SUPPLEMENTARY APPENDIX

SLP76 integrates into the B-cell receptor signaling cascade in chronic lymphocytic leukemia cells and is associated with an aggressive disease course

Nili Dezorella,[1,2,*] Ben-Zion Katz,[1,2,*] Mika Shapiro,[1] Aaron Polliack,[3] Chava Perry[1] and Yair Herishanu[1,2]

[1]Department of Hematology, Tel-Aviv Sourasky Medical Center; [2]Sackler Faculty of Medicine, Tel-Aviv University and [3]Department of Hematology, Hadassah University Hospital and Hebrew University Medical School, Jerusalem, Israel

**ND and B-Z Katz contributed equally to this work*

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Correspondence: yairh@tlvmc.gov.il

Supplement 1: Patients' characteristics

Study number	Gender/ Age (y)	Binet stage	ALC x1000/uL	*IGHV* gene % germline	TTT (months)
CLL_01	M/58	B	53.5	3-9, 99.7%	+7
CLL_02	F/81	A	41.5	3-48, 83.6%	162
CLL_03	F/64	A	32.6	1-46, 91.8%	53
CLL_04	M/69	A	18.3	5-51, 92.2%	97
CLL_05	M/61	A	135	2-5, 95.6%	119
CLL_06	F/71	A	178	3-7, 94.2%	+168
CLL_07	F/60	A	34.6	3-7, 90.2%	96
CLL_08	F/75	A	47	2-5, 96.6%	51
CLL_09	F/68	C	148.4	1-69, 94.6%	235
CLL_10	F/79	A	17.2	1-2, 85.1%	110
CLL_11	F/74	A	135.3	3-7, 90.2%	137
CLL_12	F/42	B	37.6	3-23, 98.6%	+59
CLL_13	M/64	B	38.7	4-34, 93.0%	+62
CLL_14	M/65	B	88.6	1-1, 99.7%	+44
CLL_15	M/50	B	53.3	3-7, 99.3%	46
CLL_16	M/55	B	70.2	3-33, 98.0%	+81
CLL_17	M/57	B	49.2	5-51, 98.0%	+23
CLL_18	F/81	A	210.4	5-51, 85.7%	128
CLL_19	F/59	A	36.7	3-23. 94.3%	59
CLL_20	M/69	C	113.0	3-3, 99.3%	+56
CLL_21	M/54	B	172.6	1-60, 100%	+47
CLL_22	F/62	A	181.2	3-11, 99.7%	+56
CLL_23	M/64	B	211.3	3-21, 96%	+23
CLL_24	F/87	B	205.3	ND	+158
CLL_25	F/67	B	36.45	3-64. 98.3%	+23
CLL_26	M/82	B	22.5	1-2. 99.7%	+66
CLL_27	M/80	B	104.9	3-53, 100%	+3
CLL_28	F/75	A	268.2	3-64, 99.7%	+31
CLL_29	M/70	B	50.7	3-21, 96%	+23
CLL_30	F/68	C	85.6	4-39, 92.1%	+94
CLL_31	M/71	A	54.7	1-69, 98.4%	69
CLL_32	M/75	B	57.9	1-69, 99.3%	+38
CLL_33	F/85	C	59.9	1-2, 98.6%	99
CLL_34	M/55	C	271.1	4-30, 100%	+0.8
CLL_35	F/68	C	103.3	ND	+162
CLL_36	F/70	A	80.8	3-30, 100%	124
CLL_37	M/61	A	59.5	2-5, 100%	44
CLL_38	F/61	C	46.7	3-13, 99.3%	+21
CLL_39	F/57	A	54.5	3-64, 100%	46
CLL_40	M/67	B	57.7	1-18, 99.6%	+13

ALC: absolute lymphocyte count, TTT: progression free survival in months from diagnosis to initiation of treatment , + indicates patients treated, all others remain in continued observation without treatment, ND-no data

Supplement 2: *Expanded Materials and methods*

Antibodies and Reagents

Monoclonal antibodies (MoAb) anti-human CD19-APC, CD3-FITC, CD5-APC, CD23-PE-Cy7, SLP76-PE, phospho-SLP76 (pY128)-PE, Isotype control-PE/APC/FITC, purified anti phospho-SLP76 (pY128) antibody, cytofix fixation buffer, and Phosflow perm/wash buffer I were from BD Biosciences (San Jose, CA), purified anti-human SLP76, phospho-SLP76 (pS376), Lck, BLNK, ITK, CD3ε, ZAP-70, PLCγ1, PLCγ2, BTK, Phospho-BTK (pY223), AKT(pan), phospho-AKT (pS473) antibodies were from Cell Signaling Technology (Beverly, MA). Purified anti-human LAT was obtained from eBioscience (San Diego, CA) and purified anti-human Actin from MP Biomedicals (Illkirch, France). Goat anti Rabbit IgG (H+L)-HRP conjugate and Goat anti Mouse IgG (H+L)-HRP conjugate were from Bio-Rad Laboratories (Richmond, CA). Goat F(ab')2 anti-human IgM was from Jackson Immunoresearch Laboratories, (West Grove, PA). All antibodies utilized in the study were used in concentrations according to the manufacturer's instructions. MβCD, and Cytochalasin B were from Sigma-Aldrich (St. Louis, MO), Syk Inhibitor II (SYKII), R406 and Lck inhibitor (LCKi) were from Calbiochem (Gibbstown, NJ). PP2 was from Cayman chemical (Ann Arbor, MI), Ficoll-Paque PLUS from GE healthcare (Uppsala, Sweden), dimethyl sulfoxide (DMSO) from Merck (Darmstadt, Germany). protein A/G PLUS-Agarose beads and anti β Tubulin antibody from Santa Cruz Biotechnology (Santa Cruz, CA), RPMI medium from Gibco Laboratories (Grand Island, NY), fetal calf serum (FCS), Dulbecco's phosphate buffered saline (PBS), L-glutamine, penicillin-streptomycin and gentamycin were from Biological Industries (Beit-Haemek, Israel).

Patients and samples

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation. Viable frozen cells were kept in FCS containing 10% DMSO and stored in liquid nitrogen. Before use, frozen cells were thawed and cultured at 37°C, 5% CO2, in RPMI medium supplemented with 10% FCS, penicillin, streptomycin, and L-glutamine.

Western blotting

Purified CLL cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing Phosphatase Inhibitor Cocktail 2 and protease inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Extract from cell lysates were separated on 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper was incubated with the designated antibodies and HRP conjugated secondary antibodies according to the manufacturer's instructions. bands were detected using a MicroChemi Chemiluminescence image analysis system (DNR Bio-imaging Systems, Jerusalem, Israel) and analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD), or using MyECL Imager (Thermo Scientific, Rockford, IL).

Co-immunoprecipitation

CLL cells ($1*10^7$/ml) were stimulated with goat F(ab′)2 anti–human IgM (10 μg/ml) at 37°C for 15 min. The stimulated cells and unstimulated controls were lysed in RIPA lysis buffer (Cell Signaling Technology, Beverly, MA) containing phosphatase and protease Inhibitor Cocktails. Cell lysates were incubated with purified anti human SLP76 (1:50) for 1 hour in 4°C. Following incubation, 20 µl of re-suspended volume of protein A/G PLUS-Agarose were added to cell lysates. The lysates were then incubated on rocker platform at 4°C overnight. Immunoprecipitates were collected by centrifugation at 2,500 RPM for 5 min at

4°C and washed 4 times with RIPA buffer. After final washing, immunoprecipitates were re-suspended in electrophoresis sample buffer, boiled and blotted with anti-human SLP76 and anti-human BTK antibodies.

Quantitative RT-PCR

All of the PCR reactions were performed in duplicates. The following primers were used for RT-PCR: SLP76 forward: 5'-AGA AAG CCA CGA AGA GGA CA-3' and reverse: 5' GAG CTT CCT CGT CAT TGG AG-3' , GAPDH forward 5'-ATG GGG AAG GTG AAG GTC G-3' and reverse 5'-GGG GTC ATT GAT GGC AAC AAT A-3'. The primers were purchased from HY-labs (Rehovot, Israel).

IGHV gene analysis

cDNA was amplified by polymerase chain reaction (PCR) using a mixture of 5′ oligonucleotides specific for each leader sequence of the VH1 to VH7 *IGHV* families as forward primers and a 3′ oligonucleotide complementary to either the consensus sequence of the joining region or the constant region of the IgM locus as reverse primer. PCR was performed in 25 μL reactions with Faststart Taq (Roche, IN) and 20 pmol of each primer. Products were purified with QIAquick Kit; (Qiagen, CA) and sequenced directly with the appropriate 3′ and 5' oligonucleotides by HyLab Sequencing Service (Rehovot, Israel). Nucleotide sequences were aligned to the VBase sequence directory (http://www.ncbi.nlm.nih.gov/igblast). Sequences with 2% or less deviation from any germline *IGHV* sequence were considered unmutated.

siRNA transfection

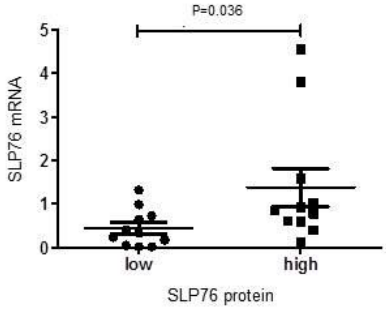
CLL cells ($7*10^6$) were resuspended in 100 μl SF Cell line 4D-Nucleofector™ X Kit (Lonza Group Ltd, Basel, Switzerland) with 0.4 μM ON-TARGETplus SMARTpool small interfering RNA (siRNA) to Human SLP76 or ON-TARGETplus Non-targeting pool as negative control (both from GE Healthcare Dharmacon Inc, Lafayette, CO). Cells were then transfected with the 4D-Nucleofectordevice, incubated for 5 min on ice and cultured for 36 hours in 24-well plates in RPMI medium 1640 supplemented with 20% FCS, penicillin, streptomycin, and L-glutamine. Thereafter cells were collected, stimulated as described above and either lysed for western blot analysis or analyzed by flow cytometry for apoptosis detection.

Cell apoptosis assay

Following SLP76 knockdown, a total of $2*10^5$ CLL cells/well were seeded into 96 well plates with or without goat F(ab′)2 anti–human IgM (10 μg/ml) for 48 h incubation at 37°C. Cells were then collected, stained with anti CD23 and anti CD5 antibodies, washed once with PBS and Cell apoptosis was detected by using MEBCYTO Apoptosis Kit. $3*10^4$ events from each sample were acquired by a FACSCalibur and analyzed using CellQuest software.

Supplement 3:

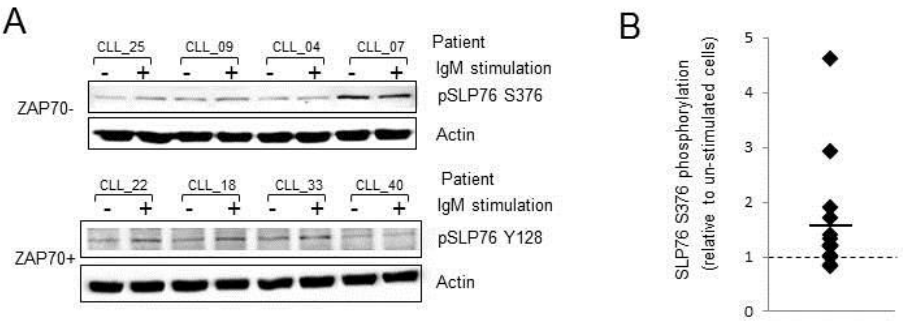
A correlation between SLP76 protein expression and mRNA level.



The level of total SLP76 protein was detected by Western blot in primary $CD19^{+}$ purified CLL cells and compared to the mRNA levels (detected by RT-PCR) of SLP-76 in those patients. n=22.

Supplement 4:

An analysis of SLP76 serine phosphorylation in ZAP-70 negative and ZAP70 positive samples.



A. Western blot analysis of SLP76 serine phosphorylation (S376) following 45 min of IgM stimulation (10 µg/ml), in ZAP-70 negative (n=4) and ZAP70 positive (n=4) patients. **B.** A summary of the serine phosphorylation levels of SLP76 (S376) after IgM stimulation (10 µg/ml) for 45 min (n=12).