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SENSITIVITY OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS TO CHEMOTHERAPEUTIC DRUGS *EX VIVO* DEPENDS ON EXPRESSION STATUS OF CELL SURFACE RECEPTORS

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Background: **Response of chronic lymphocytic leukemia (CLL) patients to classical chemoimmunotherapy that remains the main strategy in treatment of this disease is strikingly variable. This issue requires the finding of biomarkers which could predict efficiency of drug administration and choose the best treatment option for each patient individually.** ***The aim*** **of this study was to find out association between cell surface receptors expression levels and CLL B cells sensitivity to chemotherapeutic drugs** ***ex vivo*****.** ***Materials and Methods:*** **The study was performed on malignant B cells isolated from peripheral blood of primary CLL patients. Flow cytometry, qPCR,** ***ex vivo*** **drug sensitivity assay, and cell viability assay were used in this study.** ***Results:*** **The high CD5 expression level was linked to better bendamustine (BEN) and cyclophosphamide (CP) CLL B cells response in contrast to B cells with low CD5 expression. Sensitivity of CLL B cells to CP also could be predicted by high level of CD20 expression. Expression of CD38 and high levels of CD37 and CD40 showed CLL B cells resistance to BEN** ***ex vivo*****. CLL B cells sensitivity to analyzed chemotherapeutic drugs was not dependent on CD22 expression status. The CD180 expression was detected in CLL B cells which were more susceptible to fludarabine and cyclophosphamide (FC) combinatory action. CLL B cells that coexpressed CD150 and CD180 on the cell surface were characterized by significantly decreased cell viability under fludarabine (FLU) exposure alone or FC in comparison with $CD150^{-}CD180^{-}$ B cells. Cell surface expression level of CD150 was not associated with CLL B cells chemosensitivity. However, high mRNA expression level of mCD150 isoform in CLL B cells was linked to their FLU sensitivity and CP resistance, while high nCD150 mRNA expression level showed resistance to FLU. Simultaneous CD150 and CD180 ligation increased FLU resistance, but BEN susceptibility of CLL B cells. CD150 and CD180 alone or in combination are involved in upregulation of CD20 cell surface expression.** ***Conclusion:*** **Expression status of the CD5, CD20, CD37, CD38, CD40, CD150, and CD180 cell surface receptors could be used in prediction CLL B cells sensitivity to FLU, CP, BEN and FC** ***ex vivo*****. Moreover, CD150 and CD180 receptors are involved in regulation of CLL B cells susceptibility to FLU and BEN. The CD150 and CD180 are positive regulators of CD20 expression that could make $CD150^{+}CD180^{+}$ CLL B cells more responsive to CD20-based immunotherapy.**

Key Words: **cell surface receptors, chronic lymphocytic leukemia B cells, chemotherapeutic drugs, chemosensitivity,** ***ex vivo*****.**

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Chronic lymphocytic leukemia (CLL) is the most frequent form of the lymphoproliferative disorders in the Western world [1]. Despite the target therapy as a new and optimistic option in CLL treatment, classical cytotoxic drugs (fludarabine (FLU), bendamustine (BEN), chlorambucil) together with rituximab are still the gold standard in first-line therapy of CLL patients [2]. Mutation status of *IGHV, TP53* and molecular cytogenetics should be assessed in CLL patients to choose the most appropriate treatment strategy [1]. Unfortunately, determining of CLL molecular profile is not widely available due to expensive, time-consuming and technically complicated procedure that requires special skills. On the other hand, clinical response of CLL patients is heterogeneous and often remains a trial-and-error endeavor. Ineligible treatment strategy of CLL patients may lead to serious side effects taking into consideration that the median of patient' age at diagnosis is 65–70 years and they have one or more comorbidities [3]. That is why it is essential to find an easy to perform approach that would allow to predict individually patient's response to chemotherapy for improving treatment efficacy. Several methods have been developed to screen *in vitro* or *ex vivo* drugs response in line of personalized medicine including clonogenic and proliferation assays, cell viability assay, analysis of gene expression, etc. [4]. Heterogeneous molecular profile of neoplastic cells is responsible for cell behavior that partially underlay variable tumor drug response and clinical outcome. Differentially expressed signaling molecules including cell surface receptors are promising candidates for prognostic and predictive markers. On one side, pattern of cell surface receptors expression partially reflects the status of signaling network. On the other side, receptors are active players of cell signaling network and could be involved in regulation of biological properties of malignant B cells including sensitivity to chemotherapeutic drugs. The number of studies that showed possibilities to use cell surface receptors as predictive markers is limited. It was shown that expression of CD69 is associated with better CLL B cell response to BEN [5]. In contrast, CD38 positive

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Abbreviation used: BEN – bendamustine; CIT – chemoimmunotherapy; CLL – chronic lymphocytic leukemia; CP – cyclophosphamide; FC – fludarabine plus cyclophosphamide; FLU – fludarabine; M – median; mAbs – monoclonal antibodies; PBMCs – peripheral blood mononuclear cells.

cells were resistant to BEN [6]. CLL patients with low CD20 expression level on malignant B cells are refractory to rituximab therapy [7]. In our study we try to find association between *ex vivo* drug sensitivity of CLL B cells and differentially expressed cell surface receptors on malignant B cells. For *ex vivo* drugs sensitivity testing we chose FLU, cyclophosphamide (CP) and BEN, chemotherapeutic agents that are included in the first-line therapy of CLL. Based on our previous study [8] and cell surface receptors signaling properties, the following list of cell surface receptors were selected as potential predictors of response to chemotherapy: CD5, CD20, CD22, CD37, CD38, CD40, CD150, and CD180.

MATERIALS AND METHODS

Patients and PBMCs isolation. Blood samples from 50 previously untreated patients were obtained from the Department of Oncohematology of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of National Academy of Science of Ukraine (IEPOR NASU, Kyiv, Ukraine) with verified CLL diagnosis. Each patient was informed about the study, and all experimental procedures were performed in accordance with the Declaration of Helsinki, the International Review Board and Research Ethics Committees of IEPOR NASU. Peripheral blood mononuclear cells (PBMCs) from CLL patients were isolated by Lymphoprep (Axis-Shield PoCAS, Norway) density gradient centrifugation according to the manufacturer's protocol. The malignant cells represent at least 90% of PBMCs.

Flow cytometry. PBMCs from CLL patients were immunophenotyped on the subject of CD5, CD23, CD43, CD19, CD20, CD22, CD37, CD38, CD40, CD95, CD150, and CD180 as described earlier [8]. Results were analyzed according to GeoMean MFI ratio of antigen to isotype control, where GeoMean ratio < 1.3 r.u. was considered as negative and GeoMean ratio $\geqslant 1.3$ r.u. was considered as positive cell surface expression.

Ex vivo analysis of drugs cytotoxity. In order to analyze sensitivity of CLL cells to chemotherapy drugs PBMCs from CLL patients were seeded in 96-well plate in concentration $1 \cdot 10^5$ cells/well and maintained in 100 µl of RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in CO_2 incubator. The following cytotoxic drugs: FLU (S.C. Sindan-Pharma S.R.L., Bucuresti, Romania), CP (Baxter Healthcare Corporation, Germany) and BEN (S.C.Sindan-Pharma S.R.L., Bucuresti, Romania) were added to PBMCs at the beginning of culture in appropriate concentration and incubated for 48 h. PBMCs maintained in culture medium alone served as a control. To test whether CD150 and CD180 receptors could be involved in modulation of CLL B cells sensitivity to chemotherapy drugs, PBMCs were pretreated with monoclonal antibodies (mAbs) against CD150 (IPO3, IEPOR NASU) and CD180 (G28-8, kindly provided by Prof. Edward Clark, University of Washington, Seattle, WA, USA) (both at final concentration of 10 µg/ml) for 24 h. After that chemotherapy agents were added to PBMCs and incubated for 48 h.

Cell viability assay. Viability of PBMCs from CLL patients under the drugs treatment *ex vivo* was determined by resazurin assay. After 48 h of PBMCs culture with or without drugs, the 10% of resazurin stock solution (440 µM) (Sigma-Aldrich, USA) was added to each well for further 4 h. The resazurin viability assay is based on the resazurin (blue) reduction to resorufin (red) by metabolically active living cells. Resazurin reduction was detected by measuring absorbance at both 540 nm and 630 nm using Labsystems Multiskan PLUS spectrofluorimeter (USA). Cells viability was calculated according to the following formula:

$$\frac{(\varepsilon ox)\,\lambda 2 A\lambda 1 - (\varepsilon ox)\,\lambda 1 A\lambda 2 \textit{ of test drug dilution}}{(\varepsilon ox)\,\lambda 2 A'\lambda 1 - (\varepsilon ox)\,\lambda 1 A'\lambda 2 \textit{ of untreated control}} \cdot 100 = \% \textit{ of reduction},$$

where: $\lambda 1 = 540$ nm; $\lambda 2 = 630$ nm; $(\varepsilon ox)\,\lambda 2 = 34{,}798$; $(\varepsilon ox)\,\lambda 1 = 47{,}619$; $A\lambda 1$ — experimental sample absorption at $\lambda 1 = 540$ nm; $A\lambda 2$ — experimental sample absorption at $\lambda 2 = 630$ nm; A' $\lambda 1$ — control sample absorption at $\lambda 1 = 540$ nm; A' $\lambda 2$ — control sample absorption at $\lambda 1 = 630$ nm.

Results were presented as a percent of cell viability that is directly proportional to percent of resazurin reduction.

Quantitative polymerase chain reaction. Total RNA was isolated from $5 \cdot 10^6$ PBMCs of CLL patients using NucleoZOL (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to manufacturer's protocol. The RNA quality and concentration were determined with a spectrophotometer NanoDrop™ 1000 (Thermo Scientific, USA). A detailed description of cDNA synthesis and real-time polymerase chain reaction were reported elsewhere [8, 9]. The following forward (For) and reverse (Rev) primers for real-time polymerase chain reaction were used: Cyt-n CD150 (isoform containing alternative cytoplasmic tail): For 5′-TGAGAAGAAGAGCCACCTTGA-3′, Rev 5′-GGTTCGTTTTACCATGGGAAG-3′; Cyt-m CD150 (isoforms containing conventional cytoplasmic tail): For 5′-GTGTATGCTGGGCTGTTAGG-3′, Rev 5′-AGAGGTAAAACGAACCATTACCA-3′; secreted CD150: For 5′-AGACCCCTCAGGTAAAACG-3′; Rev 5′-TCTGGACTTGGGCATAGATCG-3′. As internal control gene for normalization was chosen TATA-box binding protein: For 5′-CCACTCACAGACTCTCACAAC-3′; Rev 5′-CTGCGGTACAATCCCAGAACT-3′. The Ct values for target genes were determined and normalized to Ct value of TATA-box binding protein internal control gene using comparative Ct (ddCt) method.

Statistical analysis. Statistical significance between groups was evaluated by nonparametric Mann-Whitney U test using Prism software Version 4.0. Statistical significance between examined groups was assessed as $p < 0.05$. Correlation analysis between viability of CLL cells under the treatment with chemotherapy agents and cell surface receptors expression levels were determined according to Spearman's correlation coefficient using Prism software Version 4.0. Results of CLL B cells viability under the treatment with chemotherapy drugs are presented in box plots where whiskers mean maximum and minimum values, upper and lower borders of rectangles match the third and first quartiles respectively.

RESULTS AND DISCUSSION

Initially it was essential to determine the optimal concentration values of FLU, CP, BEN, and FC for treating CLL B cells *ex vivo.* The PBMCs from 13 CLL patients that were not included in main study were cultured with different concentrations of FLU, CP and BEN ranging from 1 μg/ml to 15 μg/ml, and cell viability was measured after 48 h. It should be noted that CLL B cells demonstrated varying sensitivity to different concentration values of examined cytotoxic drugs. That is why, dose of drugs that inhibit 35–60% of CLL B cells in not less than 70% of studied cases was chosen as optimal for the next study. Thus, the effective concentration values of FLU and CP alone were 5 μg/ml and 10 μg/ml correspondingly. 10 μg/ml BEN was an optimal dose for CLL B cells *ex vivo*. To evaluate the effective concentration values of FC we titrated both drugs in 1:2 ratio that is equivalent to the optimal dose ratio for each drug alone. The effective concentration values for FC were 2 μg/ml of FLU and 4 μg/ml of CP respectively.

Malignant B cells of all studied CLL cases showed typical for this disease phenotype – $CD5^+CD19^+CD23^+$. Heterogeneous expression level of CD22, CD38, CD150, and CD180 in CLL B cells (Table 1) allowed us to divide examined CLL cases into negative and positive based on presence or absence aforementioned cell surface receptors on malignant B cells. Since CD5, CD20, CD37 and CD40 were expressed in all CLL cases (Table 1), the optimal cut off for dividing CLL cases into two comparison groups was defined at median Geo-Mean values (M) of CD5, CD20, CD37 and CD40 expression levels: cell surface receptors expression level less than median (< M) depicts low expression, and cell surface receptors expression level greater than median (> M) means high expression.

According to our study, CLL B cells of all examined cases showed variable response to chemotherapeutic drugs *ex vivo* that depended on their cell surface phenotype. Cumulative statistics for each experimental group is represented in Table 2. It should be noted that CLL B cells viability without adding any cytotoxic

Table 1. Immunophenotype of malignant B cell isolated from peripheral blood of CLL patients

Cell surface antigen	GeoMean of antigen/GeoMean of isotype control		Positive cells		Positive cases, %
	Min-Max, r.u.	Median, r.u.	Min-Max, %	Median, %	
CD5	2.62–56.72	6.29	37.54–94.89	73.87	100
CD20	1.27–16.52	2.64	14.61–85.21	43.35	100
CD22	1.19–4.59	1.79	7.91–60.01	20.93	82.1
CD37	2.71–79.33	10.71	26.87–95.80	83.31	100
CD38	1.16–1.80	1.25	5.47–34.89	11.51	50
CD40	1.34–23.06	5.08	18.82–92.53	71.62	100
CD150	1.19–3.29	1.54	6.41–58.55	13.57	51.6
CD180	1.14–1.49	1.29	5.15–27.10	10.73	38.7

Table 2. Chemosensitivity of CLL B cells according to cell surface receptors expression level

Cell surface antigen expression	Cell viability (% of control)	Chemotherapy drugs			
		FLU	CP	BEN	FC
CD5, M <	Min-Max	5.53–87.00	21.19–86.95	16.71–98.16	9.38–56.46
	Median	47.84	73.92	49.19	33.93
	Mean ± SEM	49.24 ± 5.93	67.42 ± 5.12	51.63 ± 6.48	33.42 ± 10.51
CD5, M >	Min-Max	2.48–87.60	17.11–73.42	1.4–50.49	10.13–56.88
	Median	43.80	**58.99***	**26.59***	20.73
	Mean ± SEM	45.91 ± 5.77	50.77 ± 5.0	27.41 ± 4.19	25.97 ± 5.01
CD20, M <	Min-Max	9.57–87.00	29.63–88.59	1.4–99.78	9.38–85.50
	Median	47.84	73.90	35.11	27.92
	Mean ± SEM	50.56 ± 5.89	70.11 ± 4.14	47.32 ± 7.64	38.13 ± 13.09
CD20, M >	Min-Max	2.48–94.00	17.11–69.44	9.46–98.94	10.13–87.66
	Median	45.30	**55.82#**	47.33	28.39
	Mean ± SEM	47.05 ± 5.70	49.55 ± 4.16	50.55 ± 6.50	34.00 ± 6.92
$CD22^-$	Min-Max	5.53–73.88	24.06–83.54	20.92–62.91	ND
	Median	46.29	60.47	26.59	ND
	Mean ± SEM	38.65 ± 12.10	54.32 ± 11.83	35.50 ± 7.57	ND
$CD22^+$	Min-Max	9.57–87.00	17.11–108.80	1.41–99.78	ND
	Median	50.33	72.94	36.08	ND
	Mean ± SEM	52.10 ± 5.05	68.14 ± 4.66	50.03 ± 6.38	ND
CD37, M <	Min-Max	5.53–87.00	17.11–94.71	18.33–62.91	9.38–56.88
	Median	46.22	71.67	29.69	27.30
	Mean ± SEM	43.18 ± 6.22	60.34 ± 6.77	34.06 ± 3.64	30.21 ± 12.04
CD37, M >	Min-Max	2.48–87.60	31.66–91.20	15.37–99.78	10.13–87.66
	Median	46.32	64.90	**64.75****	27.92
	Mean ± SEM	52.06 ± 5.30	63.22 ± 3.75	63.73 ± 7.54	36.76 ± 7.13
$CD38^-$	Min-Max	5.53–84.21	17.60–86.95	1.41–72.00	9.38–56.46
	Median	47.26	71.69	35.08	36.11
	Mean ± SEM	47.72 ± 6.92	60.89 ± 6.33	36.84 ± 5.58	34.51 ± 10.22
$CD38^+$	Min-Max	9.82–87.00	17.11–108.8	29.30–99.78	10.29–85.50
	Median	51.80	71.19	**51.80##**	24.67
	Mean ± SEM	51.67 ± 6.52	70.45 ± 6.04	59.30 ± 7.56	40.18 ± 13.68
CD40, M <	Min-Max	5.53–87.00	17.11–94.71	1.4–67.59	27.92–56.88
	Median	43.11	71.69	27.95	50.38
	Mean ± SEM	45.04 ± 6.80	64.32 ± 6.19	30.55 ± 4.60	46.39 ± 6.81
CD40, M >	Min-Max	28.09–87.60	29.63–91.20	29.59–99.78	10.13–42.19
	Median	46.30	60.77	**60.41*#**	23.55
	Mean ± SEM	53.06 ± 4.27	59.87 ± 4.37	64.35 ± 6.40	24.48 ± 3.98

Note: ND – not determined; *p = 0.01 compared to CD5, M <; **p = 0.01 compared to CD37, M <; #p = 0.01 compared to CD20, M <; ##p = 0.05 compared to $CD38^-$; *#p = 0.01 compared to CD40, M <.

drugs did not depend on studied cell surface receptors expression level with the exception of CD38. The CD38 positive ($CD38^+$) CLL B cells were characterized by significantly higher cell viability than CD38 negative ($CD38^-$) malignant B cells (data not shown). Moreover, $CD38^+$ CLL B cells were more resistant to BEN than $CD38^-$ cells (median of cell viability was 51.8% *vs* 35.08% after 48 h incubation with drug) (Fig. 1, *b*). CLL B cells expressing CD37 and CD40 at the level greater than median values (> M) are characterized by significantly higher cell viability under BEN exposure compared to B cells with low CD37 and CD40 (< M) expression levels (Fig. 1, *a, c*). At the same time, viability of CLL B cells that expressed CD5 at the level more than M value decreased after incubation with BEN by 22.6% in contrast to CLL cells with low CD5 (< M)

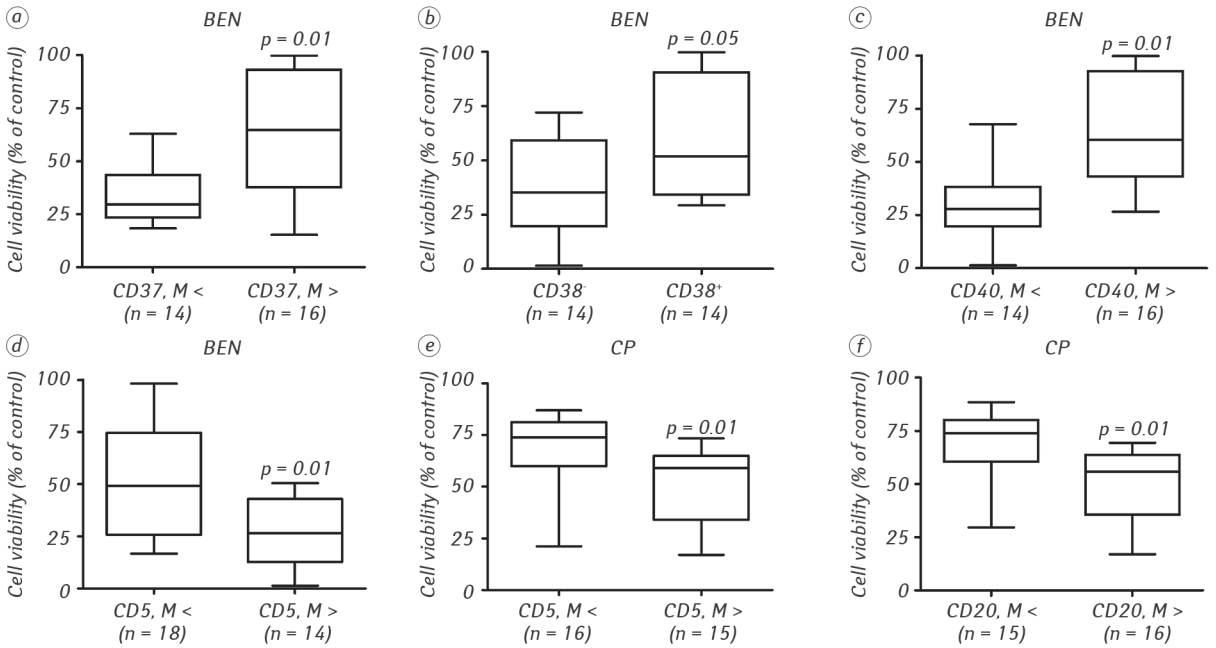

Fig. 1. Chemosensitivity of CLL B cells is dependent on expression status of CD37 (*a*), CD38 (*b*), CD40 (*c*), CD5 (*d, e*), and CD20 (*f*). Box plots show quartiles, median, minimum and maximum values of CLL B cells viability under BEN or CP action *ex vivo*

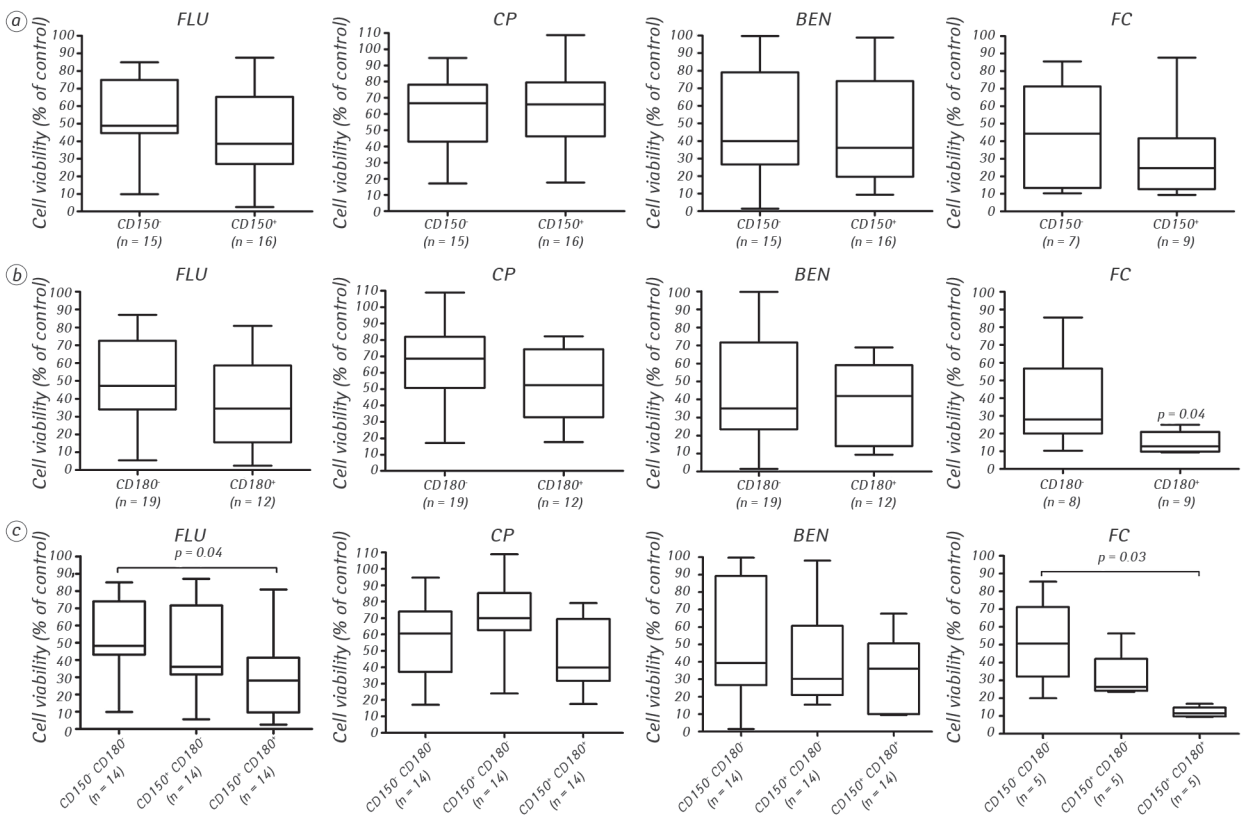

Fig. 2. Chemosensitivity of CLL B cells is dependent on expression status of CD150 (*a*), CD180 (*b*) alone, or simultaneous CD150 and CD180 expression (*c*). Box plots show quartiles, median, minimum and maximum values of CLL B cells viability under FLU, BEN, CP, or FC action *ex vivo*

(Fig. 1, *d*). *Ex vivo* CLL B cells viability under CP action was reduced in CLL cases with high CD5 and CD20 cell surface expression levels (> M) (Fig. 1, *e–f*). Expression level of CD22 was not associated with CLL B cells sensitivity to analyzed chemotherapeutic drugs. No association was found between cell surface expression of CD5, CD20, CD37, CD38, and CD40 in CLL B cells and their sensitivity to FLU or FC *ex vivo* (see Table 2). Taken together, CD5, CD20, CD37, CD38, and CD40 cell surface expression levels are associated with sensitivity of CLL B cells to BEN and CP *ex vivo*. The high CD5 expression level (> M) is linked to better BEN and CP CLL B cells sensitivity. Sensitivity of CLL B cells to CP also could be predicted by greater than median value of CD20 expression. Expression of CD38 and high levels of CD37 and CD40 showed CLL B cells resistance to BEN *ex vivo*.

Our previous study showed that CD150 and CD180 receptors possess signaling properties in CLL B cells [8, 10]. In contrast to CD150 or CD180 ligation alone, the combination of CD150 and CD180 crosslinking led to specific mutual inhibition of Akt and mitogen-activated protein kinase (MAPK) pathways affecting translational machinery and probably disrupting the spreading of pro-survival signals in CLL B cells [8]. CD150- and CD180-mediated blocking of pro-survival pathways may be a restraining factor for neoplastic CLL B cells propagation and underlie the better patient's response to chemoimmunotherapy (CIT) in more than 50% of CLL cases where these receptors are coexpressed. To clarify this issue, we try to find any correlation between CLL cells viability under chemotherapeutic drugs exposure *ex vivo* and CD150 and CD180 expression levels. Despite the fact that we did not reveal statistically significant differences in CLL B cells drugs sensitivity and CD150 cell surface expression, viability of CD150 positive CLL cells under FLU and FC action was by approximately 10% lower than in CLL cases with $CD150^-$ malignant cells (Fig. 2, *a*). Similarly, $CD180^+$ *vs* $CD180^-$ CLL B cells were more sensitive to FLU at the tendency level, while combination of FC showed the strongest cytotoxic effect in $CD180^+$ CLL cells (Fig. 2, *b*). The most important was to find whether double negative $CD150^-CD180^-$ and double positive $CD150^+CD180^+$ CLL B cells display any differences in sensitivity to chemotherapeutic drugs, since CD150 and CD180 receptors could mediate inhibition of pro-survival Akt and mitogen-activated protein kinase signaling pathways just when are co-expressed. We found that $CD150^-CD180^-$ CLL B cells are characterized by significantly higher cell viability under FLU and FC action *ex vivo* than $CD150^+CD180^+$ malignant B cells (Fig. 2, *c*). At the same time, sensitivity of CLL B cells to BEN or CP alone did not depend on CD150 or CD180 expression (see Fig. 2). Thus, CD180 expression is correlated with better CLL B cells response to FC, whereas simultaneous expression of CD150 and CD180 on the cell surface of CLL B cells is associated with their sensitivity to FLU and FC *ex vivo*, but not to BEN or CP.

Absence of significant differences between CD150 cell surface expression and CLL B cells drugs sensitivity *ex vivo* could be related to differential CD150 isoforms expression. Among several alternatively spliced isoforms of CD150 in CLL B cells were found canonical transmembrane mCD150, novel nCD150 with alternative cytoplasmic domain and soluble sCD150 without transmembrane part. Despite the fact that all main isoforms are expressed in CLL, the predominant one is mCD150 [8]. We tried to find whether the mRNA expression levels of mCD150, nCD150 and sCD150 isoforms are linked to the chemosensitivity of CLL B cells *ex vivo*. We have shown that CLL B cells viability was significantly lower in CLL cases with mRNA expression level of mCD150 isoform higher than median value compared to CLL cases with low mCD150 expression level (< M) (Fig. 3, *a*). In contrast, high mCD150 (> M) mRNA expression level correlated with CLL B cells resistance to CP *ex vivo* (Fig. 3, *c*). The mRNA expression level of nCD150 isoform below the median value was found in CLL cells more sensitive to FLU (Fig. 3, *b*). It is important to note that the expression levels of mCD150 and nCD150 isoforms mRNA showed reverse correlation with CLL B cells sensitivity to FLU suggesting possible opposite mCD150 and nCD150 signaling properties. No dependency was found between mRNA expression level of sCD150 isoform and CLL B cells sensitivity to tested chemotherapeutic drugs (data not shown). Consequently, while cell surface expression of CD150 is not associated with CLL B cells sensitivity to chemotherapeutic drugs, detection of mCD150 and nCD150 isoforms mRNA expression level could predict the response efficiency of malignant B cells to FLU and CP *ex vivo*.

Based on CD150 and CD180 signaling properties in CLL B cells and revealed corelation between expression of these cell surface receptors and CLL B cells

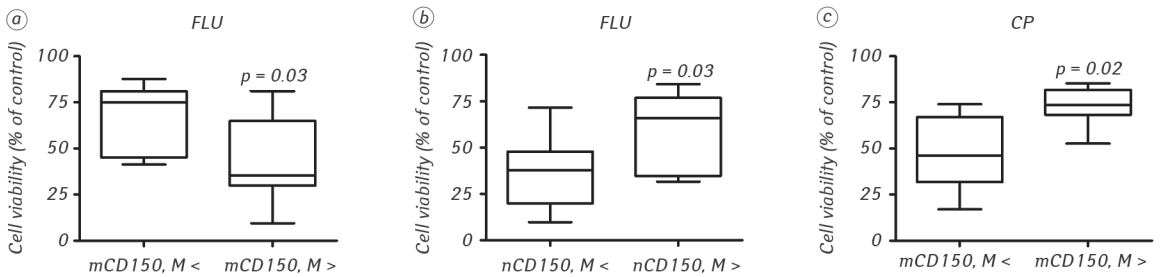

Fig. 3. The mRNA expression level of mCD150 and nCD150 isoforms is associated with CLL B cells sensitivity to FLU (*a, b*) and CP (*c*)

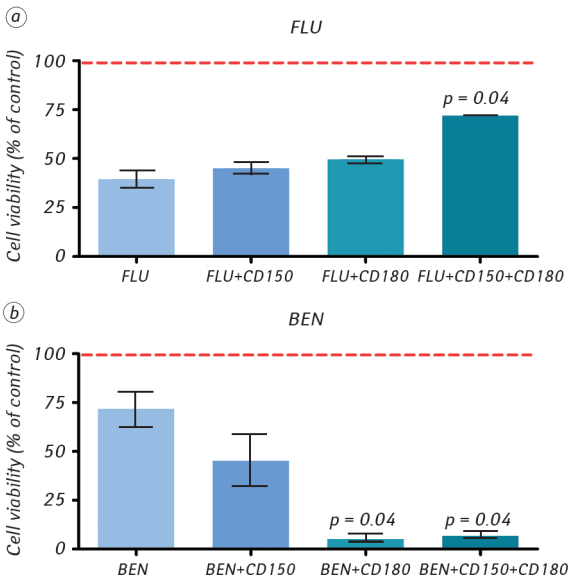

Fig. 4. Sensitivity of CLL B cells to FLU (*a*) and BEN (*b*) after ligation of CD150 and CD180 cell surface receptors. CLL B cells were pretreated with mAbs anti-CD150 and anti-CD180 during 24 h. Then chemotherapeutic drugs were added in appropriate concentrations for next 48 h. Red dashed line means viability of CLL cells without adding drugs and mAbs and serves as a control. *P*-value was calculated as compared to BEN and FLU effects without adding Abs

drugs sensitivity, we tested a hypothesis whether preincubation of CLL B cells with mAbs against CD150 and CD180 could improve the efficacy of chemotherapeutic drugs *ex vivo*. To answer this question, stimulation of CLL B cells via CD150 and CD180 alone or in combination was performed during 24 h before cytotoxic drugs were added to the culture media. CLL B cells cultured in medium alone, with mAbs against CD150 and CD180 only, and with cytotoxic drugs only served as an appropriate control. Ligation of CD150 and CD180 without addition of cytotoxic drugs had no effect on CLL B cells viability. Ligation of CD150 and CD180 did not influence the malignant B cells viability under CP action (data not shown). Similarly, no changes were observed in sensitivity of CLL B cells to FLU after preincubation with mAbs agains CD150 or CD180 alone (Fig. 4, *a*). However, simultaneous CD150 and CD180 ligation in CLL B cells with following FLU incubation increased cell viability by up to 20% in contrast to FLU action alone (see Fig. 4, *a*). Surprisingly, it was found that CD150 or/and CD180 ligation in CLL B cells followed by cultivation with BEN led to strongly decreased maligant B cells viability (more than 50%) compared to BEN effects only (Fig. 4, *b*). Therefore, CD150 and CD180 receptors are involved in regulation of CLL B cells viability under the chemotherapeutic drugs exposure *ex vivo*. Simultaneous stimulation of CLL B cells via CD150 and CD180 increased resistance of malignant B cells to FLU action. At the same time, pretreatment of CLL B cells with mAbs against CD150 or/and CD180 could be potentially used to increase BEN efficacy in CLL treatment.

Rituximab is a target drug directed against cell surface receptor CD20 and is widely used in treatment of non-Hodgkin lymphoma [11, 12]. CD20 is exclusive B-cell surface antigen which is expressed starting from pre-B stage of B-cell development and remains up to formation of plasma cells [11]. In contrast to other non-Hodgkin lymphomas, CLL is characterized by decreased CD20 expression on malignant B cells that could be associated with worse effectivity of anti-CD20 mAb-based therapy [13]. It is known that one of CD20 positive regulators, PU.1 transcription factor is downregulated in CLL [10, 14]. We have previously shown that CD150 and CD180 ligation lead to upregulation of PU.1 expression level in CLL B cells [10]. This fact allows us to hypothesize that CD150 and CD180 receptors could be linked to regulation of CD20 expression in CLL B cells. To clarify this question, *in vitro* stimulation of CLL cells via CD150 and CD180 for 48 h with following evaluation of CD20 expression level was performed. We found that CD20 expression was upregulated after CD150 ligation up to 2.25 times compared to CLL B cells cultured in medium alone (Fig. 5). CD180 ligation alone or in combination with CD150 also lead to elevation of CD20 expression in CLL B cells, but had less pronounced effect than

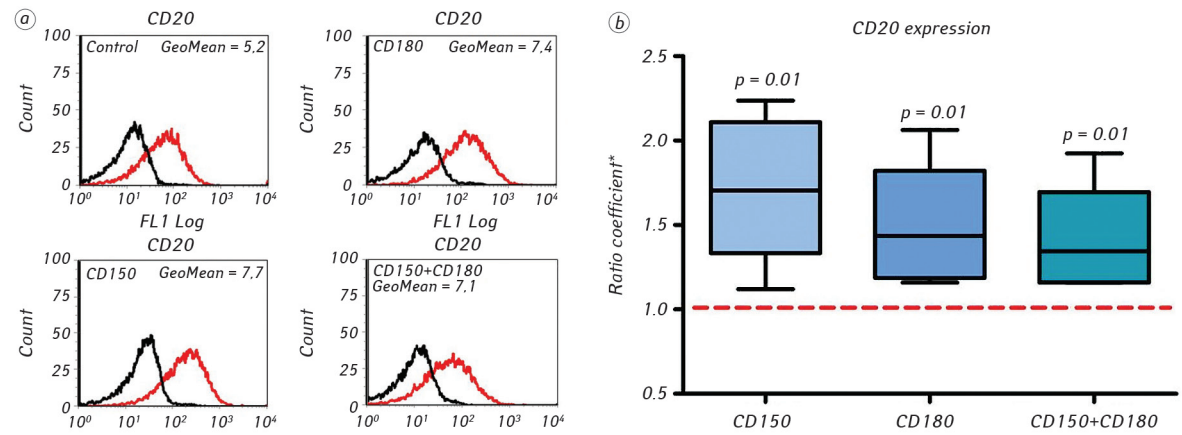

Fig. 5. CD150 and CD180 are involved in CD20 cell surface expression upregulation in CLL B cells. Flow cytometry analyses of cell surface CD20 expression on CLL B cells after CD150, CD180 and CD150+CD180 ligation. Representative histograms of CD20 expersion before and after CD150 or/and CD180 ligation (*a*). Black line marks isotype control, green line marks CD20 expression level. Summarized results from 6 independent experiments (*b*). Red dashed line means the level of CD20 expression in CLL B cells cultured without adding mAbs and serves as a control. *Ratio coefficient — GeoMean values of CD20 expression level after CD150 or/and CD180 ligation normalized to GeoMean CD20 values in cells cultured without mAbs. *P*-value was calculated as compared to control

CD150 (see Fig. 5). Thus, CD150 and CD180 are positive regulators of CD20 expression in CLL B cells. At the same time, CD150 showed the strongest effect on CD20 upregulation in CLL B cells compared to CD180 alone or in combination.

Personalized medicine is called to yield the most effective treatment option and minimize side effects on the base of patient's molecular profile [15]. This issue requires to develop a wide range of diagnostic, prognostic and predictive biomarkers that potentially could cover all individual variations. Cell surface receptors are suitable candidates for biomarkers due to their differential expression pattern that underlies phenotype and functional differences between normal cell types as well as between normal and pathological cells. Moreover, many target drugs, including anticancer drugs, are directed against cell surface receptors (e.g. CD20, CD22, CD33, CD75 etc.) [16]. Examples of cell surface receptors as predictive biomarkers are expression level of HER2/neu in breast cancer, EGFR2 in non-small-cell lung carcinoma, PD-1L and CTLA in melanoma and lung cancer, etc. [17]. Frequently, predictive biomarkers are co-developed with target drugs directed against the same molecules. In contrast, the number of response predictors to classical chemotherapeutic drugs is limited.

CLL is a strikingly variable disease [1]. Numerous prognostic markers are used to predict CLL outcome including mutational status of the *IGHV* genes, expression of ZAP-70, CD38, and CD49d [1]. Advances in sequencing technology allow to reveal genetic lesions in *NOTCH1* and *SF3B1* gene, *TP53* and *BIRC3* that depict intermediate and high risk of CLL progression correspondingly [18]. Moreover, up to now, evaluation of *TP53* aberrations in CLL B cells is the only one valid predictor of CIT effectivity. Deletion or mutation of *TP53* shows resistance to classical CIT and require target drugs application (e.g. Ibrutinib) [1]. CLL patients who carry wild type status of *TP53* in malignant B cells undergo classical scheme of first-line CIT that include FC and rituximab (< 65 years) or BEN with rituximab (> 65 years) [19, 20]. Response to CIT strategy in CLL patients is highly variable that requires searching for new biomarkers with predictive potential. *In vitro* or *ex vivo* chemosensitivity testing is not a new approach in oncology that is used to screen new anticancer agents or predict individual response to drugs [4, 21]. Many studies have shown strong relationship between *ex vivo* sensitivity of CLL cells to chemotherapeutic drugs and clinical response of CLL patients [22, 23]. In current study we showed for the first time that cell surface expression level of CD5, CD20, CD37, CD38, CD40, CD150, and CD180 is associated with chemosensitivity of CLL B cells to FLU, CP, BEN and FC *ex vivo*. Expression levels of CD5, CD37, CD38, and CD40 correlate with sensitivity of CLL B cells to BEN; CD5 and CD20 expression is linked to CP response, while expression level of CD150 and CD180 receptors predicts susceptibility of CLL B cells to FLU or FC. Determination of studied cell surface receptors expression levels could be performed at the stage of CLL diagnosis without attracting any additional analysis saving patient's time. That is why we propose to use evaluation of CD5, CD20, CD37, CD38, CD40, CD150, and CD180 cell surface expression levels as potential predictive biomarkers of CLL patient response to chemotherapeutic drugs.

CLL development and progression depends on numerous factors such as B-cell receptor signaling, *IGHV* mutational status, genetic aberrations, impairment of epigenetic regulation, intratumor heterogeneity, clonal evolution, and tumor microenvironment [24, 25]. Crosstalks between malignant cells and plethora of cellular and molecular components in local tissue microenvironments define CLL B cells behavior [24]. Cell surface receptors are not merely passive diagnostic or prognostic markers, but active players of signaling network. Activation of cytokines receptors, adhesion molecules, co-stimulatory receptors mediates B-cell receptor signaling enhancing, CLL B cells survival, proliferation, and apoptosis evasion that supports disease progression [25]. It is not surprising that profile of cell surface receptors expression in CLL B cells underlies not only cell fate but also sensitivity to chemotherapeutic drugs.

One of the surrogate prognostic markers of CLL unfavorable outcome is CD38 [26]. High rate of $CD38^+$ CLL B cells survival and resistance to BEN revealed in our study (see Fig. 1, *b;* and data not shown) could be explained taking into consideration CD38 functions. Association between CD38 expression and BEN sensitivity in CLL B cells was also described by Sara Kost *et al.* [6]. Multifunctional CD38 possesses ectoenzymatic activity by conversion of NAD into adenosine diphosphoribose and cyclic adenosine diphosphoribose and mediates intracellular signaling [26–28]. In CLL B cells CD38 forms macromolecular signaling complex with CD49d, CD44 and matrix metalloproteinase 9 that recruits ZAP-70 and activates Akt and ERK1/2 signaling pathways [29]. Moreover, CD38 is involved in enhancing CLL B cells homing by promoting CXCL12/CXCR4 signals [28]. Thus, $CD38^+$ in contrast to $CD38^-$ CLL B cells are characterized by higher migration capabilities to second lymphoid organs, where they obtain supportive pro-survival signals that drive leukemia progression and mediate resistance to therapy. Malignant B cells could obtain additional pro-survival signals via activation of CD40 and CD37. Co-stimulatory receptor CD40 induces activation of NF-kB pathway [30] while CD37 ligation leads to Akt phosphorylation [31] explaining revealed in our study BEN resistance in CLL B cells with high CD37 and CD40 cell surface expression levels (see Fig. 1, *a, c*).

Our study showed that CD5 receptor is not only "passive" diagnostic marker of CLL, but its expression level may predict malignant B cells sensitivity to BEN and CP. CLL B cells with high CD5 expression level are characterized by better BEN and CP response than cells with low CD5 expression (see Fig. 1, *d, e*). Revealed dependence could be associated with CD5-mediated negative regulation of B-cell receptor signaling [32].

Moreover, it was shown that higher CD5 expression is related to better clinical outcome of CLL patients, and specifically longer time to treatment [33]. CLL B cells with high CD5 are more susceptible to CD20-based target therapy since express higher CD20 level [34]. In our study, high CD20 level showed better CLL B cell response to CP (see Fig. 1, *f*). All these suggest that CD5 receptor is a modulator of CLL pathobiology and disease aggressiveness. Therefore, quantification of CD5 expression level may help to evaluate prognosis and predict CLL therapy outcome.

CD150 as adhesion and costimulatory molecule and CD180 as a pattern recognition receptor are active players in regulation of CLL pathobiology and microenvironment. Expression of CD150 is associated with favorable CLL outcome. In particular, patients with $CD150^+$ malignant B cells are characterized by longer time to first treatment and overall survival [35]. In current work we determined that CD150 and CD180 expression levels may be used for predicting CLL B cells sensitivity to FLU alone or FC combination. CD150 and CD180 double positive malignant B cells showed higher susceptibility to FLU and FC (see Fig. 2, *c*). CD150 and CD180 signaling properties that are realized in CLL B cells underlie their behavior. It was previously shown that CD150 together with CD180 inhibit propagation of pro-survival Akt and mitogen-activated protein kinase signaling pathways partially explaining observed phenomena [8]. CD150 alone links to CLL B cells migration in second lymphoid organs and bone marrow due to downregulation of malignant B cells chemotaxis toward CXCL12. On the other hand, CD150 makes B cell more sensitive to FLU by inducing authophagy [35]. Interesting to note that ligation of CD150 or CD180 alone does not change efficiency of FLU action in CLL B cells (see Fig. 4, *a*). Surprisingly, we detected that CD150 and CD180 coligation leads to higher CLL B cells survival under FLU action that is in disagreement with observed $CD150^+CD180^+$ malignant B cells sensitivity to FLU (see Fig. 2, *c*; Fig. 4, *a*). Partially this could be explained by revealed differences in FLU sensitivity and CD150 isoforms mRNA expression levels. High mCD150 mRNA expression is associated with better CLL B cells response to FLU while nCD150 mRNA expression level shows opposite relation (see Fig. 3, *a, b*). Both isoforms have similar extracellular part and can be activated by mAbs, however structural differences in cytoplasmic part of mCD150 and nCD150 isoforms may lead to initiation of different signaling pathways and modulate B cells biological properties in opposite manner. This hypothesis needs further study. Crosslinking of CD180 alone or together with CD150 on CLL B cells enhances BEN effectivity despite that dependency between CD150/CD180 expression and sensitivity to BEN was not revealed (see Fig. 2, *c*; Fig. 4, *b*). More likely, signaling via CD180, but not CD150 is dominant in regulation of CLL B cells sensitivity to BEN.

Rituximab as an obligatory component of first-line CIT is used in combination with FLU and CP to treat CLL patients, who are younger than 65 and have no significant comorbidities. CLL patients older than 70 with renal disfunction are usually treated by BEN and rituximab [36, 37]. In the present study we did not focus on determination of CLL B cells sensitivity to rituximab because of its mechanisms of action including complement-dependent cytotoxicity that cannot be implemented in *ex vivo* conditions. However, we found that rituximab efficiency could be potentially improved by upregulation of CD20 expression on the cell surface of malignant B cells via CD150 and CD180 ligation. Density of CD20 expression on the cell surface of malignant B cells is directly correlated with efficiency of anti-CD20 immunotherapy [11]. Low CD20 expression level in CLL B cells is associated with *NOTCH1* mutation that is observed in 5–12% of CLL cases [38]. Patients with *NOTCH1* mutation in CLL B cells were refractory to rituximab therapy [7]. Decreased CD20 expression level in other 88–95% of CLL patients could be linked to dysregulation of the transcription factors that control CD20 expression. Here we showed for the first time that CD150 and CD180 receptors alone or together are the positive regulators of CD20 cell surface expression in CLL B cells (see Fig. 5). Importantly, CD150 and CD180 are involved in upregulation of CD20 positive regulator, transcription factor PU.1 [10]. Thus, CD20 expression may be upregulated via CD150 and CD180 receptors with possible implication of PU.1 signaling axis. CD150 and CD180 ligation could be used as a potential approach to increase CLL B cells sensitivity to anti-CD20 mAb-based therapy via upregulation of the CD20 cell surface expression on malignant B cells.

In conclusion, our study showed that expression level of CD5, CD37, CD38, and CD40 cell surface receptors is associated with CLL B cells sensitivity to BEN, while CD5 and CD20 expression correlated with malignant B cells response to CP *ex vivo*. Coexpression of CD150 and CD180 receptors in CLL B cells is linked to better efficiency of FLU alone or FC. CD180 expression indicates CLL B cells susceptibility to combination of FLU and CP. High level of mCD150 isoform expression could evidence on better CLL response to FLU and resistance to CP, while high nCD150 mRNA expression is associated with malignant B cells FLU refraction. CD150 and CD180 are the positive regulators of CD20 expression making $CD150^+CD180^+$ CLL B cells more responsive to CD20-based immunotherapy.

Summarizing all of the above, expression status of the CD5, CD20, CD37, CD38, CD40, CD150, CD180 cell surface receptors could be used as predictive biomarker in CLL. Easy and quick determination of their expression level at the stage of diagnosis may help to predict sensitivity of malignant B cells to chemotherapeutic drugs and in this way enhance therapy efficiency.

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