Received: 11 April 2023 | Accepted: 13 July 2023

DOI: 10.1111/bjh.18996

BJHaem
BRITISH JOURNAL OF HAEMATOLOGY

ORIGINAL PAPER

Focal adhesion kinase activation by calcium-dependent calpain is involved in chronic lymphocytic leukaemia cell aggressiveness

Filippo Severin | **Nayla Mouawad** | **Edoardo Ruggeri** | **Andrea Visentin** | **Leonardo Martinello** | **Elisa Pagnin** | **Valentina Trimarco** | **Stefano Pravato** | **Francesco Angotzi** | **Monica Facco** | **Livio Trentin** | **Federica Frezzato**

Hematology and Clinical Immunology Unit, Department of Medicine, University of Padova, Padua, Italy

Correspondence
Federica Frezzato, Hematology and Clinical Immunology Unit, Department of Medicine, University of Padova, Via Orus 2/B, Padova 35131, Italy.
Email: federica.frezzato@unipd.it

Livio Trentin, Hematology and Clinical Immunology Unit, Department of Medicine, University of Padova, Via Giustiniani 2, Padova 35128, Italy.
Email: livio.trentin@unipd.it

Funding information
Associazione Italiana per la Ricerca sul Cancro (AIRC); Associazione italiana contro le leucemie-linfomi e mieloma (AIL); ONLUS 'Ricerca per Credere nella Vita' (RCV) odv; Gilead Fellowship Program 2018

Summary
Signalling events downstream the B-cell receptor (BCR) are central for the survival and progression of chronic lymphocytic leukaemia (CLL) cells. Focal adhesion kinase (FAK), regulated through calpain, interacts with molecules of BCR signalling, cytoskeletal modelling and disease progression, such as Src/Lyn, cortactin and HS1. Hypothesizing that FAK might play a key role in CLL pathogenesis, we observed a down-modulation of FAK whole form, associated with FAK cleavage due to calpain activity upon BCR stimulation. Patients, whose cells were able to release Ca^{++} after BCR stimulation, had less amount of full-length FAK, which translated into a higher presence of cleaved/activated form of the protein phosphorylated at Y397, these features being mostly shown by immunoglobulin heavy chain (IGHV)-unmutated poor-prognosis patients. Moreover, we found that cortactin and HS1 proteins were overexpressed in those cells, suggesting a possible interplay with FAK. Treatment with the FAK inhibitor Defactinib was able to induce apoptosis in CLL cells. In conclusion, the malignant phenotype in unfavourable-prognosis patients seems to be encouraged by the overexpression of cortactin and HS1, that, together with FAK, may be involved in a druggable pathogenetic pathway in CLL.

KEYWORDS
B lymphocytes, chronic lymphocytic leukaemia, focal adhesion kinase, signal transduction

INTRODUCTION

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase protein[1] whose overexpression and constitutive activation have been extensively found to be involved in survival, proliferation, motility and invasion of human solid cancers.[2,3] Nonetheless, little is known in human normal and leukaemic cells.

Chronic lymphocytic leukaemia (CLL) is characterized by the proliferation of mature CD5+ B cells within microenvironmental niches in secondary lymphoid organs and bone marrow which support leukaemia cell survival and disease progression.[4] Upon B-cell receptor (BCR), chemokine receptors and integrins triggering, downstream pathways contributing to cancer phenotype and therapy resistance are activated. Considering the BCR pathway, the Src-kinase Lyn[5,6] mediates the activation of downstream factors such as BTK, PI3K/AKT and NF-kB, actin-binding proteins such as HS1 and cortactin, and the calcium release from intracellular stores. Considering chemokine receptors, CXCR3 and CXCR4, and their ligands, are up-regulated in CLL cells.[7] Consequently, they drive homing of B cells to lymphoid organs, through calcium release, PI3K, MAPK and FAK. Moreover, the CD49d/α4 integrin was found to be expressed

Filippo Severin and Nayla Mouawad are co-first authors.

Livio Trentin and Federica Frezzato are co-last and co-corresponding authors.

in a subset of patients with aggressive CLL and to mediate trafficking between blood and lymphoid organs through the activation of FAK, Src and actin.[8]

As a critical mediator of signals generated in response to the engagement of integrins, chemokines and growth factor receptors, FAK activation promotes adhesion/motility, survival and proliferation in different solid tumours.[9–11] FAK is characterized by a multidomain structure: the N-terminal FERM domain containing a nuclear localization signal (NLS) and binding sites for membrane receptors and MDM2/p53, the kinase domain and the C-terminal domain which contains two proline-rich-(PR) and a focal adhesion targeting (FAT) regions mediating the binding with actin and partners such as paxillin, talin, HS1 and cortactin. Upon binding of integrins with the extracellular matrix, FAK is recruited to the cell membrane together with Src/Lyn, dimerizes and auto-phosphorylates at Y397, generating a motif recognized and phosphorylated by Src/Lyn (Y576/577) thus contributing to FAK full activation and downstream signalling.

FAK is regulated by protease calpain cleavage and, while the full-length 125 kDa-FAK contains all the three domains, the calpain-mediated cleaved-FAK lacks the FAT one.[12] The distinct roles of these two isoforms are still unclear being only known that, when FAK is cleaved/activated, the focal adhesion (FA) disassembles and tumour cell motility, invasion and metastasis are enhanced.

Although only a few studies concerning the role of FAK in haematological malignancies have been carried out,[13–17] it is evident that FAK interacts with players involved in BCR signalling and correlated with CLL poor prognosis, such as Lyn and its substrates HS1[18] and cortactin.[19,20] Intriguingly, cortactin binds directly to FAK and, in turn, FAK phosphorylates cortactin, thus inducing a cortactin-FAK interplay accompanied by FA turnover and cell movement.[21] HS1 has been shown to bear similar characteristics to cortactin in CLL patients.[22] The high sequence homology shared by HS1 and cortactin suggests that also HS1 may attend FAK signalling.

This background supports a role for FAK in CLL and warrants further investigation to support our hypothesis of FAK involvement in the CLL cell survival and death especially at the crossroads of BCR, chemokine and integrin signalling pathways. In this context, our aim is to dissect the characteristics and the role of FAK in CLL. To this purpose, by meanings of cellular biology, flow cytometry (FC), biochemical and biomolecular techniques, we herein investigated FAK expression, activation, localization and inhibition in CLL B lymphocytes.

METHODS

Patients, cell separation and culture conditions

Leukaemic B cells were obtained from untreated CLL patients, enrolled by the Hematology Division of Azienda Ospedale—Università di Padova, satisfying standard criteria for CLL. Written informed consent according to the Declaration of Helsinki and ethical approval were obtained. Normal and leukaemic B cells have been obtained as previously detailed.[23] Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific) with 2% FBS and 5% Penicillin/Streptomycin (Euroclone) at 2×10^6/mL and incubated at 37°C in a humidified atmosphere containing 5% CO_2 with or without: 5 μM Defactinib, 50 μM calpeptin, 5 μM Bafetinib (Selleckchem), 2.5 and 5 mM Calcium Lactate (Sigma-Aldrich) for 12 h or 24 h; with 10 μg/mL IgM or IgD for 5′ and 3′ respectively, according to the different experiments.

Flow cytometry

Apoptosis was assessed using the Annexin V Apoptosis Detection Kit (Valter Occhiena S.r.l.) as previously described[23] and calcium (Ca^{++}) mobilization has been performed as detailed in Martini et al.[24] HS1 and cortactin were analysed in CD19+/CD5+ cells with anti-HS1 AlexaFluor®647 (cell signalling) and anti-cortactin AlexaFluor®488 (Merck Millipore). Before staining, cells were fixed and permeabilized with FIX & PERM cell permeabilization kit for 10 min each at room temperature (Invitrogen; Thermo Fisher Scientific, Inc.).

Subcellular protein fractionation

Nuclear/cytoplasmic fractionation has been performed with the 'Subcellular Protein Fractionation Kit for Cultured Cells' (Thermo Fisher Scientific).

Western blotting analysis

Samples were prepared as previously described[25] and analysed with antibodies to FAK (Merck Millipore), FAK-Y397, calpain, PARP (Cell Signalling), HS1 (kindly provided by Prof. Brunati, Padua University), cortactin and β-actin (Sigma-Aldrich).

Chemotaxis assay

Three-micrometre-pore polycarbonate transwell inserted in 24-well plates (Corning Inc.) was used to evaluate the migration. Briefly, 2×10^6 cells were incubated in 1 mL RPMI medium with and without 5 μM Defactinib for 4 h at 37°C. 10^6 cells in 200 μL RPMI were transferred into the top chambers of transwell culture insert. In the lower chamber, 600 μL of RPMI medium containing 200 ng/mL CXCL12 (R&D Systems) were added. CLL B cells were allowed to migrate for 3 h at 37°C. Migrated cells were then collected and counted on a FACSCanto A for 60 s in duplicates.

Statistical analysis

Statistical analyses were performed using Prism 9 (GraphPad Software Inc.). Data are reported as mean ± standard deviation (SD) and p values <0.05 were considered significant.

RESULTS

FAK is down-modulated in poor prognosis CLL patients

CLL B cells exhibited a considerable variability of 125 kDa-FAK expression levels ($n = 142$, 0.56 ± 0.55) differently from normal B cells ($n = 10$, 0.67 ± 0.18; Figure 1A). Although no significant difference was detected between the two populations ($p = 0.55$, Student's t-test), there was an evident down-modulation of FAK expression in a number of patients compared to normal controls, being correlated with somatic hypermutations of immunoglobulin heavy chain (IGHV) genes.[26,27] 125 kDa-FAK resulted to be significantly down-regulated in poor-prognosis unmutated patients (U-CLL, $n = 56$; 0.37 ± 0.34) with respect to the mutated counterpart (M-CLL, $n = 67$; 0.75 ± 0.71, $p < 0.01$ ANOVA test involving the normal, M-CLL and U-CLL groups—Figure 1B).

FAK is cleaved, hence activated, by calpain protease in CLL patients

By proteolysis, calpain generates FAK fragments of different lengths.[28,29] To examine this phenomenon in CLL, we checked the expression of potential cleaved fragments in samples from 41 CLL and 10 normal B cells. As presented in Figure 1C, besides the full-length protein of 125 kDa, bands of approximatively 90, 80 and 50 kDa (arrows) were detected. Normal B cells mainly presented the full-length FAK. We observed that unmutated patients (13/41) had an increased expression of FAK cleavage fragments with respect to mutated ones (0.56 ± 0.37; $p < 0.05$, ANOVA test—Figure 1D). In agreement with Łopatniuk et al.,[30] we observed a trend according to which poor-prognosis U-CLL ($n = 15$) and

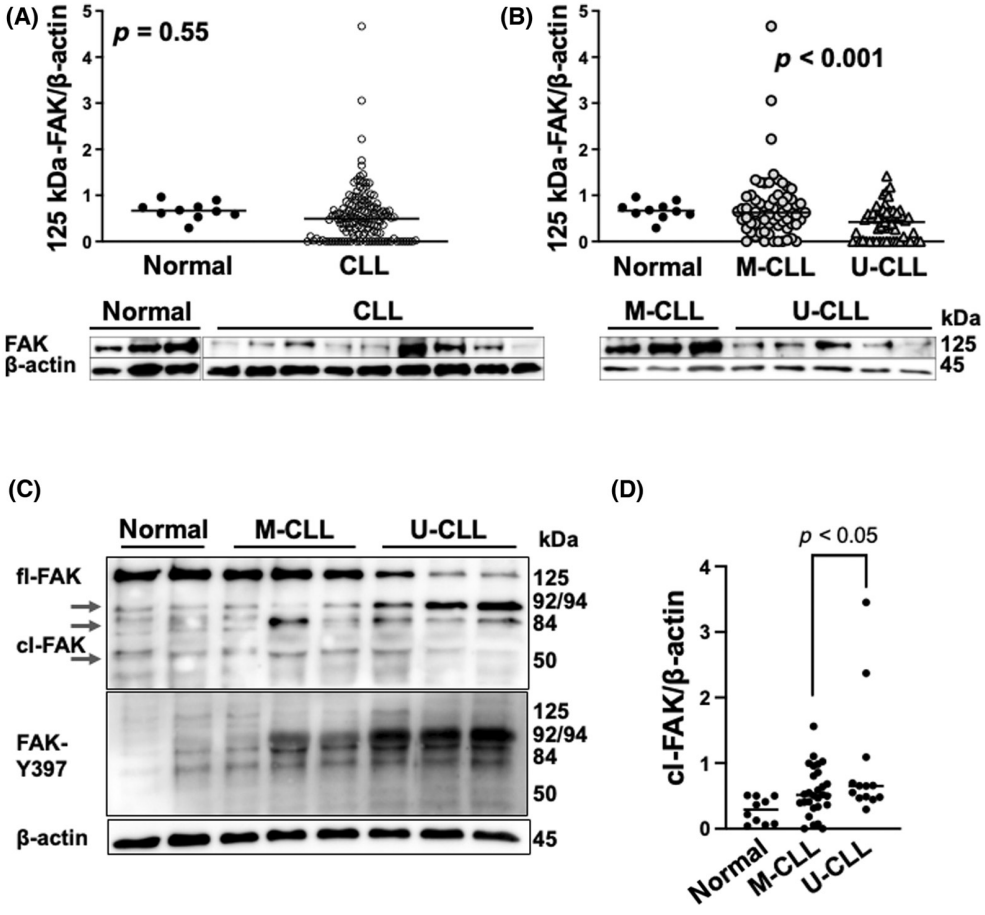

FIGURE 1 Expression of 125 kDa-focal adhesion kinase (FAK) protein in chronic lymphocytic leukaemia (CLL) B lymphocytes. Lysates obtained from normal and CLL B cells were analysed by immunostaining with anti-FAK, anti-FAK-Y397 and β-actin as loading control. (A) Densitometry of FAK/β-actin ratio of B cells from 142 CLL patients and 10 healthy subjects. (B) Patients plotted in part A have been divided according to different prognosis, mutated (M, $n = 67$) versus unmutated (U, $n = 56$) ones ($p < 0.01$, ANOVA test). Western blotting are representative of all the samples analysed. (C) WB shows full length (fl)-, cleaved (cl)-, and Y397-FAK in representative CLL ($n = 41$) and normal ($n = 10$) samples. Arrows indicate FAK cleavage fragments. (D) Densitometry of cl-FAK/β-actin ratio of B cells from 28 M-CLL, 13 U-CLL and 10 healthy subjects.

ZAP70^{pos} ($n = 11$) patients expressed calpain at higher levels compared to M-CLL ($n = 18$) and ZAP70^{neg} ($n = 14$; $p < 0.05$, Student's *t*-test—Figure 2A), this result potentially explaining the major cleavage found in U-CLL. The cleaved forms detected indicate that the protein undergoes a proteolytic cleavage mediated by calpain, which in turn is activated by Ca^{++} release from intracellular stores. Calcium lactate (CaLa), a calpain activator, is able to induce the cleavage and the phosphorylation of FAK with increasing concentrations. The activation of calpain is highlighted by its enhanced auto-catalytic activity ($n = 6$, Figure 2B,D). On the contrary, the treatment of CLL cells with the calpain inhibitor calpeptin, as assessed by the reduction of calpain auto-cleavage product, reduces both FAK cleavage and phosphorylation at Y397. As calpain is also activated by Src-kinases, the treatment of CLL cells with Bafetinib, which inhibits Lyn, is able to further decrease calpain auto-cleavage as well as FAK cleavage and phosphorylation at Y397 ($n = 6$, Figure 2C,E).

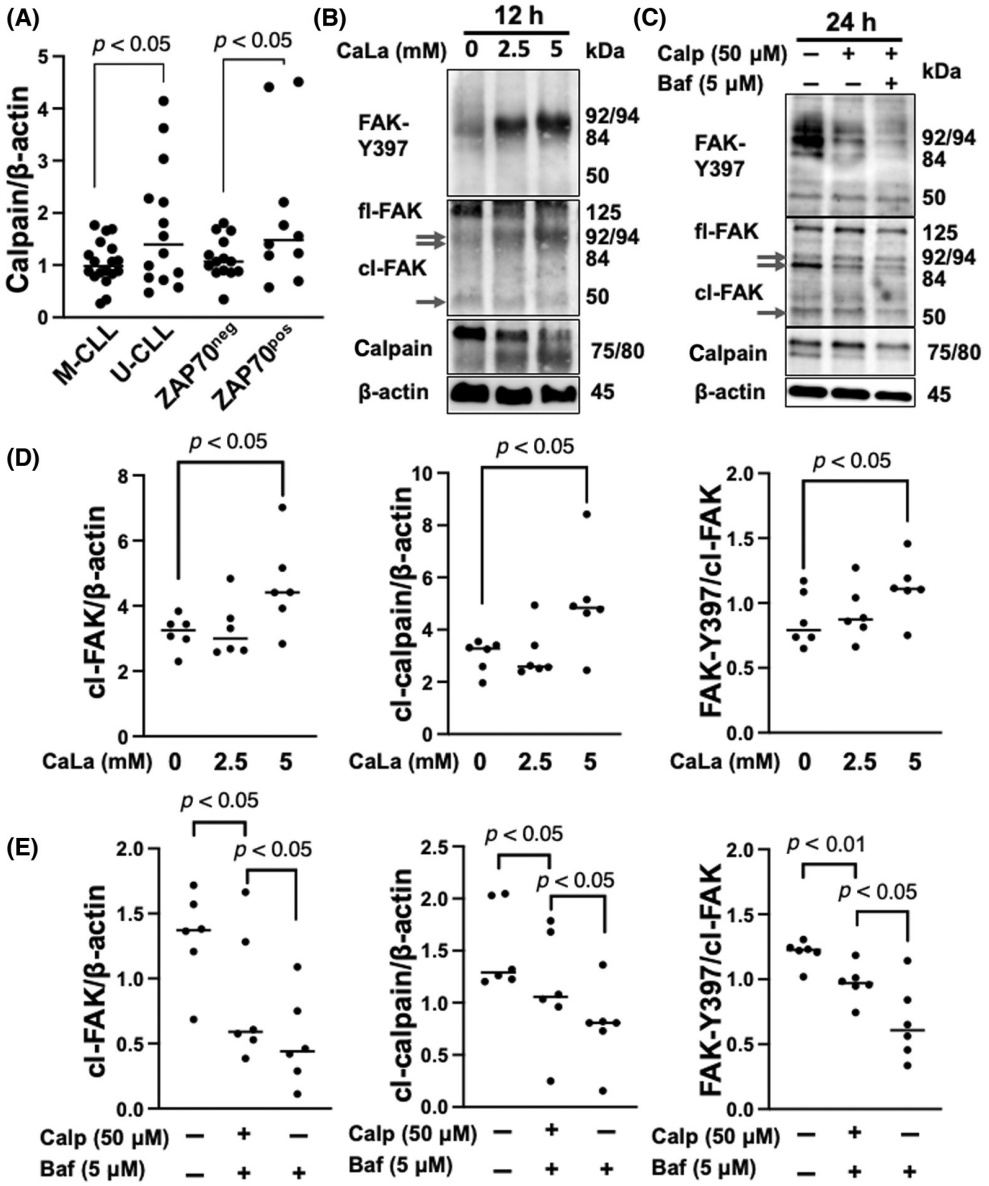

FIGURE 2 Calpain involvement in focal adhesion kinase (FAK) cleavage in chronic lymphocytic leukaemia (CLL) cells. Lysates obtained from CLL B cells were analysed by immunostaining with anti-calpain, anti-FAK, anti-FAK-Y397 and β-actin as loading control. (A) Densitometry of calpain/β-actin ratio in mutated (M, $n = 18$) versus unmutated (U, $n = 15$; left) and in ZAP70^{neg} ($n = 14$) versus ZAP70^{pos} ($n = 11$, right) patients ($p < 0.05$, Student's *t*-test). (B) Western blotting (WB) is representative of experiments with CLL cells ($n = 6$) incubated for 12 h with Calcium Lactate (CaLa) 0, 2.5 and 5 mM. (C) WB is representative of experiments with CLL cells ($n = 6$) incubated for 24 h alone, with calpeptin (Calp) 50 μM and calpeptin 50 μM plus Bafetinib (Baf) 5 μM. (D) Densitometry of cl-FAK/β-actin, cl-calpain/β-actin, and FAK-Y397/cl-FAK ratios from six different experiments as represented in B ($p < 0.05$, paired Student's *t*-test). (E) Densitometry of cl-FAK/β-actin, cl-calpain/β-actin, and FAK-Y397/cl-FAK ratios from six different experiments as represented in C ($p < 0.05$ and $p < 0.01$, paired Student's *t*-test). fl, full length; cl, cleaved. Arrows indicate FAK cleavage fragments.

FAK is cleaved in CLL patients mobilizing intracellular calcium

Calpain is stimulated by Ca^{++}, thus we hypothesized that Ca^{++} mobilization following BCR activation enhances FAK cleavage/activation. Given that CLL patients may or may not express surface immunoglobulin (sIg) (IgM and/or IgD), and being heterogeneous in terms of Ca^{++} mobilization, we evaluated both Ig expression and the ability of CLL cells to mobilize Ca^{++} using FC. In particular, we assessed the response of the BCR following the addition of anti-IgM and/or IgD. After the Ca^{++} release analysis, we divided patients into two groups based on their ability to mobilize Ca^{++} or not: (i) patients expressing surface IgM and/or IgD, that were capable of mobilizing Ca^{++} from intracellular stores upon stimulation (referred to as responsive CLL, $n = 16$), (ii) patients who do not express neither IgM nor IgD or cannot be stimulated, that were not able to release Ca^{++} (referred to as non-responsive CLL, $n = 14$; Table 1). In these groups, we assessed FAK expression and its Y397 phosphorylation. As hypothesized, cells of the responsive group showed a down-regulation of 125 kDa-FAK while they mainly expressed cleaved forms as well as phosphorylation at Y397, index of FAK activation (responsive CLL; Figure 3A—right and B). On the contrary, non-responsive group presented a major expression of 125 kDa-FAK with

TABLE 1 Biological and clinical characteristic of patients.

CLL	Age	Sex[a]	IGHV[b]	WBC (/mm^3)	CD19/5 (%)	CD19/IgM[c] (%)	CD19/IgD (%)[d]	Ca^{++} mobilization[e]
#1	74	M	M	28 750	89	Neg	Neg	N
#2	73	M	M	9860	50	Neg	Neg	N
#3	80	M	M	25 140	71	Pos	Pos	N
#4	72	M	M	21 160	71	Neg	Neg	N
#5	68	M	M	94 750	96	Neg	Neg	N
#6	83	F	M	29 950	88	Neg	Neg	N
#7	58	M	M	46 950	88	Neg	Neg	N
#8	74	M	M	28 750	89	Neg	Neg	N
#9	73	M	M	9860	50	Neg	Neg	N
#10	65	F	M	29 500	92	Neg	Neg	N
#11	72	M	M	21 160	71	Neg	Neg	N
#12	76	F	M	100 800	95	Neg	Neg	N
#13	83	F	M	29 850	88	Neg	Neg	N
#14	68	M	M	94 750	96	Neg	Neg	N
#15	69	M	U	65 260	92	Pos	Pos	N
#16	84	F	U	47 050	91	Neg	Pos	Y
#17	68	M	U	153 900	98	Neg	Pos	Y
#18	68	M	U	64 950	91	Neg	Pos	Y
#19	73	F	U	16 800	59	Pos	Pos	Y
#20	41	M	U	49 800	89	Pos	Pos	Y
#21	71	M	U	130 000	99	Neg	Pos	Y
#22	79	F	U	51 260	86	Pos	Pos	Y
#23	81	F	U	28 940	92	Neg	Pos	Y
#24	63	M	U	86 580	96	Pos	Pos	Y
#25	86	F	U	47 050	96	Neg	Pos	Y
#26	68	M	U	64 950	91	Neg	Pos	Y
#27	78	M	U	74 000	97	Pos	Neg	Y
#28	79	F	U	51 260	86	Pos	Pos	Y
#29	84	F	U	28 940	92	Neg	Pos	Y
#30	63	M	U	47 050	96	Pos	Pos	Y

Abbreviations: CLL, chronic lymphocytic leukaemia; nd, not determined; neg, negative; pos, positive.

[a]M: male, F: female.

[b]Mutated (M) was defined as having a frequency of mutations ≥2% from the germline heavy-chain-variable (VH) sequence.

[c,d]As determined by cytofluorimetric analysis (cut-off = 30%).

[e]N: no, Y: yes.

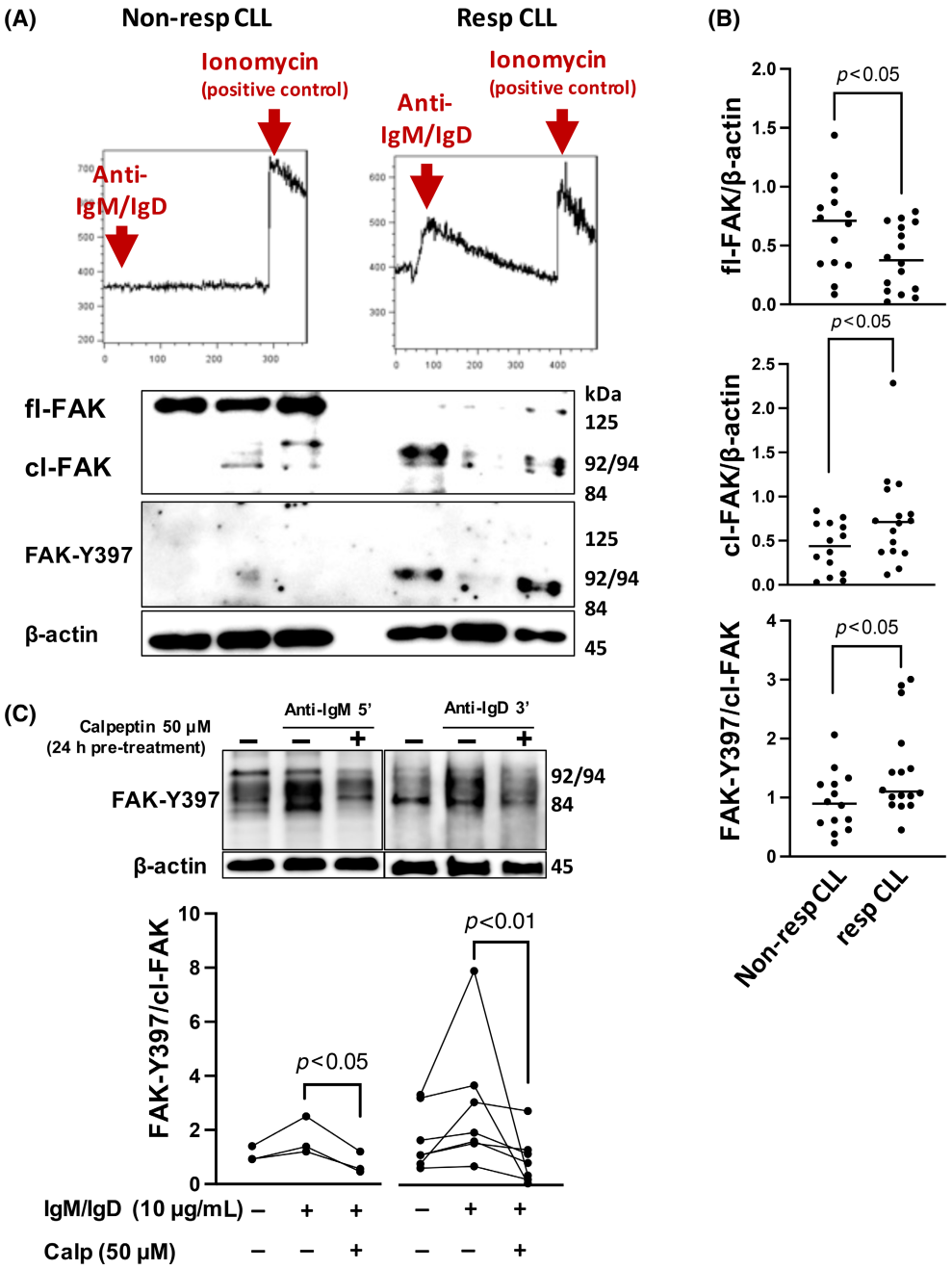

FIGURE 3 B-cell receptor (BCR) signalling response in chronic lymphocytic leukaemia (CLL) patients. (A) The ability of CLL patients to respond to BCR stimulation has been verified in terms of intracellular Ca^{++} mobilization. Therefore, patients have been divided in two groups: one comprising CLL cases which did not show any significant Ca^{++} signal after stimulation, labelled as the non-responsive CLL (non-responsive, left); and the other, identified as responsive BCR patients, consisting of patients' cells which manifested Ca^{++} mobilization after BCR engagement (responsive, right). As reported in the representative western blotting (WB), responsive BCR patients exhibit cleaved and active focal adhesion kinase (FAK). The group of non-responsive CLL ($n = 14$) exhibited barely just the non-phosphorylated full-length FAK, whereas in the responsive patients ($n = 16$) FAK was present as its active and cleaved form. (B) Densitometry of fl-FAK/β-actin, cl-FAK/β-actin and FAK-Y397/cl-FAK of all the sample analysed ($p < 0.05$, Student's t-test). (C) The effect of BCR stimulation on FAK activation has been evaluated. Representative WB evaluating FAK-Y397 in responsive CLL patients following stimulation with 10 µg/mL IgM (5′) or IgD (3′) and pretreated or not with calpeptin 50 µM. Plots represent densitometric analysis of FAK-Y397/cl-FAK ratios performed in IgM-responsive CLL ($n = 3$) and IgD-responsive CLL ($n = 7$) following calpeptin pretreatment ($p < 0.05$ and $p < 0.01$, respectively; Friedman test).

less presence of cleaved fragments and Y397 phosphorylation (non-responsive CLL; Figure 3A—left and B). In our case history, patients able to mobilize Ca^{++}, with a reduced expression of 125 kDa-FAK and a higher expression of phosphorylated fragments mostly belong to U-CLL group. In line with our previous results, calpain was mainly expressed in responsive versus non-responsive CLL (data not shown). To further evaluate whether BCR stimulation leads to FAK activation, and whether this process is mediated by calpain activity, we stimulated patients' cells with

anti-IgM ($n = 4$) or anti-IgD ($n = 10$) in vitro. We observed an increase in FAK phosphorylation at the active site Y397 upon BCR stimulation in three of four CLL patients stimulated with anti-IgM and in 7 of 10 CLL patients stimulated with anti-IgD. Notably, this increase was significantly prevented by pretreatment with the calpain inhibitor calpeptin (Figure 3C). Of note, the remaining patients who did not show an increase in FAK phosphorylation (1/4 IgM and 3/10 IgD positive) were not able to mobilize calcium (data not shown). In exploring the subcellular localization of FAK by protein fractionation, we revealed that, in the non-responsive patients ($n = 7$), FAK was mostly present in the cytoplasm. Instead, in the responsive samples ($n = 5$) FAK localized also inside the nucleus, in its cleaved/activated form. cl-FAK expression levels in the nuclear fraction of responders (0.3459 ± 0.1415) is significantly higher than in non-responders (0.096 ± 0.060; $p < 0.05$, Mann–Whitney test—Figure 4A,B).

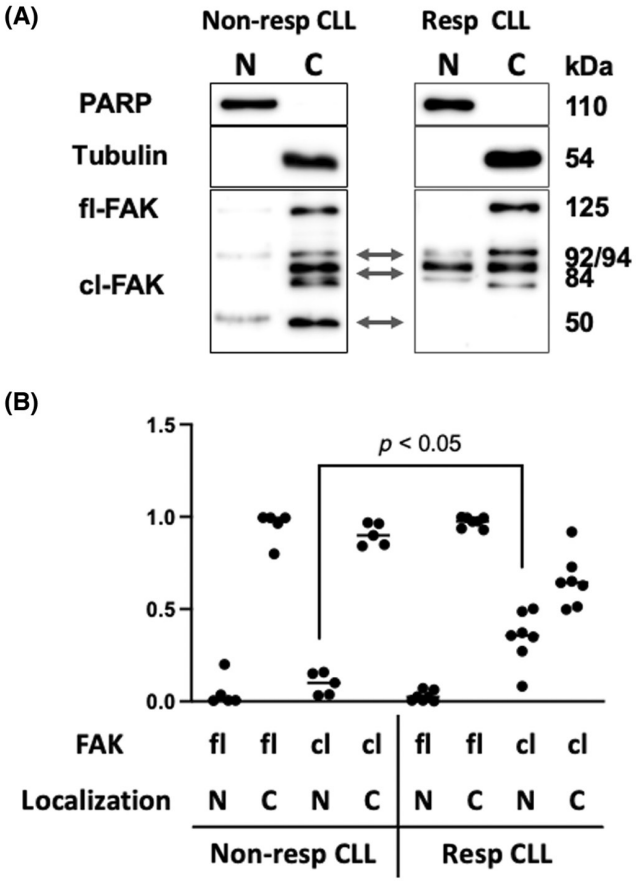

FIGURE 4 Focal adhesion kinase (FAK) subcellular localization in responders versus non-responders. Aliquots of 10^7 cells were lysed with the different buffers provided by the kit for subcellular fractionation, obtaining proteins from the cytoplasmic (C) and nuclear (N) compartments. (A) Representative blot shows FAK full-length (125 kDa) present only in the cytoplasm, while cleaved/activated FAK fragments were present also inside the nucleus of cells, particularly in patients responsive to B-cell receptor (BCR) stimulation (responsive CLL, right). fl, full length; cl, cleaved. Arrows indicate FAK cleavage fragments. (B) Plots represent values from all the patients analysed ($n = 5$ non responder and $n = 7$ responders). fl-FAK and cl-FAK expression in each fraction has been normalized on total FAK expression (fl-FAK + cl-FAK) of that specific fraction. $p < 0.05$, Mann–Whitney test between nuclear cl-FAK in responder versus non-responders.

FAK activation correlates with cortactin and HS1 expression

FAK involvement in the engagement of integrins and assembly of FA, implies a major role in cellular adhesion and invasion of various cancers. In the same processes, HS1 and cortactin[22] are relevant for cytoskeletal shaping and motility of the tumour cell, these molecules found to be associated with CLL aggressiveness[18–20] and interacting with FAK. Parallel to the presence of active/cleaved-FAK in the group of patients with positive BCR signal and aggressive disease ($n = 6$), we found an overexpression of cortactin and HS1, compared to the non-responsive cases ($n = 6$, 697 ± 399 vs. 265 ± 137 for cortactin; 1618 ± 1575 vs. 232 ± 139 for HS1; $p < 0.05$ Mann–Whitney test; Figure 5A). Another point in favour of the correlation between HS1 and cortactin overexpression and the presence of active FAK was obtained by a previous RPPA study.[31] We divided patients into two groups according to the obtained median value of FAK phosphorylation at Y397, namely FAK-Y397^{low} ($n = 28$) versus FAK-Y397^{high} ($n = 27$, Figure 5B, up). HS1 was significantly overexpressed in the phospho-FAK-high group ($29\,802 \pm 7354$) compared to the samples with decreased phospho-FAK ($25\,027 \pm 6481$; $p < 0.05$), and so was for cortactin ($365\,786 \pm 117\,921$ vs. $302\,955 \pm 122\,501$; $p < 0.05$, Student's t-test, Figure 5B, down). A significant positive correlation was demonstrated between HS1 expression levels and FAK activation ($p < 0.05$, Pearson's correlation, data not shown). In this context, we analysed by western blotting cortactin, HS1 and FAK-Y397 in the same patients ($n = 16$) thus finding a positive correlation between the expression levels of the two proteins (HS1 and cortactin) with FAK activation (as indicated by Y397 phosphorylation), this correlation being more evident for HS1 ($p < 0.0001$) than for cortactin ($p < 0.05$, Pearson's correlation; Figure 5C).

FAK inhibition leads to CLL cell apoptosis

We examined the effect of FAK inhibition on the survival of B lymphocytes from CLL patients after treatment with Defactinib and we demonstrated a lower amount of 125 kDa-FAK in the treated condition (0.47 ± 0.39) compared to the untreated cells (0.91 ± 0.90; $p < 0.001$, paired Student's t-test). In addition, FAK activation was also reduced in the treated cells (1.16 ± 0.80 vs. 1.81 ± 0.94; $p < 0.0001$, paired Student's t-test; $n = 34$, Figure 6A,B). FAK inhibition induced significant apoptosis of CLL cells as assessed by the increase in the cleaved PARP in the treated samples compared to the untreated control (Figure 6A, PARP-related bands). These data were confirmed by FC

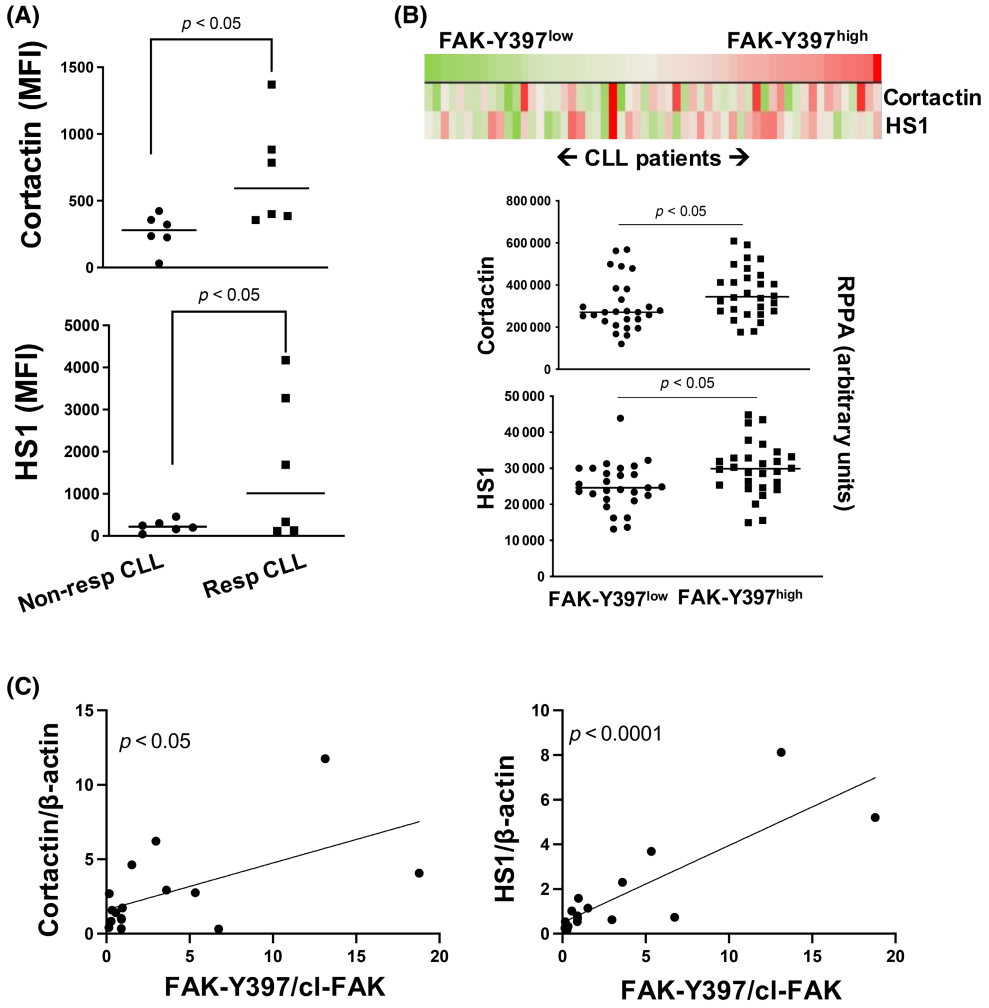

FIGURE 5 Focal adhesion kinase (FAK) connection to cytoskeletal/microenvironmental actors. (A) HS1 and cortactin expression have been evaluated by flow cytometry, using specific fluorochrome-conjugated Abs against the two cytoplasmic proteins (AlexaFluor®647 and AlexaFluor®488 respectively). Patients with responsive B-cell receptor and a more aggressive disease (responsive chronic lymphocytic leukaemia [CLL], $n = 6$; right), overexpressed cortactin and HS1 compared to the non-responsive cases (non-responsive CLL, $n = 6$; left—$p < 0.05$ for both proteins, Mann–Whitney test). (B) HS1 and cortactin expression and FAK phosphorylation on Y397 were evaluated by a previous RPPA as reported in Frezzato et al.[31] Patients were divided in two groups according to the median value of FAK phosphorylation on Y397, namely FAK-Y397^{low} versus FAK-Y397^{high}. HS1 and cortactin were found to be significantly overexpressed in the pFAK-Y397^{high} group ($p < 0.05$, Student's t-test). (C) HS1 and cortactin expression and FAK phosphorylation on Y397 were evaluated by western blotting. HS1 and cortactin have been normalized on β-actin, Fak-Y397 has been normalized on cl-FAK. Values obtained have been plotted by Pearson's correlation ($p < 0.05$, HS1 vs. FAK-Y397 and $p < 0.0001$, cortactin vs. FAK-Y397).

analysis that demonstrated a reduced number of viable cells after the treatment with the inhibitor, confirming that Defactinib is effective in CLL (62 ± 17% vs. 33 ± 24%, $n = 46$; $p < 0.0001$, paired Student's t-test; Figure 6C,D). We checked the baseline expression of 125 kDa-FAK expression in patients treated with Defactinib (data reported in 34 patients) thus observing a significant correlation ($p < 0.05$, Pearson's correlation; Figure 6E). Also in this cohort, IGHV-mutated patients present higher levels of fl-FAK ($n = 15$, 1.34 ± 1.19) with respect to IGHV-unmutated ones ($n = 9$, 0.54 ± 0.24; $p < 0.05$ Student's t-test, Figure 6F). Defactinib is more effective in U-CLL (38 ± 17%) versus M-CLL (61 ± 22%; $p < 0.05$, Student's t-test—Figure 6G). Of note, both cortactin ($n = 21$) and HS1 ($n = 19$) expression levels decreased after Defactinib treatment as demonstrated in Figure 7A,B ($p < 0.001$ and $p < 0.01$ respectively; alone vs. treated, paired Student's t-test). Moreover, Defactinib is able to counteract the chemotaxis induced by CXCL12 ($n = 8$; $p < 0.001$, paired Student's t-test; Figure 7C).

DISCUSSION

We demonstrated that, in CLL lymphocytes, FAK is present in both full-length and calpain-cleaved forms, is hyper-phosphorylated, and is correlated with cortactin and HS1,[18–20] especially in poor prognosis patients. We showed that FAK has a role in the survival potential of CLL, as supported by the induction of apoptosis by the use of its selective inhibitor, Defactinib.

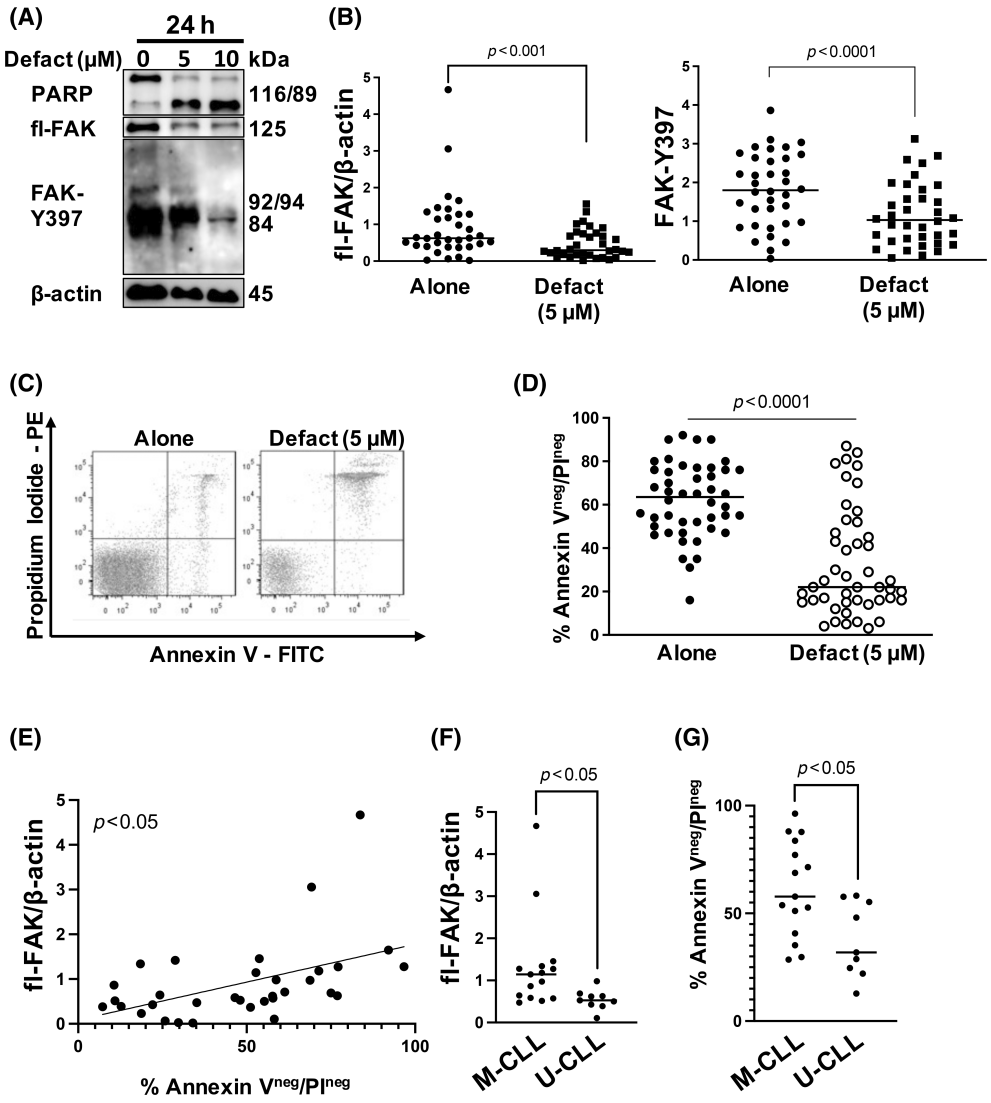

FIGURE 6 Defactinib is effective in chronic lymphocytic leukaemia (CLL) cells. (A) The representative western blotting (WB) (of 34 samples analysed) revealed that a lower amount of the full-length 125 kDa-focal adhesion kinase (FAK) as well as FAK-Y397 phosphorylation was present after treatment with 5 and 10 μM Defactinib (Defact). FAK inhibition led to the increase in cleaved PARP (89 kDa) in the treated samples compared to the untreated control, demonstrating a pro-apoptotic effect of Defactinib in CLL cells. (B) Plots represent densitometric analysis of 125 kDa-FAK/β-actin ratio (left) and of FAK-Y397 (left) performed in untreated cells versus cells treated with 5 μM Defactinib for 24 h ($n = 34$; $p < 0.001$ for 125 kDa-FAK and $p < 0.0001$ for FAK-Y397, paired Student's *t*-test). (C) Treatment with 5 μM Defactinib led to apoptosis of CLL, determined as a decrease in the percentage of alive cells as represented in the representative cytogram. (D) Cells were cultured alone or in the presence of 5 μM Defactinib and cell apoptosis was analysed by annexin V/PI flow cytometric test. Plots report the percentage of Annexin V^{neg}/PI^{neg} cells after 24 h treatment. Data are reported as mean ± SD ($n = 46$; $p < 0.0001$, paired Student's *t*-test). (E) Graph reports basal values of fl-FAK obtained by WB and normalized on β-actin and % of viable cells after 24 h treatment with 5 μM Defactinib in the same patients plotted by Pearson's correlation ($p < 0.05$). (F) Plots represent densitometric analysis of fl-FAK/β-actin ratio performed in M-CLL ($n = 15$) versus U-CLL ($n = 9$; $p < 0.05$, Student's *t*-test). (G) Plots represent % of viable cells after treatment with 5 μM Defactinib for 24 h in M-CLL ($n = 15$) versus U-CLL ($n = 9$; $p < 0.05$, Student's *t*-test).

FAK is a crucial molecule at the crossroad between extracellular environment and intracellular signalling that, once activated by interactions with integrins, growth-factor receptors and chemokine receptors, interacts with Src/Lyn thus controlling migration, proliferation and survival.[10] Differently from solid tumours,[32] little is known about FAK functions in CLL.[17] Thus, we planned to explore FAK role in CLL considering its involvement in many key signalling pathways connected to leukaemia development.

FAK has a crucial role in regulating the dynamics of FA, thus it is subjected to proteolytic cleavage by calpain proteases in turn activated by intracellular Ca^{++} release. Łopatniuk et al.[31] demonstrated that calpain levels in CLL were correlated with the clinical stage of the disease. Accordingly, we demonstrated that poor-prognosis patients followed a trend towards overexpression of the protease.

Research papers describing FAK in haematological malignancies[33–35] did not report any evidence on FAK

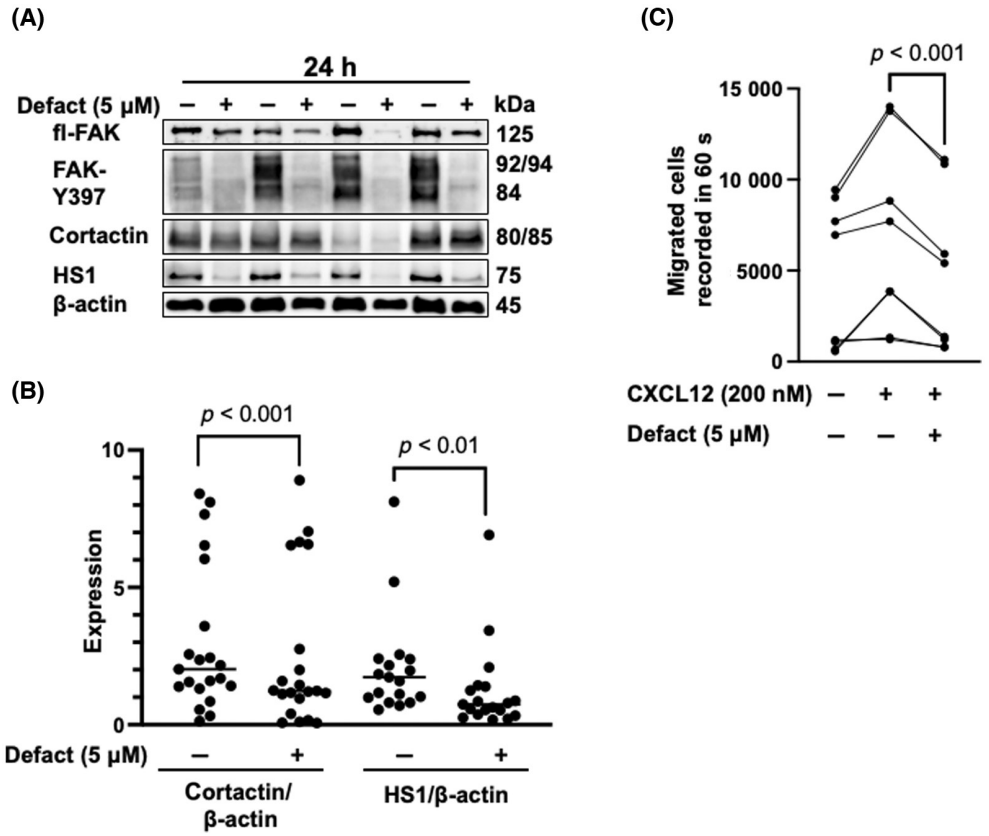

FIGURE 7 Effect of Defactinib on cytoskeletal proteins and migration of chronic lymphocytic leukaemia (CLL) cells. (A) The representative western blotting (WB) (of 21 samples analysed) revealed that a lower amount of the full-length 125 kDa-focal adhesion kinase (FAK) as well as FAK-Y397 phosphorylation was present after 24 h treatment with 5 μM Defactinib (Defact). Of note, a decrease in cortactin and HS1 expression is highlighted. (B) Plots represent cortactin and HS1 reduction in CLL cells after 24 h treatment with 5 μM Defactinib (21 and 19 samples analysed, respectively—$p < 0.001$ for cortactin and $p < 0.01$ for HS1 versus untreated condition, paired Student's *t*-test). fl, full length. (C) Graph represents the number of migrated cells, recorded in 60 s by flow cytometry, treated (+) or not (–) with CXCL12 (200 nm) and Defactinib (5 μM) ($p < 0.001$, paired Student's *t*-test).

investigation beyond the full-length form of 125 kDa. In CLL, we found that FAK was present in both full-length form of 125 kDa and also as fragments of lower molecular weight. Literature review suggested that these fragments of 92/94, 84 and 50 kDa were compatible with cleavage products by calpain.[12] We found a major amount of the calpain-mediated products of FAK in poor-prognosis U-CLL, where FAK was also hyperphosphorylated, meaning active. Our results are supported by Sundaramoorthy et al. who, in colon cancer, demonstrated that FAK cleavage by calpain, after Ca^{++} administration, increased the kinase activity thus enhancing the motility of cancer cells, synonym of adverse outcome.[36]

The absence of mutations in IGHV genes in U-CLL often reflects the presence of a responsive BCR, compared to a more anergic receptor on M-CLL cells, commonly expressing low levels of surface Igs. We validated that, in contrast to non-responsive cases, the BCRs on responsive cells were able to mobilize Ca^{++}. Since calpain is regulated by Ca^{++}, we hypothesized that the protease might be more active in the responsive patients, mainly U-CLL. This may explain our results on the increased expression of FAK cleavage products, particularly in poor-prognosis cases.

FAK possesses also kinase-independent functions especially in the nucleus[37] where FAK can suppress p53 activity acting as a scaffold protein for both p53 and MDM2 thus facilitating the degradation of p53.[38,39] As of CLL, we demonstrated that the full-length 125 kDa-FAK was present only in the cytosol, while the cleaved/active forms were localized also inside the nucleus, especially in patients with a responsive BCR. Comparably, in another model, it was found that Ca^{++} administration increased cleaved-FAK (N-FAK) levels in nuclear fractions.[36] We therefore suppose that, when calpain cleaves FAK in the two N-FAK and C-FAK fragments, N-FAK can dissociate from FA and translocate into the nucleus. Here, FAK may be directly involved in mechanisms promoting survival and proliferation. Since p53 can bind to FAK gene promoter, counteracting its expression, and considering that p53 is inactivated in about the 15% of CLL patients correlating with a remarkable unfavourable prognosis, the investigation on FAK-p53 interaction is certainly to be further explored.

We analysed FAK relationship with HS1 and cortactin, two Lyn substrates associated with CLL pathogenesis and aggressiveness.[18–20] We found that FAK expression and phosphorylation correlated with HS1 and cortactin, thus permitting to speculate the existence of a pathway involving FAK–cortactin–HS1 ultimately promoting the aggressive phenotype of CLL.

Small molecules inhibiting FAK kinase are being developed, among which the selective ATP-competitive inhibitor Defactinib completed the Phase II clinical trial in patients with KRAS-mutant non-small-cell lung cancer.[10] We demonstrated that Defactinib was effective in inducing apoptosis of CLL cells as well as downmodulating FAK expression and phosphorylation, HS1 and cortactin expression, and attenuating CXCL12-induced chemotaxis. We observed that patients with a major basal expression of fl-FAK showed also a major 'resistance' to Defactinib treatment in vitro. In other words, as IGHV-mutated patients present higher levels of fl-FAK—with respect to IGHV-unmutated ones—we can state that Defactinib is more effective in unmutated patients. The rationale behind this observation may be attributed to the fact that unmutated patients also have a more active FAK (demonstrated in terms of phosphorylation at Y397) and Defactinib acts specifically by inhibiting this FAK phosphorylation. Other studies revealed that VS-4718, another FAK inhibitor, prevented cell growth and induced apoptosis in AML cell lines.[39] Similarly, the silencing of FAK inhibited leukemogenesis in BCR/ABL-transformed ALL cells and increased apoptosis of cancer cells in combination with Imatinib.[40]

Figure 8 outlines our hypothetical pathway involving BCR-calpain-FAK activation-HS1/cortactin, eventually leading to an aggressive phenotype. Sustained by literature, we hypothesize that, in CLL, FAK can be activated by multiple pathways, besides being cleaved by calpain. In BCR responsive patients, calpain protease action results in an improved cleavage of FAK and the consequent generation of N-terminal fragments. These latter can translocate inside the nucleus where they could counteract the anti-tumoral functions of p53. Back to the cytoplasm, FAK expression and activation is correlated with overexpressed cortactin and HS1.[18–20]

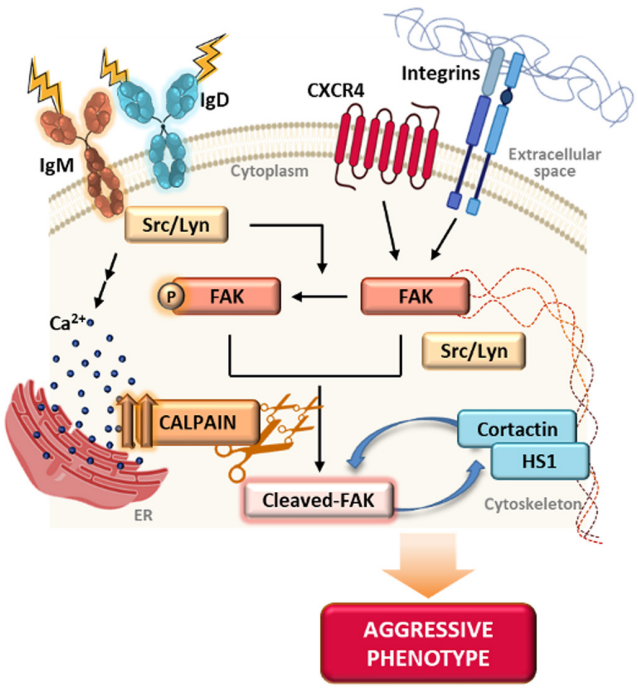

FIGURE 8 Hypothetical model of focal adhesion kinase (FAK) interactions in chronic lymphocytic leukaemia (CLL). After B-cell receptor stimulation, Ca^{++} is released in the cytoplasm and can activate calpain protease, which cleaves FAK, thus activating it. Together with HS1 and cortactin, FAK could be involved in a pathway promoting the malignant phenotype of CLL.

We therefore propose that FAK has a decisive role in the survival of poor prognosis CLL cells and could be exploited as a therapeutic target. Despite the recent developments in the search for effective treatments to eradicate the disease, CLL still remains incurable. The identification of the features of the patients more responsive to Defactinib administration may be helpful in identifying a subset of CLL cases whose therapy could be implemented with this inhibitor, alone or in combination.

AUTHOR CONTRIBUTIONS

Filippo Severin, Nayla Mouawad, Eodardo Ruggeri, Leonardo Martinello, Elisa Pagnin and Valentina Trimarco performed the research. Filippo Severin, Nayla Mouawad, Federica Frezzato and Livio Trentin designed the research study. Andrea Visentin, Stefano Pravato, Francesco Angotzi and Livio Trentin gave patient samples and retrieved patient clinical data. Federica Frezzato, Monica Facco and Livio Trentin wrote and reviewed the paper. Livio Trentin acquired funds for the research.

ACKNOWLEDGEMENTS

This work was supported by funds from Associazione Italiana per la Ricerca sul Cancro (AIRC) project to L.T. (IG-25024), Associazione italiana contro le leucemie-linfomi e mieloma (AIL), ONLUS 'Ricerca per Credere nella Vita' (RCV) odv, Padua, Italy, Gilead Fellowship Program 2018 to L.T. L.M., V.T. and E.R. received a research fellowship from AIL. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical approval has been obtained for this study from the local ethic committee.

PATIENT CONSENT STATEMENT

Written informed consent according to the Declaration of Helsinki has been obtained.

ORCID

Andrea Visentin https://orcid.org/0000-0003-0271-7200
Livio Trentin https://orcid.org/0000-0003-1222-6149
Federica Frezzato https://orcid.org/0000-0001-7093-2493

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How to cite this article: Severin F, Mouawad N, Ruggeri E, Visentin A, Martinello L, Pagnin E, et al. Focal adhesion kinase activation by calcium-dependent calpain is involved in chronic lymphocytic leukaemia cell aggressiveness. Br J Haematol. 2023;203(2):224–236. https://doi.org/10.1111/bjh.18996