RESEARCH ARTICLE

Autologous T cells expressing the oncogenic transcription factor KLF6-SV1 prevent apoptosis of chronic lymphocytic leukemia cells

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Abstract

Crosstalk between leukemic cells and the tumor microenvironment is of importance in chronic lymphocytic leukemia (CLL). T cells seem to sustain the survival of CLL cells by various mechanisms. The Krüppel-like family of transcription factors (KLFs) are identified as regulators of proliferation and cell death. In the present study, we analyzed the expression of the wild type (WT) gene KLF6 and the oncogenic splice variant 1 (KLF6–SV1) at the mRNA level in subsets of T cells from CLL patients (n = 29), multiple myeloma patients (n = 6) and normal donors (n = 10). RNA Silencing was used for wtKLF6 and KLF6–SV1. Tumor cell apoptosis was measured. A significant overexpression of wtKLF6 and KLF6–SV1 in T cells of CLL patients compared to normal donors and myeloma patients (p < 0.002). Western blot showed that both wtKLF6 and KLF6–SV1 were expressed in purified T cells from CLL patients. KLF6–SV1 siRNA transfection induced a significant downregulation of KLF6–SV1 in T cells of CLL patients compared to normal donors and myeloma patients was noted (p<0.002). Western blot showed that both wtKLF6 and KLF6–SV1 were expressed in purified T cells from CLL patients. KLF6–SV1 siRNA transfection induced a significant downregulation of KLF6–SV1 in T cells of CLL patients, which lost the capability to sustain the growth of leukemic cells. However, no such a significant effect was seen after wtKLF6 transfection of the autologous T cells. The results suggest that KLF6–SV1 may play a role in the regulation of survival CLL cells.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of CD5+, CD19+, CD23+ neoplastic small B cells in secondary lymphoid tissues and peripheral blood. In lymph
nodes, the CLL clone proliferates in distinct areas called "proliferation centers" or "pseudo follicles" [1–4], with a low proliferative rate, accounting for approximately 1% to 2% of the tumor clone [5]. Persistence of the tumor clone is sustained by the ability to resist apoptosis rather than the proliferation rate (5). However, in vitro purified CLL cells undergo rapid spontaneous apoptosis, suggesting that ex vivo conditions may lack survival factors for leukemic cells might be present in vivo and that resistance to apoptosis is not only intrinsic to the leukemic B cells [6]. Dividing CLL cells were shown to express survivin and were frequently surrounded by T cells as well as other accessory stromal cells [7]. The tumor microenvironment seems to play an important role in pathology of CLL and may also be a target for treatment strategies [8]. Such treatment approaches may include inhibition of the crosstalk between CLL cells and the supportive microenvironment to overcome stromal-mediated tumor cell survival [9, 10].

We have recently completed a thorough analysis of shown an aberrant expression of cell surface and signaling molecules in T cells of CLL patients as well as an altered gene expression profile and increased production of cytokines as IL-4 and IFN-γ were also observed [11–13]. Functionally abnormal T cells may contribute to a microenvironment in which the proliferating leukemic clone resists differentiation and apoptosis sustaining the malignant phenotype of the B cells [12, 14]. In spite of the current knowledge of T cell support for CLL cells, the mechanisms of the anti-apoptotic effects of T cells are not fully understood [4, 12].

Kruppel-like factor (KLF) 6 belongs to the KLF family consisting of 17 proteins acting as DNA-binding transcription factors [15]. Wild-type KLF6 (wtKLF6) is a tumor-suppressor gene frequently inactivated in colorectal, prostate, colon cancers as well as in astrocytic gliomas [16, 17]. KLF6 interacts with cyclin D1 to mediate growth inhibition [18].

The KLF6 splice variant 1 (SV1) has 21 unique amino acids in the C-terminal, resulting in the loss of three zinc finger DNA binding domains [19]. The KLF6-SV1 N-terminal might activate the Ras/Pi3-K/Akt proto-oncogenes [20]. Overexpression of c-Myc may act synergistically with KLF6-SV1 to increase the metastatic capacity of tumor cells [21]. KLF6-SV1 expression in tumor cells was associated with epithelial mesenchymal transition (EMT) and metastasis [17, 19]. It regulates extracellular matrix components as E-cadherins [22, 23]. The expression is associated with a poor prognosis of many types of cancers [3, 19, 24, 25]. It has also been shown that small interfering RNA (siRNA) down-regulating KLF6-SV1 reduced the capacity of tumor cells to progress in vitro and in vivo as well as enhanced the sensitivity to chemotherapy [26, 27]. KLF6-SV1 down-regulation in non-malignant cells might induce proliferation arrest, further indicating that KLF6-SV1 might have a role in cell growth [21].

In the present study we analyzed the expression of wtKLF6 and KLF6-SV1 in T cells of CLL patients and report a supportive effect of T cells expressing KLF6-SV1 on the survival of leukemic cells in vitro. No such effect was observed with T cells from patients with multiple myeloma (not considered to be dependent on T cells for tumor cell survival [28]) nor with T cells from healthy donors.

Materials and methods

Patients

Peripheral blood was collected from 29 patients with CLL, 6 patients with multiple myeloma and 10 healthy donors. The study was approved by The Regional Ethical Review Board in Stockholm: www.epn.se. The trial was performed in accordance with the Helsinki declaration on the use of human samples for clinical research. Sampling was done after written and oral informed consent was obtained from the participants. Diagnostic criteria for CLL and multiple myeloma have been described previously [29]. The characteristics of the patients are shown in Table 1.
| Patient’s number | Age | Sex | Clinical stage | M-component type & concentration (g/L) | Previous therapy | Disease phase | CD3% | Time since last therapy (mo) |
|------------------|-----|-----|----------------|----------------------------------------|------------------|--------------|------|-----------------------------|
| **CLL patients** |     |     |                |                                        |                  |              |      |                             |
| CLL1             | 71  | M   | I              | NA                                     | Non             | Indolent     | 19   | NA                          |
| CLL2             | 73  | F   | I              | NA                                     | Non             | Indolent     | 5.5  | NA                          |
| CLL3             | 72  | M   | 0              | NA                                     | Non             | Indolent     | 34   | NA                          |
| CLL4             | 58  | M   | I              | NA                                     | Non             | Indolent     | 3.5  | NA                          |
| CLL5             | 63  | M   | 0              | NA                                     | Non             | Indolent     | 17.6 | NA                          |
| CLL6             | 84  | M   | I              | NA                                     | Non             | Indolent     | 7    | NA                          |
| CLL7             | 59  | M   | 0              | NA                                     | Non             | Indolent     | 15   | NA                          |
| CLL8             | 74  | F   | II             | NA                                     | Non             | Indolent     | 1.8  | NA                          |
| CLL9             | 68  | F   | II             | NA                                     | Non             | Indolent     | 4.4  | NA                          |
| CLL10            | 78  | F   | II             | NA                                     | Non             | Indolent     | 12   | NA                          |
| CLL11            | 63  | M   | 0              | NA                                     | Non             | Indolent     | 1.5  | NA                          |
| CLL12            | 76  | M   | I              | NA                                     | Non             | Indolent     | 19   | NA                          |
| CLL13            | 68  | M   | I              | NA                                     | CLB             | Response/Plateau | 0.4 | 12                          |
| CLL14            | 71  | F   | 0              | NA                                     | None            | Indolent     | 24   | NA                          |
| CLL15            | 65  | F   | I              | NA                                     | F               | Response/Plateau | 3.4 | 1                           |
| CLL16            | 72  | M   | 0              | NA                                     | None            | Indolent     | 21   | NA                          |
| CLL17            | 73  | M   | I              | NA                                     | None            | Indolent     | 15.7 | NA                          |
| CLL18            | 86  | F   | I              | NA                                     | None            | Indolent     | 13   | NA                          |
| CLL19            | 79  | M   | I              | NA                                     | None            | Indolent     | 13.4 | NA                          |
| CLL20            | 69  | F   | I              | NA                                     | None            | Indolent     | 1.8  | NA                          |
| CLL21            | 65  | M   | II             | NA                                     | CLB             | Response/Plateau | 5.5 | 24                          |
| CLL22            | 79  | F   | I              | NA                                     | CLB             | Response/Plateau | ND  | 4                           |
| CLL23            | 73  | F   | II             | NA                                     | None            | Indolent     | 9.7  | NA                          |
| CLL24            | 75  | M   | II             | NA                                     | None            | Indolent     | 67.5 | NA                          |
| CLL26            | 66  | F   | II             | NA                                     | None            | Indolent     | 2    | NA                          |
| CLL27            | 71  | F   | 0              | NA                                     | None            | Indolent     | 6.8  | NA                          |
| CLL28            | 69  | M   | 0              | NA                                     | None            | Indolent     | 9.1  | NA                          |
| CLL29            | 78  | F   | 0              | NA                                     | None            | Indolent     | ND   | NA                          |
| **Myeloma patients Group** |     |     |                |                                        |                  |              |      |                             |
| MM1              | 73  | F   | IA             | IgA, 20                                | None            | Asymptomatic | ND   | NA                          |
| MM2              | 77  | M   | IA             | IgG, 7                                 | None            | Asymptomatic | ND   | NA                          |
| MM3              | 75  | F   | IIA            | IgG, 23                                | MP              | Response/Plateau | ND  | 49                          |
| MM4              | 83  | F   | IIIA           | IgG, 70                                | MP              | Response/Plateau | ND  | 9                           |
| MM5              | 85  | F   | IIIA           | IgG, 8                                 | MP              | Response/Plateau | ND  | 51                          |
| MM6              | 79  | F   | IIA            | IgA, 23                                | None            | Asymptomatic | ND   | NA                          |

* Rai (CLL) and ISS (MM) staging system were used
NA = not applicable, CLB = Chlorambucil, F = Fludarabine, MP = Melphalan-Prednisone, ND = not done

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Purification of T and B cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood as described [30]. CLL B cells from PBMC were depleted by filtration through a nylon wool column (Biotest, Breiech, Germany) [31] and T cells were further enriched by immunomagnetic depletion of B cells, NK cells and monocytes using MidiMACS columns and anti-CD19, anti-CD56 and anti-CD14 MACS MicroBeads (MiltenyiBiotec, Bergisch Gladbach, Germany) according to the manufacturer’s recommendations. The purity of CD3 T-cells was 93–99% as determined by flow cytometry. CD4⁺ and CD8⁺ T cells were further purified from CD3⁺ T cells using the MACS MicroBeads negative selection kit (MiltenyiBiotec, Bergisch Gladbach, Germany). The purity of CD4⁺ and CD8⁺ cells were 96% and 94%, respectively. The purity of B cells (CD19⁺) was >99% as determined by flow cytometry.

Cellular staining and flow cytometry

Surface markers were analysed by flow-cytometry using fluorochrome-conjugated monoclonal antibodies. Anti-CD3, CD4, CD8, CD19, and CD56 monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA). Appropriate concentrations of antibodies as well as isotype -matched controls were added to the cells (5 x 10⁵ cells/ tube) in 100 μL staining buffer and incubated for 25 min at 4°C in the dark. Analyses were performed using a FACS Canto II flow cytometry (BD) and the FLOW JO™ software (Tree Star, Ashland, OR, USA). A minimum of 10,000 lymphocyte-gated cells, were acquired and analyzed for CD3⁺CD4⁺, CD3⁺CD8⁺, CD19⁺ and CD56⁺ cells. Criteria for positive staining was set at a fluorescent intensity displayed by <0.5% of the cells stained by the appropriate fluorochrome-conjugated isotype control mAb.

siRNA transfection

Purified T cells from CLL patients (1x10⁵) were cultured in 96-well cell culture plates in 100 μl Accell siRNA Delivery Media (Thermo scientific, PA, USA). Sequence specific siRNA for wtKLF6 (5’-GGGGAGGCAUCGCCAUUU-3’), KLF6-SV1 (5’-CAGGGAAGGAAGAAAAAGCCUUU-3’) [27, 32] and control siRNA (5’-UGGUUUACAUUGCGACUAA-3’) (Thermo scientific Dharmacon, PA, USA) were added to the cells (1 μM siRNA in Accell siRNA delivery medium, (Thermo Fisher Scientific) and incubated for 48 hours. Cells were then harvested. Total RNA was extracted and cDNA synthesized [33]. Apoptosis was measured after 72 hours by Annexin V/PI staining using flow cytometry (see below).

Apoptosis assay

Untransfected purified CLL cells or T cells (1x10⁵) were cultured alone or after transfection with wtKLF6 siRNA, KLF6-SV1 siRNA as well as mock transfected for 72 hours. The cells were harvested and apoptosis measured by Annexin V/PI staining using a commercial kit (BD San Jose, CA, USA) according to the manufacturer’s instructions [30]. Briefly, cells were washed in PBS and stained for surface CD19 and CD3 expression. After washing in PBS, cells were resuspended in binding buffer. Annexin V/PI (BD) was added and incubated for 15 min at room temperature in the dark. Cells were analyzed using BD FACS Canto II flow cytometer (BD) and the FLOW JO™ software (Tree stars). A minimum of 10000 gated events were analyzed.

Isolation of RNA and cDNA synthesis

Total RNA was extracted from freshly isolated CD4⁺ and CD8⁺ T cells and CLL cells from CLL patients as well as from CD4⁺ and CD8⁺ T cells of multiple myeloma patients and healthy
donors using the RNaseasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The KLF6 negative cell line 293T (isolated from human embryonic kidneys, ATCC 30–2002) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FBS (heat inactivated), 2mM L-glutamine 1% and penicillin/streptomycin. The cells were washed and RNA extracted. The purity and quality of extracted RNA were confirmed by measuring the A260/A280 ratio and separation on agarose gel to ensure RNA integrity prior to cDNA synthesis. cDNA synthesis was performed by converting total RNA to cDNA, using a cDNA kit (Fermentas/Thermo) (Waltham, Massachusetts, USA) according to manufacturer’s guidelines. Briefly, 100 ng of total RNA and 50 pmol/μl of random hexamer primers and 50 pmol/μl of oligo dT primers were heated in 11 μl of RNase-free water at 65°C for 10 min and chilled in ice water. A mixture consisting of 4 μl of 5x RT buffer, 2 μl of 20 mM DTT, 1 μl of 10mM dNTPs, and 1 μl of RNase Inhibitor (RiboLock RNase Inhibitor (20U/μL); Waltham, Massachusetts, USA) were added and incubated at room temperature for 5 min. One microliter of MMLV Reverse Transcriptase (200 U/μl; thermo Scientific) was added to the reaction and incubated at 42°C for 60 min.

**Quantitative real-time RT-PCR (qRT-PCR)**

The expression of the KLF6-SV1 gene was quantified by qRT-PCR. Total RNA was extracted from purified CD4+ and CD8+ T-cells of CLL patients, multiple myeloma patients and healthy donors. qRT-PCR was performed using the TaqMan probe (Life technology, Stockholm, Sweden) and the 79000 Real Time PCR equipment (ABI, CA, USA). qRT-PCR reactions were performed in 20 μL in duplicate. Analysis of sequences of interest was performed by the comparative Ct method of relative quantification using GAPDH as endogenous control and PBMC of a normal donor as calibrator. The difference in Cq values (mean of duplicates) between the investigated transcript and the endogenous reference gene was calculated, and the mean normalized expression (MNE) reported as 2^{-ΔCq}.

**Immunoblotting**

Purified T cells from CLL patients and healthy donors (5 x 10^6) were lysed in buffer containing 1% Triton X-100, 50 mMTris–HCl pH 7.4, 150 mMNaCl, 5 mM EDTA, and 1% protease inhibitor cocktail. Gel electrophoresis was run applying 20–30 μg of cell lysate per lane using a pre-cast NuPAGE® 10% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) at 200 V for 1 hour under reducing conditions. Resolved proteins were transferred onto polyvinylidene difluoride (PVDF) microporous membranes (Millipore, Billerica, MA, USA) in a Mini Trans-Blot cell (Thermo Fisher Scientific). Membranes were blocked overnight with 5% nonfat milk (Semper, Stockholm, Sweden) in PBS with 0.05% Tween 20 (PBS-T). All immunostaining steps and washings were performed in PBS-T supplemented with 5% nonfat milk (Semper). Membranes were incubated with the primary antibody overnight. The following mAbs were used for detection of proteins: anti-KLF6 and anti-KLF6-SV1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After extensive washings, membranes were incubated with the appropriate HRP-conjugated antibody (DAKO, Glostrup, Denmark) for 1 hour. Membranes were then developed using enhanced chemiluminescence Amersham ECL Select Detection Reagents (Amersham Place, Little Chalfont, UK) according to manufacturer’s instruction. Membranes were subsequently stripped and re-probed using an anti-β-actin antibody as loading control.

**Statistical analysis**

Student’s t-test was used for comparison between the groups. The influence of variables on apoptosis was assessed by the non-parametric Mann Whitney test. The non-parametric
Wilcoxon signed rank test was used for dependent groups. A p-value < 0.05 was considered significant.

Results

Wild-type KLF6 and KLF6-SV1 gene expression in CLL

The relative gene expression of wtKLF6 was statistically significantly higher in CD4⁺ and CD8⁺ T-cells of CLL patients as compared to healthy donors (p = 0.01) (Fig 1). There was no difference between CD4⁺ and CD8⁺ T cells. Expression of wtKLF6 in CLL B-cells was low as well as in the T-cell line 239 (negative control) (www.abnova.com). The relative gene expression of KLF6-SV1 was statistically significantly higher in CD8⁺ T-cells of CLL patients as compared to CD8⁺ T cells derived of myeloma patients (p<0.05) and healthy donors (p = 0.002) (Fig 2). There was a trend towards a lower expression of KLF6-SV1 in CD4 T cells of myeloma patients as compared to CD4 T cells of CLL patients (p = 0.09) but not compared to healthy donors. There was no significant difference in KLF6-SV1 gene expression comparing CD4⁺ and CD8⁺ T cells of CLL patients. Furthermore, CD4⁺ T cells of healthy donors expressed a higher level of KLF6-SV1 as compared to CD8⁺ T cells (p<0.05). Significantly lower level of KLF6-SV1 was observed (Student’s t-test) in leukemic CLL cells as compared to both CD4⁺ and CD8⁺ T cells of CLL patients (p<0.05) (Fig 2).

KLF6-SV1 protein expression

The relative expression of the KLF6-SV1 protein was analyzed in purified CLL T cells (CD3⁺) (n = 6), compared to PBMC (n = 4) and purified T cells from healthy donors (n = 2). There was a significantly higher protein expression in CLL T cells as compared to PBMC and T cells of healthy donors (p = 0.01, Mann Whitney test) (Fig 3).

![Graph showing expression levels of KLF6-SV1 protein in different cellular contexts]
wtKLF6 and KLF6-SV1 siRNA transfection of CLL T cells and impact on CLL B-cell apoptosis

T cells from CLL patients were transfected with KLF6-SV1 and wtKLF6 siRNA with a high efficiency (Fig 4A). Transfection with KLF6-SV1 siRNA and wtKLF6 siRNA respectively induced a significant decrease in the gene and protein expression of KLF6-SV1 and wtKLF6 as exemplified in Fig 4A and 4B. No significant decrease in target mRNA and protein expression of non-transfected or control-transfected cells was noted.

After culture of purified leukemic CLL cells alone for 48 h, 38% (median) apoptotic cells were noted (Fig 5). When the leukemic cells were co-cultured with non-siRNA-transfected
autologous T cells (ratio 1:1) a significant protection from apoptosis of the leukemic CLL cells was noted. Only 16% (median) apoptotic cells was seen (p = 0.001) (Fig 5). However, when the leukemic B-cells were co-cultured with autologous T cells transfected with KLF6-SV1 siRNA the protective effect of T-cells was lost. The percentage of apoptotic leukemic cells noted after 48 h of culture was comparable to that of leukemic cells cultured alone. The difference in surviving B cells comparing B cells co-cultured with control-transfected T cells and those transfected with KLF6-SV1 siRNA was statistically significant (p = 0.005) (Fig 5). When T cells were transfected with wtKLF6 siRNA, no significant loss of the protective effect on the survival of the CLL cells was noted (p = 0.1, Mann Whitney test).

Discussion

CLL cells frequently undergo rapid and spontaneous apoptosis in vitro under conditions that support the growth of normal human B-cells. This may suggest that extrinsic factors in vivo might be of importance for CLL cell survival and that resistance to apoptosis is not only intrinsic of the CLL cells. Our previous studies have demonstrated that autologous T cells of CLL patients supported survival of leukemic cells in vitro (12). CLL T cells produced high levels of IL-4 and IL-5, which were of importance for the survival of CLL cells [12, 29–31]. We could also show that the supportive effect of the T cells was mediated through soluble factors as well as by a direct cell-cell contact in a T cell dose-dependent manner. High doses of IL-4 inhibited
apoptosis of CLL cells in vitro, but to a lower degree compared to that of autologous T cells [12]. The anti-apoptotic effects of the micro-environment were associated with upregulation of anti-apoptotic factors at BCL-XL and MCL-1 mediated by activated autologous T cells and macrophages [34].

Studies in several malignancies have shown that KLF6-SV1 regulated the tumor cell cycle and survival [19, 22, 35]. KLF6-SV1 antagonized both the tumor-suppressor wtKLF6, and the pro-apoptotic protein NOXA, by targeting those proteins inhibiting a rapid enzymatic degradation [9]. The NOXA/MCL-1 balance in CLL cells was inverted in lymph nodes compared to peripheral blood, indicative of an increased resistance of CLL cells in lymph nodes as compared to peripheral blood [34]. KLF6-SV1 may also promote EMT, contributing to the acquisition of a highly metastatic tumor phenotype. High expression of KLF6-SV1 in primary breast tumors was also a prognostic factor for increased risk of metastasis and a poor survival [9, 36].

T cell-derived cytokines, including IL-1β, TNF-α and IFN-γ, enhanced significantly TGF-β1-induced EMT in breast cancer (FMC-7) and lung cancer (A549) cell lines (33). TGF-β has also been shown to be produced both by the leukemic B cells and the T cells in CLL, as well as being detected in serum of CLL patients [37]. TGF-β may normally process growth inhibitory effects but is dysregulated in malignancies. CLL cells have been shown to be resistant to apoptotic effects of TGF-β [38]. Other mechanisms than TGFβ might also be considered to be involved in the T cell promoting survival effects on CLL cells. We have previously shown that the wtKLF6 gene was differentially expressed in CLL cells as compared to normal T cells (12). In the present study, we could confirm that CD4⁺ and CD8⁺ T cells in CLL expressed high levels of wtKLF6 compared to healthy donors. Moreover, an increased expression of KLF6-SV1 mRNA and protein in at least CD4⁺ T cells from CLL patients compared to T cells from myeloma patients and normal healthy donors was noted. Silencing of the KLF6-SV1 gene in CLL cells significantly abrogated the T cell mediated inhibition of apoptosis of CLL.

Fig 5. Apoptosis of CLL cells (CD19⁺ cells) co-cultured with siRNA control transfected, transfected KLF6-SV1 and wtKLF6 autologous T cells (n = 6) for 48 hours. The box represents the 25th to 75th percentiles with line at median. The top whisker is drawn from the value associated with the 75th to 90th percentile, and bottom from 25th to 10th percentile. P-values are indicated at the top.

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cells. It might be assumed that KLF6-SV1 may play a role in the survival supportive effects of autologous T cells for CLL cells. This effect seemed to be specific for CLL T cells as T cells from myeloma patients; another B malignancy did not express KLF6-SV1. Moreover, T cells in myeloma might not significantly contribute to the anti-apoptotic effects of myeloma plasma cells (38).

KLF6-SV1 down regulation in the ovarian carcinoma cell line SKOV-3 inhibited tumor growth in vivo [24] and silencing of KLF6-SV1 induced apoptosis in vivo and in vitro, as well as restored chemosensitivity of prostatic carcinoma cells [39]. KLF6-SV1 siRNA transfection also induced suppression of gastric cancer cell growth, colony formation, migration and invasion [27]. Apoptosis was also noted in KLF6-SV1 siRNA transfected lung cancer cells [26]. Our observation that KLF-6 SV1 expression was highly significantly lower in CLL leukemic cells compared to T cells might be interesting as overexpression of KLF6-SV1 in human cancer cells may accelerate cancer progression and metastasis in animal models as well as in human cancer [39], [19].

Our data is the first report suggesting that KLF6-SV1 may be involved in an anti-apoptotic effect exerted by T cells on CLL B cells. T cells of multiple myeloma patients, another chronic B lymphocyte malignancy, did not express high level of this oncogenic proteins further supporting the notion that KLF6-SV1 in T cells from CLL might be part of the pathobiology of CLL as also suggested by others [40]. A number of studies have highlighted the potential efficacy and specificity of future siRNA/RNAi-based therapies in malignancies through up-regulation of the pro-apoptotic protein Noxa and reduction of the anti-apoptoptic proteins MCL-1 and Bim [3, 24]. Targeting T cells might be a strategy to increase tumor cell apoptosis in CLL. Further studies are warranted to assess the role of T cell mediated regulation of the CLL clone.

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References

1. Palma M, Kokhaei P, Lundin J, Choudhury A, Mellstedt H, Osterborg A. The biology and treatment of chronic lymphocytic leukemia. Ann Oncol. 2006; 17 Suppl 10: x144–154.
2. Masoodi M, Lee E, Eiden M, Bahlo A, Shi Y, Ceddia RB, et al. A role for oleoylethanolamide in chronic lymphocytic leukemia. Leukemia. 2014; 28: 1381–1387. https://doi.org/10.1038/leu.2014.10 PMID: 24413323
3. DiFeo A, Martignetti JA, Narla G. The role of KLF6 and its splice variants in cancer therapy. Drug Resist Updat. 2009; 12: 1–7. https://doi.org/10.1016/j.drup.2008.11.001 PMID: 19097929
4. ten Hacken E, Burger JA. Microenvironment dependency in Chronic Lymphocytic Leukemia: The basis for new targeted therapies. Pharmacol Ther. 2014; 144: 338–348. https://doi.org/10.1016/j.pharmthera.2014.07.003 PMID: 25050922
5. Messmer BT, Messmer D, Allen SL, Kolitz JE, Kudalkar P, Cesar D, et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. J Clin Invest. 2005; 115: 755–764. https://doi.org/10.1172/JCI23409 PMID: 15711642
6. Burger JA, Quiroga MP, Hartmann E, Burke A, Wierda WG, Keating MJ, et al. High-level expression of the T-cell chemokines CCL3 and CCL4 by chronic lymphocytic leukemia B cells in nurselike cell cocultures and after BCR stimulation. Blood. 2009; 113: 3050–3058. https://doi.org/10.1182/blood-2008-07-170415 PMID: 19074730
7. Herman SE, Gordon AL, Hertlein E, Ramanunni A, Zhang X, Jaglowski S, et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. Blood. 2011; 117: 6287–6296. https://doi.org/10.1182/blood-2011-01-328484 PMID: 21422473
8. Caligaris-Cappio F, Bertilacci MT, Scielzo C. How the microenvironment wires the natural history of chronic lymphocytic leukemia. Semin Cancer Biol. 2014; 24: 43–48. https://doi.org/10.1016/j.semcancer.2013.06.010 PMID: 23931274
9. Zhang W, Huang P. Cancer-stromal interactions: role in cell survival, metabolism and drug sensitivity. Cancer Biol Ther. 2011; 11: 150–156. https://doi.org/10.4161/cbt.11.2.14623 PMID: 21191169
10. Ramsay AG, Johnson AJ, Lee AM, Gorgun G, Le Dieu R, Blum W, et al. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. J Clin Invest. 2008; 118: 2427–2437. https://doi.org/10.1172/JCI35017 PMID: 18551193
11. Rossmann ED, Lewin N, Jedd-Tehrani M, Osterborg A, Mellstedt H. Intracellular T cell cytokines in patients with B cell chronic lymphocytic leukaemia (B-CLL). Eur J Haematol. 2002; 68: 299–306. PMID: 12144536
12. Kiai S, Kokhaei P, Mozaffari F, Rossmann E, Pak F, Moshfegh A, et al. T cells from indolent CLL patients prevent apoptosis of leukemic B cells in vitro and have altered gene expression profile. Cancer Immunol Immunother. 2013; 62: 51–63. https://doi.org/10.1007/s00262-012-1300-y PMID: 22736254
13. Choudhury A, Derkow K, Daneshmanesh AH, Mikaelsson E, Kiai S, Kokhaei P, et al. Silencing of ROR1 and FMOD with siRNA results in apoptosis of CLL cells. Br J Haematol. 2010; 151: 327–335. https://doi.org/10.1111/j.1365-2141.2010.08362.x PMID: 20813009
14. Granziiero L, Circosta P, Scielzo C, Frisaldi E, Stella S, Geuna M, et al. CD100/Plexin-B1 interactions sustain proliferation and survival of normal and leukemic CD5+ B lymphocytes. Blood. 2003; 101: 1962–1968. https://doi.org/10.1182/blood-2002-05-1339 PMID: 12406905
15. Bieler JF. Kruppel-like factors: three fingers in many pies. J Biol Chem. 2001; 276: 34355–34358. https://doi.org/10.1074/jbc.R100043200 PMID: 11443140
16. Narla G, Heath KE, Reeves HL, Li D, Giono LE, Kimmelman AC, et al. KLF6, a candidate tumor suppressor gene mutated in prostate cancer. Science. 2001; 294: 2563–2566. https://doi.org/10.1126/science.1066326 PMID: 11752579
17. Reeves HL, Narla G, Oguniyi O, Haq A, Katz A, Benzeno S, et al. Kruppel-like factor 6 (KLF6) is a tumor-suppressor gene frequently inactivated in colorectal cancer. Gastroenterology. 2004; 126: 1090–1103. PMID: 15057748
18. Wait IW, Johnston CT, Blatchley ER, 3rd. The influence of oxidation reduction potential and water treatment processes on quartz lamp sleeve fouling in ultraviolet disinfection reactors. Water Res. 2007; 41: 2427–2436. https://doi.org/10.1016/j.watres.2007.02.057 PMID: 17448518
19. Hatami R, Sieweverts AM, Izadmehr S, Yao Z, Qiao RF, Papa L, et al. KLF6-SV1 drives breast cancer metastasis and is associated with poor survival. Sci Transl Med. 2013; 5: 169ra112.

20. Pearson R, Fleetwood J, Eaton S, Crossley M, Bao S. Kruppel-like transcription factors: a functional family. Int J Biochem Cell Biol. 2008; 40: 1996–2001. https://doi.org/10.1016/j.biocel.2007.07.018 PMID: 17904406

21. Anvekar RA, Asciori JJ, Lopez-Rivera E, Floros KV, Izadmehr S, Elkhori R, et al. Sensitization to the mitochondrial pathway of apoptosis augments melanoma tumor cell responses to conventional chemotherapeutic regimens. Cell Death Dis. 2012; 3: e420. https://doi.org/10.1038/cddis.2012.161 PMID: 23152056

22. DiFeo A, Narla G, Hirshfeld J, Camacho-Vanegas O, Narla J, Rose SL, et al. Roles of KLF6 and KLF6-SV1 in ovarian cancer progression and intraperitoneal dissemination. Clin Cancer Res. 2008; 12: 3730–3739. https://doi.org/10.1158/1078-0432.CCR-06-0054 PMID: 18778100

23. Teng MS, Brandwein-Gensler MS, Teixeira MS, Martignetti JA, Duffy DC. A study of TRAIL receptors in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg. 2005; 131: 407–412. https://doi.org/10.1001/archotol.131.5.407 PMID: 15897419

24. DiFeo A, Feld L, Rodriguez E, Wang C, Beer DG, Martignetti JA, et al. A functional role for KLF6-SV1 in lung adenocarcinoma prognosis and chemotherapeutic response. Cancer Res. 2008; 68: 965–970. https://doi.org/10.1158/0008-5472.CAN-07-2604 PMID: 18250346

25. Li J, Cai A, Wang D, Chen C, Ni Y. Structure analysis of aerobic granule from a sequencing batch reactor for organic matter and ammonia nitrogen removal. Int J Environ Res Public Health. 2014; 11: 2427–2436. https://doi.org/10.3390/ijerph.1103.002427 PMID: 24577284

26. Sangodkar J, Shi J, DiFeo A, Schwartz R, Bromberg R, Choudhri A, et al. Functional role of the KLF6 tumour suppressor gene in gastric cancer. Eur J Cancer. 2009; 45: 666–676. https://doi.org/10.1016/j.ejca.2008.11.009 PMID: 19101139

27. Chen H, Chen L, Sun L, Zhen H, Li X, Zhang Q. A small interfering RNA targeting the KLF6 splice variant, KLF6-SV1, as gene therapy for gastric cancer. Gastric Cancer. 2011; 14: 339–352. https://doi.org/10.1007/s10120-011-0049-9 PMID: 21538018

28. Joshua D, Suen H, Brown R, Bryant C, Ho PJ, Hart D, et al. The T Cell in Myeloma. Clin Lymphoma Myeloma Leuk. 2016; 16: 537–542. https://doi.org/10.1016/j.clml.2016.08.003 PMID: 27601001

29. Kokhaei P, Rezvan Y MR, Virving L, Choudhury A, Rabbani H, Osterborg A, et al. Apoptotic tumor cells are superior to tumor cell lysate, and tumor cell RNA in induction of autologous T cell response in B-CLL. Leukemia. 2003; 17: 894–899. https://doi.org/10.1038/sj.leu.2402913 PMID: 12750703

30. Kokhaei P, Choudhury A, Mahdian R, Lundin J, Moshfegh A, Osterborg A, et al. Apoptotic tumor cells are superior to tumor cell lysate, and tumor cell RNA in induction of autologous T cell response in B-CLL. Leukemia. 2004; 18: 1810–1815. https://doi.org/10.1038/sj.leu.2403517 PMID: 15385926

31. Kokhaei P, Palma M, Hansson L, Osterborg A, Mellstedt H, Choudhury A. Telomerase (hTERT 611–612). https://doi.org/10.1158/1058-8241.CAN-06-0054 PMID: 16778100

32. Abdalla AO, Kokhaei P, Hansson L, Mellstedt H, Osterborg A. Idiotype vaccination in patients with myeloma reduced circulating myeloma cells (CMC). Ann Oncol. 2008; 19: 1172–1179. https://doi.org/10.1093/annonc/mdn017 PMID: 18272909

33. Slinger E, Wensveen FM, Guikema JE, Kater AP, Eldering E. Chronic lymphocytic leukemia development is accelerated in mice with deficiency of the pro-apoptotic regulator NOXA. Haematologica. 2016; 101: e374–377. https://doi.org/10.3324/haematol.2016.142323 PMID: 27479816

34. Andreoli V, Gehrau RC, Bocco JL. Biology of Kruppel-like factor 6 transcriptional regulator in cell life and death. IUBMB Life. 2010; 62: 896–895. https://doi.org/10.1002/iub.396 PMID: 21154818

35. Vetter D, Cohen-Naftaly M, Villanueva A, Lee YA, Kocabayoglu P, Hannivoort R, et al. Enhanced hepatocarcinogenesis in mouse models and human hepatocellular carcinoma by coordinate KLF6 depletion and increased messenger RNA splicing. Hepatology. 2012; 56: 1361–1370. https://doi.org/10.1002/hep.25810 PMID: 22535637

36. Kremer JP, Reisbach G, Nerl C, Dormer P. B-cell chronic lymphocytic leukemia cells express and release transforming growth factor-beta. Br J Haematol. 1992; 80: 480–487. PMID: 1581232

37. Douglas RS, Capocasale RJ, Lamb RJ, Nowell PC, Moore JS. Chronic lymphocytic leukemia B cells are resistant to the apoptotic effects of transforming growth factor-beta. Blood. 1997; 89: 941–947. PMID: 9028925
39. Narla G, DiFeo A, Fernandez Y, Dhanasekaran S, Huang F, Sangodkar J, et al. KLF6-SV1 overexpression accelerates human and mouse prostate cancer progression and metastasis. J Clin Invest. 2008; 118: 2711–2721. https://doi.org/10.1172/JCI34780 PMID: 18596922

40. Vardi A, Vlachonikola E, Karypidou M, Stalika E, Bikos V, Gemenetzis K, et al. Restrictions in the T-cell repertoire of chronic lymphocytic leukemia: high-throughput immunoprofiling supports selection by shared antigenic elements. Leukemia. 2017; 31: 1555–1561. https://doi.org/10.1038/leu.2016.362 PMID: 27904140