cancer. Cancer Metastasis Rev 2003;22:359—74.
[17] Cance WG, Harris JE, Iacocca MV, Roche E, Yang X, Chang J, et al. Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: correlation with preinvasive and invasive phenotypes. Clin Cancer Res 2000;6:2417—23.
[18] Tremblay L, Hauck W, Aprikian AG, Begin LR, Chapdelaine A, Chevalier S. Focal adhesion kinase (pp125FAK) expression, activation and association with paxillin and p50CSK in human metastatic prostate carcinoma. Int J Cancer 1996;68:164—71.
[19] Owens LV, Xu L, Dent GA, Yang X, Sturge GC, Craven RJ, et al. Focal adhesion kinase as a marker of invasive potential differentiated human thyroid cancer. Ann Surg Oncol 1996;3:100—5.
[20] Judson PL, He X, Cance WG, Van Le L. Overexpression of focal adhesion kinase, a protein tyrosine kinase, in ovarian carcinoma. Cancer 1999;86:1551—6.
[21] Natarajan M, Hecker TP, Gladson CL. FAK signaling in anaplastic astrocytoma and glioblastoma tumors. Cancer J 2003;9(2):126—33.
[22] Weiner TM, Liu ET, Craven RJ, Cance WG. Expression of focal adhesion kinase gene and invasive cancer. Lancet 1993;342:1024—5.
[23] Fujii T, Koshikawa K, Nomoto S, Okochi O, Kaneko T, Inoue S, et al. Focal adhesion kinase is overexpressed in hepatocellular carcinoma and can be served as an independent prognostic factor. J Hepatol 2004;41:104—11.
[24] Recher C, Ysebaert L, Beyne-Rauzy O, Mansat-De Mas V, Ruidavets JB, Cariven P. Expression of focal adhesion kinase in acute myeloid leukemia is associated with enhanced blast migration, increased cellularity, and poor prognosis. Cancer Res 2004;64:3191—7.
[25] Kornberg L, Fleigel J. The effects of inducible overexpression of FAK-related non-kinase (FRNK) on a transformed epithelial cell line. Anticancer Res 2003;23:91—7.
[26] Duxbury MS, Ito H, Benoit E, Zinner MJ, Ashley SW, Whang EE. RNA interference targeting focal adhesion kinase enhances pancreatic adenocarcinoma gemcitabine chemosensitivity. Biochem Biophys Res Commun 2003;311:786—92.
[27] Beinke C, Van Beuningen D, Cordes N. Ionizing radiation modules of the expression and tyrosine phosphorylation of the focal adhesion-associated proteins focal adhesion kinase (FAK) and its substrates p130cas and paxillin in A549 human lung carcinoma cells *in vitro*. Int J Radiat Biol 2003;79:721—31.
[28] Parkin DM, Pisani P, Ferlay J. Global cancer statistics. CA Cancer J Clin 1999;49:33—64.
[29] Wingo PA, Ries LA, Parker SL, Heath Jr CW. Long-term cancer patient survival in the United States. Cancer Epidemiol Biomarkers Prev 1998;7:271—82.
[30] Falleni M, Pellegrini C, Marchetti A, Oprandi B, Buttitta F, Barassi F, et al. Survivin gene expression in early-stage non-small cell lung cancer. J Pathol 2003;200:620—6.
[31] Golubovskaya V, Kaur A, Cance W. Cloning and characterization of the promoter region of human focal adhesion kinase gene: nuclear factor kappa B and p53 binding sites. Biochim Biophys Acta 2004;1678:111—25.
[32] Schlaepfer DD, Mitra SK, Ilic D. Control of motile and invasive cell phenotypes by focal adhesion kinase. Biochim Biophys Acta 2004;1692:77—102.

[33] Lark AL, Livasy CA, Calvo B, Caskey L, Moore DT, Yang X, et al. Overexpression of focal adhesion kinase in primary colorectal carcinomas and colorectal liver metastases: immunohistochemistry and real-time PCR analyses. Clin Cancer Res 2003;9:215—22.
[34] Rovin JD, Frierson Jr HF, Ledinh W, Parsons JT, Adams RB. Expression of focal adhesion kinase in normal and pathologic human prostate tissues. Prostate 2002;53:124—32.
[35] Livasy CA, Moore D, Cance WG, Lininger RA. Focal adhesion kinase overexpression in endometrial neoplasia. Appl Immunohistochem Mol Morphol 2004;12:342—5.
[36] McLean GW, Avizienyte E, Frame MC. Focal adhesion kinase as a potential target in oncology. Expert Opin Pharmacother 2003;4:227—34.
[37] Duxbury MS, Ito H, Zinner MJ, Ashley SW, Whang EE. Focal adhesion kinase gene silencing promotes anoikis and suppresses metastasis of human pancreatic adenocarcinoma cells. Surgery 2004;135:555—62.
[38] Oktay MH, Oktay K, Hamele-Bena D, Buyuk A, Koss LG. Focal adhesion kinase as a marker of malignant phenotype in breast and cervical carcinomas. Hum Pathol 2003;34:240—5.
[39] Itoh S, Maeda T, Shimada M, Aishima S, Shirabe K, Tanaka S, et al. Role of expression of focal adhesion kinase in progression of hepatocellular carcinoma. Clin Cancer Res 2004;10:2812—7.
[40] Ilic D, Kovacic B, McDonagh S, Jin F, Baumbusch C, Gardner DG, et al. Focal adhesion kinase is required for blood vessel morphogenesis. Circ Res 2003;92(3):255—7.
[41] Haskell H, Natarajan M, Hecker TP, Ding Q, Stewart Jr J, Grammer JR, et al. Focal adhesion kinase is expressed in the angiogenic blood vessels of malignant astrocytic tumors *in vivo* and promotes capillary tube formation of brain microvascular endothelial cells. Clin Cancer Res 2003;9:2157—65.
[42] Kornberg LJ. Focal adhesion kinase and its potential involvement in tumor invasion and metastasis. Head Neck 1998;20(8):745—52.
[43] McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. The role of focal-adhesion kinase in cancer—a new therapeutic opportunity. Nat Rev Cancer 2005;5(7):505—15.