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Cortactin, another player in the Lyn signaling pathway, is overexpressed and alternatively spliced in leukemic cells from patients with B-cell Chronic Lymphocytic Leukemia

Short title: Overexpression of cortactin in CLL patients

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Running heads: Overexpression of cortactin in CLL patients.

Running head: Cortactin is overexpressed in CLL- B lymphocytes.

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Key words: B cell chronic lymphocytic leukemia, cortactin overexpression, cortactin splice variants, poor prognosis.

The ethic approval for our study was obtained on April 20, 2007 by the Local Ethic Committee of "Regione Veneto on Chronic Lymphocytic Leukemia" (trial registration number: 257/06) and on May 13, 2008 by Ethic Committee for the Clinical Experimentation (trial registration number: 190).

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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 overexpressed 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 overexpressed in leukemic cells of patients (1.10±0.12) with respect to normal B lymphocytes (0.19±0.06, $p=0.0065$). Fifty-three % of our patients expressed the WT mRNA and p80/85 protein isoforms, usually lacked 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 level of cortactin with a particular overexpression of the WT isoform that is lacking in normal B cells, and a correlation to poor prognosis of patients, 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 largely been 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 type.[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 overexpressed, 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 overexpressed in leukemic cells from CLL patients as compared to normal B lymphocytes and it is *in vivo* 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 6t^{h} 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 overexpressed 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 work was to identify the downstream targets of Lyn kinase, which could sustain the anomalous signaling of Lyn pathway and the altered behaviour of neoplastic B cells. We previously found that Lyn is overexpressed, constitutively activated and involved in the resistance

to apoptosis in CLL. To better understand the survival signals mediated 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 overexpressed 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 subject. 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 cells separation

Blood samples were collected from 15 healthy donors and 106 patients who satisfied standard morphologic and immunophenotipic criteria for CLL B cells. Informed consent was obtained from all patients according to the Declaration of Helsinki. The ethic approval for our study was obtained from the local ethic committee of "Regione Veneto on chronic lymphocytic leukemia". Patient characteristics are summarized in Table I and detailed in supplementary Table IS. Cells were separated from peripheral blood as detailed in supplemental materials.

Flow cytometry analysis

Purified populations (CD5+/CD19+ leukemic cells; CD19+ B lymphocytes; CD3+ T lymphocytes; CD16+ NK cells or CD14+ monocytes) were reanalyzed for purity and viability by flow cytometry as detailed in supplemental materials.

Western blotting analysis (wb)

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. Procedure is detailed in supplemental materials.

Confocal Microscopy Analysis

Cells were plated in polylisine coated glass for 15min at room temperature and fixed in 4% paraformaldehyde for 10min. The fixed cells were washed twice with PBS 1X and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 4min. Non-specific protein binding was blocked by incubating slides for at least 30min in 2% BSA. Cells were stained with diluted mouse monoclonal anti-cortactin Alexa-488 conjugated, (Millipore) at least 1h 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.

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'. Procedure is detailed in supplemental materials.

Two-dimensional gel electrophoresis (2DE)

2DE was performed as detailed in supplemental materials.

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 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 accepted as 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 accepted as statistically significant. The data are expressed as mean ± standard error (SE).

RESULTS

The protein cortactin is overexpressed in leukemic B cells

By western blotting analysis (Figure 1A and 1B) 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 overexpressed 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 *vs* 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 quantified by real time 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 and **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, reported in the above 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 (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 killers cells-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, 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 allowed to exclude the cross-reaction of cortactin antibody with HS1 protein (see supplemental materials, 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 normals, 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 *vs* 0% of normal subjects (Figure 3B, Fisher's exact test, p=0.008). Densitometry quantification of cortactin p80/85 isoforms in group 2 showed that they are expressed at 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, Anova test *p=0.0001, Figure 3C). The overexpression of p80/85 form with respect to 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 favourable prognosis and normal subjects. In particular, cortactin level was overexpressed in unmutated (1.52±0.27) *vs* mutated (0.90±0.11, p<0.01), in ZAP-70 positive (1.00±0.24) *vs* ZAP-70 negative patients (0.82±0.22, p<0.01), in CD38 positive (1.43±0.35) *vs* CD38 negative patients (1.03±0.10, p<0.01), in 12+/11q-/17p- (1.31±0.57) *vs* normal karyotype and 13q- patients (1.00±0.14, p<0.01). When we considered in our patients the need of therapy and overall survival, 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 level of the protein in patients dying from CLL (1.29±0.26) *vs* 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 *vs* the absence of the prognostic markers. Even the "p" did not reach a statistically significant value, 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 that we discovered in our CLL patients, we used two-dimensional polyacrylamide gel electrophoresis (2DE) that allows to separate the proteins 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, 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 published sequences on pubmed site (see Materials and Methods) and we did not find any alteration or mutation in cortactin mRNA both in patients and controls and 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) (unpublished data). 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 the protein p80/85, the SV1 mRNA gives rises 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 pharmacologic treatment

To investigate whether cortactin could be involved in pharmacologic resistance of patients, we quantified the expression level of cortactin by flow cytometry before and after in *vivo* treatment. We enrolled six 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, p=ns Student's t-test). We also divided patients (n=3) with higher expression of cortactin (ΔMFI: 6.47±1.27) and with lower level (3.93±0.05). The analysis of clinical parameter of patients showed that in the group with lower expression of cortactin the frequency of white blood cell decrease (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 high level of cortactin could play a role in resistance to pharmacologic treatment in CLL patients.

DISCUSSION

In this paper we found that cortactin is overexpressed in neoplastic B cells from patients with CLL. Moreover, we correlated its overexpression with negative prognostic factors for CLL, as absence of somatic hypermutation in the immunoglobulin heavy-chain variable region (IgV_H), expression of ZAP-70, CD38, abnormal karyotype, the requirement of therapy, and death for CLL. Cortactin is an actin binding protein found to be overexpressed 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 tumour cell dissemination.[25, 35-42] Thus our data suggest that cortactin may be a founded 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 considerable 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 overexpressed 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 pharmacologic 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 both proteins expression in the neoplastic clone is of particular interest. Both cortactin overexpression and HS1 hyperphosphorylation correlate to poor prognosis of CLL patients.

Another question deals with 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 paper 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 implicated in resistance to apoptosis. These observations clearly indicate that these two molecules are similar in terms of their structure but 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 tumour spreading, rather then in apoptosis resistance, thus contributing in this way to aggressiveness of neoplastic cell and to poor prognosis of patients. 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 bad prognosis of patients. 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 overexpressed 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 quote 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 investigated how this protein, under Lyn kinase regulation, is implicated in CLL pathogenesis and aggressiveness of neoplastic CLL clone.

Authorship and Disclosures

C.G. performed most of the *in vitro* research, analyzed the data and wrote the manuscript; F.F. performed confocal microscope and wb analysis and participated in analysis of data; V.M. performed cytometric analysis for patients' immunophenotype; V.T. processed blood samples and performed PCR analysis of cortactin; M.F. contributed clinical patient samples and reviewed the manuscript; E.T. and F.Z. performed Two-dimensional gel electrophoresis (2DE) investigation; A.M.B. provided intellectual input into the study; R.Z. and M.C. contributed clinical patient samples; G.S. provided intellectual input into the study and reviewed the manuscript; L.T. designed the research, contributed clinical patient samples, participated in analysis of data and reviewed the manuscript. The authors declare no competing financial interests.

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Table 1. Patient Characteristics

Patients	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.

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

Legend to Figures

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 *vs* 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.

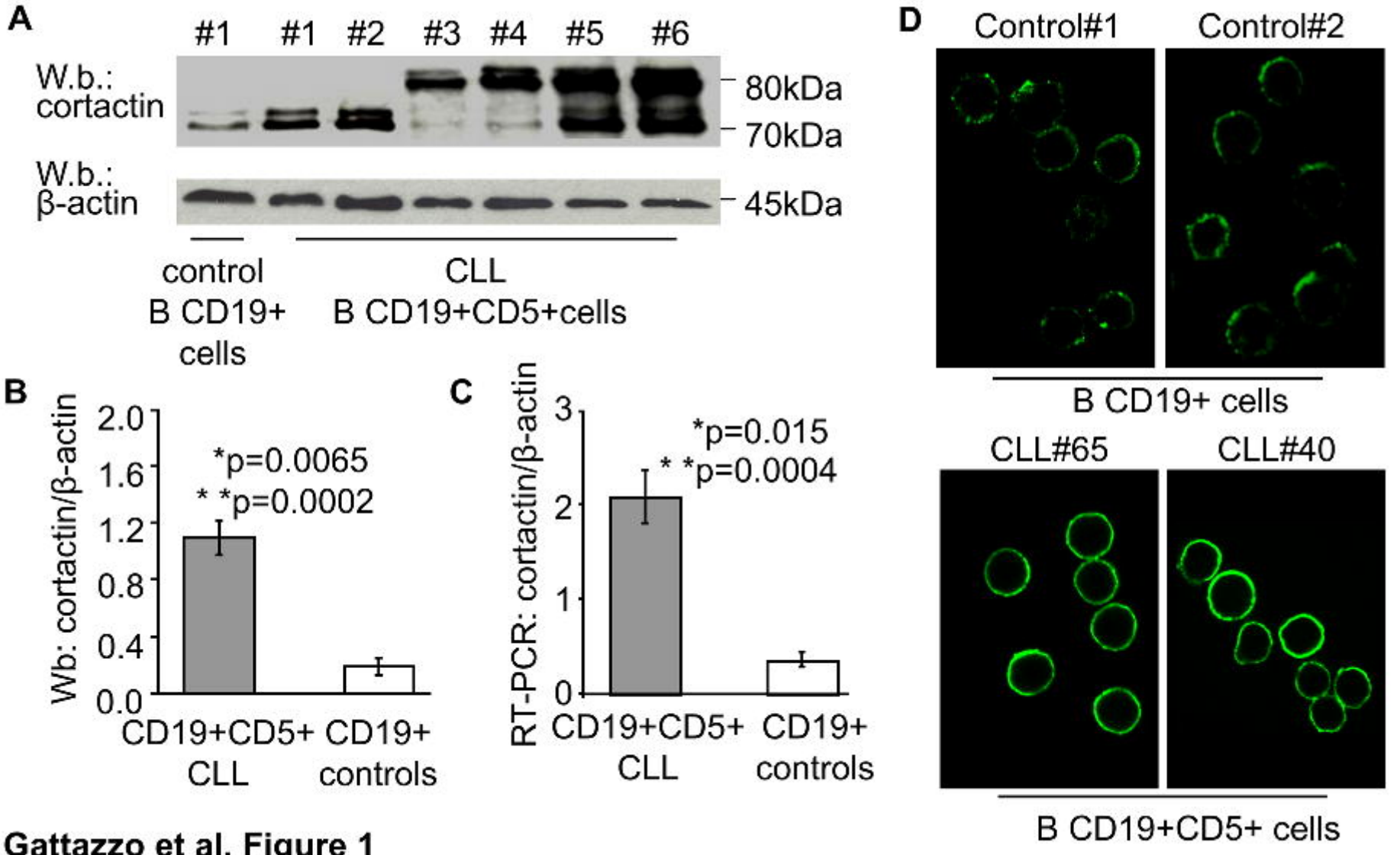
Figure 3. Expression of different cortactin isoforms in patients and healthy controls. A) Results of cortactin western blotting analysis in platelets (Plts), monocytes (Mo), NK and T cells from healthy subjects, and cell lines K562, Jurkat and Raji. **B)** Percentage of patients and controls expressing 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 three representative patients (CLL#86, #90, #08) and one control (Ctr). In the middle panel is reported the quantification of phosphorylated cortactin in Tyr421, in the lower panel is reported quantification level of total cortactin.

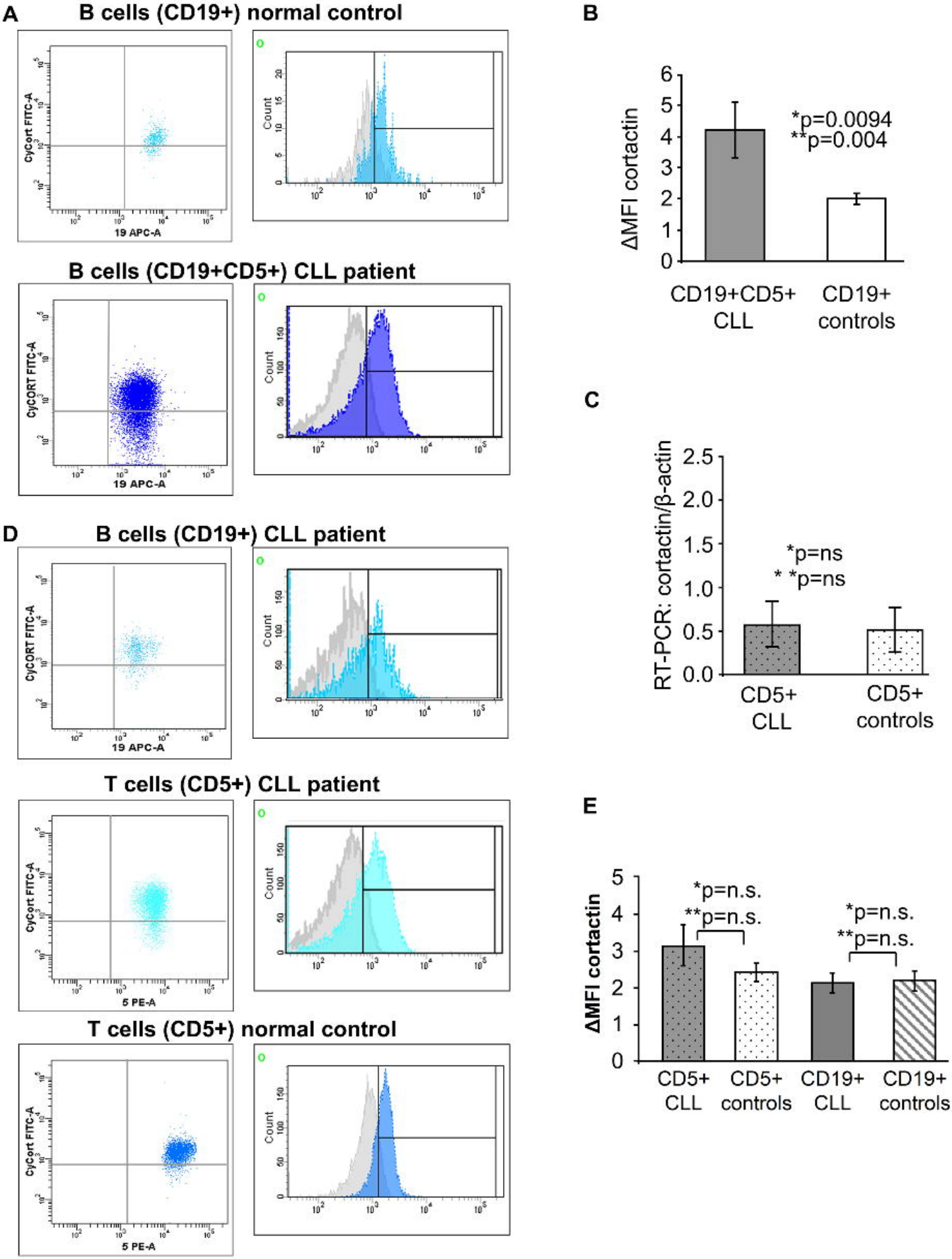
Figure 5. Investigation of cortactin phosphorylation by Src kinase Lyn. Patient cells ($5x10^5$) were lysed and subject to 2DE electrophoresis and western blotting analysis before (alone) and after treatment with the PP2 inhibitor (PP2, 30'). **A)** Evaluation by western blotting of Lyn phosphorylation 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-$6^{1/2}$) 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 two 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.



Gattazzo et al, Figure 1



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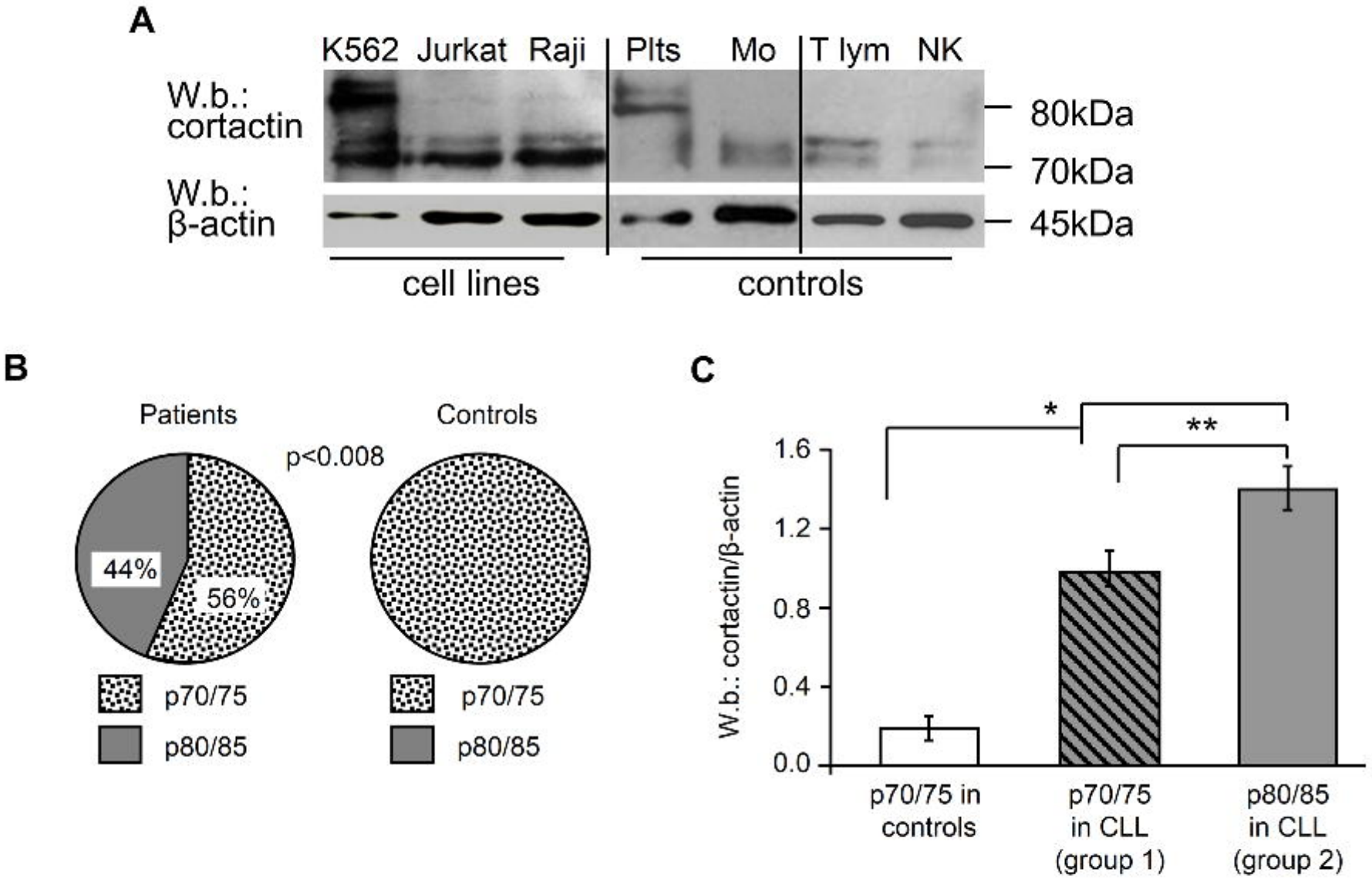

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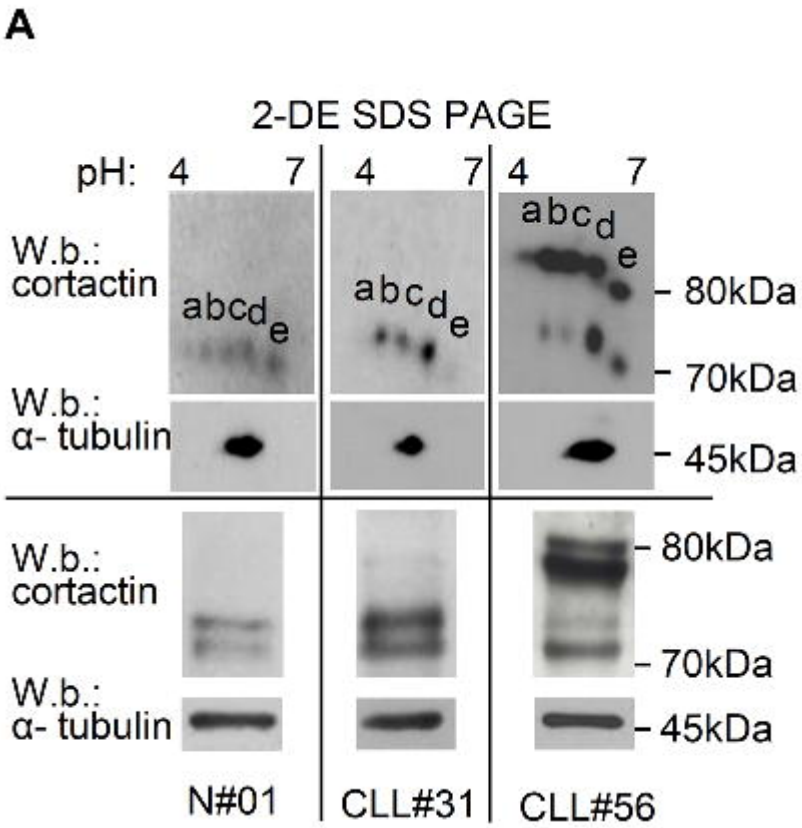

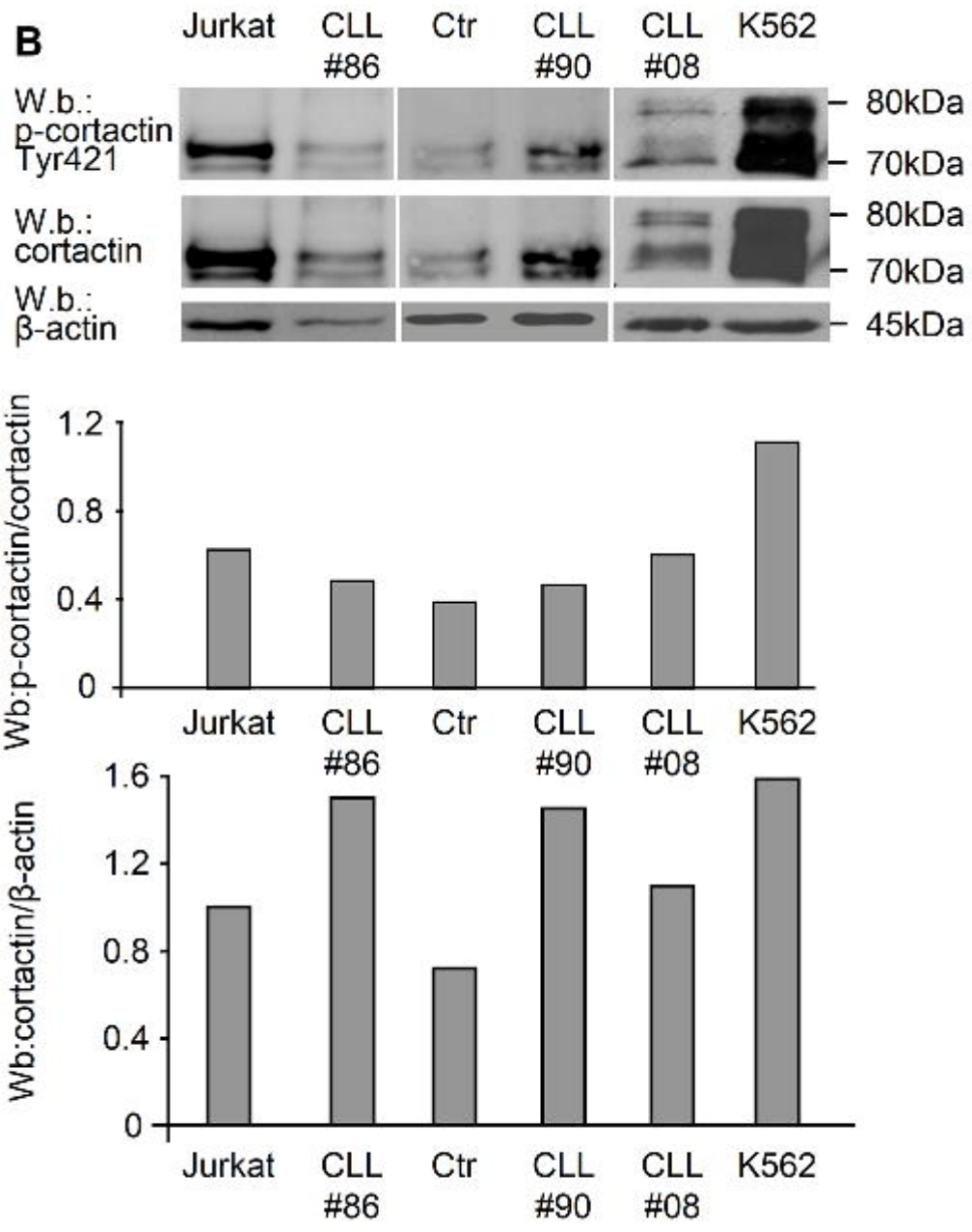

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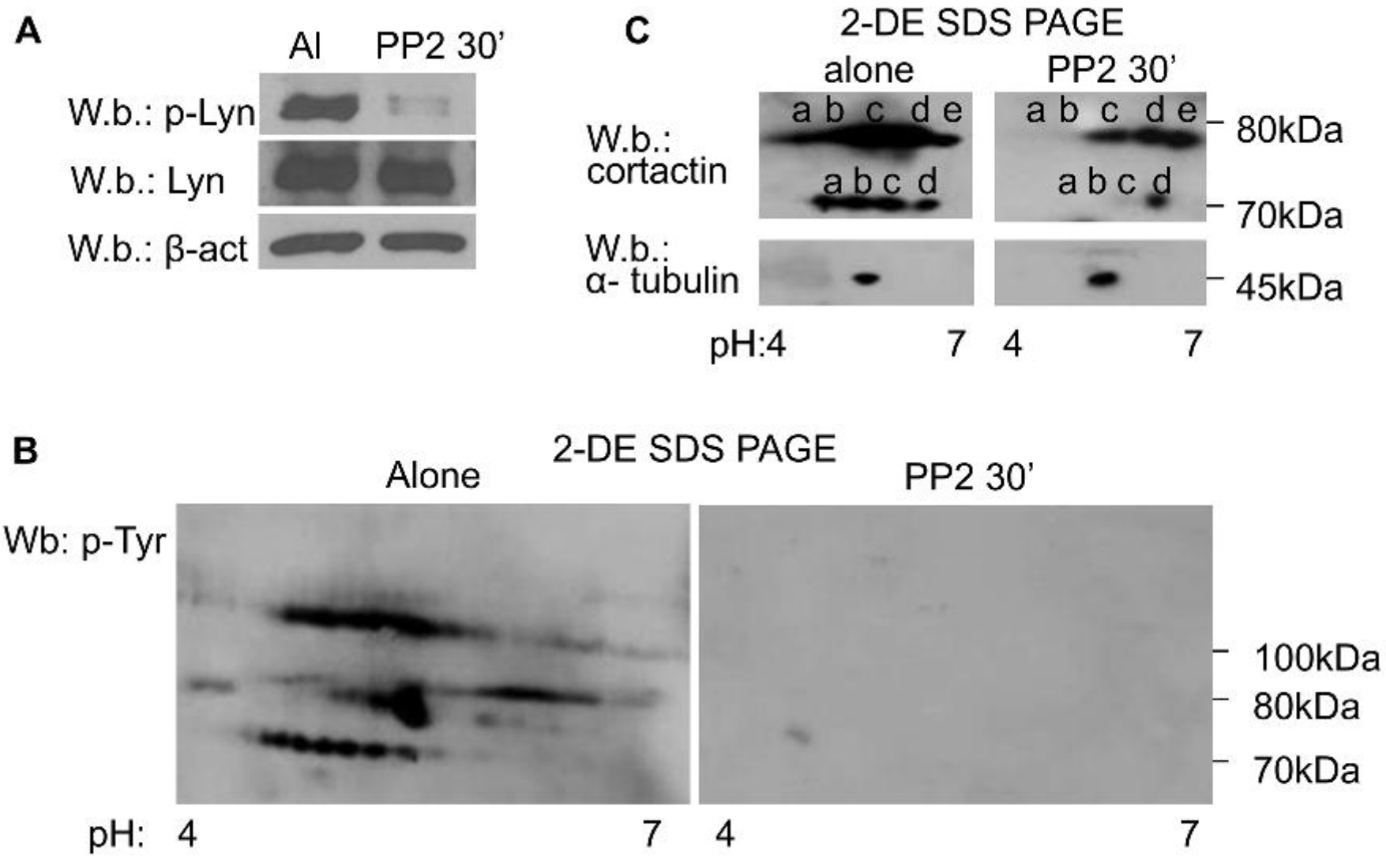

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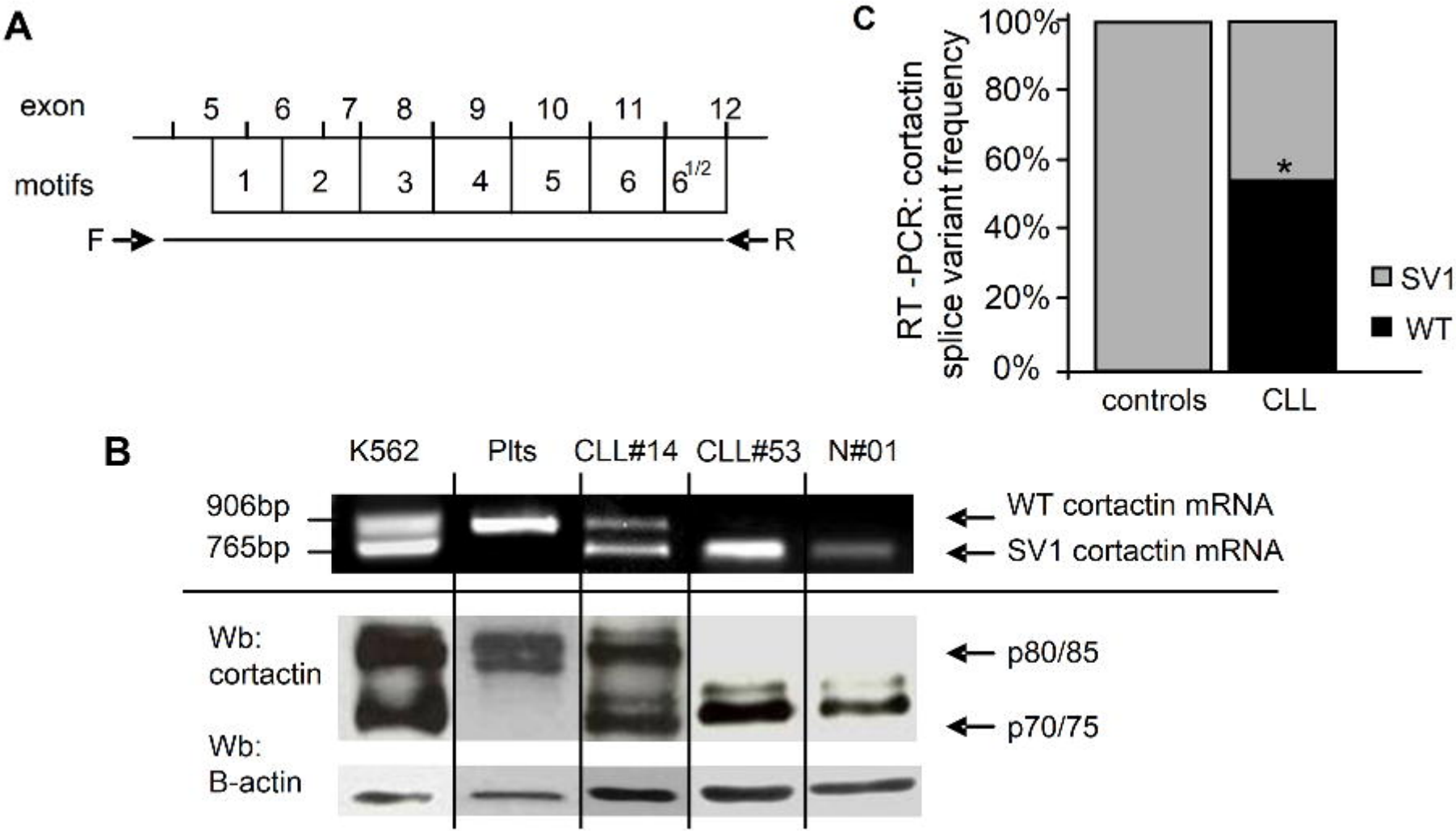

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MATERILAS AND METHODS

Patients and cells separation. Blood samples were collected from 15 healthy donors and 106 patients which satisfied standard morphologic and immunophenotypic criteria for CLL B cells. Informed consent was obtained from all patients according to the Declaration of Helsinki. The ethic approval for our study was obtained from the local ethic committee of "Regione Veneto on chronic lymphocytic leukemia". Patient characteristics are summarized in Table I and detailed in supplementary Table IS. Untouched peripheral blood cells (CD19+, CD5+/CD19+, CD3+, CD16+), were isolated from peripheral blood mononucleate cells (PBMCs) by negative selection using the *RosetteSep* cells isolation kit (StemCell Technologies; Vancouver, CND), specific for the listed cells and separated by Ficoll gradient centrifugation (Amersham Biosciences; Buckinghamshire, UK). Monocytes (CD14+) were isolated by plating whole PBMCs and collecting adherent cells after 2 hour of incubation. The samples that were used had at least 95% of purity as assessed by flow-cytometry analysis.

Flow cytometry analysis. Purified populations above described (CD5+/CD19+ leukemic cells; CD19+ B lymphocytes; CD3+ T lymphocytes; CD16+ NK cells or CD14+ monocytes) were reanalyzed for purity and viability by flow cytometry and a multiparametric approach. Briefly, cells were stained with anti-CD5 FITC, anti-CD16 PE, anti-CD19 PerCP and anti-CD3 APC monoclonal antibodies (mAbs), monocyte preparations were set using forward *versus* side scatter (SSC) morphological parameters display. Purified samples were analyzed by FACSCalibur cytometer (Becton Dickinson; Mountain View, CA), data were processed using CELLQuest Pro Software (Becton Dickinson) and the purity and viability of all samples resulted both >95%.

Staining for cell surface antigen

Whole blood was stained for surface antigens using CD5-fluoresceiniso-thiocyanate (FITC), CD19-Phycoerythrin (PE), CD16-PE, CD19-peridinin chlorophyll protein cytochrome 5.5(PerCP-Cy5.5), CD3-allophyco-cyanin (APC) and CD19-APC. All antibodies were purchased from Becton Dickinson Biosciences. The tubes were gently mixed and incubated at RT in the dark for 15 min. Next, cells were resuspended in 2 ml/tube of Lyse solution, centrifuged at 500 g for 5 min, and the superna-ant was discarded.

Staining for cytoplasmic Cortactin

Preceding the staining of cytoplasmic Cortactin the Fix & Perm kit (Invitrogen,) was used for cell fixation and permeabilization, strictly according to the manufacturer's instructions. When using Fix & Perm, the surface-stained cells were resus-pended in 100ul of Solution A, gently mixed, and incu-bated for 15 min at RT in the dark. Next, the cells were washed in 2-ml of PBS1X, centrifuged

at 500 g for 5 min, resuspended in 100ul of Solution B, and incubated directly with FITC conjugated anti-Cortactin antibody or with FITC conjugated mouse IgG1 (UpState….) at RT in the dark for 30 min. Finally, the cells were washed once in 2-ml of PBS1X and resuspended in 100ul of PBS1X and analyzed by FACS Canto I (Beckton Dickinson).

Flow cytometry analysis

Samples were gated on intact cells by forward light scatter (FSC) vs right-angle light scatter (SSC) (R1 = Lym), and residual lymphocytes were excluded when this population was apparent by FSC vs SSC. For analysis of expression of Cortactin samples labeled for CD19APC and CD5PE and a second gating step was used (R2 = CD19+CD5+, CD19+CD5-, CD19-CD5+). The double-gated events (R1*R2) were analyzed for Cortactin expression in the CD19+CD5+ B-CLL cells, the normal CD19+ and CD5+ cells (fig..). Here, we used a ratio of mean Cortactin fluorescence intensity (ΔMFI) of Cortatin and IgG1 normal control both patients and normal controls. A minimum of 30,000 total events were acquired per tube. Data were acquired with Diva (BD Biosciences) and analyzed with FACS Diva 7 software (BD Bioscence).

Western blotting analysis (Wb). Cells ($5x10^5$ for each assay) were prepared by cell lyses with Tris 20mM, NaCl 150mM, EDTA 2mM, EGTA 2mM, Triton X-100 0.5% supplemented with complete protease inhibitor cocktail (Roche; Mannheim, Germany) and sodium orthovanadate 1mM (Calbiochem; Gibbstown, NJ). Samples were then subjected to SDS/PAGE (10% gels), transferred to nitrocellulose membranes, immunostained with anti-cortactin polyclonal antibody (Santa Cruz Biotechnology, Inc; Santa Cruz, CA), anti-cortactin polyclonal antibody (Sigma-Aldrich; Milano, Italy), anti-cortactin monoclonal antibody (Millipore, Milano, Italy) and anti-β-actin monoclonal antibody (Sigma-Aldrich). Blots were revealed using an enhanced chemiluminescent detection system (Amersham Biosciences; Buckinghamshire, UK), acquired with the CHEMI DOC XRS supply (Bio-Rad Laboratories; Milan, Italy) and analyzed by Image J launcher software. Quantization of cortactin/β-actin, expressed as arbitrary units, were normalized on Jurkat cell line cortactin level.

Cortactin mRNA expression by Real-Time PCR analysis. Total cellular RNA from patient samples were extracted from $5\text{-}10x10^6$ leukemic cells using RNeasy Mini Kit (Qiagen; Hilden, Germany), according to the manufacturer's protocol and treated with DNase (Qiagen). First strand complementary DNA (cDNA) was generated from 1μg total RNA using oligo-dT primer and the AMV reverse transcriptase (Reverse Transcription System, Promega Corporation; Madison, WI). Real-Time quantitative PCR amplifications reaction were carried out in an ABI Prism 7000 sequence detection system (Applied Biosystems; Foster City, CA) in a 15μl volume. SYBR Green

PCR Master Mix was purchased from Applied Biosystems (P/N 4309155), containing AmpliTaq Gold DNA Polymerase and optimized buffer components. A fraction of 5μM primers and 1,5μl of cDNA were added to SYBR Green master mix to make a final 15μl reaction volume. The primers used for cortactin and β-Actin amplifications are: cortactin Forward 5'- CCG CAG AGG ACA GCA CCT A -3' and Reverse 5'- GGC TGT GTA CCC CAG ATC GTT -3'; β-actin Forward 5'- CCA GCT CAC CAT GGA TGA TG -3' and Reverse 5'- ATG CCG GAG CCG TTG TC -3'. These primers were obtained using the Primer Express computer software (Applied Biosystems). PCR reactions were performed under the following conditions: initial denaturation at 95°C for 10min followed by 95°C for 15s and 60°C for 1s cycled 45 times. Each quantization target was amplified in duplicate samples. A no template control for each master mix and two standard curves were generated for cortactin and β-actin using Jurkat cDNA in a serial dilution 1, 1:5, 1:25 and 1:125. The relative amounts of mRNA was determined by comparison with standard curves. For each sample, results were normalized for β-actin expression. To distinguish specific amplicons from non-specific amplifications, a dissociation curve was generated.

Two-dimensional gel electrophoresis (2DE). The first-dimensional isoelectric focusing (IEF) was carried out on an IPGphor system (Amersham Biosciences). The immunoprecipitates were dissolved in 50μl 2D specific lysis buffer (9M urea, 4% CHAPS, 40mM Tris-base, 40mM DTT) and loaded onto an immobilized pH gradient (IPG) strip (pH 4-7, 24cm, GE Healthcare) in a total volume of 450μl rehydration solution (8Murea, 2% [wt/vol] CHAPS, 10mM DTT, 0.5% [vol/vol] IPG buffer [pH 4-7], trace amount of bromophenol blue) for 10h. After rehydration, IEF was initially carried out at 200V. The voltage was gradually increased to 10,000V and then kept constant for 5-6 h at 20ºC (approximately 60–80kVh in total). After IEF, the IPG strip was immediately equilibrated in 5ml SDS equilibration buffer (6M urea, 50mM Tris-HCl [pH 8.8], 30% glycerol, 2% SDS, 1%[wt/vol] DTT, trace amount of bromophenol blue) for 15min with gentle shaking. The second dimensional separation was carried out on 8% SDS-PAGE gels. Western blotting analyses were performed using anti-cortactin polyclonal antibody (Santa Cruz Biotechnology, Inc; Santa Cruz, CA), and anti-β-tubulin monoclonal antibody (Sigma-Aldrich). The blots were acquired with the CHEMI DOC XRS supply (Bio-Rad Laboratories).

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 using the RNeasy isolation kit (QIAGEN). First strand complementary DNA (cDNA) was generated from 1μg total RNA using oligo-dT primer and the AMV reverse transcriptase (Reverse Transcription System, Promega Corporation; Madison, WI). Semi quantitative RT-PCR was performed using 3μl of cDNA in 10mM Tris-HCl (pH 8.4), 50mM KCl, 0.06% bovine serum albumin, 10mM

dithiothreitol, 0.2mM deoxynucleoside triphosphate, 2mM $MgCl_2$, 1.5 units of *Taq*DNA polymerase (Invitrogen; Paisley, UK), and 6pmol of primers F and R, (F 5'-GTCTTTCAAGAGCATCAGACCC-3', R 5'-CTCTTTCTCCTTAGCGAGGTTTTC).[11] PCR was carried out for 33 cycles at 94°C for 1min, 56°C for 1.30min, and 72°C for 2.30min, 20µl of PCR products were electrophoresed on 1% agarose gel for 1.5h at 65V in 40mM Tris Acetate and 2mM EDTA buffer. The gels were stained with ethidium bromide, and the images of the UV-illuminated gels were captured using the CHEMI DOC XRS supply (Bio-Rad Laboratories).

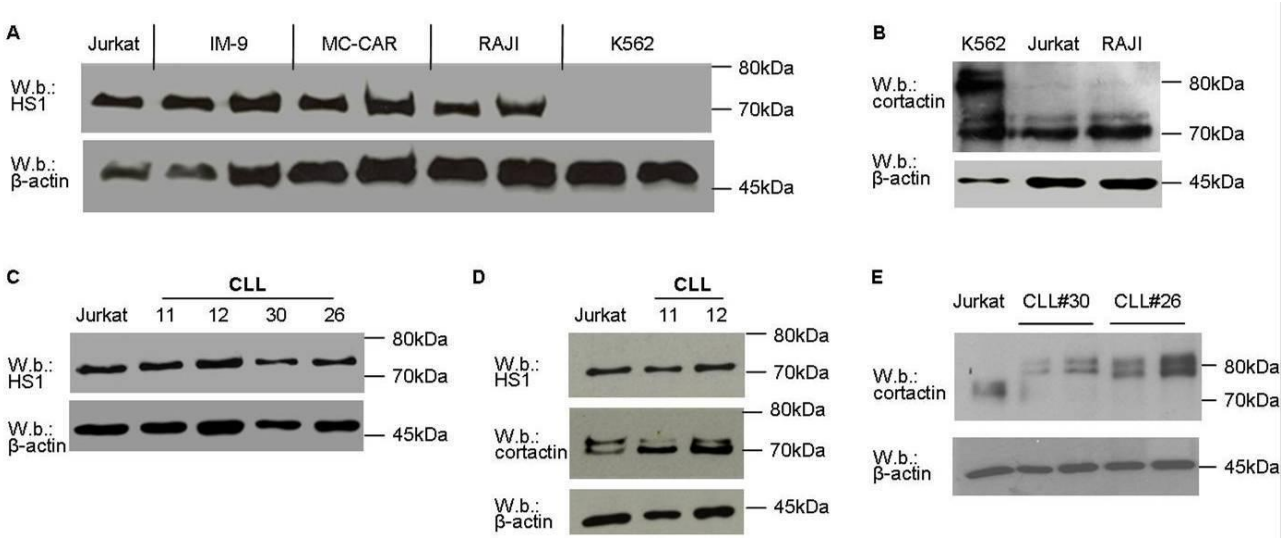

Figure 1S. Evaluation by western blotting of cortactin and HS1 expression in cell lines and patients with CLL. A) Results of immunblotting for HS1 in the cell lines Jurkat, IM-9, MC-CAR, Raji and K562. As it is possible to observe, the cell line K562 does not express the protein HS1. **B)** Results of immunblotting for cortactin in the cell lines K562, Jurkat and Raji. As it is possible to observe, the cell line K562 express all isoforms of cortactin (p70/75 and p80/85). The fact that the cell line K562 does not express HS1 (see panel A), excludes the possibility that the form p70/75, recognized by the antibody against cortactin, could be HS1 recognized because of a cross-reaction of antibody. **C)** Results of immunblotting for HS1 in patients with CLL. **D)** Results of immunblotting for HS1 and cortactin in the same patients, #11 and #12. As it is possible to observed in blots results, HS1 protein shows always a sharp band whereas cortactin shows two near bands with an completely different appearance from HS1, excluding again a cross-reaction of cortactin antibody with HS1 protein. **E)** Western blotting results using anti cortactin antibody in two patients which do not express the forms p70/75 of cortactin. Here, it is possible to observe that the antibody against cortactin does not cross-react with HS1 since at molecular weight of 70 kDa no bands are detected.

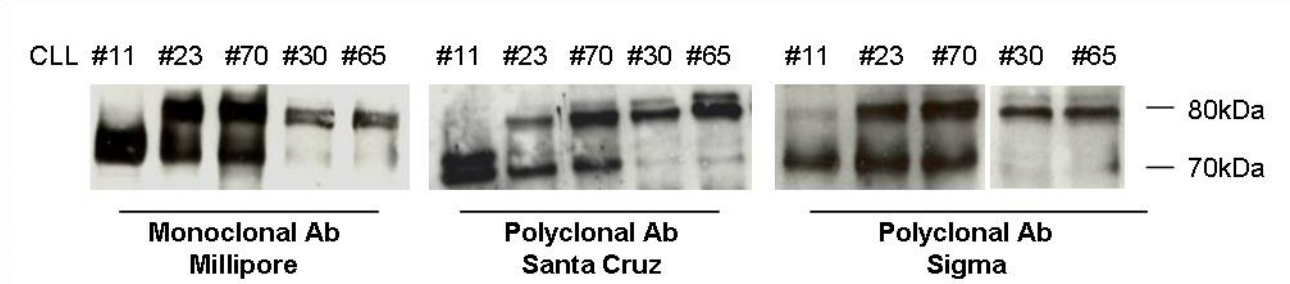

Figure 2S. Evaluation of cortactin expression level with different antibodies. Western blotting analysis were performed with three different anti-bodies against cortactin, purchased from Millipore (Monoclonal Ab), Santa Cruz (Polyclonal Ab) and Sigma (Polyclonal Ab) in five B-CLL patients. All different anti-bodies identified the same forms of cortactin in the analyzed patients.

Table 1S. Patients' characteristic.

Patient	Age	Sex	RAI stage	wbc count /mm3	mutational status	ZAP70	CD38
1	49	M	0	29,600	nd	-	+
2	65	F	0	12,790	M	+	-
3	49	M	0	16,000	M	-	-
4	56	M	0	10,500	M	-	-
5	80	F	0	26,000	nd	-	-
6	58	M	0	156,200	U	-	-
7	64	F	0	20,000	U	-	-
8	74	M	0	16,840	M	-	-
9	71	M	0	9,610	M	+	-
10	48	F	0	32,700	U	-	-
11	63	F	0	48,300	M	+	+
12	54	M	0	22,460	nd	-	nd
13	80	M	0	9,300	M	-	+
14	57	M	0	23,600	U	-	+
15	83	F	0	12,900	nd	-	-
16	81	M	0	50,100	nd	-	-
17	55	M	0	12,700	M	+	-
18	47	M	0	11,300	U	+	-
19	50	F	0	8,300	nd	-	-
20	61	F	0	19,400	nd	-	+
21	61	M	0	9,000	M	+	-
22	53	F	0	11,500	M	-	-
23	58	F	0	37,500	U	-	+
24	62	M	0	106,100	M	-	-
25	68	M	1	73,800	M	+	-
26	58	M	1	21,910	M	-	-
27	65	F	1	51,000	M	-	-
28	59	M	1	11,500	M	-	-
29	67	F	1	29,500	M	-	-
30	62	M	1	10,200	M	-	-
31	70	F	1	17,780	M	-	-
32	44	F	1	55,800	M	-	-
33	54	F	1	20,370	M	-	-
34	68	M	1	18,500	U	+	+
35	62	M	1	27,400	M	-	-
36	66	M	1	16,800	U	-	+
37	64	F	1	23,000	M	-	-
38	65	F	1	13,330	M	-	-
39	67	M	1	33,280	nd	+	+
40	67	M	1	8,300	M	+	-
41	74	F	1	24,850	U	+	+
42	61	F	1	29,200	M	+	-
43	49	F	1	100,600	M	-	-
44	57	F	1	48,500	nd	+	-
45	58	M	1	10,400	M	-	-
46	63	M	1	33,000	U	+	-
47	74	M	1	12,000	nd	-	-
48	64	F	2	48,700	M	-	-
49	63	F	2	25,600	M	-	-
50	57	M	2	25,500	U	-	-
51	62	F	2	66,000	M	+	-
52	48	F	2	44,000	U	+	-
53	65	M	2	113,000	M	-	-

Patient	Age	Sex	RAI stage	wbc count /mm3	mutational status	ZAP70	CD38
54	63	F	2	27,700	U	-	-
55	74	F	3	14,200	M	-	-
56	95	F	3	40,000	M	-	-
57	84	M	4	22,530	M	-	-
58	74	M	4	19,100	M	+	-
59	76	M	4	34,800	U	+	+
60	84	M	4	56,000	U	-	+
61	32	F	4	21,600	U	-	+
62	71	M	4	139,600	M	-	-
63	63	M	4	51,700	M	+	-
64	69	M	4	12,900	M	-	-
65	73	M	4	39,100	U	-	nd
66	43	M	4	11,220	U	+	+
67	76	M	4	162,000	U	+	nd
68	67	M	4	41,100	U	+	nd
69	76	M	4	50,600	M	-	-
70	68	M	4	2,910	U	+	+
71	78	F	4	48,000	nd	+	-
72	83	M	4	50,100	U	-	-
73	77	M	4	41,000	nd	+	-
74	78	M	4	80,000	U	+	-
75	80	M	4	125,700	M	+	+
76	69	F	4	70,600	U	+	+
77	79	M	4	35,000	M	-	-
78	57	M	4	330,000	U	+	-
79	64	F	4	17,500	M	+	+
80	74	M	4	17,830	M	-	+
81	55	M	4	77,000	M	-	+
82	73	F	nd	20,000	U	-	-
83	69	F	nd	55,000	U	-	+
84	59	M	nd	39,400	U	-	+
85	57	M	nd	32,890	U	+	-
86	70	M	nd	95,300	U	+	-
87	58	M	nd	14,800	M	-	-
88	74	F	nd	29,100	U	-	nd
89	82	M	nd	195,000	M	+	nd
90	59	M	nd	73,640	M	-	-
91	74	M	nd	19,490	U	+	+
92	58	M	nd	8,310	U	-	-
93	52	F	nd	8,530	U	-	-
94	66	M	nd	n	M	+	nd
95	53	M	nd	62,000	U	-	+
96	60	F	nd	19,030	M	-	-
97	64	M	nd	27,680	M	-	nd
98	67	M	nd	125,800	U	-	nd
99	70	M	nd	13,860	U	-	nd
100	64	M	nd	142,000	U	-	-
101	79	F	nd	15,720	M	+	-
102	68	F	nd	28,520	M	-	-
103	61	M	nd	9,730	M	-	-
104	63	M	nd	9,970	U	+	+
105	49	M	nd	33,500	M	-	-
106	71	M	nd	26,780	U	+	+