

# Expression of Focal Adhesion Kinase in Acute Myeloid Leukemia Is Associated with Enhanced Blast Migration, Increased Cellularity, and Poor Prognosis

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## ABSTRACT

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase playing an important role in cell motility and survival. However, very little is known about FAK in normal and leukemic myeloid cells. In this study, FAK protein expression and mRNA were detected in 25 of 60 cases (42%) of acute myeloid leukemia (AML). Whereas FAK was expressed in 46% of CD34<sup>+</sup> AML cells, it was not detected in normal purified CD34<sup>+</sup> cells. Conversely, the FAK homologue proline-rich tyrosine kinase 2 (PYK2) was found to be expressed both in normal and leukemic myeloid cells. When expressed, FAK displayed phosphorylation on Tyr-397, an important step for its activation. Moreover, FAK expression was correlated with the phosphorylation of PYK2 on Tyr-881, a critical site for the PYK2 function in cell migration. FAK<sup>+</sup> AML cells displayed significantly higher migration capacities and resistance to daunorubicin, compared with FAK<sup>-</sup> cells. The implication of FAK in both cell motility and drug resistance was demonstrated by small interfering RNA experiments with the FAK-positive KG1 cell line. However, adhesion on fibronectin efficiently protected FAK<sup>-</sup> AML cells from daunorubicin-mediated killing, suggesting that cellular adhesion mediated-drug resistance is not mediated by FAK. Finally, in a retrospective cohort of 60 AML patients, FAK expression was significantly correlated with high blast cell count, early death, and shorter survival rate. Altogether, this study shows that FAK is aberrantly expressed and activated in about half of the cases of AML and suggests that FAK may contribute to the regulation of AML cell transit from the marrow to blood compartment and that it may influence clinical outcome.

## INTRODUCTION

Normal and leukemic hematopoietic progenitor cells (HPCs) express high levels of extracellular matrix receptors including fibronectin receptors, very late antigen-4 and very late antigen-5, which are transiently activated by cytokines to interact with matrix and stroma cells (1). Focal adhesion kinase (FAK), a nonreceptor protein tyrosine kinase, localizes to regions of the cell that attach to the extracellular matrix, the focal adhesions. FAK coordinates signals from integrins, cytokines, growth factor receptors, and oncogenes (2). In tumor cells, FAK has been found to be overexpressed and/or constitutively activated and correlated with increased motility, invasiveness, and proliferation (3, 4). More recently, it has been documented that FAK exerts a potent antiapoptotic effect and protects tumor cells against cytotoxicity induced by  $\gamma$ -radiation, drugs, and oxidative stress (5). Invasive and antiapoptotic functions of FAK depend on its kinase activity. The major site of autophosphorylation of FAK is Tyr-397, a residue that is critical for its function (2). FAK signals for both phosphoinositide-3 kinase and mitogen-activated protein kinase, two

pathways that have been involved in FAK-mediated survival and motility. FAK shares structural and sequence homology with proline-rich tyrosine kinase 2 (Pyk2), another focal adhesion tyrosine kinase, which has also been involved in cell motility (6). However, unlike FAK, Pyk2 can display a proapoptotic function and, for example, has been found to be essential for apoptosis induced by drugs (7, 8) or cytokines (9).

FAK distribution and function in human normal and leukemic myeloid cells have received little attention. In a previous study, it has been reported that FAK mRNA was weakly expressed in murine immature myeloid precursors and up-regulated in liquid culture after stimulation by granulocyte-macrophage colony-stimulating factor (10). More recently, it has been described that FAK protein is not detectable in human CD34<sup>+</sup> cells, whereas FAK is expressed in neutrophils but not in macrophages (11–14). Altogether, these results suggest that, in human myeloid cells, FAK expression correlates with cell differentiation.

To the best of our knowledge, the expression of FAK in acute myeloid leukemia cells (AML) has not yet been documented. However, based on the role of FAK in integrating extracellular matrix signals, motility, and survival, this issue could be of interest. The present study was aimed to evaluate the expression and phosphorylation status of FAK in AML cells as well as its functional consequences in terms of adhesion, motility, and drug resistance.

## MATERIALS AND METHODS

**Antibodies.** Monoclonal antibodies against FAK and polyclonal antibodies directed to FAK phosphorylated at Y397 came from Upstate Biotechnology (Charlottesville, VA). Monoclonal antibodies against Pyk2 were from BD Transduction Laboratories. Phospho-specific antibodies against Pyk2 phosphorylated at Y402 and Y881 were from Biosource International (Camarillo, CA). Horseradish-peroxidase-conjugated secondary antibodies against mouse and rabbit immunoglobulins were from Cell Signaling (Beverly, MA).

**Cells.** Fresh AML cells were obtained from the bone marrow of 60 patients, using Ficoll-Hypaque density-gradient centrifugation after those patients gave informed consent. Blasts were immediately cryopreserved in Iscove's modified Dulbecco medium with dimethyl sulfoxide and FCS. All patients were diagnosed according to WHO classification at the Hematology Department of Toulouse University Hospital (Toulouse, France). Leukemias were characterized in terms of morphology (French-American-British classification), karyotype, immunophenotyping, and *FLT3* gene mutation (*FLT3*-internal tandem duplication, *FLT3/ITD*) mutation. Bone marrow samples contained >80% leukemic blasts after processing. For *in vitro* experiments, blasts were immediately resuspended in RPMI 1640 with 10% FCS after thawing. Cells were then washed twice in RPMI 1640 containing 10 mM HEPES and 0.2% BSA. Only samples with >80% of cell survival estimated by trypan blue were processed. Normal bone marrow CD34<sup>+</sup> HPCs were obtained from healthy donors after informed consent. Mononuclear cells from bone marrow were obtained by density gradient centrifugation over Ficoll-Hypaque after which isolation of HPCs was performed by positive selection of CD34 expressing cells. Briefly, CD34<sup>+</sup>-HPCs were magnetically labeled using MACS CD34 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) then isolated by positive selection through mass spectrometry separation column (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the CD34<sup>+</sup> cells was

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evaluated by flow cytometry using a CD45/CD34 gating and reached 85–98%. The human leukemic cell lines KG1a, KG1, HL-60, and U937 were purchased from American Type Culture Collection (Rockville, MD). A human microvascular endothelial cell line (HBMEC) from adult bone marrow was kindly provided by Dr. B. B. Weksler (15) and used for cell migration assays.

**Cell Culture.** KG1, KG1a, U937, and HL60 cells were grown at 37°C in 5% CO<sub>2</sub> in Iscove's modified Dulbecco medium or in RPMI 1640 containing 20% or 10% FCS, respectively; 50 µg/ml penicillin, and 50 µg/ml streptomycin. HBMEC cells were maintained in DMEM supplemented with 10% FCS, 10 mM HEPES (pH 7.4), 7 IU/ml heparin, 7.5 µg/ml endothelial cell growth supplement (ECGS; Sigma) and antibiotics at the same concentrations as above. Cells (HBMEC) were plated on 0.5% gelatin-coated culture flasks.

**Western Blotting.** For Western blot, 1–3 × 10<sup>6</sup> cells resuspended in cell culture medium without serum were reduced in Laemmli sample buffer. After boiling for 10 min, proteins were resolved on SDS-PAGE and then transferred to nitrocellulose (membrane Hybond-C super; Amersham Pharmacia Biotech) using a liquid transfer apparatus (Amersham Pharmacia Biotech). The membrane was blocked overnight at 4°C in Tris-buffered saline containing 1% fat-free milk and 1% BSA. The proteins were detected by blotting with the appropriate monoclonal or polyclonal antibodies in Tris-buffered saline, 0.1% Tween, 1% fat-free milk, and 1% BSA, followed by incubation with either antimouse or antirabbit IgG antibody coupled to horseradish peroxidase. Detection was achieved using a chemoluminescent probe (Amersham Pharmacia Biotech).

**Reverse Transcription (RT)-PCR Analysis of FAK mRNA.** The level of mRNA encoding FAK was evaluated by RT-PCR. Total RNA was isolated by Trizol Reagent (Invitrogen, Cergy Pontoise, France). Semi-quantitative RT-PCR was performed using 400 ng of total RNA, amplified in a Biometra thermal cycler using oligonucleotides (0.5 µM of each) 5'-TGGGTGAGCTCATCAACAAG-3' and 5'-AGTTGGAGCTGTGAGTGCTG-3' in a 50-µl reaction volume using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech). β-Actin mRNA levels were used for the normalization of RNA. After RT at 42°C for 20 min followed by 5 min at 95°C, 30 amplification cycles were carried out (94°C for 30 s, 55°C for 45 s, and 72°C for 1 min). The amplification products were electrophoresed in 2% agarose gels, and products of RT-PCR were visualized with UV detection (292 bp FAK; 641 bp β-actin).

**Transfection of Small Interfering RNA (siRNA).** KG1 cells were transfected using the Amaxa nucleofection technology (Amaxa, Koeln, Germany). Briefly, cells were resuspended in Amaxa solution kit R, following the Amaxa guidelines for cell line transfection that we have optimized for KG1 cells. KG1 cells (2 × 10<sup>6</sup>) in 100 µl of solution kit R were mixed or not with 200 nM siRNA FAK or with 200 nM siRNA scramble (Dharmacon Inc., Lafayette, CO) and immediately nucleofected with an Amaxa Nucleofector apparatus (program T01; Amaxa). Cells were then immediately transferred into wells containing 37°C prewarmed culture medium in 12-well plates. After transfection, cells were cultured from 24 to 48 h before analyzing with Western blotting. Decrease of FAK was observed at 24 h but was maximal at 48 h. Therefore, drug sensitivity and migration tests have been performed at 48 h after nucleofection.

**Maturation Assays.** Normal bone marrow CD34<sup>+</sup> HPCs were grown in Iscove's modified Dulbecco medium supplemented with 10% BIT (BSA, insulin, transferrin; Stem Cell Technologies, Vancouver, Canada) and in the presence of 100 ng/ml stem cell factor, 100 ng/ml flt3 ligand (flt3-L), 1 IU/ml interleukin 3, and 10 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor for up to 13 days. Cytokines were from R&D Systems (Oxon, United Kingdom). The cell concentration was maintained at 5 × 10<sup>5</sup> cells/ml by diluting the cells with fresh medium and cytokines every 2 to 3 days. Cell viability was assessed by trypan blue exclusion. After checking for CD34 expression by cytofluorometric analysis (EPICS XL-MCL; Beckman Coulter, Villepinte, France), cells were processed for Western blotting analysis or RT-PCR as described above.

**Adhesion Assays.** 96-well microtiter plates (MaxiSorp Immuno Plate, Nunc, Denmark) were coated overnight at 4°C with 40 µg/ml human fibronectin (Roche Molecular Biochemicals, Mannheim, Germany) in a final volume of 50 µl in PBS and subsequently blocked with 1% fatty acid-free BSA in PBS, 1 h at room temperature. Cells (4 × 10<sup>5</sup>) were resuspended in RPMI 1640 supplemented with 10 mM HEPES and 0.2% BSA. Cells were then labeled with 200 µCi of <sup>51</sup>Cr/ml at 37°C for 1 h. After two washes, 3 × 10<sup>4</sup> cells in a final volume of 100 µl were added per well in RPMI with HEPES and BSA.

Cell adhesion assays were performed in triplicate for 1 h at 37°C. After two washes, adherent cells were lysed in 150 µl of lysis buffer (1% SDS, 0.1 M NaOH) for 2 min under shaking. One hundred µl of lysate from adherent cells or total cell suspension were counted in a gamma counter. Cell adhesion was quantified by radioactivity ratio of lysates from adherent cells and total cell suspension.

**Migration Assays.** HBMEC cells (3 × 10<sup>5</sup>)/well were plated on a gelatin-coated 24-wells FALCON companion plate (Becton Dickinson, Le Pont de Claix, France). Confluent HBMEC monolayers were obtained over 3 days without change of medium. FALCON cell culture inserts were coated with 50 µg/ml fibronectin for 30 min at 37°C. Leukemic cells (6 × 10<sup>5</sup>) were serum-starved for 1 h, resuspended in RPMI 1640 (blasts and U937) or in Iscove's modified Dulbecco medium (KG1), and were labeled for 1 h with 5 µM Cell Tracker Green cellular adhesion mediated-drug resistance (5-chloromethylfluorescein diacetate; Molecular Probes; Eugene, OR). After two washes with PBS, cells were allowed to adhere on fibronectin-coated cell culture inserts for 1 h at 37°C. Nonadherent cells were recovered to quantify adhesion efficiency. Then, cell culture inserts were put in endothelial cells containing companion plates. Cell migration was thus initiated in triplicate and continued for 5 h. At the end of the incubation period, all compartments of the cell migration system (insert, bottom, medium, and well) were recovered to measure fluorescence. After lysis with 1% Triton X-100, 100 µl were analyzed in a fluorescence microplate reader at emission and excitation wavelengths for FITC. Data were expressed as the percentage of total leukemic cells that had migrated through the cell endothelial-conditioned medium.

**Drug Cytotoxicity Studies.** AML cells were serum starved for 1 h and then allowed to adhere on fibronectin-coated microtiter plate 96 wells (10<sup>5</sup> cells/well) for 1 h at 37°C or maintained in suspension. Cells in suspension or adherent were treated with 0.5 µM daunorubicin (DNR; Laboratoire Roger Bellon, Neuilly-sur-Seine, France) for 1 h at 37°C. At the end of the incubation period, cells were washed and incubated in serum containing medium for 24 h at 37°C. Cell viability was then quantified by methyl thiazolyl tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Sigma). Experiments were performed in triplicate.

**Clinical Characteristics.** Patients with a confirmed diagnosis of AML were included in this analysis, except patients with acute promyelocytic leukemia because of different treatment and outcome. Clinical and biological data were available for all patients, except one lost to follow-up after complete remission. Patients were treated by standard induction chemotherapy DNR-aracytine (DNR-Ara-C; *n* = 51; 60 mg/m<sup>2</sup> DNR on day 1, 2, and 3 in association with 100 or 200 mg/m<sup>2</sup> Ara-C as a continuous infusion for 7 days for patients over or under 60 years of age, respectively); amsacrine and Ara-C (*n* = 2), oral idarubicin (*n* = 1) or methylgag-Ara-C and 6-thioguanine (*n* = 1) or supportive care only (*n* = 5). Consolidation therapy consisted in chemotherapy (*n* = 14), autologous (*n* = 8) or allogeneic stem cell transplantation (*n* = 16).

**Statistical Analysis.** Statistical analyses were performed using the SAS statistical software release 8.2. Patient characteristics and complete remission rates were compared between FAK status groups using the χ<sup>2</sup> test for categorical variables and the Fisher's exact test for binary variables when necessary; *t* tests were performed for continuous variables. The influence of FAK status on WBC count was analyzed with multivariate linear regression model after adjustments for confounding variables. Overall survival time was calculated from the date of diagnosis until death or last date of follow-up examination for right-censored subjects. Survival curves were estimated using the product-limit method of Kaplan-Meier and were compared using the log-rank test. Similar to the category of age, the initial degree of leukocytosis and cytogenetic status are known to be prognostic factors for complete remission achievement, duration of complete remission, or survival, comparisons were adjusted for these three potential independent prognostic factors. All adjustments were performed with the Cox model tested by the likelihood-ratio test. Student *t* test was used for *in vitro* experimental analysis.

## RESULTS

**FAK Expression in AML.** We have investigated the expression of FAK family proteins, FAK and Pyk2, in leukemic blasts derived from 60 patients with AML classified according to French-American-Brit-

ish immunophenotypic and cytogenetic criteria (Table 1). FAK was undetected in 35 of 60 samples (58%) as assessed by Western blotting. Among this group, RT-PCR was performed in 16 cases and in each case did not reveal FAK transcript (illustrated in Fig. 1). FAK was detected by Western blotting in 25 of 60 samples (42%) including immature CD34<sup>+</sup> AML samples. Among this group, RT-PCR was performed in seven cases, and in each case it did reveal FAK transcripts (illustrated in Fig. 1). However, in this group, FAK expression level was found to be variable. Pyk2, another focal adhesion tyrosine kinase, was more widely distributed because it was present in 81% of cases (Fig. 1).

**FAK Expression in Normal Purified CD34<sup>+</sup> Cells.** To check whether the detection of FAK in CD34<sup>+</sup> AML could result from a deregulated expression, we studied FAK expression in normal CD34<sup>+</sup> HPCs. In four samples, we were unable to detect FAK protein in normal CD34<sup>+</sup> cells by using Western blot analysis (Fig. 2, A and B). Moreover, after stimulation with cytokines, FAK protein remained undetectable up to 13 days of culture (Fig. 2B). By contrast, Pyk2 was detected in normal CD34<sup>+</sup> HPCs (Fig. 2A).

**Phosphorylation Status of FAK in Leukemic Cells.** FAK phosphorylation status was investigated. In fresh AML cells, FAK (*n* = 8) and Pyk2 (*n* = 16) were always phosphorylated on Tyr-397 and Tyr-402, respectively (Fig. 3A). Interestingly, Pyk2 phosphorylation on Tyr-881, an SH2-binding site for GRB2 and c-Abl, associated significantly with FAK expression in fresh AML cells (Fig. 3A; Table 2). FAK and Pyk2 expression was also studied in leukemic cell lines

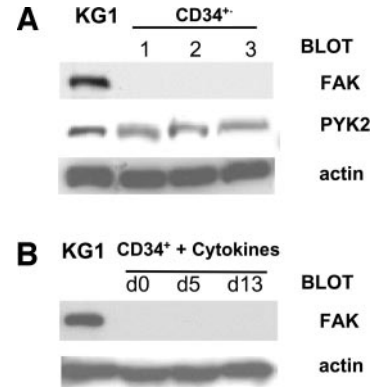


Fig. 2. Focal adhesion kinase (FAK) expression in unstimulated purified bone marrow CD34<sup>+</sup> cells and after stimulation with cytokines. A, cell lysates (10<sup>6</sup> cells) from three healthy donors were submitted to Western blotting with anti-FAK or anti-proline-rich tyrosine kinase 2 (anti-Pyk2) antibodies. KG1 was used as a positive control. B, purified CD 34<sup>+</sup> cells from a healthy donor were stimulated with 100 ng/ml stem cell factor, 100 ng/ml flt3-L, 1 IU/ml interleukin 3, and 10 ng/ml granulocyte-macrophage colony-stimulating factor for 13 days. Cell lysates (10<sup>6</sup> cells) were submitted to Western blotting with anti-FAK antibody at baseline (day 0), day 5, and day 13.

Table 1 Characteristics of AML patients at diagnosis according to FAK expression

	Total	FAK <sup>-</sup>	FAK <sup>+</sup>	P
Patients, no. (%)	60	35 (58)	25 (42)	
Median age, (years)	53 (12–80)	56 (12–77)	52 (17–80)	ns
WBC (×10 <sup>9</sup> /liter)	78 (1.3–409)	57 (1.3–250)	143 (3.1–409)	<0.05
FAB subtypes				ns
M <sub>0</sub>	5	2	3	
M <sub>1</sub>	19	13	6	
M <sub>2</sub>	9	7	2	
M <sub>4</sub>	13	7	6	
M <sub>5</sub>	9	3	6	
Secondary AML	5	3	2	
Cytogenetic, no. (%)				ns
Favorable	2 (3)	1 (3)	1 (4)	
Intermediate	43 (72)	27 (75)	16 (67)	
Unfavorable	15 (25)	8 (22)	7 (29)	
FLT3-ITD, no. (%)	26/55 (47)	15/31 (48)	11/24 (46)	ns
CD34 <sup>+</sup> , no. (%)	37/52 (71)	20/29 (69)	17/23 (74)	ns
CD117 <sup>+</sup> , no. (%)	34/44 (77)	18/25 (72)	16/19 (84)	ns

AML, acute myeloid leukemia; FAK, focal adhesion kinase; ns, not significant. FAB, French-American-British; FLT3-ITD, FLT3-internal tandem duplication.

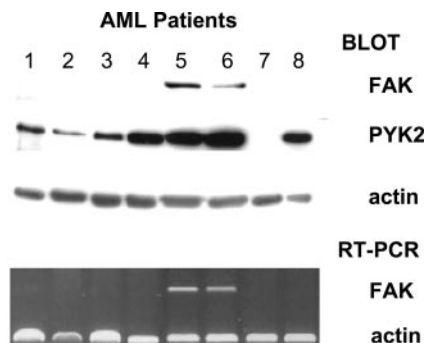


Fig. 1. Focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2) expression in acute myeloid leukemia (AML) blasts. Cell lysates (10<sup>6</sup> cells) from eight AML patients were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted sequentially with monoclonal antibodies directed to FAK, Pyk2, or actin. FAK gene expression of eight AML bone marrow samples was detected by reverse transcription-PCR, and then electrophoresed in 2% agarose gels and visualized with UV detection. Actin mRNA levels were used for the normalization of RNA.

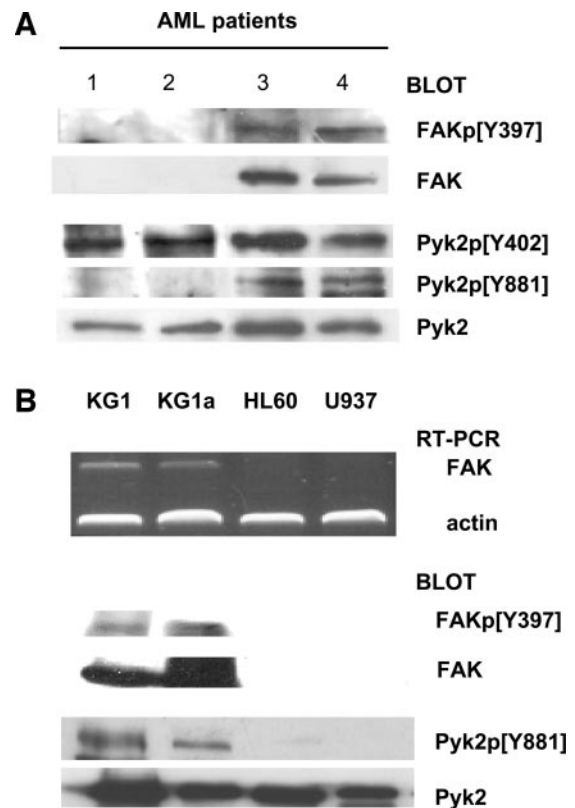


Fig. 3. Focal adhesion kinase (FAK), proline-rich tyrosine kinase 2 (Pyk2) phosphorylation in acute myeloid leukemia (AML) blasts and leukemic cell lines. A, cell lysates (10<sup>6</sup> cells) from four AML patients were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted sequentially with the indicated antibodies to evaluate FAK and Pyk2 status. B, analysis of FAK expression in KG1, KG1a, HL60, and U937 leukemic cells (10<sup>6</sup> cells) by Western blotting and reverse transcription-PCR as described in the legend to Fig. 1. FAK and Pyk2 phosphorylation status were evaluated as described in A.

(Fig. 3B). FAK was not detected in HL60 and U937 cells, whereas KG1 and KG1a cells displayed high level of FAK expression. FAK was found phosphorylated on Tyr-397. As shown in Fig. 3B, FAK protein expression in cell lines correlates with FAK mRNA results. HL-60, U937, KG1, and KG1a cells expressed PYK2 that constantly displayed phosphorylation on Tyr-402 (not shown) and phosphorylation on Tyr-881 only when coexpressed with FAK (Fig. 3B).



**Influence of FAK Expression on AML Cell Adhesion and Migration.** Cell adhesion and migration play a key role in drug resistance and tumoral invasion; we therefore investigated the influence of FAK expression on AML cell adhesion and migration. Adhesion and migration were evaluated in 18 and 12 AML samples, respectively. As shown in Fig. 4A, FAK<sup>+</sup> (*n* = 7) and FAK<sup>-</sup> (*n* = 11) AML cells displayed similar adhesion capacities on fibronectin whereas FAK<sup>+</sup> AML cells (*n* = 6) displayed significantly higher migration efficiency, compared with FAK<sup>-</sup> cells (*n* = 6; *P* < 0.01).

Table 2 Association between Pyk2<sup>a</sup> phosphorylation on Tyr-881 and FAK expression

		FAK <sup>-</sup>	FAK <sup>+</sup>	<i>P</i> <sup>a</sup>
Pyk2 Tyr-881	-	9	4	0.04
Pyk2 Tyr-881	+	3	9	

<sup>a</sup> Pyk2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase.

<sup>b</sup> *P* was from Fisher's exact test (two-sided).

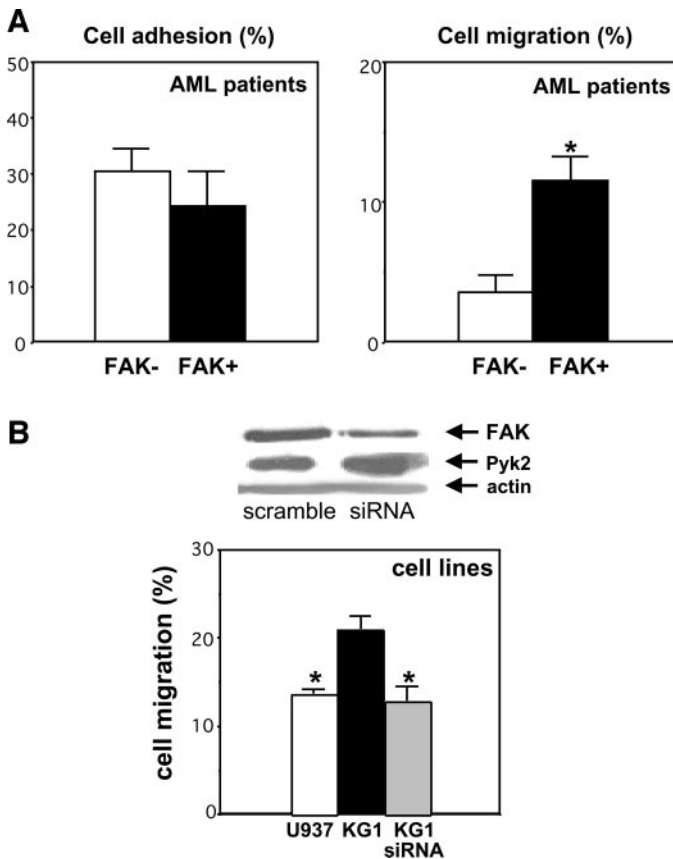


Fig. 4. Cell adhesion/migration capacities and focal adhesion kinase (FAK) expression of acute myeloid leukemia (AML) cells. *A*, cell adhesion, AML cells ( $3 \times 10^4$  cells) were serum starved, labeled with 200  $\mu$ Ci of  $^{51}\text{Cr}$ /ml and allowed to adhere on fibronectin-coated wells for 1 h at 37°C. Adhesion was quantified by radioactivity ratio between adherent cell and total cell suspension lysates. Experiments were performed in triplicate and data are from 11 FAK<sup>-</sup> and 7 FAK<sup>+</sup> AML patients, respectively. Results shown are means  $\pm$  SE. Cell migration, AML cells from patients or cell lines ( $6 \times 10^5$  cells) were serum starved, labeled with 5  $\mu$ M Cell Tracker Green cellular adhesion mediated-drug resistance and allowed to adhere on fibronectin-coated cell culture inserts for 1 h at 37°C. Migration was initiated by putting inserts in endothelial cells containing companion plates. Experiments were performed for 5 h in triplicate, and data are from six FAK<sup>-</sup> and six FAK<sup>+</sup> AML patients. Results shown are means  $\pm$  SE and comparison between FAK<sup>+</sup> and FAK<sup>-</sup> samples. \*, *P* < 0.01; Student's *t* test, unpaired. *B*, KG1 or U937 cell migration was studied as described in *A*. KG1 cells were transfected by electroporation with a FAK small interfering (si)RNA as described in "Materials and Methods." Control was obtained with a scrambled siRNA. Western blotting showing decrease of FAK expression in KG1 by siRNA after 48 h is shown. As controls, Western blotting of proline-rich tyrosine kinase 2 (Pyk2) and actin is shown. Efficiency of KG1 cell migration was not influenced by electroporation treatment. Results shown are means  $\pm$  SE of three experiments and comparison between U937 and KG1 or KG1 and KG1 siRNA. \*, *P* < 0.05; Student's *t* test, unpaired.

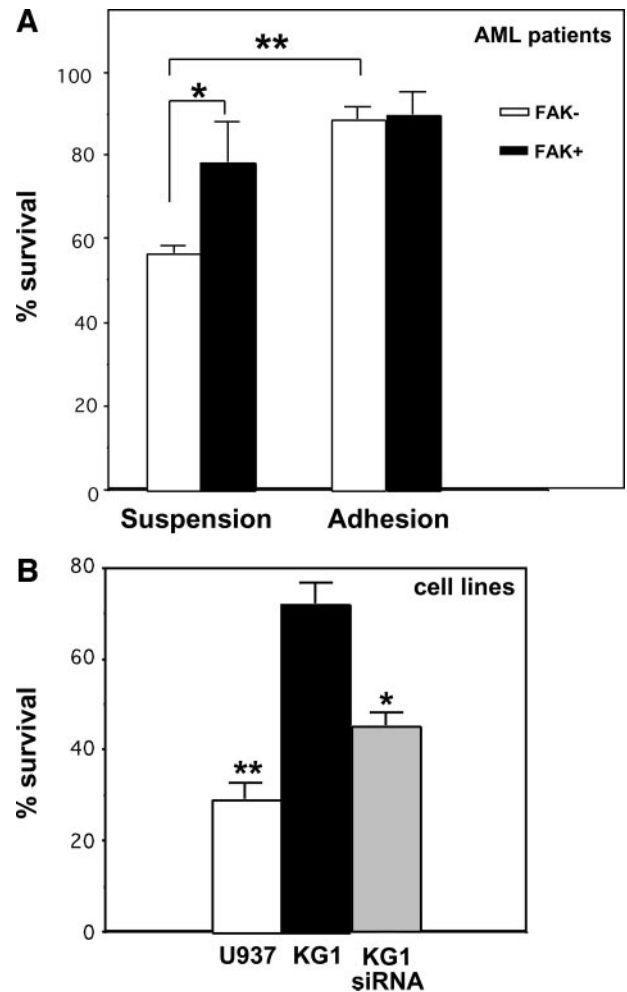


Fig. 5. Sensitivity to daunorubicin (DNR) and focal adhesion kinase (FAK) expression of acute myeloid leukemia (AML) cells. *A*, AML cells ( $10^5$ ) were serum starved and then allowed or not to adhere on fibronectin-coated wells for 1 h at 37°C. Cells in suspension or adherent were treated with 0.5  $\mu$ M DNR for 1 h at 37°C, washed at the end of the incubation period and incubated in serum containing medium for 24 h at 37°C. Cell survival was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Experiments were performed in triplicate, and data are from seven FAK<sup>-</sup> and seven FAK<sup>+</sup> AML patients. Results shown are means  $\pm$  SE and comparison between suspension and adhesion FAK<sup>-</sup> samples (\*\*, *P* < 0.001) or between suspension FAK<sup>-</sup> and suspension FAK<sup>+</sup> (\*, *P* < 0.05; Student's *t* test). *B*, U937 or KG1 cells sensitivity to DNR was studied as described in *A*. KG1 cells were transfected with FAK small interfering (si)RNA or not, as described in the legend to Fig. 4. Results shown are means  $\pm$  SE of three experiments and comparison between U937 and KG1 or KG1 and KG1 siRNA. \*, *P* < 0.05, \*\*, *P* < 0.01; Student's *t* test, unpaired.

Interestingly, the FAK<sup>+</sup> leukemic cell line KG1 displayed higher migration efficiency as compared with the FAK<sup>-</sup> leukemic cell line U937 (Fig. 4B). Moreover, when FAK expression was strongly decreased by siRNA in KG1 cells, a significant decrease of their migration capacities was observed (Fig. 4B). As a control, level of Pyk2 expression was not modified by FAK siRNA treatment (Fig. 4B). These results highlight the prominent role of FAK in migrating leukemic cells.

**Influence of FAK Expression on AML Sensitivity to DNR.** Chemosensitivity to 0.5  $\mu$ M DNR for 1 h was evaluated in 14 AML samples. When AML cells were cultured in suspension, FAK<sup>+</sup> samples (*n* = 7) displayed significant lower sensitivity to DNR, compared with FAK<sup>-</sup> samples (*n* = 7; *P* < 0.05; Fig. 5A). The FAK<sup>-</sup> leukemic cell line U937 was found significantly more sensitive to DNR than the FAK<sup>+</sup> leukemic cell line KG1 (Fig. 5B). A role for FAK in drug resistance of KG1 cells was demonstrated by siRNA experiments (Fig. 5B) because DNR-induced apoptosis of KG1 cells was signifi-

cantly increased after down-regulation of FAK expression, despite the well-known chemoresistance phenotype of this cell line.

When FAK<sup>-</sup> AML cells were layered on fibronectin for 1 h before treatment, they displayed significant lower sensitivity to DNR, compared with suspension-treated FAK<sup>-</sup> AML cells ( $P < 0.001$ ; Fig. 5A). This result showed that cellular adhesion mediated-drug resistance can apply to fresh AML cells. However, FAK expression did not correlate with cellular adhesion mediated-drug resistance because FAK<sup>-</sup> AML cells as well as FAK<sup>-</sup> U937 cells (not shown) appeared to be efficiently protected by adhesion.

**Correlation between FAK Expression and Clinical Features and Outcome.** No correlation was found between FAK expression and French-American-British subtypes, cytogenetics, FLT3 status, and immunophenotype, including CD34 and CD117 (Table 1). Indeed, contrasting with the lack of FAK expression in normal CD34<sup>+</sup>, FAK was present in 46% of CD34<sup>+</sup> AML cells. However, we found a significant correlation between FAK expression and high WBC count at diagnosis. The initial median WBC counts were  $143 \times 10^9$ /liter in FAK<sup>+</sup> patients versus  $57 \times 10^9$ /liter in FAK<sup>-</sup> patients ( $P < 0.05$ ; Fig. 6). In this cohort of unselected patients, the initial WBC count, as well as the frequency of FLT3/ITD, was unusually high. In as much as FLT3/ITD is generally associated with leukocytosis, we used linear regression analysis to confirm that the impact of FAK on leukocytosis was independent from the FLT3/ITD ( $P < 0.05$ ). Among patients with FLT3/ITD<sup>-</sup> phenotype, 7 of 20 (35%) displayed a WBC count at diagnosis superior at 30,000 in the FAK<sup>-</sup> group versus 9 of 14 (64%) in the FAK<sup>+</sup> group.

Among the 51 patients treated with DNR-Ara-C-standard regimen, we found that, whereas FAK expression does not influence response rate (70% versus 74% for FAK<sup>+</sup> and FAK<sup>-</sup>, respectively), it significantly correlated with a higher early death rate (30% versus 3% for FAK<sup>+</sup> and FAK<sup>-</sup>, respectively;  $P < 0.04$ ; Table 3). It should be noted that among the 6 FAK<sup>+</sup> patients who died early, only one was positive for FLT3/ITD. Moreover, multivariate analysis was performed to study the influence of FAK expression on overall survival. Three models of analysis have been applied to the cohort of patients treated with standard regimen ( $n = 51$ ). The first model indicated that FAK expression was correlated with reduced overall survival independently of age, sex, treatment, WBC, and cytogenetic status (Cox model,  $P = 0.04$ ). Secondly, after inclusion of FLT3/ITD in param-

Table 3 Clinical outcome in DNR/Ara-C<sup>a</sup>-treated patients

	Total	FAK <sup>+</sup>	FAK <sup>-</sup>	P
CR	37/51 (73%)	14/20 (70%)	23/31 (74%)	0.7
Relapse <sup>b</sup>	18/36 (50%)	9/14 (64%)	9/23 (39%)	0.1
Early death <sup>c</sup>	7/51 (14%)	6/20 (30%)	1/31 (3%)	<0.04
Death in CR	4/37 (11%)	1/4 (7%)	3/4 (13%)	0.5

<sup>a</sup> DNR, daunorubicin; Ara-C, aracytin; FAK, focal adhesion kinase; CR, complete remission.

<sup>b</sup> One patient lost to follow up in CR.

<sup>c</sup> Early death is defined by death occurring less than 35 days after initiation of therapy; Fisher's exact test

eters, FAK expression (hazard ratio, CI 95%: 2.66 [1.19–5.93]) was still correlated with overall survival ( $P < 0.02$ ). However, in this model, collinearity between FLT3/ITD and WBC was observed. In the third model where WBC has been excluded from parameters, FAK remained highly correlated with overall survival independently of FLT3/ITD and other variables ( $P = 0.01$ ). As shown in Fig. 7, median survival from diagnosis was significantly shorter in the FAK<sup>+</sup> group (10.8 months) compared with the FAK<sup>-</sup> group (24 months; log-rank test;  $P = 0.049$ ).

## DISCUSSION

This study shows that FAK is frequently expressed and activated in AML cells, and that FAK expression correlates with enhanced migratory properties, drug resistance, high leukocytosis, and reduced survival. Several reports have implicated FAK in the motility, invasiveness, proliferation, and survival of human solid tumor cells. This property may be linked to FAK overexpression because of gene amplification (16) or FAK constitutive activation (4). In contrast, the distribution and the function of FAK in normal and leukemic hematopoietic cells have received little attention. Previous studies have described that, whereas FAK is expressed in polymorphonuclear cells, FAK protein is undetectable in bone marrow CD34<sup>+</sup> cells (11–13). However, the expression of FAK in fresh AML cells has not yet been documented. In this study, we show for the first time that FAK is expressed at both gene and protein level in about half of the cases of AML patients, including CD34<sup>+</sup> AML cell samples. This result suggests that, in AML cells, FAK is aberrantly regulated at the transcriptional level. The mechanism by which FAK is expressed in AML cells needs further investigation. However, it has been recently documented that, in AML cells, 8q22–8q24 chromosomal region is amplified in about 20% of cases (17). Because this region contains FAK gene, it is possible that 8q amplification accounts for aberrant FAK expression in AML cells because it has been proposed for melanoma cells (16).

In AML cells, FAK is constitutively phosphorylated on Tyr-397 residue, a critical site for its function (2). The mechanism by which FAK is activated has not been investigated.

However, previous studies have established that FAK phosphorylation can be regulated by integrins, chemokines, small GTPase, oncogenes and tyrosine kinases receptors (for a review, see Ref. 18). Therefore, it is conceivable that aberrant integrin (19) or chemokine-receptor signaling pathways (20, 21), expression of oncogenic products such as Ras (22), or autocrine production of hematopoietic growth factors such as stem cell factor (23), could result in FAK phosphorylation in AML cells. Alternatively, it is possible that FAK phosphorylation is facilitated by deregulated activity of regulatory phosphatase, such as SHP-2 or PTEN (24, 25), which can be mutated in some myeloid leukemic cells (26, 27). Whatever the mechanism of FAK phosphorylation, enhanced FAK activity may contribute to the activation of downstream signaling pathways involved in cell survival or invasion, such as extracellular signal-regulated kinase and phos-

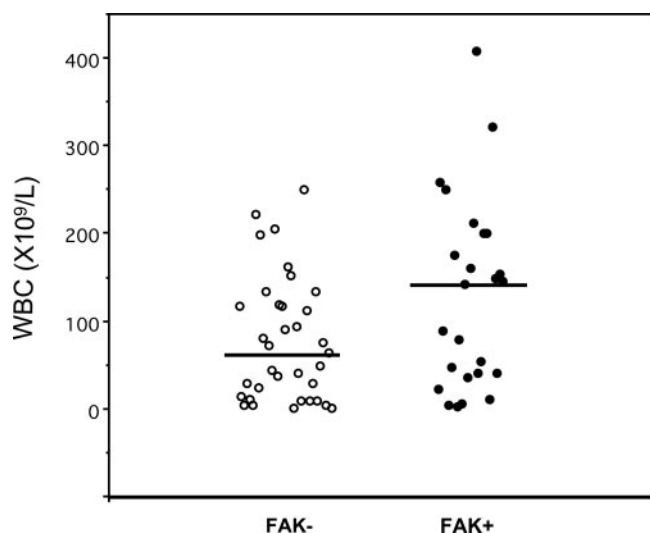


Fig. 6. Dot plot analysis of the initial WBC count in acute myeloid leukemia (AML) patients focal adhesion kinase (FAK<sup>+</sup>;  $n = 25$ ) or FAK<sup>-</sup> ( $n = 35$ ). The initial median WBC count is indicated for FAK<sup>+</sup> and FAK<sup>-</sup> groups of patients.

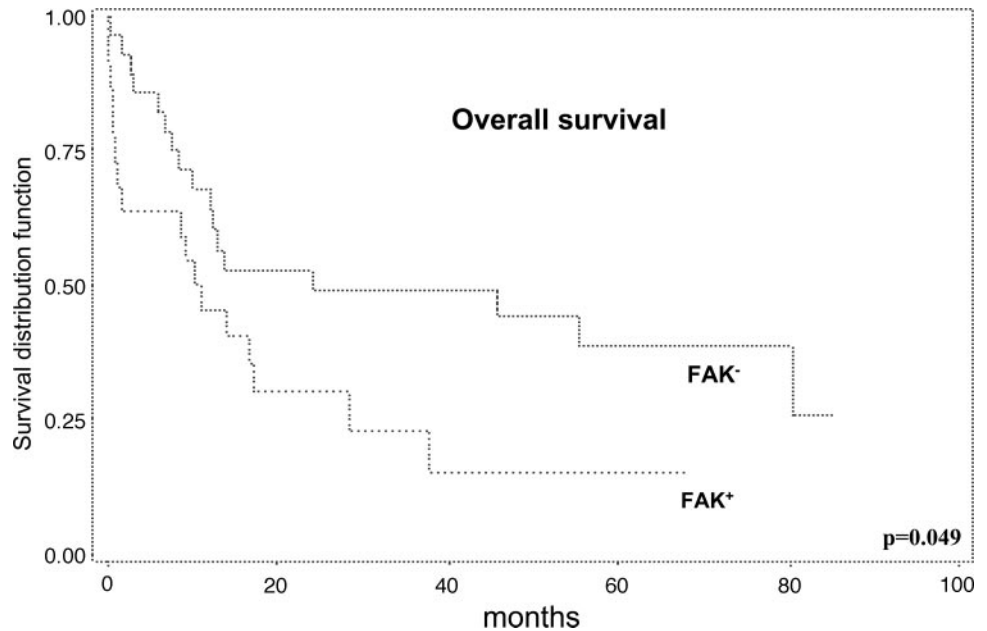


Fig. 7. Overall survival and focal adhesion kinase (FAK) expression in acute myeloid leukemia (AML) patients. Survival from diagnosis was analyzed in 51 AML patients treated with daunorubicin-aracytin (DNR-Ara-C) standard regimen. Cases were analyzed by the product limit method of Kaplan-Meier and were compared using the log-rank test.

phoinositide-3 kinase/Akt, both of which are being constitutively activated in AML cells (28, 29).

FAK expression correlated with enhanced AML cells migration and drug resistance *in vitro*. Strikingly, down-modulation of FAK expression by siRNA significantly decreased migration and resistance to DNR of the CD34<sup>+</sup> leukemic cell line KG1. Our results are in line with previous *in vitro* studies performed in nonhematopoietic cells that linked FAK overexpression with motility. Indeed, FAK accelerated the turnover of focal adhesion contacts (30), whereas FAK-deficient cells displayed reduced motility in response to chemotactic stimuli (31). Moreover, FAK overexpression protected from etoposide-induced apoptosis *in vitro* (5) and FAK siRNA potentiated gemcitabine-induced cytotoxicity *in vitro* and *in vivo* (32).

FAK expression negatively influences survival in AML patients. Indeed, although our cohort is heterogeneous, it should be noted that among the 51 patients treated with the standard regimen DNR-Ara-C, FAK correlates with a high risk of early death, most likely because of increased blast cell count at presentation. The regulatory function of FAK on cell migration may explain the correlation between FAK expression in AML cells and leukocytosis. Shorter survival might also be related to FAK-mediated drug resistance as suggested by our results *in vitro*.

However, adhesion onto fibronectin of AML cells confers a significant protection toward DNR independently from FAK expression. This result suggests not only that CAMDR is applicable to fresh AML cells (33, 34) but also that FAK plays no role in cellular adhesion mediated-drug resistance of AML cells, as has been suggested for myeloma cells (35, 36).

Pyk2, another focal adhesion tyrosine kinase, was found to be expressed in most AML cells as it has been described in normal unstimulated CD34<sup>+</sup> cells (11). In each case, Pyk2 was found phosphorylated on Tyr-402, a required step for full catalytic activation (2). However, Pyk2 was also, although less consistently, phosphorylated on Tyr-881, a Grb2- and c-Abl-binding site (37). Interestingly, Tyr-881 phosphorylation correlated with FAK expression, suggesting that Pyk2 is a target for FAK as described previously (38). The mechanism by which Pyk2 is activated has not been investigated. However, previous studies have established that Pyk2 phosphorylation can be regulated not only by integrins but also by growth factors, including vascular endothelial growth factor and basic fibroblast growth factor

(39). Therefore, it is conceivable that accumulation of vascular endothelial growth factor and basic fibroblast growth factor, as documented in AML, could result in Pyk2 phosphorylation (40, 41).

Recent articles devoted a pro-migratory role to Pyk2 in cell migration of normal (42) or transformed cells (37, 43). Pyk2 phosphorylation on Tyr-402 and Tyr-881 seems to be critical for its function in cell locomotion (37). However, the specific roles of Pyk2 and FAK in the migration of cells that express both kinases are not known. Like FAK, Pyk2 activation may contribute to extracellular signal-regulated kinase activation through Grb2- and c-Abl regulation (6), but it can also regulate specific signaling pathways such as CDC42 activation (44), an important step for cell polarization (45). Moreover, the regulation of Pyk2 by FAK may influence the pro- or antiapoptotic pathways downstream of activated Pyk2 (7, 8, 9). Depending on the microenvironment, the balance between Pyk2 and FAK activities might represent an important factor in regulating the migratory and survival behavior of leukemic cells.

Finally, we show that aberrant expression of FAK is frequent in AML cells and that FAK enhances migration of leukemic cells from marrow to the circulating compartment, confers drug resistance, and negatively influences clinical outcome. This study designates FAK as a potentially important risk factor at the initiation of AML therapy and provides rationale for the development of strategies leading to FAK inhibition to sensitize AML cells to chemotherapy. It remains to determine whether Pyk2 could play a specific role in AML, notably in cellular adhesion mediated-drug resistance.

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