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Key Words: *B-cell chronic lymphocytic leukemia (CLL), interleukin-2 (IL-2), CD25, STAT, transcription factors, IL-2-STAT5 pathway.*

THE IL-2-STAT5 PATHWAY IS BLOCKED IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

Aim: *to study the status of the interleukin-2 (IL-2) pathway in chronic lymphocytic leukemia (CLL) cells, to understand functional differences between CLL and the normal mature B-cells.* ***Objects and methods:*** *samples of peripheral blood of patients with CLL, RNA isolation, analysis of expression of transcription factors, using RT2 profiler assay and quantitative polymerase chain reaction, bioinformatics analysis of publicly available data bases on expression.* ***Results:*** *we have found that the JAK-STAT5 pathway is largely inactivated in CLL cells. Despite elevated expression of STAT2 and STAT5 genes at the mRNA level, STAT5-responsive genes, such as BCL2L1 (BCL-XL), CCND2 (Cyclin D2), HIF1A, ID1, MCL1, and MYC are down-regulated in CLL cells, compared to peripheral blood B-cells of healthy individuals.* ***Conclusions:*** *the inactivation of the JAK-STAT5 pathway could be explained by the high levels of soluble IL-2RA, as was reported earlier. Another possibility could be inhibition of STAT5 phosphorylation, leading to inability to form the active transcriptionally protein heterodimers. The phosphorylation status of STAT proteins in CLL cells should be further illuminated.*

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common type of leukemias in Europe and USA (about 30%). The morbidity rate is approximately 3.5 per 100 000 people (5.0 for men and 2.5 for women in USA) [1]. In Ukraine, the disease rate was 3.57/100 000 in 2016. The predisposition to the disease is often hereditary — the risk of developing CLL in immediate relatives is 7 fold higher than the median of a population [2]. Most of the cases of CLL, if not all, are preceded by the monoclonal B-cell lymphocytosis, which occurs in 5–10% of people over the age of 40 and progresses to CLL with a frequency of about 1% per year [2]. The average age of men at the time of the disease is 70 years, women — 74 years. In people, younger than 50 years old, CLL is found very rarely and it was not diagnosed in children [3, 4].

CLL is represented by monomorphic small round B-lymphocytes with an admixture of pre-B-cells and a presence of characteristic pseudo-follicular centers of proliferation (revealed by an analysis of histological samples) [5]. Diagnosis is usually made on the base of infiltration of a bone marrow by clonal population of B-cells and the high content of B-cells in peripheral blood ($> 5–10 \cdot 10^9$) [6].

In spite of long history of CLL study, mechanisms of emergence and development of this disease are still not fully understood.

One of the hypotheses on the CLL origin is the accumulation of long-lived immunologically incompetent B-lymphocytes, which divide very rarely (mature B-cells) (Fig. 1, *a*). It was proposed that a proportion of CLL cells bearing immunoglobulins variable (IgV) region without mutations arise from the mature CD5-positive B-cells [7]. The heavy-water studies have shown that a small subset of the immortal cells is proliferating. However, the number of the new cells formed per day is very small, ranging from 0.1 to 0.35% of the total number of cells in the clone [8].

On a surface of CLL cells the CD5, IgM/IgD and CD19 antigens were simultaneously detected, that lead to a hypothesis that CLL arise from naïve B-cells (Fig. 1, *b*). This was supported by a finding of a post germinal center B-cells, expressing the CD5 and CD27 and possessing the mutated IgV. It was concluded, that CLL with mutated IgV could be originated from such B-cells [7].

Another possibility is that CLL represents transitional B1-cells that are also CD5-positive (Fig. 1, *c*). B1-cells are very poorly characterized. It is known, however, that CD5 expression is not a main feature of these cells [9]. Recently, it was shown that a subset of the B1-cells can produce antibodies [10], in contrast to behavior of CLL cells.

It seems that the first hypothesis correctly characterizes CLL cells as the B-cells, incapable to respond to different stimuli from the microenvironment (Fig. 1, *d*). At the normal conditions, there is an ideally tuned balance between stimuli for cells to proliferate and/or undergo apoptosis. Such signals could be transduced via B-cell receptor (BCR), expressing on a surface of mature B-cells, and also by chemokine and cytokine receptors or by direct contact with other cells.

T-cells are among the main players in the CLL microenvironment. Surprisingly, the different sets of T-cells were detected in CLL-patients, compared with healthy individuals. Thus, the absolute count of regulatory T-cells (Tregs) was dramatically increased in the peripheral blood of CLL patients [11, 12]. Also, the number of helper T-cells (Th), especially Th17, was simultaneously decreased [13]. One of the main inducers of T-cell activation and differentiation is interleukin-2 (IL-2) [14, 15].

Of note, the huge increase (up to more than 100 fold) of soluble interleukin-2 receptor alpha (sIL-2RA) was measured in sera of CLL patients, especially in patients with the advance stage of the disease [16].

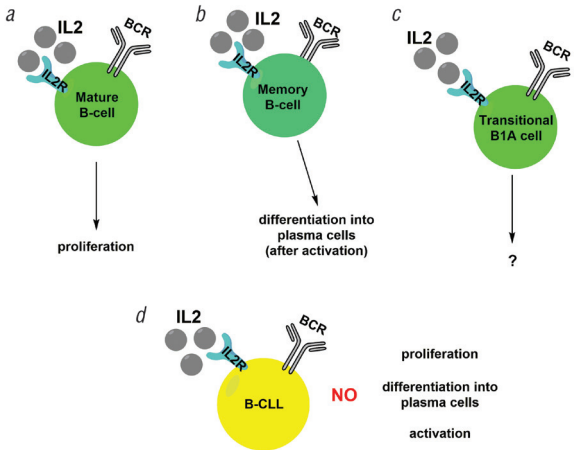

Fig. 1. A scheme representing a putative origin of CLL cells: *a* — a mature B-cell; *b* — the memory B-cell; *c* — the transitional B1A cell; *d* — the typical CLL cell. The scheme was prepared with the help of a ChemOffice® Professional 17 free trial copy

We asked a question what is the status of the IL-2 pathway in CLL cells, with an aim to understand how CLL cells differ from the mature B-cells.

MATERIALS AND METHODS

Clinical specimens

Samples of peripheral blood of CLL patients were obtained at the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of NAS of Ukraine (from 19.11.2014 till 31.12.2016). The study included 29 patients with B-cell CLL diagnoses. The main types and cytological variants of diseases were diagnosed, according to the WHO classification [17]. For control, B-cells were isolated from the peripheral blood of 3 healthy donors. All experimental work was performed, according to protocols, approved by the Committee on Bioethics at R.E. Kavetsky IEPOR.

RNA isolation and c-DNA synthesis

CLL cells were isolated from the peripheral blood by Ficoll-Paque gradient centrifugation, resuspended in TRIzol reagent (Gibco BRL, USA) and stored at −20 °C until further use. The total RNA was isolated, using the RNeasy Mini Kit (Qiagen Inc., Germany), according to the manufacturer's instructions. The cDNAs were synthesized, using M-MLV Reverse Transcriptase and RNAse inhibitor (Invitrogen, USA), according to the manufacturer's protocol. 2 μg of total RNA was used for cDNA synthesis.

Analysis of transcription factors expression, using RT2 profiler assay

The expression profile of 84 transcription factors was studied, along with 12 positive and negative controls (a total of 96 genes) on two identical PARN-075Z plates. The RNA mixture consisted of 25 μl (a half) of each RNA solution, isolated from the samples. The control group was a mixture of RNA, isolated from peripheral blood B-cells of 3 healthy donors. q-PCR was performed, using 2 μg cDNA and the SYBR Green Master Mix (Thermo Fisher Scientific Inc., USA) on PCR System 7500 (Applied Biosystem, USA). CT was not measured after 35 cycles. The obtained CT values were downloaded to the manufacturer's website (Thermo Fisher Scientific Inc., USA) for online analysis of expression of transcription factors.

Bioinformatic data analysis

In order to analyze expression of genes at a mRNA level, several publically available databases were used: the GTEx Portal (http://www.gtexportal.org/home); the Broad-Novartis CCLE (Cancer cell line encyclopedia) portal at the Broad Institute website (https://portals.broadinstitute.org/ccle); the Oncomine, which contains published data that has been collected, standardized, annotated and analyzed by Compendia Bioscience (www.oncomine.com, September 2017, Thermo Fisher Scientific, Ann-Arbor, MI, USA).

RESULTS AND DISCUSSION

Expression of 84 transcription factors was studied, using the PARN-075Z platform. To reveal the differences of gene expression in cells of CLL patients, a mixture of RNA isolated from the peripheral blood B-cells of 29 CLL patients and RNA samples from three healthy donors was used. Only few genes were expressed at a higher level in CLL cells compared with B cells of healthy donors, namely, *POU2, ATF1*, *NFAT5*, *NFATC1*, *JUNB*, *JUN*, and *RELB* (see also our earlier publication [18]). We have found that several genes, stimulating proliferation, such as *MYC* and *NFkB1*, were downregulated (3.26 and 4.23 fold, respectively) in CLL cells. This corresponds to the nature of CLL cells, which do not show a proliferative activity. Not only *MYC*, but also *ID1* and *HIF1A* transcription factors expressed at very low levels in CLL cells (Fig. 2, *a*). To validate our results, an analysis of the open publicly available data bases was performed. Thus, we have found that the *ID1* gene was downregulated in CLL, compared with peripheral blood B-cells of the healthy donors (Fig. 2, *b*, *c*). Results presented in Fig. 2, *b*, *c* were obtained by analysis of the data at the Oncomine portal [19] and [20], respectively. The same trend was shown by the *MYC* gene (Fig. 2, *d*, *e*), as was shown by a study on various tumor types and lymphomas [19] and [21], respectively.

Noteworthy, all the above-mentioned genes — *HIF1A*, *ID1* and *MYC* — are responsive genes of an IL-2 induced JAK-STAT5 signaling pathway [22]. Therefore, next we have analyzed expression of other STAT5-responsive genes, namely *MCL1*, *BCL2, BCL-XL*, Cyclin D2 (*CCND2*), and *Cyclin D1* and cyclin-dependent kinase inhibitors p21 (*CDKN1A*) and p16 (*CDKN2A*) as controls.

Expression of the *MCL1* gene was down-regulated (Fig. 2, *f*, according to [20]), as well as *BCL2* (Fig. 3, *a*), *BCL-XL* (Fig. 3, *b*) and *Cyclin D2* (Fig. 3, *f*). Noteworthy, expression levels of *p16*, *p21* and *Cy-*

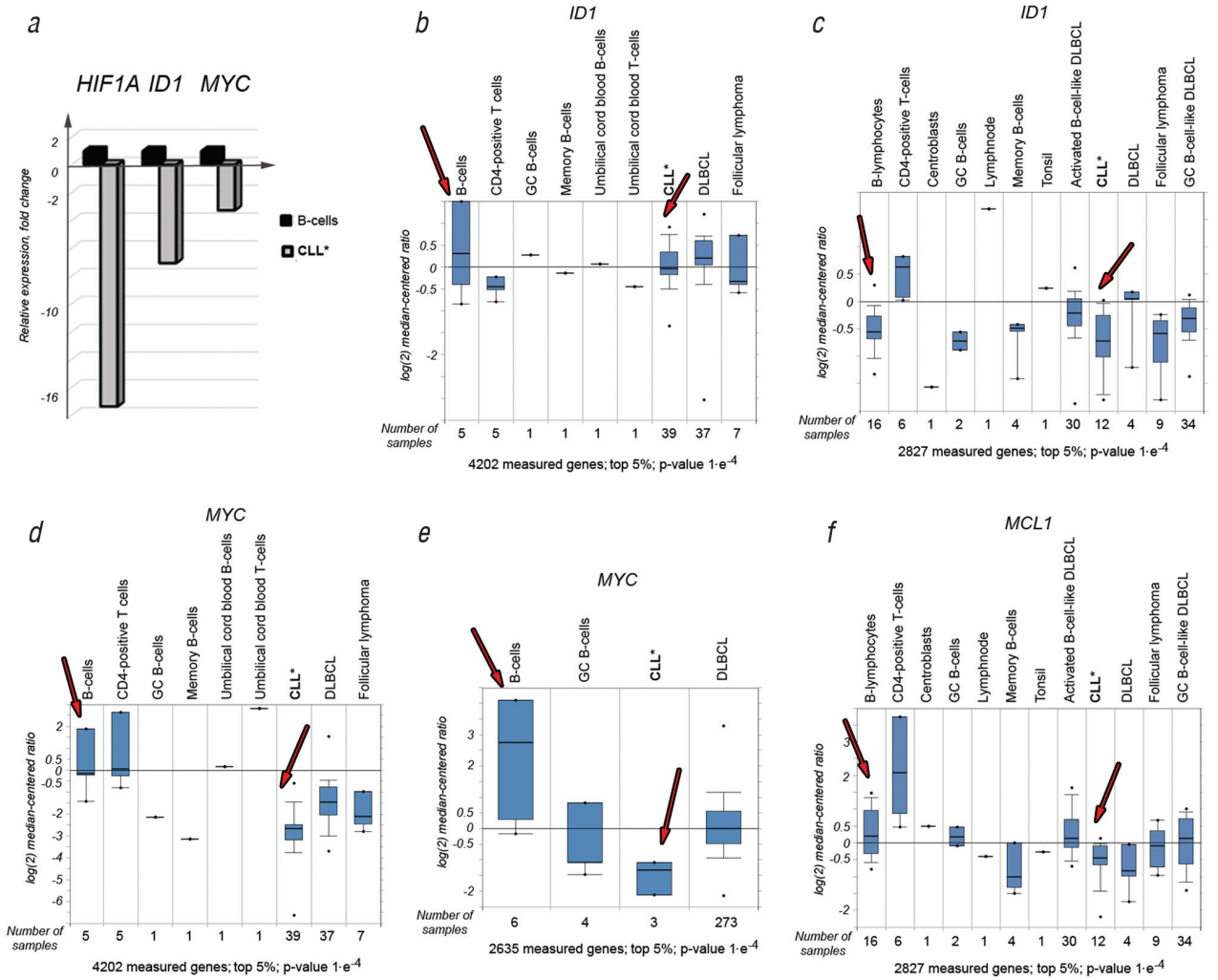

Fig. 2. Expression levels of STAT5-responsive genes: *a* — relative expression at the mRNA level, assessed by q-PCR; *b* — the *ID1* gene expression at the mRNA levels, assessed by microarray technique [19]; *c* — the *ID1* gene expression at the mRNA levels, assessed by microarray technique [20]; *d* — the *MYC* gene expression at the mRNA levels, assessed by microarray technique [19]; *e* — the *MYC* gene expression at the mRNA levels, assessed by microarray technique [21]; *f* — the *MCL1* gene expression at the mRNA levels, assessed by microarray technique [20]

clin D1 have not changed at the same time (Fig. 3, *c, d*, *e*, respectively).

Next question was asked, what is the status of *STAT* genes in CLL cells of a studied cohort of patients. We have found, that expression of *STAT* genes was not altered, except for up-regulation of *STAT2* and *STAT5A* and dramatic down-regulation of *STAT4* (Fig. 4, *a*). Noteworthy, similar trend for *STAT5A* and *STAT5B* was reported earlier by [20] (Fig. 4, *b*, *c*). Importantly, mRNA levels of *STAT5A* and *STAT5B* are quite high in many lymphoid organs and in tumor cell lines of B-cell origin (Fig. 5).

Taking into consideration, that STAT proteins function as transcription factors only as heterodimers of phosphorylated proteins [22], the down-regulation of the responsive genes could be due to lack of phosphorylation, absence of *IL-2RA* expression, or no IL-2 in the peripheral blood.

In order to monitor the levels of IL-2RA mRNA, we analyzed the publicly available databases. *IL-2RA* expression was quite high in many lymphoid tumors, as well as in lymphoid organs and blood (Fig. 6, *a*, *b*). Moreover, CLL cells and peripheral blood B-cells demonstrated similar *IL-2RA* expression (Fig. 6, *c*, *d*).

As was mentioned above, in the serum of CLL patient the concentration of soluble IL-2RA was 100 fold higher, compared to the blood of healthy individuals [16]. It is reasonable to conclude, that even a special set of T-cell secrets IL-2, this cytokine is inactivated by sIL-2RA, and CLL cells do not get a signal to differentiate into plasma cells, or antibody producing cells. Therefore, we propose that inactivation of the JAK-STAT5 pathway might be due to lack of free IL-2 (Fig. 7), or due to inability of STAT proteins to become phosphorylated and form heterodimers. The latter will be a subject of our future work.

CONCLUSIONS

We have found that the JAK-STAT5 pathway is largely inactivated in CLL cells. Despite elevated expression of *STAT2* and *STAT5* genes at the mRNA level, STAT5-responsive genes, such as *BCL2L1 (BCL-XL)*, *CCND2*

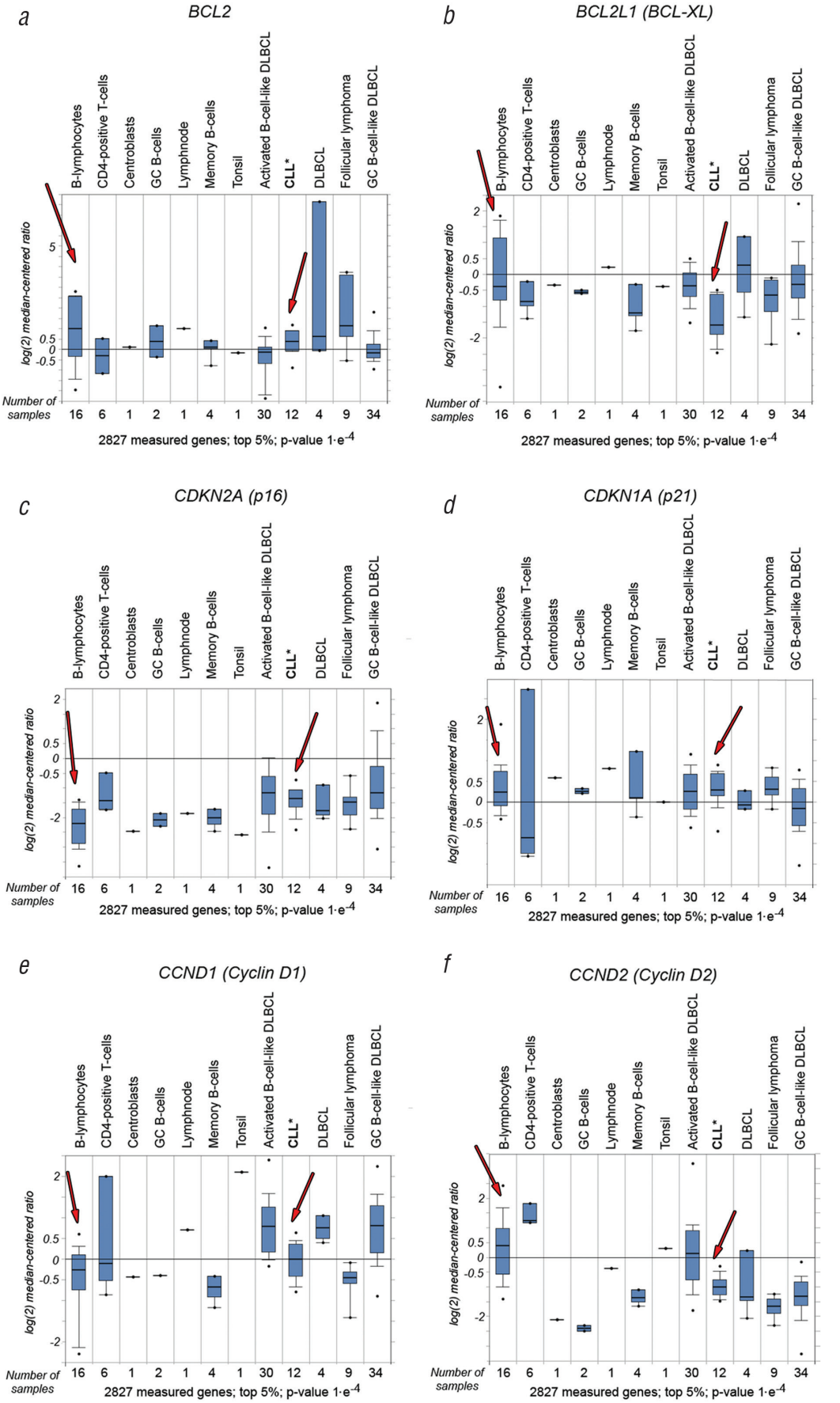

Fig. 3. Expression levels of *BCL2* (*a*), *CDKN2A* (*c*), *CDKN1A* (*d*), *CCND1* (*e*) and STAT5-responsive genes *BCL2L1* (*b*) and *CCND2* (*f*), assessed by microarray technique [20]. Notice the significant down-regulation of STAT5-responsive genes, *BCL2L1* and *CCND2*

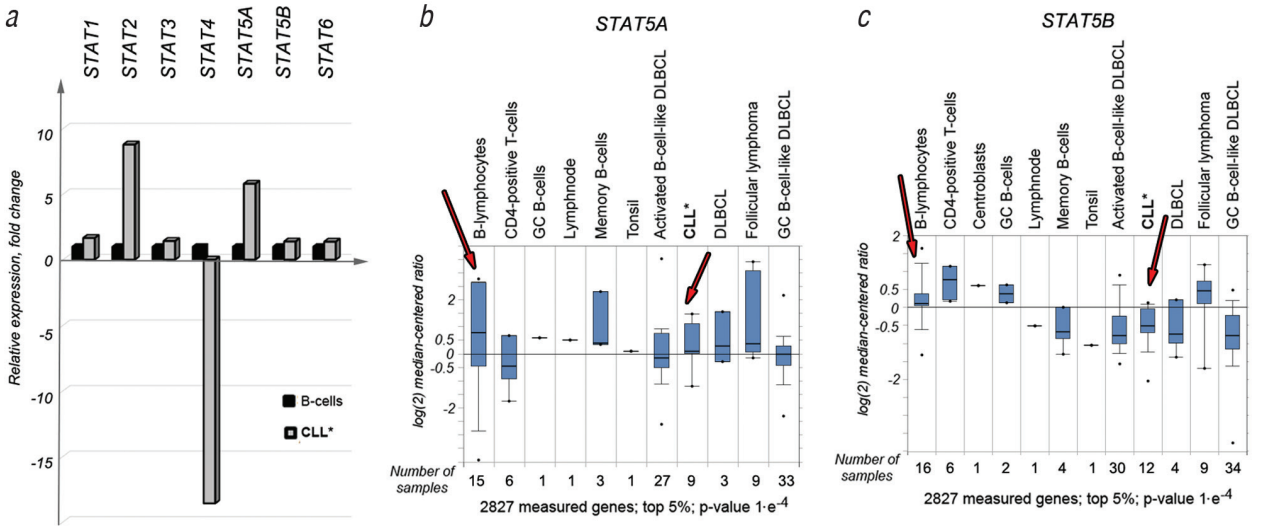

Fig. 4. Relative expression levels of *STAT* genes at the mRNA level, assessed by q-PCR (*a*). *STAT5A* (*b*) and *STAT5B* (*c*) expressed at the similar levels in CLL cells and B-lymphocytes, as was shown by the microarray technique [20]

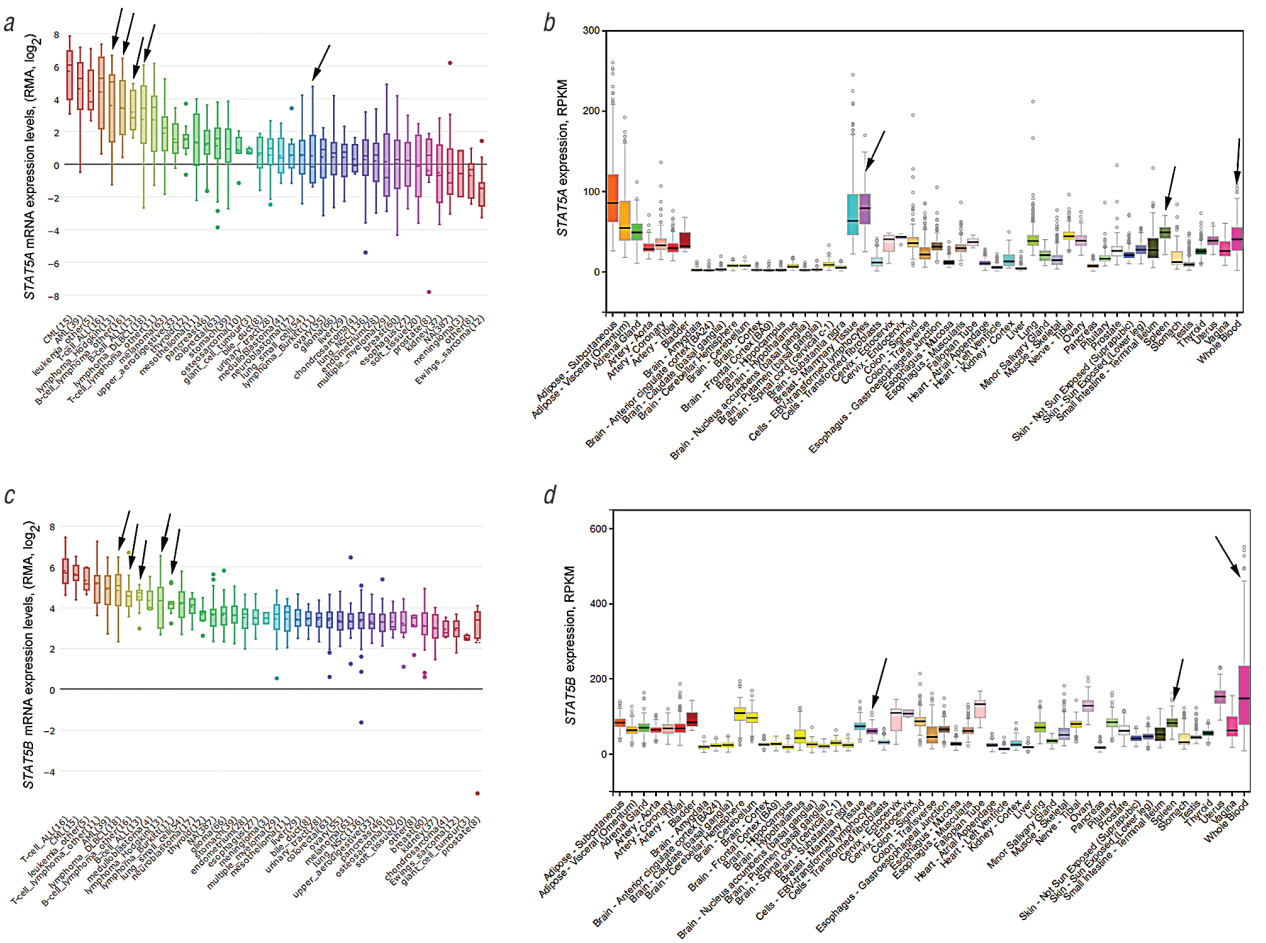

Fig. 5. Relative expression levels of *STAT5A* (Entrez Gene ID: 6776) (*a*) and (*b*) and *STAT5B* (Entrez Gene ID: 6777) (*c*) and (*d*) genes at the mRNA level. An analysis of expression in B-cell originated tumor cell lines (indicated with arrows on (*a*) and (*c*)) was performed on September 15th, 2017 at https://portals.broadinstitute.org/ccle. RMA — Robust multichip averaging, relative units of the signal intensity. Noteworthy, the high levels of the *STAT5A* were detected in whole blood and spleen, as well as in EBV-transformed B-lymphocytes (indicated with arrows on (*b*)), as was assessed on September 15th, 2017 at http://www.gtexportal.org/home. Simultaneously, the highest levels of *STAT5B* were detected in whole blood (indicated with arrows on (*d*)). RPKM — read per kilobase per million mapped reads, normalized to the gene length

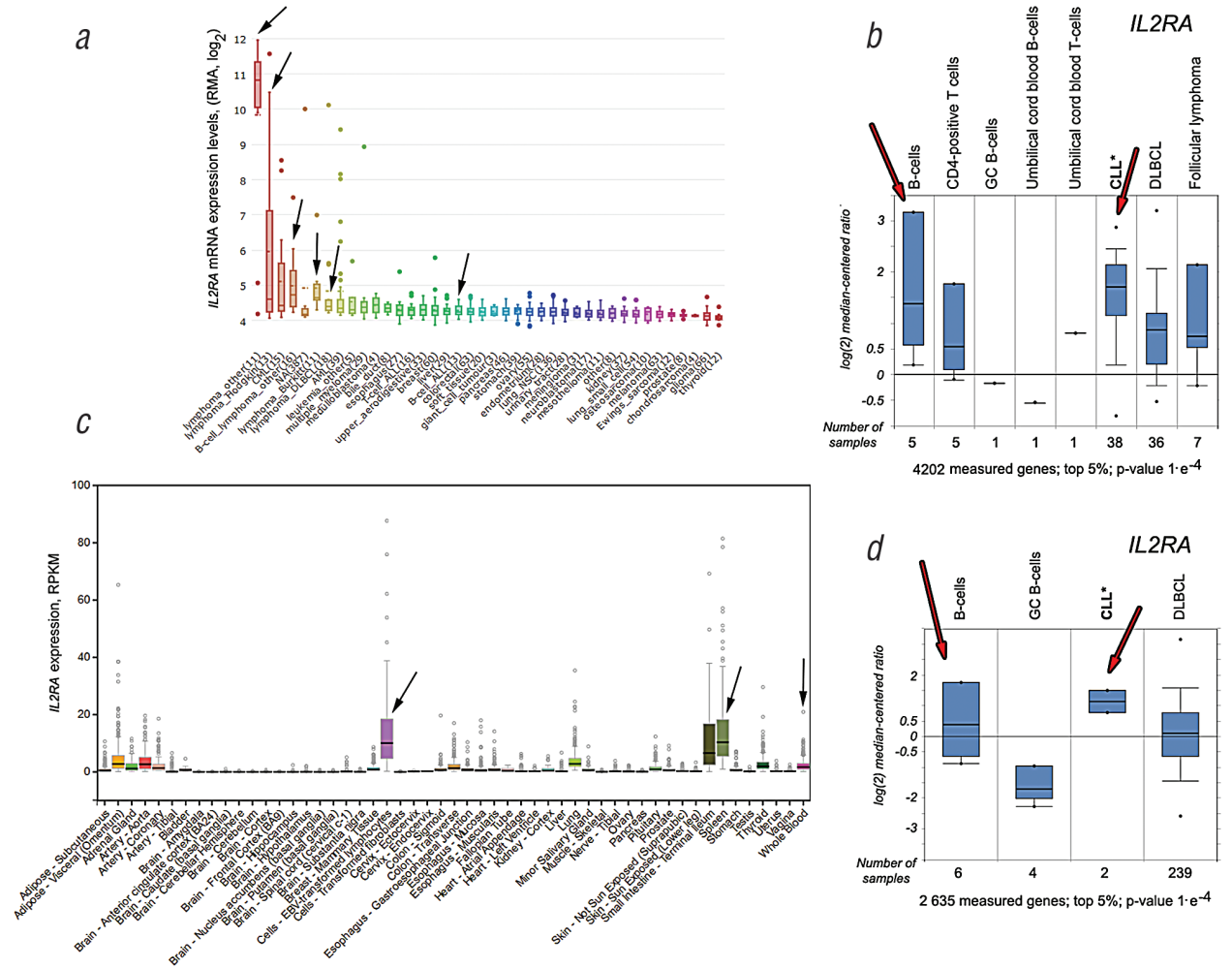

Fig. 6. Relative expression levels of *IL-2RA* (Entrez Gene ID: 3559) at the mRNA level. An analysis of expression in B-cell originated tumor cell lines (indicated with arrows on (*a*)) was performed on September 15th, 2017 at https://portals.broadinstitute.org/ccle. RMA — Robust multichip averaging, relative units of the signal intensity. The high levels of the *IL-2RA* were detected in spleen and in EBV-transformed B-lymphocytes (indicated with arrows on (*c*)), as was assessed on September 15th, 2017 at http://www.gtexportal.org/home. RPKM — read per kilobase per million mapped reads, normalized to the gene length. *IL-2RA* expressed at the similar levels in CLL cells and B-lymphocytes, as was shown by the microarray technique (*b*) — analysed data from [19], *d* — analysed data from [21]

(Cyclin D2), *HIF1A*, *ID1*, *MCL1,* and *MYC* are down-regulated in CLL-cells, compared to peripheral blood B-cells of healthy individuals.

The inactivation of the JAK-STAT5 pathway could be explained by the high levels of soluble IL-2RA, as was reported earlier. Another possibility could be inhibition of STAT5 phosphorylation, leading to inability to form the active transcriptionally protein heterodimers. The phosphorylation status of STAT proteins in CLL cells should be further illuminated.

ACKNOWLEDGMENTS

We thank Professor Danylo Gluzman (R.E. Kavetsky IEPOR of NAS of Ukraine) for the fruitful discussions over this manuscript.

This work was supported by the Swedish Cancer Society, by matching grants from the Concern Foundation (Los Angeles, US) and the Cancer Research Institute (New York, US), and by the Ministry of Education and Science of Ukraine (No. 0116U005456).

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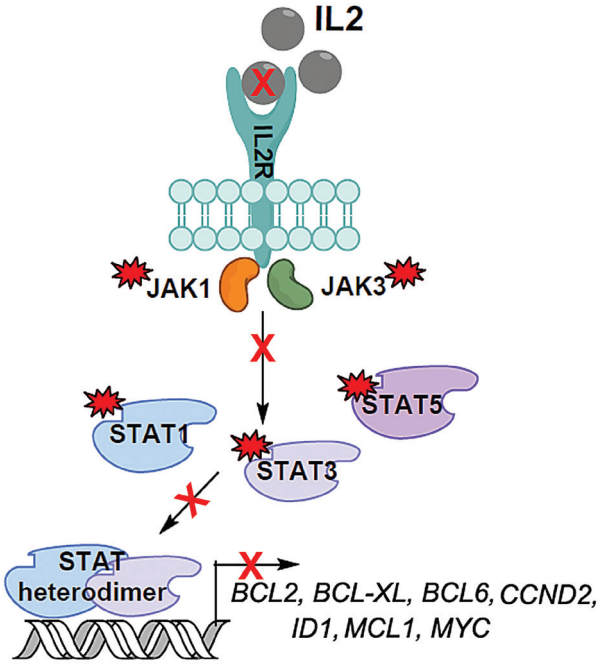

Fig. 7. A schematic view on an IL-2-STAT5 pathway. The IL-2 molecule binds to IL-2R, and JAKs kinases are phosphorylated. Then, JAKs phosphorylate STAT proteins, which can form heterodimers. These heterodimers bind to a specific DNA sequence and activate transcription of the responsive genes. A set of STAT5 responsive genes (*BCL2L1, MYC*, *ID1*, *MCL1*, *CCND2*) showed diminished expression, despite high expression of *STAT5A* and *STAT5B* in CLL cells, compared with peripheral blood B-cells. This might be due to lack of STAT and/or JAK phosphorylation, or low concentration of available IL-2 in serum of CLL patients. The scheme was prepared with the help of a ChemOffice® Professional 17 free trial copy

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СИГНАЛЬНИЙ ШЛЯХ IL-2-STAT5 ЗАБЛОКОВАНО У КЛІТИНАХ ХРОНІЧНОГО ЛІМФОЛЕЙКОЗУ

А. Матвєєва, Л. Ковалевська, А. Поліщук, О. Кашуба

Резюме. ***Мета:*** *вивчити стан шляху інтерлейкіну-2 (IL-2) у клітинах хронічного лімфоцитарного лейкозу (ХЛЛ), щоб зрозуміти функціональні відмінності між ХЛЛ і зрілими В-клітинами.* ***Об'єкти і методи:*** *зразки периферичної крові хворих на ХЛЛ, ізоляція РНК, аналіз експресії транскрипційних факторів із використанням аналізу плати RT2 та кількісної полімеразної ланцюгової реакції, біоінформатичний аналіз загальнодоступних баз даних щодо експресії генів.* ***Результати:*** *виявлено, що сигнальний шлях JAK-STAT5 переважно інактивований в клітинах ХЛЛ. Незважаючи на підвищену експресію генів STAT2 та STAT5 на рівні мРНК у клітинах ХЛЛ експресія генів, які трансактивуються STAT5, таких як BCL2L1 (BCL-XL), CCND2 (циклін D2), HIF1A, ID1, MCL1 та MYC, знижена порівняно з цим показником у В-клітинах периферичної крові здорових донорів.* ***Висновки:*** *інактивація шляху JAK-STAT5 може бути пояснена високим рівнем розчинного IL-2RA, як було показано раніше. Також є можливим інгібування фосфорилювання протеїну STAT5, що робить неможливим утворення гетеродимерів білків, які є транскрипційно активними. Стан фосфорилювання білків STAT у клітинах ХЛЛ потребує додаткового вивчення.*

Ключові слова: В-клітинний хронічний лімфоцитарний лейкоз (ХЛЛ), інтерлейкін-2 (IL-2), CD25, STAT, фактори транскрипції, шлях IL-2-STAT5.

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Submitted: October 27, 2017