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THE TGF-BETA — SMAD PATHWAY IS INACTIVATED IN CRONIC LYMPHOCYTIC LEUKEMIA CELLS

*A. Matveeva[1], L. Kovalevska[1], I. Kholodnyuk[2], T. Ivanivskaya[1], E. Kashuba[1, 3, *]*

[1]*R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine*

[2]*A. Kirchenstein Institute of Microbiology and Virology, Riga Stradins University (RSU), Riga LV-1067, Latvia*

[3]*Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Box 280, Stockholm S-17177, Sweden*

***Aim*: To study the status of the tumor growth factor beta (TGFB) pathway in chronic lymphocytic leukemia (CLL) cells and to uncover molecular details underlying CLL cell genesis. *Objects and Methods*: The study was conducted on peripheral blood samples of patients with CLL using the following methods: RNA isolation, analysis of expression of transcription factors using RT2 profiler assay, bioinformatics analysis of publicly available data bases on expression. *Results*: We have shown that the TGFB — SMAD canonical pathway is not active in CLL cells. SMAD-responsive genes, such as *BCL2L1 (BCL-XL)*, *CCND2 (Cyclin D2),* and *MYC,* are down-regulated in CLL cells compared with peripheral blood B cells of healthy donors. *Conclusions*: The TGFB-mediated signaling is not active in CLL cells due to low (or absent) expression of *SMAD1, -4, -5, -9,* and *ATF-3*. Expression and phosphorylation status of SMAD2 and -3 should be further elucidated in the future studies.**

***Key Words*: B-cell chronic lymphocytic leukemia, tumor growth factor beta, SMAD proteins, TGFB — SMAD pathway.**

Chronic lymphocytic leukemia (CLL) is represented by small B-lymphocytes, accumulating in bone marrow, spleen, lymph nodes and other tissues. B-cell count in peripheral blood in CLL can reach very high numbers, more than $5–10 \cdot 10^6$/ml [1]. Noteworthy, CLL cells express various surface markers, characteristic for mature and/or naïve B cells, such as B-cell receptor (BCR), CD5, CD19, CD23 antigens, IgM/IgD *etc*. [2]. However, in contrast to normal B cells, CLL cells can not be activated. Moreover, they are incapable to differentiate into plasma cells and do not produce antibodies [3]. Therefore, it is widely accepted that CLL cells are immunologically incompetent mature B-lymphocytes.

From other hand, CLL cells express a set of cytokine receptors, namely interleukin receptors, — IL-2R, -4R, -6R, -10R, -13R [4–7], and also receptors for tumor necrosis factor alpha (TNFA) [5, 8], interferon alpha (INFA) [9] and gamma (INFG) [10] and also for tumor growth factor beta (TGFB) [11]. We have to mention, that CLL cells, despite being non-proliferating, secrete still many cytokines that are described above.

One of the most important cytokines is TGFB, which has a dual role in carcinogenesis [12]. In lymphoid cells the active canonical TGFB pathway leads to apoptosis. Hence, it was shown that activation of the TGFB pathway resulted in induction of pro-apoptotic *BMF*, *BIM*, and, as consequence, *BAX* [13]. In Burkitt's lymphoma cells, anti-apoptotic *BCL2L1 (BCLXL)* and *BCL2* were downregulated upon the TGFB-pathway activation [14]. However, in CLL cells, levels of *BCL2* did not differ from normal peripheral blood B cells.

We asked a question, what is the status of the TGFB pathway in CLL cells, with the aim to uncover molecular details underlying CLL pathogenesis.

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*Correspondence: E-mail: lenakash@yahoo.com; Elena.Kashuba@ki.se

Abbreviations used: ATF – activating transcription factors; BCR – B-cell receptor; CD – cluster of differentiation; CLL – chronic lymphocytic leukemia; IL – interleukin; ILR – interleukin receptor; INF – interferon; INFA – interferon alpha; INFG – interferon gamma; TNF – tumor necrosis factor; TNFA – tumor necrosis factor alpha; TGF – tumor growth factor; TGFB – tumor growth factor beta.

MATERIALS AND METHODS

Clinical specimens. Samples of the peripheral blood from patients were obtained at the Department of Oncohematology of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of NAS of Ukraine (in the period 19.11.2014–31.12.2016). The study included 38 patients. Among them, CLL was diagnosed in 29 individuals, 4 persons had CLL of the mixed type, 1 patient had B-cell lymphoma, 1 — marginal zone lymphoma and 3 patients had T-cell lymphoma. The non-CLL cases will be analyzed individually in the future studies. The main types and cytological variants of lymphoid tumors were diagnosed, according to the WHO classification [15]. Mononuclear cells were isolated from peripheral blood, using centrifugation with the Ficoll-Paque density gradient media. The cell content was usually high (80–99% of B-cells in a population of mononuclear cells in patients with CLL).

For control, B lymphocytes were isolated from the peripheral blood of 3 healthy donors, using the Ficoll-Paque density gradient media to separate mononuclear cells with the following "rosetting" of T-cells by erythrocytes of sheep blood.

All experimental work was performed, according to the protocols that have been approved by the Committee on Bioethics at IEPOR. Also, all patients were thoroughly informed about the study.

RNA isolation and cDNA synthesis. CLL cells were isolated from peripheral blood of patients by gradient centrifugation using the Ficoll-Paque density gradient media.

Cells were resuspended in TRIzol reagent (GibcoBRL, 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 2 μg of total RNA, M-MLV Reverse Transcriptase and RNAse inhibitor (Invitrogen, USA), according to the manufacturer's protocol.

Analysis of the transcription factors expression using RT2 profiler assay. The expression profile of 84 transcription factors was determined in duplets using two identical PARN-075Z plates. Along with positive and negative controls, a total of 96 genes were analyzed. The RNA mixture consisted of 25 μl (a half) of each RNA solution isolated from the sample. The control represented a mixture of RNAs isolated from the peripheral blood B cells of three healthy donors. q-PCR was performed, using 2 μg cDNA and the SYBR Green Master Mix (Thermo Fisher Scientific Inc., USA), on the PCR System 7500 (Applied Biosystem, USA). CT was not measured after 35 cycles. The obtained CT values were downloaded into the manufacturer's website (Thermo Fisher Scientific Inc., USA) for the online analysis of the transcription factors expression.

Bioinformatic data analysis. In order to analyze expression of genes at the mRNA level, a publically available data base Oncomine was used. This data base contains published data that have been collected, standardized, annotated and analyzed by Compendia Bioscience (www.oncomine.com, September 2017, Thermo Fisher Scientific, Ann-Arbor, MI, USA).

RESULTS AND DISCUSSION

A PARN-075Z platform was used to assess expression of 84 various transcription factors. A mixture of RNAs isolated from the peripheral blood cells of 38 patients was used to perform these experiments, in comparison with the RNAs mixture isolated from the peripheral blood B-lymphocytes of three healthy individuals. We have shown that the majority of TFs were downregulated, in patient mononuclear (presumably CLL) cells [16]. For example, both *MYC* and *NFkB1* genes were downregulated 3.26 and 4.23 fold, respectively. We also found that many genes, which are usually induced upon activation of the IL-2 — STAT5 pathway (Matveeva et al., 2017, submitted), were downregulated in CLL cells. We have shown that *ID1*, *HIF1A, MCL1*, *BCL2, BCL2L1,* and *Cyclin D2* (*CCND2*) genes are expressed in CLL cells at lower levels than in B cells of healthy donors. These data suggest that the IL-2 — STAT5 pathway is inactivated in CLL cells, probably, due to the lack of STAT phosphorylation.

Among other pathways that are crucial for regulation of B cell fate, very important is the TGFB signaling. Noteworthy, activation of the TGFB pathway *in vitro* did not lead to increased apoptosis in CLL cells, in contrast to the peripheral blood B cells [17]. Moreover, TGFB is secreted by CLL cells and could be measured in patient blood sera. Of note is that the TGFB receptors (*TGFBR*) are expressed at the similar level in CLL cells and peripheral blood B-lymphocytes.

It is known that the active TGFB ligand binds to homodimers of receptors, TGFBRI and TGFBRII [12]. A tetramer of these receptors is formed then and auto-phosphorylated TGFBRII can transfer a phosphate group to TGFBRI starting the signal transduction through SMAD proteins (Fig. 1 , *a*, *b*).

SMADs are homologs of *D. melanogaster* proteins, named Mothers against decapentaplegic (MAD). It was shown that mutations in *MAD* led to repression of the gene called *Decapentaplegic* (*Dpp*) in a fruit fly embryo. *Dpp* is a morphogen and is responsible for correct growth of many tissues in drosophila.

The SMAD protein family consists of at least 8 members. SMAD1, -2, -3, -5, and -8 are regulatory proteins (R-SMADs) that could be phosphorylated [18]. SMAD8 is encoded by the *SMAD9* gene. Importantly, only SMAD2 and SMAD3 function as R-SMADs in the canonical TGFB pathway (Fig. 1, *c*).

SMAD4 (also called co-SMAD) forms the complexes with phosphorylated SMAD2 and SMAD3 (Fig. 1, *d*). These heterodimers, containing SMAD4, are imported to a nucleus, where they bind to DNA, together with

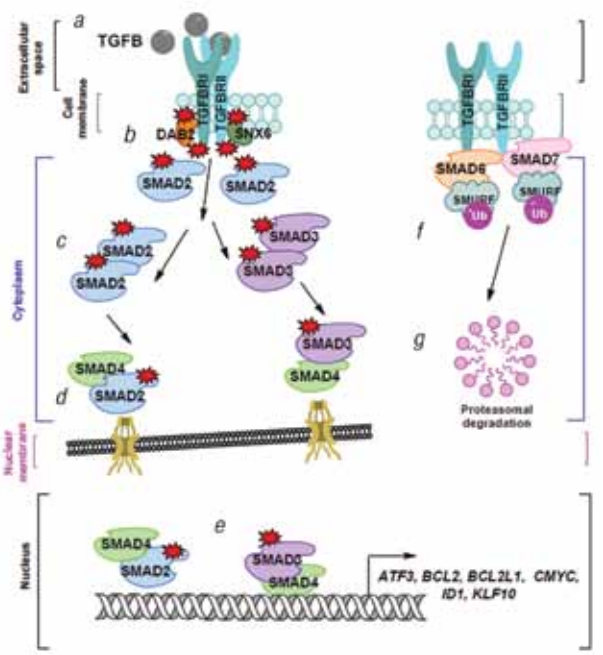

Fig. 1. An oversimplified scheme depicting the TGFB pathway. The active TGFB ligand binds to homodimers of receptors, TGFBRI and TGFBRII (*a*). A tetramer of these receptors is formed then and auto-phosphorylated TGFBRII can transfer a phosphate group to TGFBRI (*b*). SMAD2 and SMAD3 are phosphorylated then (*c*). SMAD4 forms the complexes with the phosphorylated SMAD2 and SMAD3 (*d*). These heterodimers are imported to a nucleus. They bind to DNA and transactivate a set of responsive genes (*e*). SMAD6 and SMAD7 can bind to a SMAD-specific E3 ubiquitin ligase SMURF (*f*). Together, they form complexes with TGFBR and that results in proteasomal degradation of the latter (*g*). The scheme was prepared with the help of a ChemOffice® Professional 17 free trial copy

other co-factors, and transactivate (or, alternatively, repress) a set of responsive genes, namely *ATF3*, *ID1*, *CMYC*, etc (Fig. 1, *e*).

SMAD6 and SMAD7 are the inhibitory SMADs (I-SMADs). They can bind to a SMAD-specific E3 ubiquitin ligase SMURF (Fig. 1, *f*). Together, they form complexes with TGFBR that results in proteasomal degradation of the latter [18] (Fig. 1, *g*).

Taking into consideration that the TGFB pathway might be inactivated in CLL cells, we assessed expression of several SMADs in CLL cells in comparison with the peripheral blood B cells. We have found that all the studied *SMAD*s (*SMAD1*, *-4*, *-5*, and *-9*) were downregulated at the mRNA level in CLL in comparison with B cells (Fig. 2, a left panel). Noteworthy, *SMAD1* and *SMAD9* were hardly detectable; their expression was lower in CLL cells up to 20 folds (18.2 and 18.1, respectively). *SMAD4* expression was diminished 1.6 fold and *SMAD5* — 2.8 fold. It is obvious to conclude that the alternative TGFB pathway, where SMAD1, -5, and -6 play an important role, could be inactivated due to low expression of these regulatory molecules.

To corroborate our results, we have analyzed a set of the experimental data that have been published earlier and deposited to the Oncomine portal. *SMAD1*, *-2*, -5, *-6*, and *-9* are expressed at very low levels in B cells (Fig. 3). Importantly, their expression at mRNA levels diminished further in CLL cells: almost 7 fold for *SMAD1* [19], 2.5 fold for *SMAD2* [20], 2.5 fold for *SMAD6* [21] and 2 fold for *SMAD9* [19]. The *SMAD5* levels were similar in B and CLL cells, according to [21].

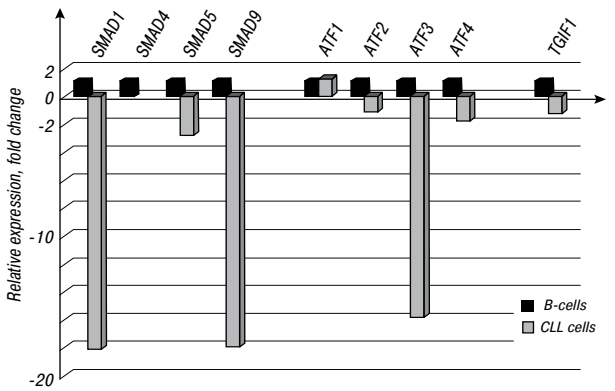

Fig. 2. Relative gene *mRNA* expression assessed by q-PCR. An expression study was performed for two RT^2 plates. Median values are presented here. The standard deviation is no more than 30% of the median value. Expression of *SMAD*s is shown on the left panel; *ATF*s — on the middle; *TGIF1* — on the right. BL — Burkitt's lymphoma; GC — germinal center; DLBCL — diffuse large B-cell lymphoma

SMAD3 and *SMAD4* showed higher signals in B cells, compared with other *SMAD*s, but their levels dropped dramatically in CLL cells as well: 6 fold for *SMAD3* [19] and 2.4 fold for *SMAD4* [21]. Importantly, these data are in concordance with the obtained in our study results. Thus, we were expecting that SMAD responsive genes will be not induced.

As was mentioned above, expression of *BCL2L1*, *CMYC,* and *ID1* genes were diminished in CLL cells compared with the peripheral blood B cells of healthy

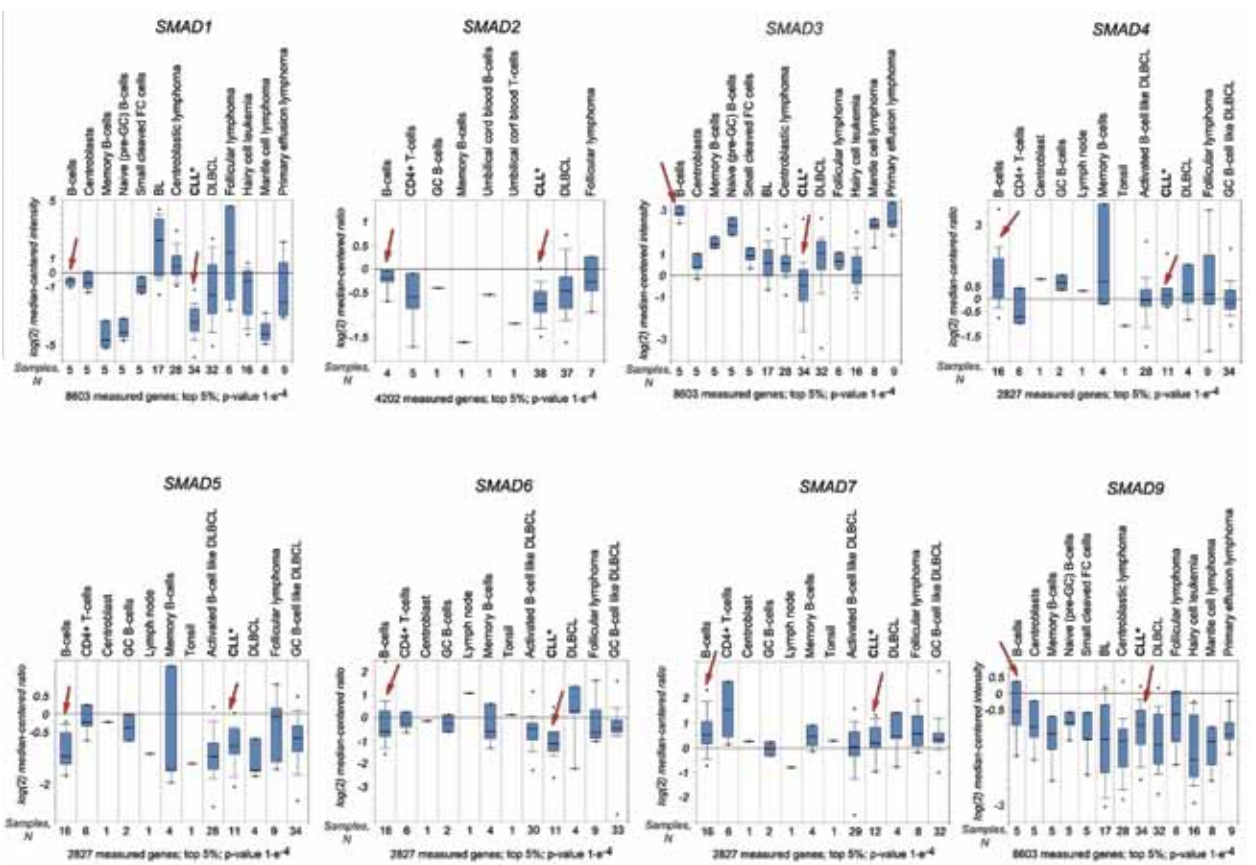

Fig. 3. Expression levels of *SMAD*s assessed by microarray technique. Note the significant down-regulation of the studied genes (indicated by arrows): *SMAD1* [19], *SMAD2* [20], *SMAD3* [19], *SMAD4, SMAD5*, *SMAD6, SMAD7* [21], and *SMAD9* [19]. BL — Burkitt's lymphoma; GC — germinal center; DLBCL — diffuse large B-cell lymphoma

donors (Matveeva et al., 2017, submitted). Expression of other SMAD-induced genes were also studied (Fig. 2, 4 and 5). Thus, members of a family of activating transcription factors (ATF), namely *ATF3* and *ATF4,* were downregulated in CLL cells: *ATF3* — 15.9 fold and *ATF4* — 1.8 fold. Expression of *ATF1* was similar in CLL and B cells, and *ATF2* was diminished in CLL cells; however, the differences were not significant (Fig. 2, the middle panel). Importantly, *ATF3* should be induced by the active TGFB — SMAD pathway. ATF3 can bind to SMAD2 and SMAD3, resulting in repression of the *ID1* transcription [22]. However, in our case, induction of *ATF3* was not detected. Moreover, the analysis of the Oncomine data base corroborated our results (Fig. 4). Thus, the *ATF3* expression was 6 fold (median value) lower in CLL.

No big difference in expression of the TGFB-induced factor (*TGIF1*) was found in CLL cells (approximately 2 fold) (Fig. 2, the right panel). *TGIF1* encodes another co-repressor of the SMAD2/3-dependent transcription [23].

Of note, the levels of the TGFB-inducible early gene (*TIEG,* also called *KLF10*) were downregulated dramatically in CLL cells versus normal B cells (Fig. 5). Thus, expression was diminished 17 fold comparing with the published data [19, 20]. We have to mention that overexpression of KLF10 in pancreatic cancerous cells resulted in their apoptosis [24]. Importantly, it was shown that expression of *KLF10* is quite high in spleen and peripheral blood leukocytes compared to other tissues [25].

Thereby, many TGFB — SMADs responsive genes are not induced in CLL cells. It can be due to the low expression of SMADs or the lack of their phosphorylation.

CONCLUSIONS

We have found that the TGFB — SMAD2/3 pathway is not active in CLL cells. Expression of the *SMAD* genes were diminished in CLL cells compared with the peripheral blood B cells. Moreover,

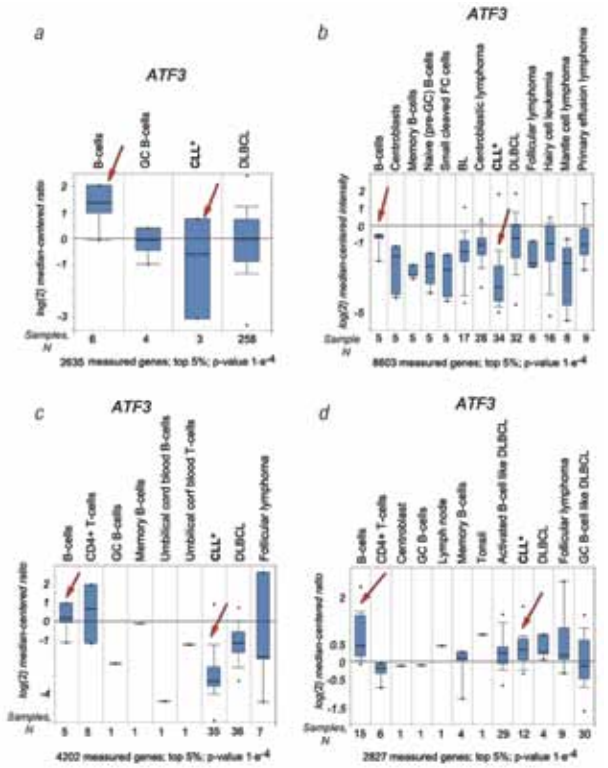

Fig. 4. Expression levels of *ATF3* assessed by microarray technique. Note the significant down-regulation of the *ATF3* gene (indicated by arrows) in the studies [26] (*a*), [19] (*b*), [20] (*c*), and [21] (*d*). BL — Burkitt's lymphoma; GC — germinal center; DLBCL — diffuse large B-cell lymphoma

the TGFB — SMAD-responsive genes, namely *BCL2L1 (BCL-XL)*, *CCND2 (Cyclin D2)*, *ID1*, *MYC, ATF3, TGIF1,* and *KLF10* (*TIEG*), are down-regulated in CLL cells as well.

Why the TGFB — SMAD2/3 pathway is inactivated, it is an open question. Beside low expression of SMADs, there might be the lack of the SMAD2 and SMA3 phosphorylation involved. Expression and the phosphorylation status of SMAD2 and -3 should be further elucidated in the future studies.

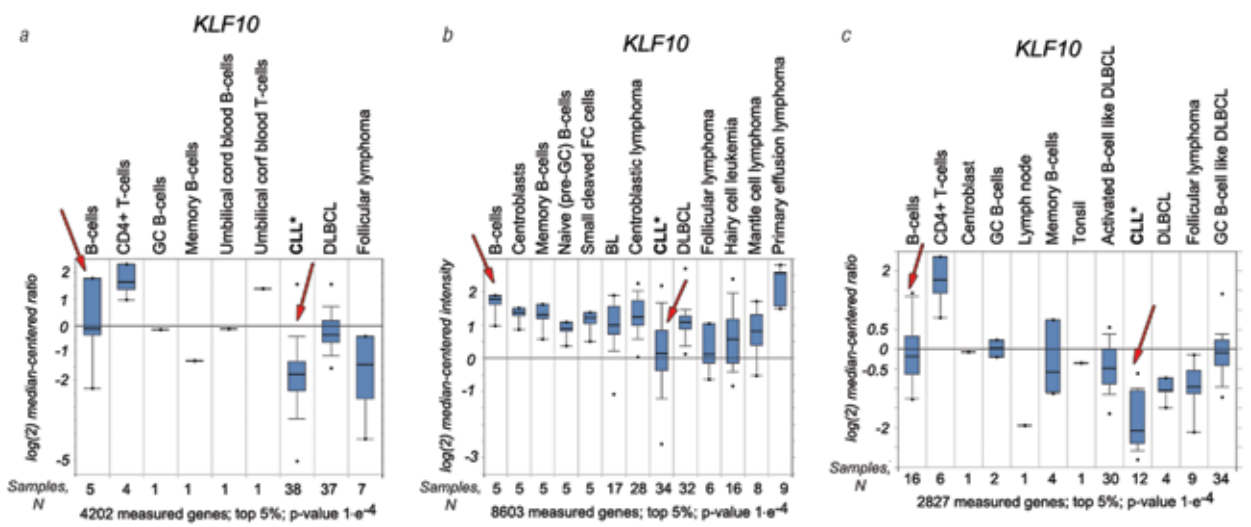

Fig. 5. Expression levels of *TIEG (KLF10)* assessed by microarray technique. Note the significant down-regulation of the *KLF10* gene (indicated by arrows) in the studies [20] (*a*), [19] (*b*), and [21] (*c*). BL — Burkitt's lymphoma; GC — germinal center; DLBCL — diffuse large B-cell lymphoma

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