SUPPLEMENTARY APPENDIX

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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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).

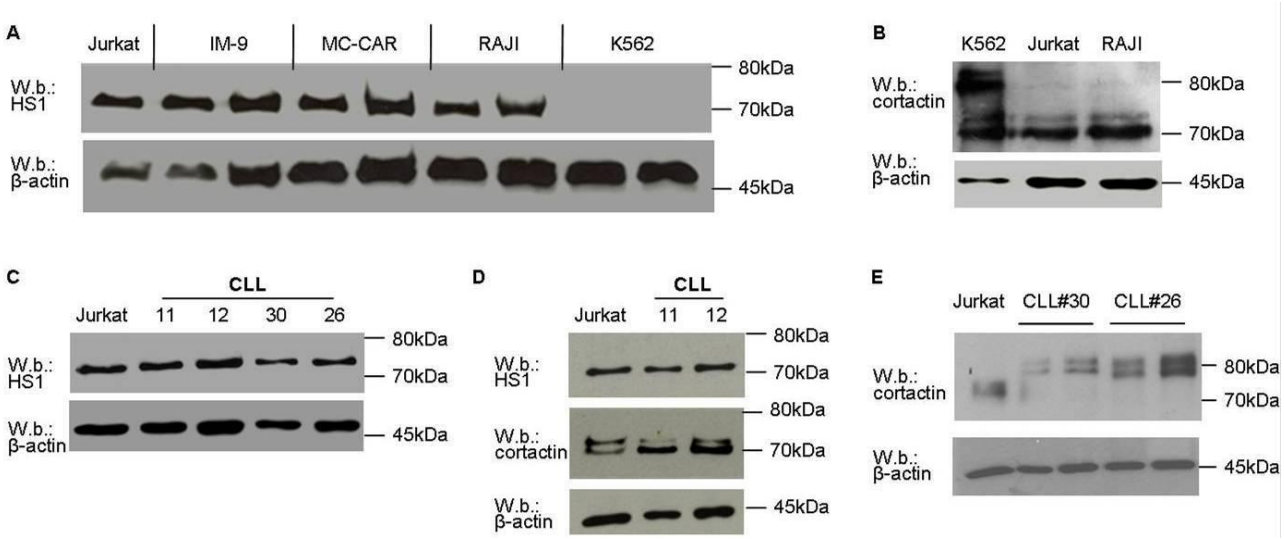

Figure 1S. Evaluation by western blotting of cortactin and HS1 expression in cell lines and patients with CLL. A) Results of immunblotting for HS1 in the cell lines Jurkat, IM-9, MC-CAR, Raji and K562. As it is possible to observe, the cell line K562 does not express the protein HS1. **B)** Results of 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.

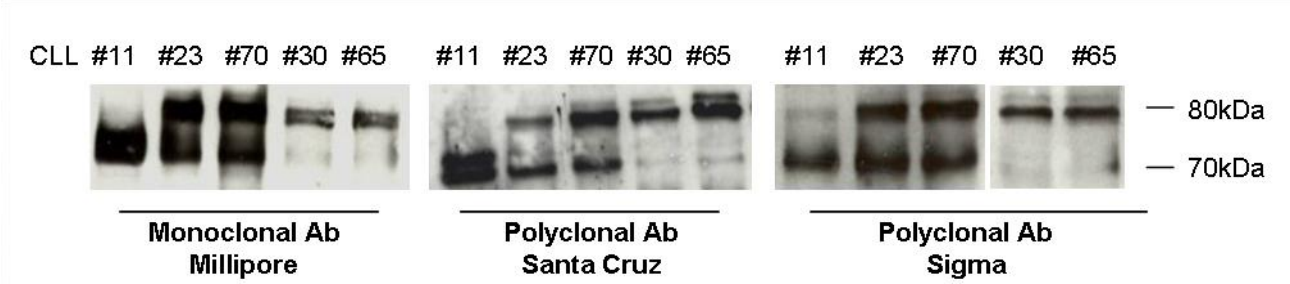

Figure 2S. Evaluation of cortactin expression level with different antibodies. Western blotting analysis were performed with three different anti-bodies against cortactin, purchased from Millipore (Monoclonal Ab), Santa Cruz (Polyclonal Ab) and Sigma (Polyclonal Ab) in five B-CLL patients. All different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