POTENTIAL GENETIC AGENT BFL1 FOR TARGETED THERAPY IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Abstract

Background: Many prognostic factors have been identified in chronic lymphocytic leukemia (CLL) but new ones are still desired. The biological characterization of CLL is now being translated into novel treatment strategies. One new prognostic factor, and therapeutic target, may be BFL1. It is both a serum and a molecular marker that contributes to the progression of CLL and its resistance to chemotherapy. The aim of this study was to evaluate the prognostic value of BFL1 and to assess its correlation with other known prognostic markers in CLL for the cladribine and cyclophosphamide regimen (CC). **Methods:** qPCR TaqMan® Low Density Array was used for gene expression measurements. Assessment of CD38, ZAP70 and BFL-1 proteins expression was done by means of flow cytometry. Serum TK activity was measured by immunoassay. **Results:** Protein BFL1 expression was found to be significantly higher in CLL patients than healthy volunteers (p=0.001). Moreover its level was significantly higher in patients with no response (NR) to CC therapy (p=0.009). The expression of BFL1 was considerably down regulated during CC treatment and *BFL1* mRNA levels were inversely correlated with apoptotic response. In addition, protein BFL1 expression was found to be similar to thymidine kinase (TK) concentration regarding treatment response. As far as other markers are concerned, a positive correlation was identified between BFL1 and TK (r=0.52, p=0.01). **Conclusions:** Our findings suggest that BFL1 contributes to chemoresistance and may be a co-existing prognostic factor in CLL in the future.

Keywords: Chronic Lymphocytic Leukemia, BFL1, thymidine kinase, microarray gene expression, cladribine

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent leukemia in the western world. The disease affects mostly elderly CLL patients (81% of patients are over 60), but recently it has been observed more frequently in younger people. Recent advances in understanding the biology of CLL have resulted in the development of novel agents targeting key prosurvival pathways in the neoplastic B cell.

Among prognostic factors in CLL, the most important are clinical stage of the disease, markers of tumour load, such as thymidine kinase (TK), cellular protein expression (e.g., CD38 and ZAP70), as well as genetic parameters including immunoglobulin heavy chain variable gene segment (IGHV) mutational status, genomic aberrations and individual gene mutations (Oscier, et al., 2002) (Zenz, et al., 2010).

Among serum markers, TK deserves special attention. TK takes part in DNA synthesis and catalyses the phosphorylation of thymidine to thymidinomonophosphate (Wintersberger, 1997). It has been already indicated that the activity of TK is a reliable marker of tumor cell proliferation (Ke & Chang, 2004), mostly a cytoplasmic (TK1) isoenzym (Wintersberger, 1997), (Ke & Chang, 2004), (Pflug, et al., 2014). The other serum factor, which correlates with prognosis, is basic fibroblast growth factor (bFGF). It exists in basement membranes as well as in the subendothelial extracellular matrix in normal blood vessels. As long as no signal peptide is present, bFGF remains bound to the membrane. However, during tumor development, the action of heparan sulphate-degrading enzymes activates bFGF and thus triggers an angiogenesis, which enhances the proliferation of epithelial cells and fibroblasts involved in the regulation of cellular replication (Kühn, et al., 2012), (Bertolini, Mancuso, Shaked, & Kerbel, 2007).

Although many prognostic factors in CLL have been already identified, new ones are still being revealed. One such factor might be the activity of BFL1, which could be either a serum marker or a molecular marker, which contributes to disease progression and resistance to chemotherapy (Morales, et al., 2005), (Olsson, et al., 2007). *BFL1* is an antiapoptotic member of the *BCL2* family. The protein encoded by this gene has been previously reported as a regulatory marker of the other members of BCL2 family proteins. Working in antagonism, the antiapoptotic proteins BCL2, BCL-X_L, MCL1 or BFL1 (also called BCL2A1 or A1) inhibit BAX and BAK activation and apoptosis (Danial, & Korsmeyer, 2004). Of the antiapoptotic proteins, BFL1 has the highest affinity to BID (Kim, et al., 2006), (Chen, et al., 2005). BFL1 has been reported as a direct transcriptional target of NF-κB (Chen, Edelstein, & Gelinas, 2000). In lymphocytes B, BFL1 is induced by CD40 ligation and has been shown to protect from apoptosis triggered by ligation of the B-cell receptor or FAS (Craxton, Chuang, Shu, Harlan, & Clark, 2000). Higher *BFL1* expression has been found in several types of cancer, for example diffuse large B-cell lymphoma (DLBCL), acute myeloid leukemia (AML), lymphoma and melanoma (Shipp, et al., 2002), (Piva, et al., 2006), (Haqq, et al., 2005). Moreover, it is thought to be required for tumor initiation, maintenance and chemoresistance. The *BFL1* gene has been found to be overexpressed in B-CLL cells with an apoptosis-resistant phenotype (Morales, et al., 2005), (Olsson, et al., 2007), (Aguilar-Santelises, Rottenberg, Lewin, Mellstedt, & Jondal, 1996) (Kitada, et al., 1998).

Several chemotherapies have been used for the treatment of CLL, such as alkylating agents, antracyclines, corticosteroids, purine nucleoside analogues (PNAs) and monoclonal antibodies. Purine analogues (cladribine,

fludarabine and pentostatin) were introduced in the 1980s. Among the class of PNAs an important role is played by fludarabine (FA) and cladribine (2-CdA), which currently are the most frequently used in CLL therapy, in our hematology clinic cladribine has been used since 1995. The chemical structure of both drugs is similar to adenosine. They require phosphorylation to activate metabolites, and the induction of apoptosis, as well as cytotoxicity, depends on their accumulation of their triphosphate form (PNA-TP). The mechanism of action of both drugs is similar, however minor differences are observed. Gene expression profiling (GEP) under the influence of both drugs is also similar, which proof the similar mechanism of action (Franiak-Pietryga, et al., 2012a).

Although, antibody anti-CD20 is standard today in CLL treatment, we have already checked the GEP under the influence on RCC (Franiak-Pietryga, et al., 2012b). In this paper we would like to evaluate only CC regimen and compare to previous study.

The aim of this study was to evaluate the value of BFL1 and to assess the correlation between BFL1 and other known markers in CLL for the cladribine and cyclophosphamide regimen.

Materials and Methods

Patients

Peripheral blood samples from 24 previously untreated progressive CLL patients (Rai 3 and 4) were obtained after informed consent was given. The diagnosis of CLL was based on IWCLL standard criteria (Hallek, et al., 2008). The Ethics Committee of the Medical University of Lodz, Poland (RNN/196/07/KE) approved the study. The characteristics of the patients are given in Table 1. Ten healthy volunteers were enrolled into the study as a negative control.

Table 1. Characteristics of the CLL patients

Patient No.	Age/ Gender	Months since diagnosis	Blood lymphocyte count ($x10^9l^{-1}$)	*IGHV* mutation	CD38	Cytogenetic abnormalities	Treatment response
CLL 1	74/M	0	503	n.d.	-	del(13)(q14), del(11)(q22)	CR
CLL 2	53/M	12	120	unmutated	+	del(13)(q14), del(11)(q22)	CR
CLL 3	69/M	0	216	mutated	-	del(13)(q14)	CR
CLL 4	70/M	0	163	unmutated	-	del(11)(q22)	NR, †
CLL 5	65/M	15	75	unmutated	-	del(13)(q14), del(11)(q22)	NR
CLL 6*	53/M	38	130	unmutated	-	del(13)(q14)	PR
CLL 7	53/M	14	84	unmutated	-	del(13)(q14), del(17)(p13)	NR, †
CLL 8	70/F	156	86	n.d.	-	del(13)(q14)	CR
CLL 9	66/M	28	157	n.d.	+	del(13)(q14), del(11)(q22)	PR
CLL 10	63/M	0	34	mutated	-	del(13)(q14), del(17)(p13)	CR
CLL 11	41/F	3	75	n.d.	-	del(13)(q14)	CR
CLL 12	75/F	6	115	n.d.	-	n.d.	PR
CLL 13*	76/F	43	120	unmutated	+	del(13)(q14), del(11)(q22)	NR, †
CLL 14	51/M	0	109	unmutated	-	del(11)(q22), trisomy 12	CR
CLL 15	74/M	28	97	unmutated	+	del(13)(q14), del(17)(p13)	CR
CLL 16	68/M	32	159	n.d.	-	del(13)(q14)	CR
CLL 17*	72/M	69	106	unmutated	-	del(13)(q14)	CR
CLL 18*	75/F	36	290	unmutated	+	del(13)(q14), del(11)(q22)	CR
CLL 19*	60/M	1	52	unmutated	-	del(13)(q14)	CR
CLL 20*	66/M	0	634	unmutated	-	del(13)(q14)	NR
CLL 21*	63/F	1	55	unmutated	+	n.d.	NR, †
CLL 22*	77/M	5	24	unmutated	+	del(13)(q14), del(11)(q22)	NR, †
CLL 23	70/M	2	30	n.d.	+	del(13)(q14), trisomy 12	CR
CLL 24	58/M	0	315	n.d.	+	n.d.	NR, †

M, male; F, female; *IGHV*, immunoglobulin heavy chain variable gene;
NR, no response; CR, complete remission; PR, partial remission; †, death
n.d., not determined; *, patients selected to microarray analysis

CLL therapy

All the patients received cladribine and cyclophosphamide (CC) treatment by i.v. infusion according to the following schedule: cladribine 0.12mg/kg – days 1-3, cyclophosphamide 600mg/m^2 – days 1-3. The regimen was repeated every 4 weeks for 6 courses.

Isolation of mononuclear cells and serum collection

Blood samples were collected from all patients before and 2 weeks after the first CC cycle. Peripheral blood mononuclear cells (PBMNCs) were separated from EDTA blood as previously described (Franiak-Pietryga, et al., 2012b). Mean B-cell (CD19+) purity was >95% as measured by flow cytometry (FACS). The serum obtained from blood was stored at -80°C until assayed for TK and bFGF.

PCR amplification of IGHV-D-J rearrangements; immunoglobulin sequence analysis and cytogenetics

IGHV mutational status and cytogenetic abnormalities were assessed according to previously published methods (Franiak-Pietryga, et al., 2012b). IGHV rearrangements were performed on genomic DNA (gDNA) extracted from peripheral blood. Monoclonal PCR products were purified on low-melting-point agarose gel and than subjected to sequencing on an ABI sequencer. The sequence data was analysed using the international ImMunoGeneTics information system (IMGT®) database and tools. Sequences with a germline identity of 98% or higher were considered unmutated, and those with an identity less than 98% were considered mutated.

Cytogenetic aberrations were evaluated using interphase fluorescence *in situ* hybridization (FISH) with the standard panel of probes (Abbott Molecular, USA) (Franiak-Pietryga, et al., 2012b).

mRNA quantification by real-time reverse transcription polymerase chain reaction

Total RNA was extracted with the use of the RiboPure Blood Kit (Ambion, USA) according to the manufacturer's instructions. Total RNA (0.5μg) was reversed transcribed to complementary DNA (cDNA) using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The *BFL1* mRNA levels were determined in a Rotor-Gene®Q using a predesigned Assay probe (Hs00187845_m1 probe, Applied Biosystems). The relative expression of each gene was quantified by the comparative cycle threshold method (ΔΔCt), using β-actin (Hs99999903_m1 probe, Applied Biosystems) as an endogenous control, and untreated samples as a calibrator.

cDNA expression microarray

The 384 TaqMan® Low Density Array Human Apoptosis Panel (Applied Biosystems, USA) containing primers without cDNA and other necessary reagents for PCR analysis of 96 gene transcripts (93 examined, 3 controls) was used as described previously (Franiak-Pietryga, et al., 2012b). Prepared cDNA samples from 0.5 μg of RNA were subjected to real-time PCR in duplicate in the TaqMan® 7900HT Sequence Detection System (Applied Biosystems).

The complete list of microarray data is available at Gene Expression Omnibus (accession no. GSE55686).

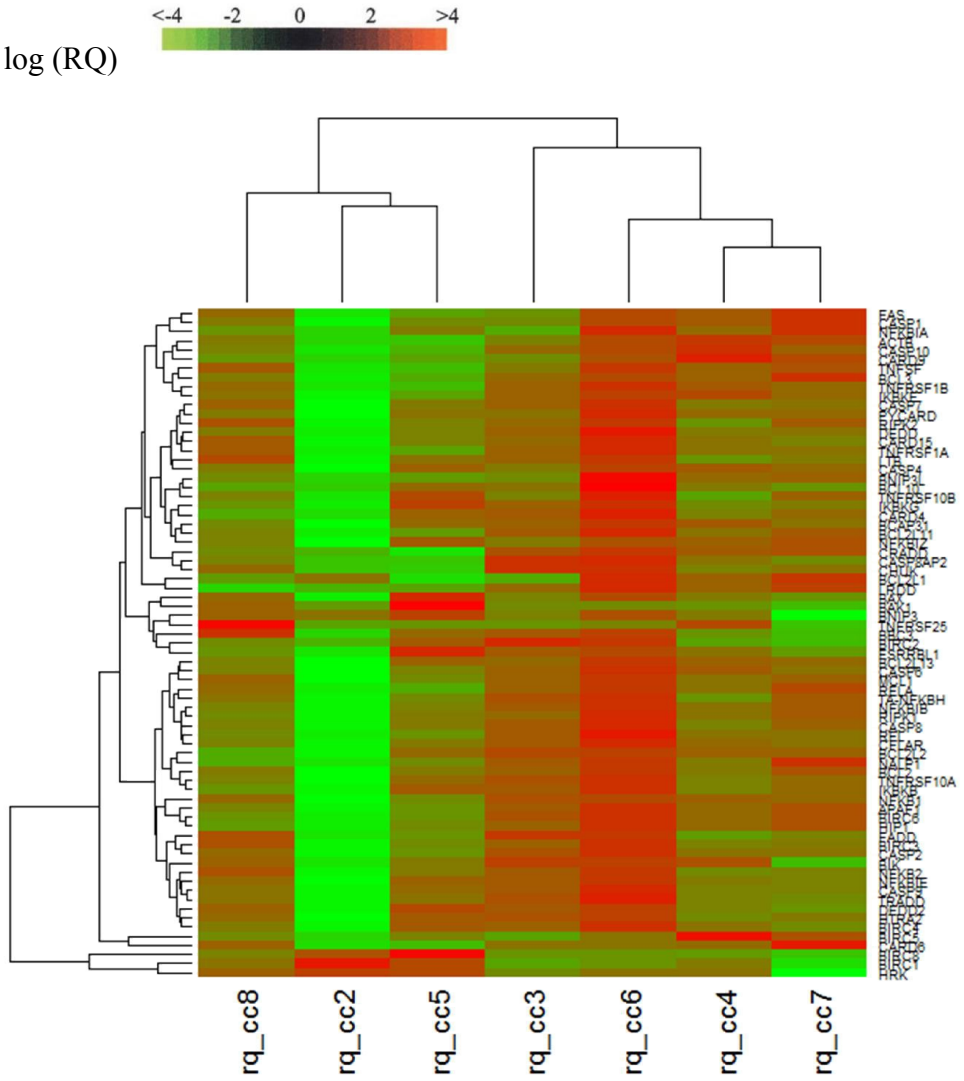
Gene expression profile analysis

The relative expression of each gene was quantified by the comparative cycle threshold method (□□Ct, ddCt), using 18S as an endogenous control (a housekeeping gene) (Franiak-Pietryga, et al., 2012b).

The difference in expression of a certain gene was considered significant only if the fold change values (RQ), defined as the differential gene expression before and after treatment, were >1.0. The purpose of data analysis was to identify genes with the most differential expression under CC treatment.

The dendrogram and matrix showing the hierarchical clustering of 80 genes are based on gene expression data generated from 7 CLL samples (Fig. 1). The hierarchical clustering algorithm was evaluated in accordance with the average-linkage clustering method (Hartigan, et al., 1975), (Eisen, Spellman, Brown, & Botstein, 1998). The rows represent individual genes, and the columns represent individual mRNA samples. The relative level of gene expression is depicted according to the colour scale for the matrix. Microarray analysis was used as a screening.

Fig. 1. Heatmap of relative gene expression levels (log ratio of expression after treatment to expression before treatment, log RQ).



Assessment of CD38, ZAP70 and BFL-1 expression

CD38 and ZAP70 expression were measured immediately prior to the start of the CC regimen using the FACS method. BFL1 expression was assessed before and 2 weeks after the first CC cycle. PBMNCs at a concentration of $1x10^6$ were incubated with anti-CD38-FITC or anti-ZAP70-FITC or anti-BFL1-FITC. Samples were analysed in a Becton Dickinson FACSCanto ™ II system equipped with an argon laser (λ=488 nm), using $1x10^4$ cells for each determination. The cut-off values for CD38 and ZAP70 were 30% and 20%, respectively. In BFL-1 case mean fluorescence intensity (MFI) level was calculated.

Serum TK activity

The s-TK assay was performed with a commercially available quantitative immunological assay (Biomedica, Poland). This is a non-radioactive assay. In this technique 3'-azido-2',3'-deoxythymidine (AZT) is first phosphorylated to AZT 5'-monophosphate (AZTMP) in the sample by TK1. AZTMP is measured by immunoassay with anti-AZTMP antibodies and AZTMP-labeled peroxidase. The assay runs in a closed system using laboratory equipment from DiaSorin LIAISON® (USA). The TK1 activity is expressed in units per liter, with 7 U/l as the upper level in healthy subjects.

Serum bFGF concentration

Serum bFGF concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems Inc., USA). The assay employs quantitative sandwich enzyme immunoassay technique. Standards and samples were assayed as duplicates. Serum before measurements was stored at -70°C. Assay sensitivity was less than 3.0 pg/ml. The procedure is described in detail at *www.rndsystems.com/pdf/dfb50.pdf*.

Response criteria

Treatment efficacy was assessed according to IWCLL guidelines at least 2 months after completion of the therapy (Hallek, et al., 2008). A complete response (CR) required the absence of symptoms and organomegaly, a normal blood cell count (leukocytes count 1.5 x 10^9/L (1500/μL) or more), untransfused hemoglobin concentration > 11.0 g/dL and platelet count > 100 x 10^9/L (100 000/ μL), lymph nodes should not be larger than 1.5 cm in diameter. Partial response (PR) was defined as a decrease in the number of blood lymphocytes by ≥ 50% from the value before therapy and/or a decrease in lymphadenopathy, splenomegaly and hepatomegaly by ≥ 50% from the value before therapy. Hematologic toxicity was evaluated according to the criteria of IWCLL guidelines (Hallek, et al., 2008).

Statistical analysis

Comparisons of values were made with the Wilcoxon rank test and X^2 test using STATISTICA v. 8.0. Results were considered statistically significant when $P < 0.05$. Correlations were assessed by the Spearman rank test.

Results

Characteristics of CLL patients

All 24 patients enrolled into the study were treated with a CC regimen as the first line therapy. Thirteen patients achieved CR, 3 patients

PR and 8 patients did not respond (NR) to therapy. In the NR group, 6 patients died. One patient died because of infection, while the other 5 due to progression of CLL.

In 16 out of 24 patients *IGHV* mutation status was assessed. Two patients were *IGHV*-mutated, while 14 were *IGHV*-unmutated. *IGHV*-mutated patients attained CR. Among *IGHV*-unmutated patients, the following treatment response was observed: 6 patients CR, 1 patient PR, and 7 patients NR. CD38 was assessed in the whole group of patients. Nine out of 24 patients were CD38(+). In this group, 4 patients achieved CR, 1 patient PR and 4 patients NR. In the group of patients with CD38(-) the following treatment response was observed: 9 patients CR, 2 patients PR and 4 patients NR (Table 1). Among cytogenetic abnormalities, that were assessed in 21 patients, del(17) was evaluated in 3 patients, 19 patients had del(13) and 8 patients had del(11). The characteristics of the group of patients selected for microarray analysis is shown in Table 2. All patients in microarray group were *IGHV*-unmutated.

Table 2. Characteristics of the CLL patients selected for microarray analysis.

Patients			***IGHV mutation*** [a]	***ZAP70*** [a]		***CD38*** [b]		***Cytogenetic abnormalities*** [c]
Nr	***Sex***			***% cells***	***status***	***% cells***	***status***	
Age								
cc2 (CLL 19)	M	unmutated		0.28	–	1.36	–	del(13q)
60								
cc3 (CLL 21)	F	unmutated		0.23	–	32.09	+	n.d.
63								
cc4 (CLL 6)	M	unmutated		3.83	–	4.72	–	del(13q)
53								
cc5 (CLL 22)	M	unmutated		6.91	–	74.45	+	del(13q),del(11q)
77								
cc6 (CLL 17)	M	unmutated		7.94	–	1.76	–	del(13q)
72								
cc7 (CLL 13)	F	unmutated		13.85	–	26.86	+	del(13q), del(11q)
76								
cc8 (CLL 20)	M	unmutated		19.48	–	4.74	–	del(13q)
66								

CC, cladribine cyclophosphamide; ZAP70, zeta-associated protein 70; *IGHV*, immunoglobulin heavy chain variable gene.

[a,b] The values demonstrate the percentage of cells defined as ZAP70 or CD38 positive.

[c] Cytogenetic variations were measured by means of FISH method

Serum markers in the response to treatment

Thymidine kinase (TK) and *Basic fibroblast growth factor (bFGF)*

The serum TK and bFGF levels were significantly higher in CLL patients as compared with those of healthy volunteers (p=0.001) (Table 3).

Table 3. The median values of BFL1 expression, bFGF and TK serum concentrations in CLL patients before and after 2 weeks of the first CC cycle and in controls.

Parameter	**Healthy donors Median (Range)**	**Before treatment Median (Range)**	**After treatment Median (Range)**	**Statistical comparison (p)**
	(3)	**(1)**	**(2)**	**(4)**
BFL1 (MFI)	248.48 (179.25-320.3) n = 20	746.41 (242.13-1885.57) n = 22	667.77 (333.18-1678.87) n = 22	1 vs. 2 = 0.03 1 vs. 3 = 0.001
bFGF (pg/ml)	9.4 (0-20) n = 20	43.94 (0-348.65) n = 19	6.27 (0-93.35) n = 19	1 vs. 2 = 0.0009 1 vs. 3 = 0.001
TK (U/l)	7.75 (1.1-15.1) n = 20	30.45 (6.5-100) n = 24	26.40 (6.4-100) n = 24	1 vs. 2 = 0.03 1 vs. 3 = 0.001

n – number of patients; MFI – Mean Fluorescence Intensity
TK – thymidine kinase bFGF – basic fibroblast growth factor

Table 3a. Values of BFL1 expression, bFGF and TK serum concentrations for particular single CLL patient.

	BCL2-A1 (BFL-1)		**Thymidine kinase (TK)**		**FGF (pg/ml)**	
Patient	**Before treatment**	**After treatment**	**Before treatment**	**After treatment**	**Before treatment**	**After treatment**
CLL1	628.81	493.05	13.8	17.2	31.6	6.93
CLL2	640.11	661.29	NA	NA	NA	NA
CLL3	645.06	800.25	24.7	26.3	0	0
CLL4	824.01	724.7	49.8	42.7	NA	NA
CLL5	927.75	883.2	84.1	100	6.93	5.6
CLL6	976.43	768.27	35	30.5	90.93	2.93
CLL7	727.77	873.56	NA	87.5	NA	NA
CLL8	1750.47	1040.98	14.9	19.5	226.93	2.27
CLL9	665.13	560.48	19.2	8	27.18	0
CLL10	809.84	674.24	47.2	60.9	NA	NA
CLL11	543.95	754.09	18.8	10.5	248.27	14.93
CLL12	631.11	387.32	35.9	12.9	76.6	18.93
CLL13	1013.98	595.65	97.6	6.4	42.47	4.93
CLL14	938.84	365.62	55.1	32.7	NA	NA
CLL15	943.34	1678.87	80.1	80.5	NA	NA
CLL16	242.13	333.18	18.3	12.6	NA	2.93
CLL17	630.26	1231.09	7.9	13.9	53.65	45.12
CLL18	660.77	480.91	15.2	20.5	751	93.35
CLL19	1521.41	381.56	100	100	34.82	17.47
CLL20	1885.57	558.17	6.5	100	51.88	30.71
CLL21	977.47	353.36	100	100	43.94	53.94
CLL22	268.53	737.88	25.9	26.5	38.06	29.24
CLL23	765.05	NA	16.7	12.7	0	0
CLL24	NA	NA	88.02	100	0	0
CLL25	NA	NA	30.45	28.6	NA	NA
CLL26	725.99	NA	72.6	21.1	348.65	29.24
CLL27	NA	NA	12.4	19.3	219.82	0
Median	**746.41**	**667.77**	**30.45**	**26.4**	**43.94**	**6.27**
Range	**242-1885**	**333-1678**	**6-100**	**6-100**	**0-348**	**0-93**

The median basic TK concentration was 30.45 U/l, but this fell to 26.40 U/l after the 1st CC cycle (p=0.03). The decrease in TK concentration in relation to baseline values after completed treatment was statistically significant in each group: CR, PR and NR (p=0.001). The median TK value was 18.55 U/l in CR patients, 35 U/l in PR patients and 84.1 U/l in NR patients. While the median TK level increased in CLL non-responders, decreased in CR patients after the 1st cycle.

The median basic bFGF concentration was 43.94 pg/ml, but this fell to 6.27pg/ml (p=0.001) after the 1st CC cycle, which is lower than that observed in the control group (9.4pg/ml, p=0.001). The median basic concentration of bFGF in the group of patients with the following treatment response was observed: 140.29 pg/ml in CR patients, 76.6 pg/ml in PR patients, and 42.47 pg/ml in NR patients. The greatest decreased of concentration was calculated between CR and NR groups (p=0.0017).

The bFGF levels fell after the first cycle of treatment in all but one patient (CLL21), who died during the chemotherapy schedule after the 3rd CC cycle. In this case, very high levels of bFGF, TK and BFL1 protein and gene expression were observed before treatment (Table 3, 4).

Table 3a presents together three measured serum markers expression for particular single patient, which could give the proper view about patient markers expression reduction as a result of CC administration. This could prove a proper value of this potential marker in diagnostics for particular patients. If we observe both factors TK and bFGF over-expressed in one patient we can assume an active disease with high proliferation index and it could be a good marker for active proliferative disease status or disease conversion to active secondary cancer, what could happened sometimes even after aggressive chemotherapy.

Table 4. Relationship between *BFL1* gene and protein expression.

Patient	Gene		Protein
	ΔΔCt	**RQ**	**Mean MFI variance**
CLL 1	1.85	0.27	⇓ 135.96
CLL 2	0.20	1.64	⇑ 21.18
CLL 5	0	1.00	⇓ 44.55
CLL 6	3.01	0.10	⇓ 208.16
CLL 8	− 0.87	**4.77**	⇓ 709.49
CLL 9	− 0.55	1.46	⇓ 104.65
CLL 10	1.20	0.60	⇓ 135.60
CLL 11	− 1.38	**2.60**	⇑ 210.14
CLL 12	0.94	0.78	⇓ 243.79
CLL 13	3.63	0.05	⇓ 418.33
CLL 15	− 0.28	**2.65**	⇑ 735.53
CLL 16	1.24	0.42	⇑ 91.05
CLL 17	− 0.64	**3.79**	⇑ 600.83
CLL 18	− 0.21	**2.47**	⇓ 179.86
CLL 20	5.87	0.01	⇓ 1327.4

CLL 21	− 0.74	**4.19**	⇓ 624.11

Upregulated gene (RQ ≥ 2.5) is in bold.
⇓ - Down and ⇑ - upregulation of expression.
ΔΔCt - comparative cycle threshold method
MFI – mean fluorescence intensity; RQ – fold change

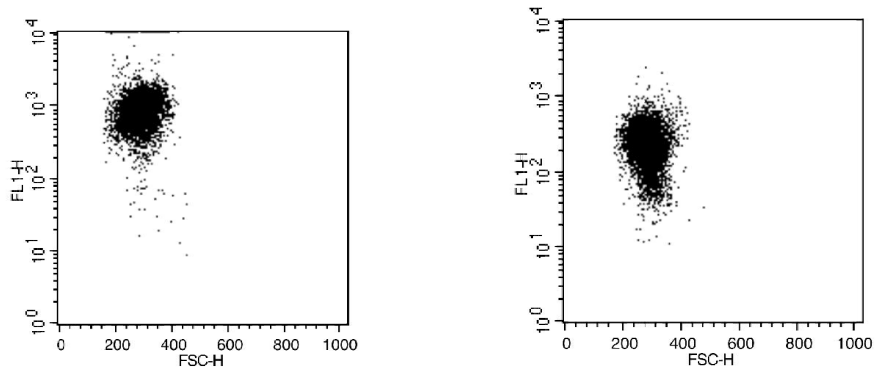
BFL1 expression

Protein expression

BFL1 expression was significantly higher in CLL patients than healthy volunteers (p=0.001). Its basic median expression was 746.41 MFI, which fell to 667.77 MFI after the 1st CC cycle (p=0.03), but it still did not reach the control group value (248.48 MFI). The median basic expression of BFL1 in the group of patients with CR, PR and NR was 660.77 MFI, 665.13 and 927.75 MFI respectively. The decrease in BFL1 protein expression after the 1st cycle of treatment compared between groups of patients who responded to treatment (CR+PR) and NR was statistically significant (p=0.009) as well as the difference between CR and NR group (p=0.01) (Table 3, Fig. 2).

Fig. 2. The cytometric analysis of BFL-1 protein changes after CC treatment

A. B.



A. Before treatment; B. After 2 weeks of the infusion of CC first cycle

Correlations between markers

The following statistically significant correlations between studied markers were found: a positive correlation between BFL1 and TK (r=0.52, p=0.01) median measured after 1st CC cycle.

Gene expression studies

qPCR array analysis

The purpose of this analysis was to identify genes with the greatest differential expression (DE) in the group of 7 CC patients (economic reasons) before and after 2 weeks of the first cycle treatment. The analysis

was based on the RQ measurement, which can be interpreted as the ratio of gene expression after treatment to gene expression before treatment (Franiak-Pietryga, et al., 2012b).

Most of the examined genes were down-regulated after treatment. The up-regulation was observed in 37 different genes at least in one patient due to treatment. The greatest up-regulation was detected in subsequent genes: *BAK, BAX, BIRC1* (Table 5). Although some variations in the expression of particular genes between CLL patients were evident, the unsupervised hierarchical clustering, revealed two distinct expression patterns: the first one represented by patients cc2, cc5, cc8 and the second one by cc3, cc6, cc7 (Fig. 1). The cc2 patient could be regarded as an outlier because indicated down-regulation in expression in almost all genes. The first group contains the following genes in overexpression: *HRK, BIRC1, BIRC8* as well as *BNIP3* and *BCL2L2*.

Table 5. The greatest differences in expression of particular genes in CLL patients treated with CC.

Gene	***MCL1***		***BCL2***		***BCL2A1***		***BCL2L1***		***BCL2L2***	
Patient	**ddCt**	**RQ**	**ddCt**	**RQ**	**ddCt**	**RQ**	**ddCt**	**RQ**	**ddCt**	**RQ**
cc2	3.21	0.08	3.79	0.05	3.72	0.05	-0.04	2.09	2.59	0.15
cc3	0.02	1.95	0.40	1.34	-0.30	2.70	0.97	0.76	0.43	1.31
cc4	0.44	1.29	0.91	0.80	-0.53	3.39	-0.28	2.66	0.67	1.02
cc5	0.88	0.83	0.89	0.82	0.37	1.38	1.77	0.34	0.82	0.88
cc6	-0.79	4.39	-0.38	2.94	-0.27	2.63	-1.23	6.87	0.28	1.51
cc7	0.03	1.94	0.15	1.72	-1.26	7.05	-0.93	5.08	0.68	1.02
cc8	0.04	1.91	1.05	0.70	-0.29	2.68	0.73	0.96	1.69	0.37

BFL1 mRNA RT-PCR validation

BFL1 mRNA expression was assessed in 16 patients before and after treatment. It was upregulated 2.5-fold in 6/16 cases. In 13/16 cases, the expression of *BFL1* mRNA was correlated with the protein. The down-regulation in mRNA *BFL1* and lower protein expression was identified in 10/16 patients after the 1st CC cycle (Table 4).

Discussion

CLL is a lymphoproliferative disorder in which BCL2 is expressed from an unrearanged locus (Schena, et al., 1992). Leukemic B-cells circulate in the peripheral blood but also exist in lymph nodes, where they demonstrate down-regulation of BCL2 and other pro-survival proteins, BCL2L1 and BFL1, are induced. Similar changes occur during normal B-cell maturation (Willimott, Baou, Naresh, & Wagner, 2007), (Vogler, et al., 2009). Although peripheral blood B-cells express BCL2, it is repressed following the encounter with antigens and T-cells on entry to the germinal center, and BCL2L1 and BFL1 are induced (Vogler, et al., 2009). BCL2 inhibits cell cycle entry and its overexpression reduces proliferation (Korz, et

al., 2002), (Huang, O'Reilly, Strasser, & Cory, 1997), (O'Reilly, Harris, Tarlinton, Corcoran, & Strasser, 1997).

Knowledge of the regulation of BCL2 is controversial. While *BCL2* mRNA has been detected in situations in which there is no BCL2 protein, suggesting that BCL2 is post-transcriptionally regulated, microarray and real-time PCR gene expression studies have confirmed the presence of an association between mRNA and protein levels (Chleq-Deschamps, et al., 1993), (Kondo, et al., 1992), (Husson et al., 2002), (Klein, et al., 2003), (Shen, Iqbal, Huang, Zhou, & Chan, 2004). Our findings indicate that in most cases, BFL1 protein expression parallels mRNA level after treatment.

Although this study included too few patients to draw final conclusions, BFL1, TK and bFGF levels were found to be higher in NR patients than in CR and PR patients. Olsson et al. have also shown high *BFL1* mRNA expression in leukemic cells from chemotherapy refractory B-CLL patients (Olsson, et al., 2007). Using microarrays, they report a pattern of high expression levels of *BFL1*, *BCL2* and *MCL1* mRNA in apoptosis-resistant B-CLL cells (Morales, et al., 2005). Other studies have also confirmed significantly higher expression of *BCL2* in the refractory patients (Olsson, et al., 2007). Although most patients with NR in the present study were found to have very high levels of BFL1 protein and mRNA, others were also found with low basic level of BFL1 (CLL22), which increased after treatment. Our microarray study revealed *BFL1* overexpression in the cluster of patients with unmutated-*IGHV* and no response. The strongest difference between non-responding and responding patients was observed when at least one of the two genes (*BFL1* and *BCL2* considered together) was overexpressed, as characterized by the NR group. In contrary, low expression of both studied genes was typical for patients who responded to therapy. This is in agreement with Olsson et al. (2007). Our microarray data confirm the above-mentioned data. These findings indicate that the expression of both *BFL1* and *BCL2*, as well as other members of *BCL2* family, for example *MCL1*, may be a good predictor for chemotherapy response (Olsson, et al., 2007), (Rogalińska, et al., 2013). However, there is some dose of uncertainty whether high mRNA *BFL1* expression levels is predictive of chemotherapy outcome or if is it a result of resistance development.

Five of the eight NR (CLL4, 7, 13, 21, 22) patients in the present study demonstrated parallel elevation of BFL1 and TK. The protein BFL1 expression acted similar to TK concentration in reference to treatment response. In 9 patients, expression decreased after 2 weeks of the 1st CC cycle, whereas higher expression was observed in 5 cases. This correlation may be characteristic of CLL patients with very poor prognosis.

A number of serological parameters such as TK and β2-M have been shown to provide information on CLL outcome (Hallek, et al., 1999). It has been shown that TK levels correlate with the proliferative activity of CLL cells and elevated levels predict disease progression (Zenz, Fröhling, Mertens, Döhner, & Stilgenbauer, 2010). Some studies have also shown that the serum TK level could be an independent predictor of the duration of the progression-free interval in CLL (Stilgenbauer, et al., 2009), (Hallek, et al., 1996) and could stratify patients to the smouldering and non-smouldering stages of CLL (Hallek, et al., 1999). It still remains unclear whether the TK level can be used to predict response and the length of survival in patients with CLL. Raimondo et al., (2001) measured the TK level in a cohort of patients with active or advanced CLL (treated with fludarabine), correlated the serum TK level with other presenting features, and assessed the TK level as a prognostic indicator of both response and survival. They confirm that serum TK level in CLL patients provides useful prognostic information regarding both response to therapy and length of survival and should be used in planning appropriate therapy. In particular, patients with a TK level of ≥ 10 U/l had a poor prognosis and should be considered for aggressive treatment (Raimondo et al., 2001). The results of our study indicate that not only elevated TK levels before treatment but also an increase in TK serum level after the first chemotherapy cycle can be useful in predicting response to therapy and survival. TK median level in CLL non-responders increased, whereas in CR patients it decreased after 1st cycle of chemotherapy.

In this study, markedly higher bFGF levels were found in CLL patients than in control group. Moreover, a negative correlation was observed between bFGF and CD38 before treatment ($r= -0.63$, $p<0.004$). It had been already reported that bFGF induce apoptotic resistance through up-regulation of BCL2 (König, et al., 1997), (Bairey, Zimra, Shaklai, & Rabizadeh, 2001). Moreover, the elevation of bFGF correlates with stage of CLL and it associated with resistance to fludarabine (Menzel, et al., 1996). In a previous study, Gora-Tybor at al. (2002) found that the purine analog cladribine used in monotherapy downregulated the bFGF levels as well as other angiogenic factors in patients with CLL. Our results confirm that cladribine decreases bFGF serum concentration, not only in monotherapy but also when combined with cyclophosphamide.

Conclusion

This analysis demonstrates that the CC regimen induces a relatively high response rate in patients with previously untreated CLL patients. High basic TK concentrations and overexpression of BFL1/*BFL1* are associated with inferior response to therapy. BFL1 contributes to chemoresistance and may be a co-existing prognostic factor in CLL in the future. Further studies

on a bigger group of patients are needed to elucidate the role of BFL1 as a predictive marker in CLL.

Although many discoveries have been made in CLL biology, interaction with the microenvironment is of pivotal importance in the search for new drug targets. The biological characterisation of CLL has led to great progress being made in outcome prediction, and novel treatment strategies are being found based on a growing number of agents, opening entirely new therapeutic approaches.

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