Cortactin, another player in the Lyn signaling pathway, is over-expressed and alternatively spliced in leukemic cells from patients with B-cell chronic lymphocytic leukemia

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ABSTRACT

Cortactin, an actin binding protein and Lyn substrate, is up-regulated in several cancers and its level is associated with increased cell migration, metastasis and poor prognosis. The identification that the Src kinase Lyn and its substrate HS1 are over-expressed in B-cell chronic lymphocytic leukemia and involved in resistance to chemotherapy and poor prognosis, prompted us to investigate the role of cortactin, an HS1 homolog, in the pathogenesis and progression of this disorder. In this study, we observed that cortactin is over-expressed in leukemic cells of patients (1.10±0.12) with respect to normal B lymphocytes (0.19±0.06; P=0.0065). Fifty-three percent of our patients expressed the WT mRNA and p80/85 protein isoforms, usually lacking in normal B lymphocytes which express the SV1 variant and the p70/75 protein isoforms. Moreover, we found an association of the cortactin overexpression and negative prognostic factors, including ZAP-70 (P<0.01), CD38 (P<0.01) and somatic hypermutations in the immunoglobulin heavy-chain variable region (P<0.01). Our results show that patients with B-cell chronic lymphocytic leukemia express high levels of cortactin with a particular overexpression of the WT isoform that is lacking in normal B cells, and a correlation to poor prognosis, suggesting that this protein could be relevant in the pathogenesis and aggressiveness of the disease.

Introduction

The intracellular signaling cascades involving protein tyrosine kinases of Src family (SFK) has been widely investigated in the last few years. The family consists of eight members (Lyn, Hck, Lck, Blk, Src, Fyn, Yes and Fgr) involved in signaling networks regulating metabolism, viability, proliferation, differentiation and migration of different cell types.[1-4] In particular, Lyn plays a key role in many signaling pathways as the most relevant SFK in B cells. Particularly this kinase plays a key role in CLL pathogenesis and progression. In fact, in CLL cells, Lyn is over-expressed, anomalously present in the cytosol, and displays a high constitutive activity, compared with normal B lymphocytes.[5,6] Recently our attention focused on two homolog proteins, substrates of Src kinase: HS1 and cortactin.

Literature data report that HS1 undergoes a process of sequential phosphorylation synergistically mediated by Syk[7] and Lyn[8] and its downmodulation contributes to a defective proliferation and antigen receptor-induced apoptosis.[9] We also found that this protein is over-expressed in leukemic cells from CLL patients as compared to normal B lymphocytes and *in vivo* it is down-regulated by FLU-Cy therapy.[10] Its homolog cortactin, rather than in apoptosis and chemotherapy resistance, is involved in the regulation of several actin-dependent processes[11-13] such as endocytosis, cell migration and invasion,[14-16] trafficking of the key invadopodia metallo-proteases[17,18] and intracellular transducer downstream of kinase-mediated cell signaling upon phosphorylation.[15] Cortactin migrates in 2 different bands with a molecular weight of 80-85kDa, where the p85 isoform originates from the p80 because of tyrosine phosphorylation by various different tyrosine kinases.[19-21] Van Rossum *et al*. described the identification of two alternative splice variants affecting the F-actin binding domain of human cortactin, i.e. SV1-cortactin, lacking the 6th repeat (exon 11), and SV2-cortactin, lacking the 5th and 6th repeats (exon 10 and 11). They showed that cells expressing SV1- and SV2- mRNA cortactin differ significantly in their capability to bind and cross-link F-actin, promoting actin polymerization *in vitro* and cell migration, when compared with cells expressing WT-mRNA cortactin.[22] Finally, cortactin is over-expressed in several tumors,[23,24] most frequently through chromosomal amplification of the 11q13.3 region.[25] However, the overexpression has also been reported in tumors without that amplification.[26,27] *In vivo* and *in vitro* studies suggest that this overexpression increases tumor aggressiveness, possibly through promotion of tumor invasion and metastasis.

The aim of this study was to identify the downstream targets of Lyn kinase, which could sustain the anomalous signaling of Lyn pathway and the altered behavior of neoplastic B cells. We previously found that Lyn is over-expressed, constitutively activated and involved in the resistance to apoptosis in CLL.[5] To better understand the survival signals mediated

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by Lyn in CLL B cells, we investigated its downstream molecules HS1 and cortactin. In particular, herein we focused our attention on cortactin. We found that cortactin is over-expressed in neoplastic B lymphocytes with respect to normal controls and that leukemic cells express the isoforms p80/85 of cortactin, which are never expressed in healthy subjects. In addition, we also found that the overexpression of cortactin, with a particular overexpression of the p80/85 isoform, correlated to negative prognostic factors and bad prognosis of patients.

Methods

Patients and cell separation

Blood samples were collected from 15 healthy donors and 106 patients who satisfied standard morphological and immunophenotipic criteria for CLL B cells. Informed consent was obtained from all patients according to the Declaration of Helsinki. Approval for our study was obtained from the local ethics committee of "Regione Veneto on chronic lymphocytic leukemia". Patients' characteristics are summarized in Table I and detailed in *Online Supplementary Table S1*. Cells were separated from peripheral blood as detailed in the *Online Supplementary Appendix*.

Flow cytometry analysis

Purified populations ($CD5^+/CD19^+$ leukemic cells; CD19+ B lymphocytes; $CD3^+$ T lymphocytes; $CD16^+$ natural killer (NK) cells or $CD14^+$ monocytes) were re-analyzed for purity and viability by flow cytometry as detailed in the *Online Supplementary Appendix*.

Western blotting analysis

Cells from 106 CLL patients, 15 healthy donors and cell lines were prepared by cell lyses. Samples were subjected to SDS/PAGE, transferred to nitrocellulose membranes, immunostained with anti-cortactin antibodies, anti-Lyn antibodies and anti-β-actin antibody. Quantization of cortactin/β-actin, expressed as arbitrary units, were normalized on Jurkat cell line cortactin level. The procedure is detailed in the *Online Supplementary Appendix*.

Confocal microscopy analysis

Cells were plated in polylisine coated glass for 15 min at room temperature and fixed in 4% paraformaldehyde for 10 min. The fixed cells were washed twice with PBS 1X and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 4 min. Non-specific protein binding was blocked by incubating slides for at least 30 min in 2% BSA. Cells were stained with diluted mouse monoclonal anti-cortactin Alexa-488 conjugated, (Millipore) at least 1 h at room temperature or overnight at 4°C. Background staining with control antibodies was routinely compared with positively stained cells and was not visible using identical acquisition settings. Slides were mounted with cover slips and fluorescence was detected using the UltraView LCI confocal system (Perkin Elmer) equipped with a fluorescence filter set for excitation at 488 nm.

Table 1. Patients' characteristics.

Patients	N=106
Median age, years (range)	59 (32-87)
Male/female	63/38
Wbc count/mm^3 (range)	42,593 (2,910-330,000)
% Lymphocytes (range)	73 (38-99)
Mutated[1]	57
ZAP-70 positive[2]	32
CD38[3] positive	27
Karyotype (N[4]/13q-/12+/11q-/17p-)	12/23/3/3/1

[1] *"Mutated" was defined as having a frequency of mutations greater than 2% from germline VH sequence.* [2] *As determined by flow cytometry analysis (cut-off: 20%).* [3] *As determined by flow cytometry analysis (cut-off: 30%).* [4] *N: normal karyotype.*

Cortactin mRNA expression by real-time PCR analysis

The primers used to evaluate cortactin and β-actin mRNA levels are: cortactin F 5'-CCG CAG AGG ACA GCA CCT A-3' and R 5'-GGC TGT GTA CCC CAG ATC GTT-3'; β-actin F 5'-CCA GCT CAC CAT GGA TGA TG-3' and R 5'-ATG CCG GAG CCG TTG TC-3'. Details of the procedure are to be found in the *Online Supplementary Appendix*.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2DE) was performed as detailed in the *Online Supplementary Appendix*.

Analysis of cortactin splice variants

Total RNA was isolated from cell line K562, B lymphocytes purified from peripherals blood of 7 healthy controls and 21 patients with CLL. Semi-quantitative real-time polymerase chain reaction (RT-PCR) was performed as elsewhere described.[22]

Statistical analysis

The comparison of cortactin expression levels was performed using Student's t-test, Mann-Whitney test (two-tailed test) or Anova test; $P<0.05$ or $P<0.01$ were considered statistically significant. The frequency of cortactin protein p80/85 or p70/75 isoforms and the frequency of WT or SV1 mRNA splice variants were determined by direct counting and significance estimated by Fisher's exact test; $P<0.01$ was considered statistically significant. The data are expressed as mean ± standard error (SE).

Results

The protein cortactin is over-expressed in leukemic B cells

Using Western blotting analysis (Figure 1A and B) and RT-PCR (Figure 1C), we evaluated the expression of cortactin in $CD19^+CD5^+$ cells from patients with CLL and in $CD19^+$ lymphocytes from healthy subjects. We found that this protein was over-expressed in patients with respect to controls, both at protein level (patients: 1.10±0.12 *vs.* normal controls: 0.19±0.06; $^*P=0.0065$ Student's t-test; $^{**}P=0.0002$ Mann-Whitney test) and at mRNA level (patients: 2.08±0.28 *vs.* normal controls: 0.36±0.08; $^*P=0.015$ Student's t-test; $^*P=0.0004$ Mann-Whitney test). Moreover, by microscopy analysis (Figure 1D) and flow cytometry (Figure 2), we investigated whether cortactin was expressed in all normal and neoplastic B lymphocytes. We found that cortactin was detectable in almost all analyzed cells (Figure 2A) and, by calculating the fold change in the mean fluorescence intensity (ΔMFI) in normal B $CD19^+$ cells versus neoplastic B $CD19^+CD5^+$ lymphocytes, we confirmed its overexpression in leukemic cells (2.00±0.18 *vs.* 4.21±0.89, respectively; $P=0.0094$ Student's t-test; $^{**}P=0.004$ Mann-Whitney test) (Figure 2B). Furthermore, we did not observe any intraclonal pattern of expression.

In order to verify whether, in CLL, the overexpression of cortactin was a peculiarity of neoplastic B cells, we quan-

tified by RT-PCR the levels of cortactin mRNA in T CD5$^+$ lymphocytes purified from 5 patients with CLL and 5 healthy controls. We observed that cortactin is equally expressed in CD5$^+$ cells purified from patients and normal controls (respectively 0.51±0.25 *vs.* 0.56±0.27; *P=ns Student's t-test; **P=ns Mann-Whitney test) (Figure 2C), suggesting that its overexpression is restricted to neoplastic B cells. Results were confirmed by flow cytometry considering the ΔMFI between T lymphocytes, gated on CD5$^+$, and B lymphocytes, gated on CD19$^+$, in patients and controls (Figure 2D). We found that the ΔMFI of cortactin in T and B lymphocytes of patients is comparable with the ΔMFI in normal controls (T CD5$^+$: patients 3.08±0.68 *vs.* normal controls 2.25±0.21; *P=ns Student's t-test; **P=ns Mann-Whitney test. B CD19$^+$: patients 2.05±0.22 *vs.* normal controls 2.17±0.21; *P=ns Student's t-test; **P=ns Mann-Whitney test).

EMS1 *gene encodes for proteins with different molecular weight in neoplastic B cells and in normal B cells*

Results from Western blotting analysis (Figure 1A) showed that cortactin presented different molecular weight forms, respectively 70/75 and 80/85 kDa. The data were validated with three different antibodies and we found that all anti-cortactin antibodies identified the same forms of protein in the analyzed samples (*Online Supplementary Figure 1S*). Since it has been reported that in platelets cortactin migrates with the molecular weight of 80/85kDa,[28] we investigated by SDS-PAGE the molecular weight of cortactin in different hematopoietic cells purified from 5 healthy donors (platelets (Plts), natural killer (NK), monocytes (Mo) and T lymphocytes (T lym)) and three human hematopoietic cell lines (K562, Jurkat and Raji). In platelets from healthy subjects, as expected,[28] we found that this protein migrated in SDS-PAGE in the p80/85 form (Plts, Figure 3A). In T, NK, Mo cells, but also in Raji and Jurkat cell lines, the protein migrated with the isoforms p70/75 (Figure 3A). Finally, the K562 cell line showed the expression of both forms (Figure 3A). These results suggested that the molecular weight of cortactin is related to cell type. Since the cortactin homolog HS1 has a MW of 75kDa, to rule out that the antibody against cortactin p70/75 isoform does not cross react with HS1, we used the cell line K562 that does not express HS1 but expresses all cortactin forms (p70/75 and p80/85). In addition, some CLL patients who express HS1 but express only the p80/85 or only the p70/75 forms of cortactin were investigated. Results from Western blotting excluded the cross-reaction of cortactin antibody with HS1 protein (*Online Supplementary Figure 2S*).

On the basis of molecular weight, patients were subdivided into two groups: group 1 (characterized by the p70/75 form similarly to what observed in normal) and group 2 (characterized by the expression of the p80/85 form, alone or associated to p70/75). We observed that p80/85 form was detectable in 44% of CLL patients *versus* 0% of normal subjects (Fisher's exact test, P=0.008) (Figure 3B). Densitometry quantification of cortactin p80/85 isoforms in group 2 showed that they are

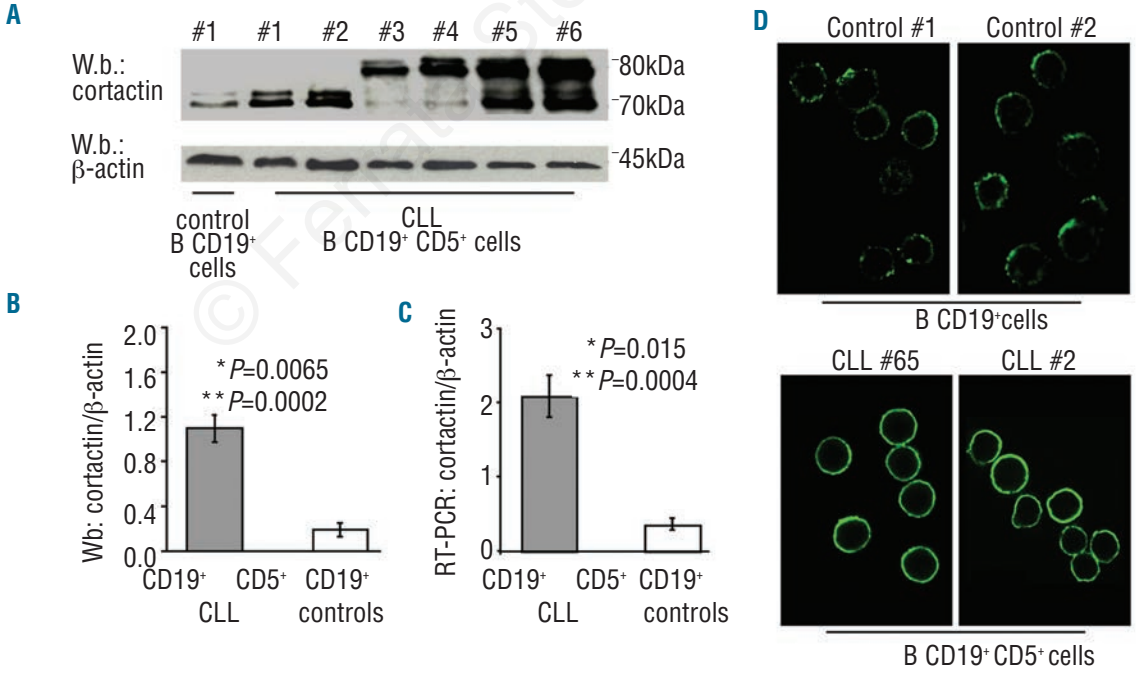

Figure 1. Evaluation of cortactin expression level in patients and healthy subjects. (A) Expression of protein cortactin was evaluated by Western blotting analysis in B CD19$^+$CD5$^+$ lymphocytes of 106 CLL patients and 15 normal CD19$^+$ controls. Figure reports one representative healthy subject (control B CD19$^+$ cells #1) and six representative B CLL patients (CLL B CD19$^+$CD5$^+$ cells #1, #2, #3, #4, #5, #6). (B) Densitometry analysis (arbitrary unit) of cortactin level in CLL patients *versus* controls. All isoforms of cortactin, presented in Western blotting results of Figure 1A, were measured densitometrically. (C) Evaluation of cortactin expression was assessed at mRNA levels by real-time RT-PCR. (D) Representative confocal microscopy analysis in normal B CD19$^+$ (n=5) and B CD19$^+$CD5$^+$ (n=15) cells of cortactin protein (Alexa-488, green). Original magnification, 60X. Fluorescence was detected using the UltraView LCI confocal system (Perkin Elmer) equipped with a fluorescence filter set for excitation at 488 nm.

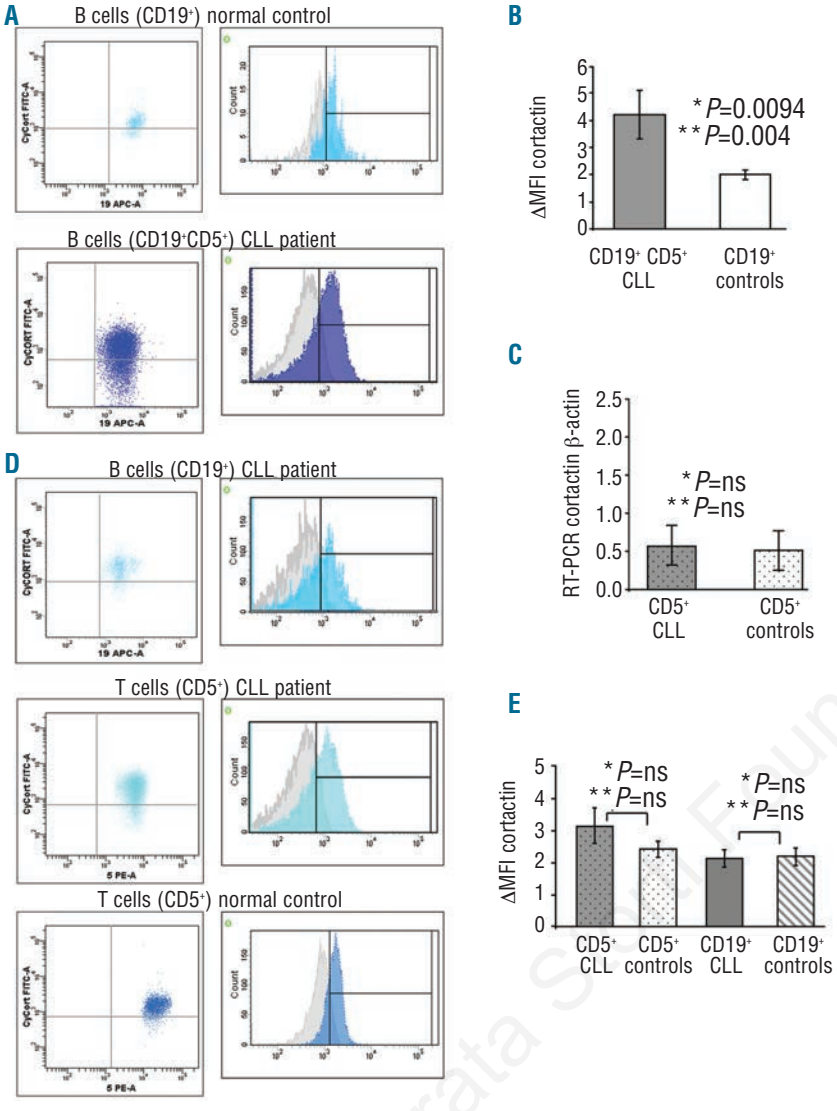

Figure 2. Evaluation of cortactin by flow cytometry. (A). Cortactin expression was evaluated in normal CD19$^+$ cells (n=10) and neoplastic CD19$^+$CD5$^+$ lymphocytes (n=40) by flow cytometry. In Figure are reported one representative plot of healthy control and one of representative patient. The left panel shows the dot plot, the right panel reports the overlay histogram plot of MFI. (B). Mean of cortactin ΔMFI evaluated in patients (CD19$^+$CD5$^+$ CLL) and in controls (CD19$^+$ controls). (C). Level of cortactin was evaluated by real-time RT-PCR in T lymphocytes isolated from patients (n=5) and healthy controls (n=5). (D). Cortactin expression was evaluated in B CD19$^+$ and T CD5$^+$ lymphocytes of patients (n=15) and T CD5$^+$ lymphocytes of normal control (n=10) by flow cytometry. One representative plot of healthy control and one of representative patient are reported. The left panel shows the dot plot, the right panel reports the overlay histogram plot of MFI. (E). ΔMFI of cortactin was evaluated in T CD5$^+$ and in B CD19$^+$ of patients and controls.

expressed at a higher level (1.31±0.13) with respect to p70/75 form expressed in group 1 (0.94±0.11) and normal controls (0.19±0.06; *P=0.0001 Anova test) (Figure 3C). The overexpression of p80/85 form with respect to the p70/75 form expressed in patients was also confirmed by Student's t-test (**P=0.025) (Figure 3C). We also analyzed whether cortactin levels (Table 2) and its isoforms (p70/75 and p80/85) (Table 2) correlated with clinical parameters and prognostic factors. Considering the cortactin levels (Table 2), we observed higher cortactin expression in patients with negative prognostic factors with respect to patients with favorable prognosis and normal subjects. In particular, cortactin level was over-expressed in unmutated (1.52±0.27) *versus* mutated (0.90±0.11; P<0.01), in ZAP-70 positive (1.00±0.24) *versus* ZAP-70 negative patients (0.82±0.22; P<0.01), in CD38 positive (1.43±0.35) *versus* CD38 negative patients (1.03±0.10; P<0.01), in 12+/11q-/17p- (1.31±0.57) *versus* normal karyotype and 13q- patients (1.00±0.14; P<0.01). When we considered the

Table 2. Evaluation of cortactin expression in correlation with clinical parameters in CLL patients.

	Wb cortactin analysis*	% of p80/85 cortactin forms**
Healthy subjects	0.19±0.05	0
Patients		
Unmutated	1.52±0.27	38
Mutated	0.90±0.11	40
ZAP-70 pos	1.00±0.24	50
ZAP-70 neg	0.82±0.22	37
CD38 pos	1.43±0.35	48
CD38 neg	1.03±0.10	37
12/11q/17p	1.31±0.57	57
13q/N	1.00±0.14	29
Treated	1.11±0.14	46
Untreated	0.79±0.15	39
Dead	1.29±0.26	48
Alive	0.92±0.10	43

*Anova test P<0.01; ** Fisher's exact test P=ns.

need for therapy and overall survival in our patients, we observed an overexpression of cortactin in patients who needed therapy (1.11±0.14) with respect to patients who do not require therapy (0.79±0.15; $P<0.01$). We also found high levels of the protein in patients dying from CLL (1.29±0.26) *versus* patients still alive (0.92±0.10; $P<0.01$). For each group of prognostic factors analyzed, we also considered the number of patients expressing the p80/85 form of cortactin comparing the presence *versus* the absence of the prognostic markers. Even the P value did not reach statistical significance; the percentage of p80/85 form was increased in the groups with the negative prognostic markers (ZAP-70, 12+/11q-/17p- karyotype, CD38) with respect to groups with the absence of negative prognostic factors (Table 2).

p85 and p75 isoforms of cortactin originate from the p80 and p70 forms after post-transcriptional modifications

To further investigate the different forms of cortactin

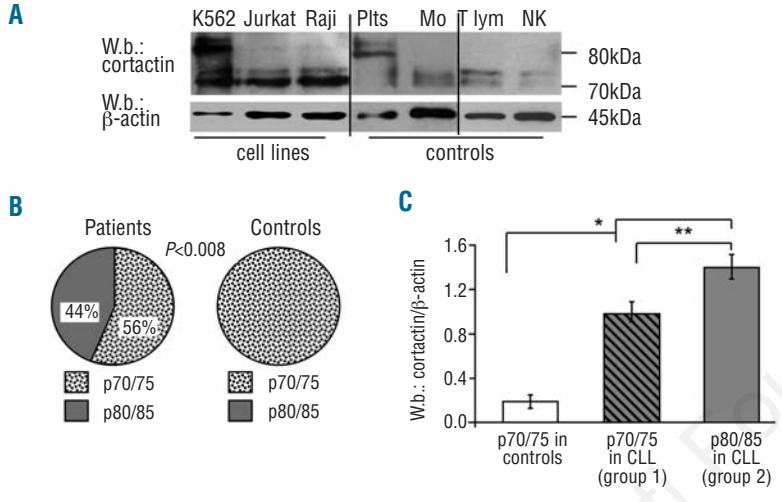

Figure 3. Expression of different cortactin isoforms in patients and healthy controls. (A) Results of cortactin Western blotting analysis in platelets (Plts), monocytes (Mo), NK and T cells from healthy subjects, and cell lines K562, Jurkat and Raji. (B) Percentage of patients and controls expressing the p70/75 or p80/85 form of cortactin. Percentages were calculated by direct count. (C) Densitometry evaluation from Western blotting analysis of p80/85 forms of cortactin expressed in patients (group 2) and of p70/75 forms expressed in patients (group 1) and in healthy subjects (controls).

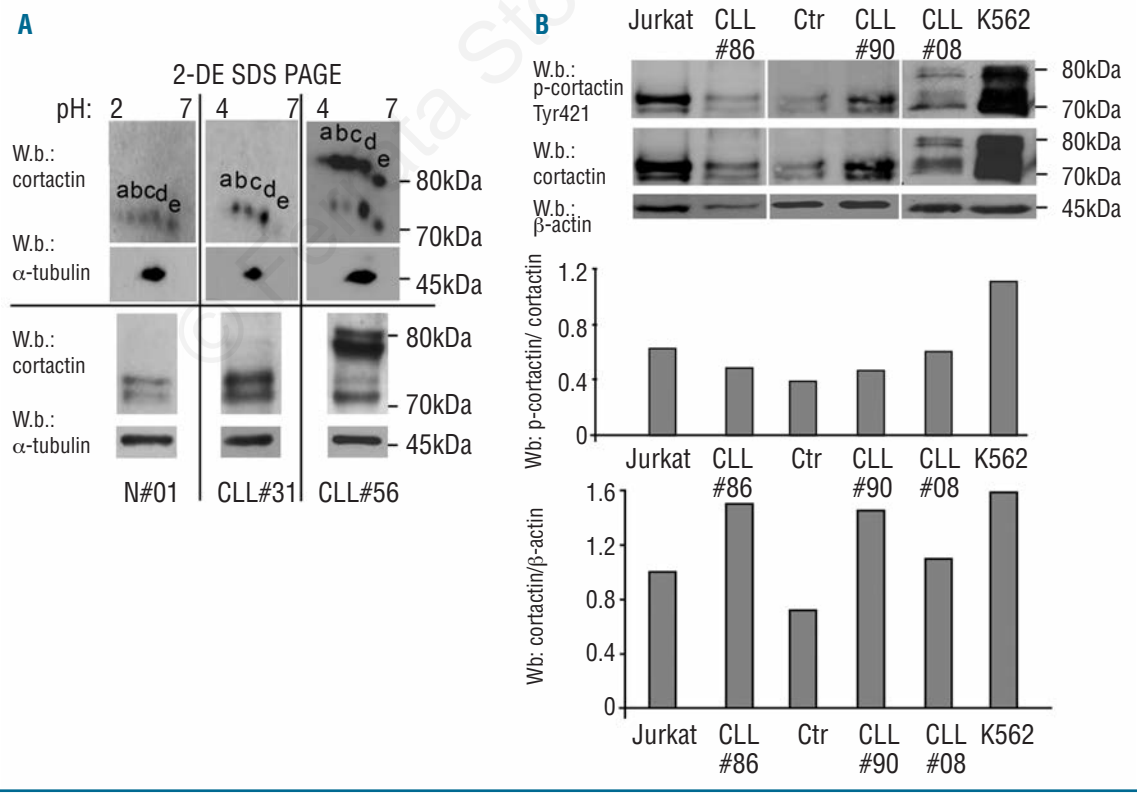

Figure 4. Investigation of cortactin forms phosphorylation. (A) B cells ($5x10^5$) were lysed and subject to 2DE electrophoresis (upper panel) and western blotting analysis (lower panel). (B) Results of Western blotting analysis for Tyr421 phosphorylated cortactin in the cell line Jurkat and K562, in 3 representative patients (CLL#86, #90, #08) and one control (Ctr). Middle panel reports quantification of phosphorylated cortactin in Tyr421, in the lower panel is reported quantification level of total cortactin.

that we discovered in our CLL patients, we used two-dimensional polyacrylamide gel electrophoresis (2DE) that the proteins to be separated according to their molecular weight and to their pI, since pI is modified by the presence of phosphoric or acetyl groups. For this investigation we selected some patients, who in SDS-PAGE presented a migration of cortactin in 2 bands (p70/75, n=4) or in 4 bands (p70/75 and p80/85, n=3). As control we used normal B cells (n=3), where cortactin always migrated in the p70/75 form.

In normal B cells, we observed that cortactin migrated with a molecular weight of about 70 kDa and in a train of spots (a, b, c, d, e) accordingly to its pI (Figure 4A, N#01, upper panel). Also patients which in SDS-PAGE presented the p70/75 cortactin bands, similarly to controls, showed in 2DE a cortactin migrating at 70kDa and in discrete spots (Figure 4A, CLL#31). Conversely, patients who in SDS-PAGE had a migration of cortactin in 4 bands, in 2DE presented a migration in 2 different molecular weight with a train of spots per molecular weight (a, b, c, d, e, Figure 4A,

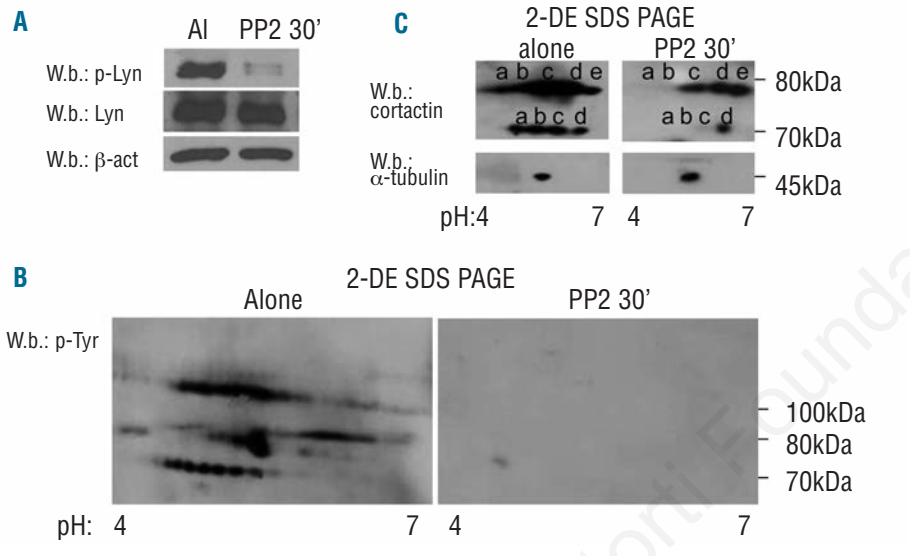

Figure 5. Investigation of cortactin phosphorylation by Src kinase Lyn. Patient cells ($5x10^5$) were lysed and subject to 2DE electrophoresis and Western blotting analysis before (alone) and after treatment with the PP2 inhibitor (PP2, 30'). (A) Evaluation by Western blotting of Lyn phosphorylation level in Tyr396, before and after PP2 treatment. (B) Effect of PP2 treatment on total tyrosine phosphorylation evaluated by 2DE electrophoresis. (C) Effect of PP2 treatment on cortactin migration in 2DE electrophoresis.

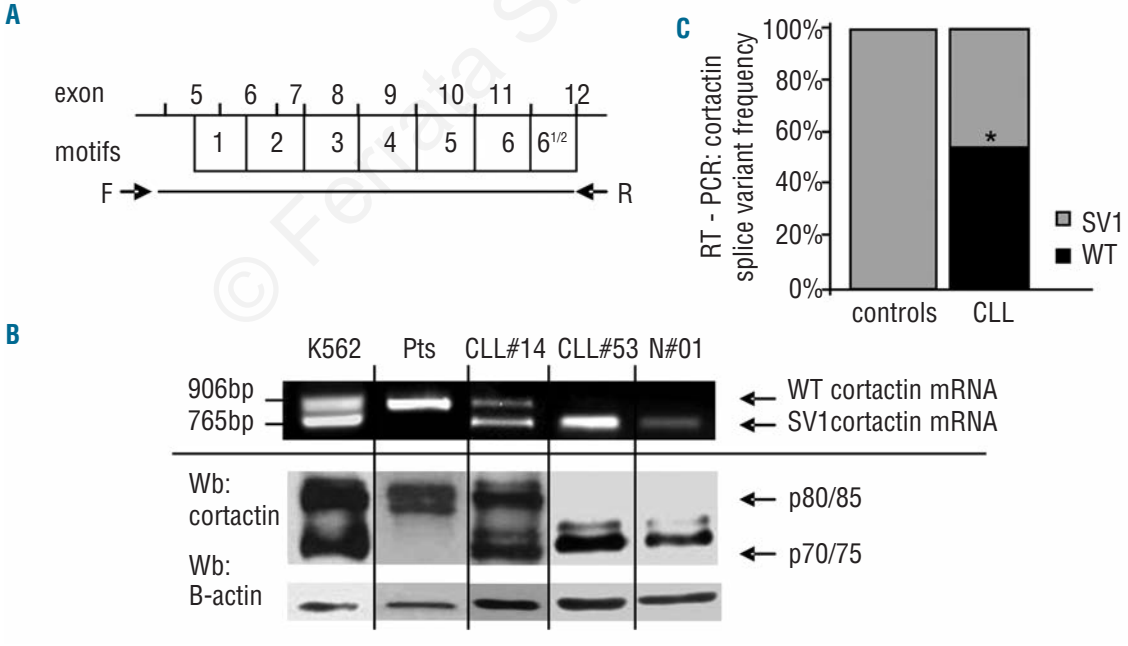

Figure 6. Exon map and alternative splicing variants of the acting binding domain of the human EMS1/cortactin gene. (A) Exon map of the acting binding repeat domain of human EMS1 gene in relation to the protein sequence derived from the cDNA nucleotide sequence.[22] The boxes (1-61/2) represent the 37 amino acid repeat motifs in the actin binding domain (motifs). The exons encoding the different motifs are indicated in the upper panel (exon). The position of primer sets used in this study for PCR is indicated. (B) Expression of WT and SV1 –cortactin mRNA variants (upper panel), using primers F-R on cDNA derived from the cell line K562, platelets and B lymphocytes of one representative healthy control (n=7) and 2 representative CLL patients (n=21), were compared to protein forms in Western blotting analysis (lower panel). (C) Frequency for WT cortactin mRNA in patients with respect to controls. K562: cell line; Plts: normal platelets; N: normal B lymphocytes; CLL: neoplastic B lymphocytes from patients with CLL.

LLC#56). In summary, we observed a molecular weight correspondence between isoforms obtained by SDS-PAGE (Figure 4A, lower panel) and by 2DE migrations (Figure 4A, upper panel). Considering that p85 form has been observed to originate from the p80 because of phosphorylation events, we investigated the phosphorylation status of cortactin in CLL cells and K562 cell line. By Western blotting assay using an antibody against Tyr 421 phosphorylated cortactin, we observed that the p70/75 form is phosphorylated in a similar way to the p80/85 proteic form detected in CLL patients, controls and the Jurkat and K562 cell lines (Figure 4B). We also investigated whether cortactin is phosphorylated by Lyn. By treating patients' purified neoplastic B lymphocytes with the Src inhibitor PP2, we found that the phosphorylation level of Lyn in Tyr 397 decreased after treatment, thus proving the inhibition of its activity (Figure 5A). In 2DE, after PP2 treatment, we observed a decreased of total tyrosine phosphorylation in the whole lysate (Figure 5B). Moreover, most of the revealed spots with the anti cortactin antibody shifted to a basic pH after treatment with PP2 (left vs right panel), confirming that tyrosine phosphorylation of the protein was at least in part dependent on Lyn activity (Figure 5C).

Our results suggest that cortactin is expressed in 2 main forms with different molecular weight (p70 and p80) and that these two forms undergo post-translational modification, i.e. phosphorylation probably by Src kinase Lyn, that would explain the migration of each form in two bands in SDS-PAGE and in different spots per form in 2DE.

Characterization of alternative splice variants of human cortactin

In human, three different splice variants (WT, SV1 and SV2), affecting the F-binding domain of cortactin and cell migration of cells, have been identified.[22] Semi-quantitative RT-PCR analysis on mRNA, using primers[22] that flank the region encoding the entire acting binding domain (Figure 6A) allowed us to identify which mRNA splice variants were expressed in B cells purified from 7 controls and 21 patients with CLL (Figure 6B). We found that 53% of analyzed patients co-expressed the mRNA WT and SV1 (Figure 6C). By contrast, none of controls was WT, bearing the expression only of SV1 isoform (100%); the SV2 transcript was hardly detectable (none of the patients and only one control). Considering the key role of actin binding domain for cortactin activity, we sequenced the WT and SV1 variants that we amplified by semi-quantitative RT-PCR. We compared the sequences that we obtained with the sequences available on Pubmed (*see* Methods) and we did not find any alteration or mutation in cortactin mRNA either in patients and controls or also in K562 cell line that we considered for this investigation (WT mRNA, ID: NM_005231.3, SV1 mRNA, ID: NM_138565, GENE IDE: 2017 CTTN) (*C. Gattazzo, V. Martini, F Frezzato, V. Trimarco, M. Facco, L. Trentin, unpublished data, 2013*). Up to now, a correlation between protein forms of cortactin in Western blotting analysis and splice variants of mRNA in PCR has never been investigated. The isoform WT encodes for a protein of 551aa, with a calculated molecular weight of 61kDa, whereas the isoform SV1 codifies for a proteins of 514aa, with a calculated molecular weight of 57 kDa. In platelets, that express only the WT mRNA of cortactin, the protein migrates in SDS-PAGE with an apparent molecular weight of 80-85kDa (Figure 2C). Therefore, it is plausible that while WT mRNA gives rise to the protein p80/85, the SV1 mRNA gives rises to the protein that in SDS-PAGE migrates with a molecular weight of 70-75kDa. In fact, we observed an intriguing relationship between mRNA (Figure 6B, upper panel) and protein molecular weight (Figure 6B, lower panel). In subjects where the WT-SV1 splice variants were expressed, proteins migrated in the forms p80/85 and p70/75, whereas in cases where only the SV-1 mRNA was expressed, cortactin presented only the proteic form p70/75.

Cortactin and pharmacological treatment

To investigate whether cortactin could be involved in pharmacological resistance of patients, we quantified the expression level of cortactin by flow cytometry before and after *in vivo* treatment. We enrolled 6 patients: 2 patients in therapy with bendamustine, 2 patients with ofatumumab, one patient with ibrutinib and one with R-CF. Blood samples were collected before the treatment and after 30 days. We evaluated the ΔMFI of cortactin in $CD19^{+}CD5^{+}$ neoplastic cells. We found that expression level of cortactin was unaffected by the therapy (before therapy: 5.20±0.80 *vs.* after therapy: 5.28±0.91; Student's t-test, *P*=ns). We also divided patients (n=3) with higher expression of cortactin (ΔMFI: 6.47±1.27) and with lower levels (3.93±0.05). The analysis of clinical parameter of patients showed that in the group with lower expression of cortactin the frequency of the decrease in white blood cells (0.36±0.18) was less than in patients with higher level of cortactin (0.96±0.11; *P*=0.038 Student's t-test), suggesting that a high level of cortactin could play a role in resistance to pharmacological treatment in CLL patients.

Discussion

In this study, we found that cortactin is over-expressed in neoplastic B cells from patients with CLL. Moreover, we correlated its overexpression with negative prognostic factors for CLL, such as absence of somatic hypermutation in the immunoglobulin heavy-chain variable region (IgVH), expression of ZAP-70, CD38, abnormal karyotype, the requirement of therapy, and death for CLL. Cortactin is an actin-binding protein found to be over-expressed in several solid tumors,[29-31] and its overexpression seems to provide a selective advantage to the development and progression of solid tumors. In fact, it has been shown to enhance cell motility in a variety of assays, including transwell migration and single cell motility.[29,32-34] Moreover, it was observed that, in several pathologies, cortactin overexpression correlated to bad prognosis, higher pathological stage, lymph node involvement and metastasis, decreased survival, and that it works in the relatively late stages of disease progression to promote tumor cell dissemination.[25,35-42] Thus our data suggest that cortactin may be an important prognostic marker for CLL aggressiveness.

Literature data report that cortactin is expressed in all cell types with exception of most hematopoietic cells.[43] Here we found that the overexpression of cortactin in CLL is a peculiar finding of neoplastic B cells, since it is expressed at low levels in T cells from the same patients and in B and T lymphocytes from healthy subjects. In this regard, cortactin differs considerably from its homolog, the hematopoietic lineage cell-specific protein 1 (HS1).[44] HS1 is mainly expressed in hematopoietic cells[7] and recently we demonstrated that it is over-expressed in

leukemic cells from CLL patients as compared to normal B lymphocytes, and in particular, its levels correlate with poor prognosis of these patients.[10] Although HS1 and cortactin are homolog proteins, they have been found to be expressed in different cell types and to be involved in different functions. In particular, cortactin has been initially reported to be involved in cytoskeleton remodeling and cell migration, whereas HS1 seems to play a major role in resistance to pharmacological treatment and to apoptosis.[10,44] As a matter of fact, the roles of cortactin and HS1 have been proven to overlap, particularly concerning their role in cell migration.[45] Moreover, it has been recently demonstrated that HS1 activity is tightly regulated by its phosphorylation level, with high HS1 phosphorylation being associated to poor clinical course.[46] The identification of expression of both proteins in the neoplastic clone is of particular interest. Both cortactin overexpression and HS1 hyperphosphorylation correlate to poor prognosis of CLL patients.

Another question is presented by the localization of cortactin. Cortactin is a cytoplasmic protein but, whereas cortactin has never been found in the nucleus, in CLL patients HS1 translocates to the nucleus after tyrosine phosphorylation.[47] This is because HS1, but not cortactin, contains a nuclear localization signal (NLS).[48,49] In agreement with this result, in this study we found that cortactin is usually localized in the cytoplasm, although it is abundantly phosphorylated at tyrosine residues. Moreover, we found that HS1 is localized with the Src kinase Lyn in an aberrant cytosolic complex in an active conformation, contributing to the unbalance between cell survival and pro-apoptotic signals,[6] while cortactin was not found localized in this complex and was never reported to be implicated in resistance to apoptosis. These observations clearly indicate that these two molecules are similar in terms of their structure but that they are functionally different, even if they have been claimed to be crucial in tumor progression. Actually, the major role of cortactin in cell metabolism is carried out in protein trafficking and cell motility. Cortactin, as HS1, is involved in Arp2/3 mediated actin polymerization *in vitro*, although HS1 is less efficient than cortactin.[50] Overexpression of cortactin has been correlated to tumor spreading rather than in apoptosis resistance, thus contributing in this way to aggressiveness of the neoplastic cell and to poor prognosis. Cortactin regulates F-actin polymerization and dynamics by means of six N-terminal "cortactin repeats". Van Rossum *et al*. reported the identification of different cortactin mRNA splice variants, WT, SV1 and SV2[22] that differ significantly in their ability to: i) bind F-actin; ii) cross-link F-actin; iii) activate Arp2/3 mediated actin polymerization; and iv) induce cell migration in vitro. Authors also report that SV2 variant was hardly detectable, whereas SV1 and WT were abundantly expressed in HNSCC.[22] Here we found that WT cortactin mRNA was expressed only in CLL patients, whereas normal B lymphocytes expressed only the SV1 variant. The expression of SV1 mRNA was correlated at protein level to the expression of a shorter form of protein, with a molecular weight of p70/75. Van Rossum et al. reported that the reduced ability of SV1- and SV2-cortactin to cross-link F-actin cannot be ascribed to altered tyrosine phosphorylation. In agreement with these results, by 2DE and Western blotting analysis we found that both forms, p70/75 and 80/85, resulted phosphorylated at Tyr421 residue in a similar way. The alternative splicing of cortactin represents a mechanism to modulate actin dynamics and cell migration, and the overexpression of the WT cortactin, that we observed in neoplastic B cells, could represent another important aspect of abnormal metabolism of B-CLL cells and be, in part, responsible for neoplastic B-lymphocyte aggressiveness and poor prognosis. In fact, progression of many diseases, including cancer invasion and metastasis, is driven also by aberrant regulation of cell migration.

In conclusion, cortactin is a pivotal protein in modulation of F-actin dynamics and cell migration. The observation that cortactin is over-expressed in B cells of patients with CLL, and that this overexpression is correlated to the presence of somatic hypermutations and negative prognostic factors, combined with the discovery that an important proportion of patients express the WT cortactin mRNA, never expressed in normal controls, suggest that overexpression of cortactin could promote disease progression in CLL. Precisely the mechanism through which cortactin facilitates these processes has remained elusive and further studies are in progress to investigate how this protein, under Lyn kinase regulation, is implicated in CLL pathogenesis and aggressiveness of the neoplastic CLL clone.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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