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Characterization of the Focal Adhesion Complex in Human Non-small cell Lung Cancer Cell Lines

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Abstract. *Background: The important metastatic potential of lung cancers is directly correlated with cell adhesion. Cell-extracellular matrix interactions occur in specialized structures termed focal adhesion (FA) complexes. Our aims were to investigate: i) the expression of the major FA components in three lung cancer cell lines (non metastatic: A549, or metastatic: Calu-1 and H460), ii) the modifications of the FA complex occurring when apoptosis was induced by Vinorelbine in the A549 cells. Materials and Methods. The FA complex was characterized by flow cytometry, immunocytochemical staining and Western blot. Results: The expressions of α3, β1, paxillin, p-paxillin and Grb2 varied depending on the histological type of the tumor. In apoptotic cells, the expressions of the PYK2, p-p38, PI3K and Grb2 adhesion proteins were increased. Conclusion: Our data suggest that these adhesion proteins may be implicated in the transduction of death signals.*

Lung cancers are the most frequent tumors in the world and represent the first cause of death by cancer (1). The bad prognosis of these tumors is related to late diagnosis and a strong metastatic potential, which is directly correlated with cell adhesion and detachment, important steps in tumor metastasis. Cell-cell and cell-extracellular matrix (ECM) interactions play a key-role in the invasive mechanism. Moreover, in most cancers, adhesion proteins are mutated and induce cell signaling dysfunctions (2). This study was focused on the cell-ECM interactions that occur in the highly regulated and specialized structures called focal adhesions (FA). FA represent contact zones between the exterior (ECM) and the interior (cytoskeleton) of the cell (2, 3), permitting the attachment of the cellular membrane to its substrate and the transduction of signals. Within the cell, signal transduction occurs through numerous and inter-related pathways, consisting mainly of successive phosphorylations on FA proteins. Integrins are heterodimeric proteins (composed of α and β chains) and represent the first components of FA. When these transmembrane receptors are bound to their ECM-ligand they mediate cell survival signals, whereas the loss of integrin-mediated cell attachment induces apoptosis (4, 5). However, integrins do not possess any enzymatic activity, but they initiate signaling through association with non-receptor kinases such as focal adhesion kinase (FAK) and the Src family of kinases. These kinases, in turn, activate several downstream pathways (3). FAK is a key-component of the FA, representing the converging point of different signaling pathways; FAK can interact with structural proteins such as paxillin, talin, vimentin, or regulator proteins such as PYK2 (prolin-rich tyrosine kinase 2), Grb2 (growth factor receptor-bound 2), PI3K (phosphatidyl inositol-3-kinase), Akt and ERK/MAPK (2, 6-9). Signals are finally transduced to the nucleus, where they induce the activation of transcription factors and thus determine cell behaviors such as cell attachment, which is essential to survival in many cell types (2). Moreover, changes occur in the FA complex during apoptosis. For instance, it has been shown that caspase-3, the final effector of apoptosis, can cleave FAK (10, 11).

The aims of our study were: i) to characterize the FA complex in three human non-small cell lung cancer (NSCLC) cell lines: A549 (non metastatic), Calu-1 and H460 (metastatic), and ii) to investigate the modifications of the FA complex when apoptosis was induced in the A549 cell line.

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Materials and Methods

Human NSCLC cell lines. Three cell lines, all provided by the ATCC, were used: A549 (adenocarcinoma), H460 (large cell carcinoma), both cultured in RPMI-1640 medium and Calu-1 (epidermoid carcinoma), cultured in Mc Coy's medium. All cell lines were maintained at 37˚C in 5% CO_2–95% air and the culture medium was changed every 2-3 days. Adherent cells were detached by incubation with trypsin (Sigma, Saint-Louis, MO, USA).

Flow cytometry. After fixation and permeabilization (Intrastain kit, Dako, Glostrup, Denmark), the cells were incubated with the primary antibody (see below), followed by addition of the secondary antibody conjugated with FITC: anti-mouse (Silenus, Les Ulis, France, diluted 1/50 in PBS) or anti-rabbit (Caltag Laboratories, San Francisco, CA, USA, diluted 1/10 in PBS). The cells were finally resuspended in 300 μL formaldehyde 1%. A negative control was performed by replacing the primary antibody with an isotypic antibody. The results were expressed as a percentage of positive cells and mean of four independent experiments.

Immunocytochemical staining with alkaline phosphatase. The cells were fixed with acetone and stained with the Vectastain ABC-AP (Alkaline Phosphatase Universal) and Vector Red Alkaline Phosphatase Substrate kits (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. The primary antibody (see below) was diluted 1/50 in PBS. A positive reaction was characterized by a red coloration. A negative control was performed replacing the primary antibody by PBS.

Western blot. The cells were harvested and protein extraction was performed, followed by electrophoresis (8-10% acrylamide gel). Protein transfer was performed on PVDF membranes (Bio-Rad, Hercules, CA, USA) at 0.25 A. The membranes were saturated with skim milk in TBST (0.075% TBS-Tween 20, Sigma). The primary antibody diluted in TBST + 1% skim milk was added and, following overnight incubation at 4˚C, the membranes were rinsed with TBST. The secondary antibody conjugated with peroxidase (Dako), diluted 1/2000 in TBST, was added. Detection was performed with the Super Signal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) and a High Performance Chemiluminescence film (Amersham, Buckinghamshire, UK), and the film was developed and fixed (Kodak, GBX Developer and GBX Fixer, Sigma, Rochester, NY, USA).

Stimulation of intracellular signaling by the induction of apoptosis. To induce apoptosis, cells from the A549 cell line were incubated with Navelbine (NVB, Vinorelbine Ditartrate, Pierre Fabre, France) (100 μg/mL) for 8 hours. The culture medium was changed and the cells were incubated overnight.

Primary antibodies. The primary antibodies used were: isotypic control anti-IgG1, anti-IgG2a and anti-IgM (Immunotech, Marseille, France), isotypic control anti-rabbit (Dako), monoclonal anti-FAK and monoclonal anti-PYK2 (Transduction Laboratories, Bedford, MA, USA), monoclonal anti-α3, anti-α4, anti-α5, anti-β1, anti-β3 integrin subunits (Immunotech), monoclonal anti-vimentin (Dako), monoclonal anti-paxillin (Oncogene, La Jolla, CA, USA), monoclonal anti-PI3K, polyclonal anti-phosphorylated paxillin,

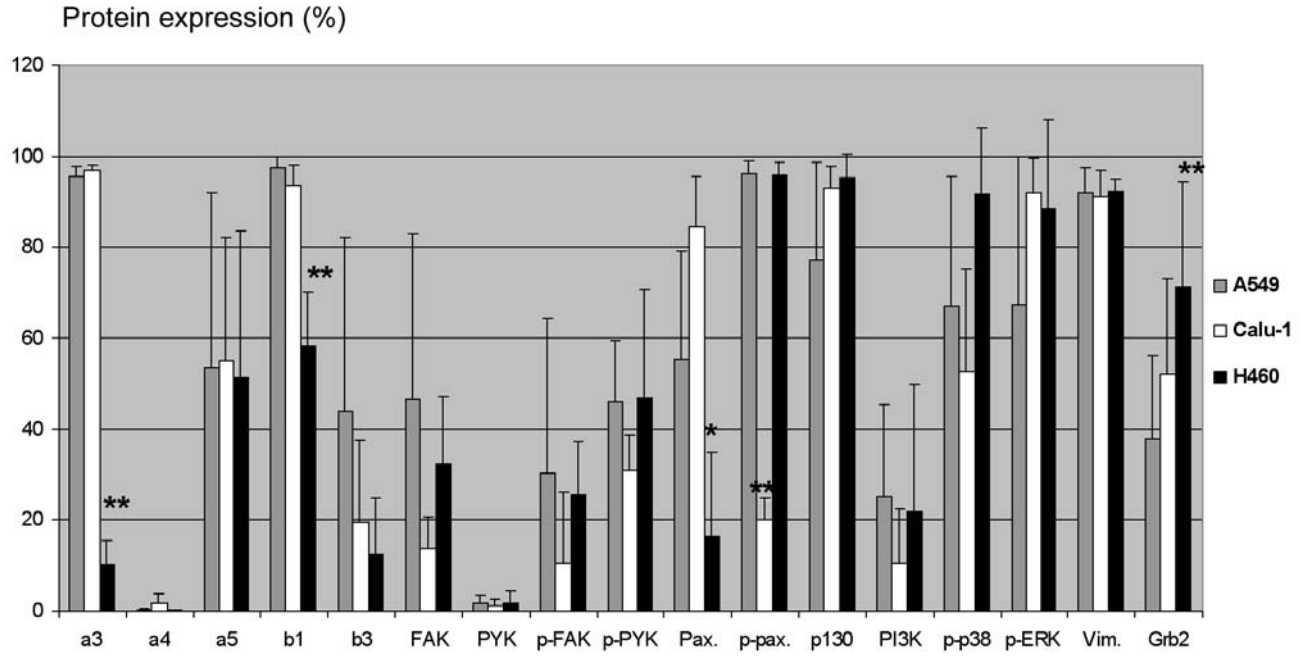

Figure 1. *Characterization of the focal adhesion complex proteins in three non-small cell lung cancer cell lines: A459, Calu-1 and H460, as determined by flow cytometry. Results are expressed as the mean of four independent experiments. Standard deviation and statistical analysis (between A549 and the two other metastatic cell lines) are also indicated (*: $p<0.05$, **: $p<0.01$).*

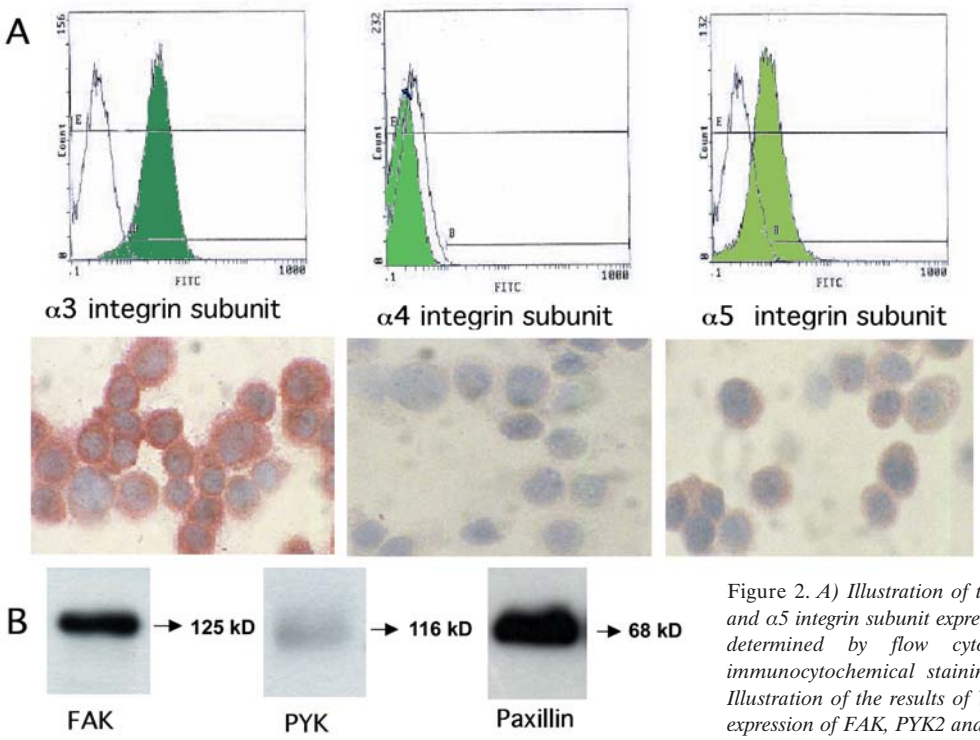

Figure 2. *A) Illustration of the correlation of the α3, α4 and α5 integrin subunit expression in the A549 cell line as determined by flow cytometry analysis and by immunocytochemical staining. Magnification x400. B) Illustration of the results of Western blot analysis for the expression of FAK, PYK2 and paxillin in A549 cells.*

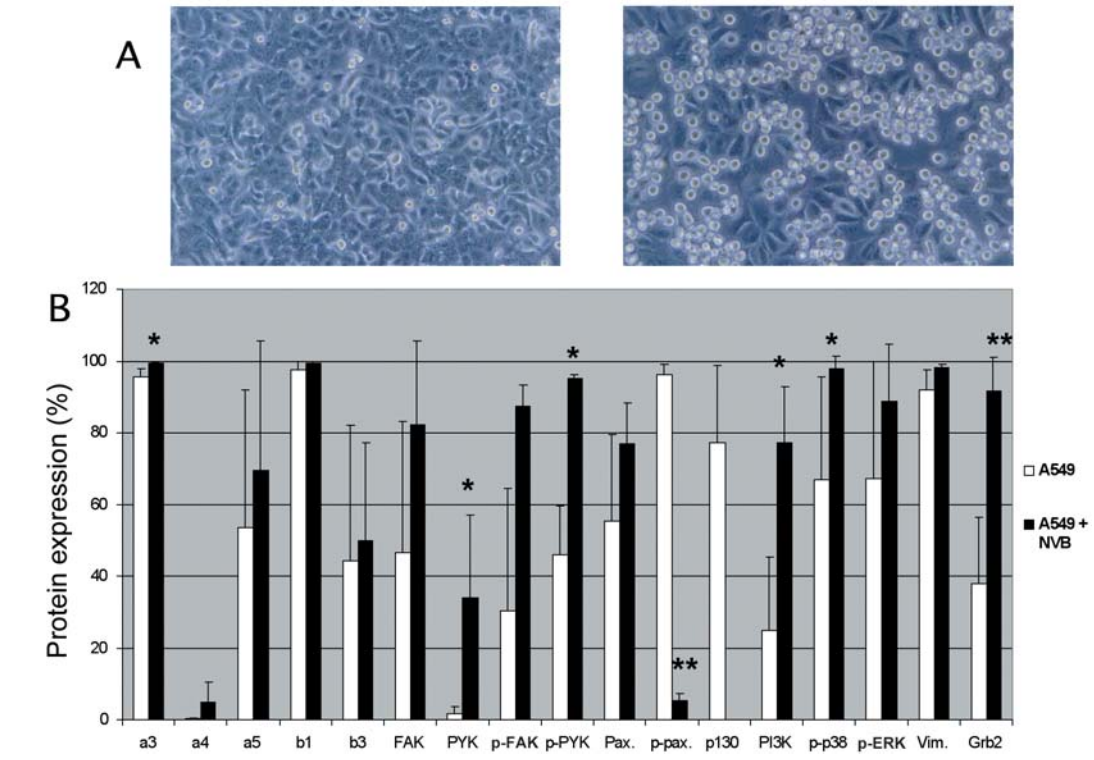

Figure 3. *A) Morphology of A549 cells in normal culture conditions (left) and in the presence of NVB (right). Induction of apoptosis can be observed: cells rounded and progressively detached from their substrate. Magnification x200. B) Comparison of the expression of some focal adhesion proteins in the A549 cells in normal culture conditions (□) and in those cells incubated with NVB (■). Standard deviation and statistical analysis are also indicated (*: $p<0.05$, **: $p<0.01$).*

polyclonal anti-Grb2, polyclonal anti-phosphorylated ERK, polyclonal anti-phosphorylated p38, polyclonal anti-p130cas (Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-phosphorylated FAK, polyclonal anti-phosphorylated PYK2 (Biosource, Camarillo, CA, USA) and polyclonal anti-cleaved caspase-3 (Cell Signalling, Berverly, MA, USA).

Statistical analysis. The results are expressed as the mean of four independent experiments. A Student's *t*-test was performed to determine significant differences ($p<0.05$) in the expression of FA proteins between the A549 and Calu-1 cell lines, between the A549 and H460 cell lines and between the groups A549 and A549 treated by NVB.

Results

Expression pattern of FA proteins in three human NSCLC cell lines. The results obtained by the different techniques were similar, although those obtained by flow cytometry analysis are particularly more detailed. The levels of expression of FA proteins in the A549, Calu-1 and H460 cell lines are compared in Figure 1. A variable expression of the different integrins was observed, depending on the subunit considered and on the cell line. For instance, in the A549 cell line α3 and β1 expression was strong, that of α5 and β3 less strong, while very little α4 could be detected. The expression of α3, β1 and paxillin was significantly lower in the H460 cell line than in the A549 cell line ($p=0.00001$, 0.004 and 0.022, respectively). On the contrary, Grb2 expression was significantly higher in H460 than in A549 cells ($p=0.005$). Similarly, FAK was moderately expressed, whereas PYK2, although structurally similar, presented negligible expression. The expression of the other FA proteins varied greatly: from 10% (PI3K in Calu-1) to 96% (p-paxillin in A549).

The correlation of results between flow cytometry and immunocytochemical staining for the expression of α3, α4 and α5 integrin chains in the A549 cell line and an illustration of the expression of FAK, PYK2 and paxillin in the A549 cell line as determined by Western blot are provided in Figure 2A and 2B, respectively.

Changes in the FA complex during apoptosis in the A549 cell line. Cell death was induced in the A549 cell line by NVB. Apoptosis can be observed morphologically in that cells incubated with NVB became round and progressively detached (Figure 3A), and was confirmed by an increased expression of cleaved caspase-3 from 0.61% in untreated cells to 29.31% in those exposed to NVB. The expression pattern of the FA proteins was then determined and compared to that of A549 (Figure 3B). When cells were incubated with NVB, the expressions of α3, PYK2, p-PYK2, PI3K, p-p38 and Grb2 increased significantly compared to those in A549 cells in normal culture conditions ($p=0.03$, 0.038, 0.017, 0.040, 0.048 and 0.003, respectively). Only p-paxillin presented a significantly decreased expression in NVB-treated A549 compared to untreated cells.

Discussion

Most lung cancers are characterized by a high metastatic potential, which is directly related to cell adhesion since cell detachment is one of the first steps of the invasive process. When the expression pattern of the major components of the FA in the A549 cell line was compared with those of two other NSCLC cell lines known to be metastatic *in vivo*, a variable expression of some proteins was revealed (Figure 1). For instance, although structurally-related, FAK and PYK2 expressions were very different: FAK was moderately-expressed (14 to 47% of positive cells) whereas PYK2 expression was very low (about 2%). This could be related to their antagonist functions; it is well known that PYK2 mediates apoptotic signals, whereas FAK is involved in cell survival and plays a key role in the invasive potential of tumors (8-12). Indeed, it has been well documented that FAK-mediated signals are critical for efficient cell migration in response to growth factor receptor and integrin stimulation (12). FAK has also been shown to be expressed in invasive breast cancer, regulating antiapoptotic signaling (13). Moreover, elevated expression of FAK in human tumors has been correlated with increased malignancy and invasiveness (12, 14). A variable expression of α3 and β1 between the A549 and H460 cell lines was also observed. This is consistent with literature data that have demonstrated that the expression pattern of integrins is correlated with the histological type of tumors (15). Moreover, the malignant transformation is generally accompanied by a modification of the expression of some integrin subunits (16, 17). For instance, an altered expression of β1 allows the cells to become more adhesive, invasive and thus increases the metastatic potential (17). Based on this statement, it could be hypothesized that H460 is the most aggressive of the three cell lines studied here since it presented a lower level of β1 expression. The α3β1 protein has also been shown to play a central role in tumor progression (18), and a decreased expression of this integrin was found to constitute a factor of poor prognosis in patients with adenocarcinoma (19). This is consistent with our previous hypothesis since H460 cells presented a significantly decreased expression of α3, suggesting once again that this cell line could potentially be the most invasive. In all cell lines considered, a very low expression of α4 (less than 2%) and a medium expression of α5 (51 to 55%) were observed, in corroboration with previous studies that have shown that in lung, normal tissues generally do not express α5, whereas a wide variety of tumors do. Moreover, antagonist functions can be distinguished depending on the kind of α integrin subunit. Thus, α4β1

generally mediates cell death signals, while α5β1 is involved in the transduction of cell survival or proliferation signals (20). Thus, malignant transformation is often associated with a loss of α4 expression.

Changes in the FA complex, occurring during apoptosis, were studied in the A549 cell line. When apoptosis was induced by NVB (morphologically observed and indicated by an increased level of cleaved caspase-3), the expression of some FA proteins was altered. For example, the expression of PYK2 was significantly increased, both in its inactive and its phosphorylated forms, confirming the implication of PYK2 in the transduction of death signals, as previously discussed. The level of expression of paxillin was not significantly different in normal and apoptotic cells, however, the expression of the active form: p-paxillin, was significantly decreased in cells incubated with NVB. This suggests that, in our model, the dephosphorylation of paxillin could be an important step in the signaling of cell death. Similarly, the levels of expression of p-p38, PI3K and Grb2 were significantly altered in apoptotic cells, suggesting that these proteins could play an important role in the intracellular transduction of death signals.

In conclusion, we have shown that the expression patterns of α3, β1, paxillin, p-paxillin and Grb2 differ among the cell lines considered. However, a difference between the non-metastatic cell line and the metastatic cell lines could not be established, and thus this difference of expression is probably related to the histological type of the tumors and does not signify a direct implication of these adhesion proteins in the invasive process. It would be interesting to characterize the FA complex *in vivo*, in order to investigate the influence of the micro-environment. It has also been demonstrated in this model that the transduction of apoptotic signals could involve PYK2 (inactive and phosphorylated forms), p-paxillin, PI3K, p-p38 and Grb2 proteins. The results of the present study could serve as the basis for the analysis of downstream signaling pathways in these cell lines.

Acknowledgements

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