Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent neoplastic disorder in children, that was a virtually incurable disease before the 1960-ies. These days, about 90% of children with ALL experience long-term survival in industrialized high-income countries using fine-tuned risk-tailored combined chemotherapy protocols. The Hungarian national group, including the Debrecen Center is working within the frame of the 'ALL Intercontinental BFM' (ALLIC) consortium. ALLIC is a network of national groups and single centers of the international Berlin-Münster-Frankfurt Study Group (iBFM-SG) with restricted resources. ALLIC members do not have a regular access to the most advanced molecular technologies required from participants of recent ALL clinical trials [*Hunger SP. J Clin Oncol 2014;32:169-170.; Acute Lymphoblastic Leukaemia Committee (ALL) – International BFM Study Group. Available at: https://bfminternational.wordpress.com/structure/committees/acute-lymphoblastic-leukaemia-committee-all*]. Yet, within the frames of the ALL IC-BFM 2002 clinical trial ALLIC consortium demonstrated successful co-operation of participating national groups and centers from more than one continent resulting in competent event-free survival (EFS) and overall survival (OS) rates which exceeded historical results of the members of the consortium [*Stary J. et al. J Clin Oncol 2014;32:174-84*]. However, toxic mortality rates in ALL IC-BFM 2002 clinical trial were higher than in parallel running clinical trials of the Children's Oncology Group (COG) and other BFM consortia. This fact highlights the need for the identification of further potent biomarkers allowing a more precise stratification of risk groups among children with ALL. WHO classifies subtypes of ALL according to recurrent genetic alterations whenever this type of information is available [*Arber DA et al. Blood 2016;127:2391-2405*]. A broad spectrum of chromosomal abnormalities, submicroscopic structural genetic alterations and sequence mutations gained a role as prognostic and predictive biomarkers in childhood ALL, in addition to conventional risk factors, such as age, sex, initial white blood cell count (WBC), and response-to-treatment measures [*Inaba H et al. Lancet 2013;381:1943-55.; Moorman AV. Haematologica 2016;101:407-16.; Iacobucci I et al. J Clin Oncol 2017;35:975-83.*]. Cell surface and intracellular proteins present in the leukemic cells may provide additional valuable prognostic biomarkers, contribute to improved minimal residual disease (MRD) detection, and offer therapeutic targets [*Mirkowska P et al. Blood 2013;121:e149-e159.; Sedek L et al. J Immunol Methods 2018. doi:10.1016/j.jim.2018.03.005.*]. Identification of novel leukemia-associated protein biomarkers is of special interest to ALLIC members. The ALLIC consortium developed a multiparameter flow cytometry (FC)-based MRD detection method as a pilot project of the ALL IC-BFM 2002 clinical trial [*Mejstrikova E et al. Pediatr Blood*

Cancer 2010;54:62-70.]. Result of mid-induction, day 15 bone marrow (BM) FC-MRD was incorporated in the risk assessment approach of the next ALLIC clinical trial, ALL IC-BFM 2009 (EuDract: 2010-019722-13, unpublished data). ALLIC FC laboratories became members of the International BFM Flow-network and participate in the internal quality control program developed for the network and ALLIC FC laboratories [*Avgeris M et al. Clin Chem Lab Med 2018. [Epub ahead of print] doi:10.1515/cclm-2018-0507.; St. Anna Children's Cancer Research Institute Science Report 2009-2010. available at: http://science.ccri.at/fileadmin/content/3Research/6Scientific_Reports/CCRI_ScienceReport_2009-2010.pdf*].

The present investigations were based on the historical results of ALLIC-BFM 2002 clinical trial and were performed as pilot projects of the BFM ALL-IC 2009 randomized, prospective multi-center clinical trial. BFM ALL-IC 2009 has been started with the institutional sponsorship of the University of Debrecen in 2010 in some of the ALLIC member of national group and in 2011 in Hungary [***Kiss C., Kovács G., Nagy AC., Gáspár I., Kappelmayer J., Zimmermann M., Campbell M. Az MRD-alapú nemzetközi, multicentrikus BFM ALL-IC 2009 klinikai tanulmány akut lymphoblastos leukaemiás gyermekek kezelésében elért előzetes eredményei Hematológia-Transzfuziológia. 2015; 48:42***.]. The study had to face hardships to solve. Because of the national legal regulations, the Polish group was not allowed to randomize, and they performed some pre-selected arms of the clinical trial and could not be included in the analysis of randomized trial results. The Turkish and the Ukrainian national groups left the study because of financial and political hardships. The Slovak national group finished the participation in BFM ALL-IC 2009 in 2016 and joint to the AIEOP-BFM 2009 clinical trial. These changes resulted in substantial losses of patients which were enrolled in BFM ALL-IC 2009. These losses were partially compensated by the joining of the Greek national group to BFM ALL-IC 2009 in 2015. Patient enrolment was extended until March 31, 2018 in the clinical trial. Therefore, final results of the clinical trial have not yet been evaluated and published and, according to study rules, neither single institutions nor national groups are allowed to publish their own results. Pilot studies not directly related to the main study questions can be evaluated and published before the joint report of combined BFM ALL-IC 2009 data. Some results of the present research supported by the "OTKA K108885" grant will be released only after that point; however, once published, the grant support will be clearly marked in the publication.

Expression of subunit A of coagulation factor XIII (FXIII-A) does not represent one of the major study questions of BFM ALL-IC 2009. We hypothesized; however, that FXIII-A may

prove a useful prognostic and predictive biomarker in childhood B-cell progenitor (BCP) ALL and a useful parameter to define leukemia associated immunophenotype (LAIP) in this subtype of acute leukemia. Should our hypothesis prove valid, determination of FXIII-A expression by FC can be incorporated in a new ALLIC clinical trial which is going to succeed BFM ALL-IC 2009. Expression of FXIII-A can be evaluated by FC, a method available for all members of the ALLIC consortium in a validated and quality-controlled way. Previously, our group described that FXIII-A was expressed in leukemic BCP lymphoblasts in addition to platelets, megakaryocytes, monocytes and macrophages [*Kiss F et al. Thromb Haemost 2006;96:172-82.; Kiss F et al. Cytometry A. 2008;73:194-201.*].

The key questions and aims of the original grant proposal were the followings:

1. Expression of FXIII-A in BCP ALL may allow a more accurate definition of LAIP with a potential of increasing specificity and sensitivity of FC-MRD detection.
2. The level of expression of FXIII-A may be influenced by in vivo prednisone single-drug treatment.
3. FXIII-A expression may prove a new independent prognostic factor in ALL.
4. FXIII-A expression may define a new subpopulation in ALL with a potential overlap with the BCR-ABL-like subtype.

Recruitment figures and demographic and clinical characterization of the patients:

We retrospectively analyzed the results of 48 consecutive BCP-ALL cases of children between 2003 and 2011 at the University of Debrecen as well as 7 test samples of similar patients from the Borsod-Abaúj-Zemplén County Hospital and University Hospital with excess diagnostic bone marrow. The patients were treated according to BFM ALL-IC 2002 protocol. The bone marrow (BM) samples were obtained by aspiration in course of routine diagnostic interventions and were anticoagulated with EDTA. We conducted the study adhering to the Declaration of Helsinki. Written informed consent was obtained from parents/legal guardians to include these children in the BFM ALL-IC 2002 trial, which encompassed immunophenotype analyses by FC and genetic investigations. Clinical characteristic of patients is shown in Table 1 [***Kárai B., Hevessy Z., Szánthó E., Csáthy L.Ujfalusi A., Gyurina K. Szegedi I., Kapplemayer J., Kiss C. Expression of coagulation factor XIII subunite A correlates with outcome in childhood acute lymphoblastic leukemia. Pathol. Oncol. Res. 2018; 24:345–352***].

Table 1.

	ALL
Number of patients	55
Age (years)	4.7 (1.3-17.9)
Sex (female/male)	32/23
WBC (x10^9/L)	8.5 80.8-637.8)
Poor prednisone response	6 (12%)
Risk group as defined BFM ALL-IC 2002 (SR/IR/HR/NA)	18/24/9/4
Genetic risk categories (low/intermediate/poor)	34/18/3
Recurrent genetic categories t(12;21)/*TEL-AML1* high hyperdiploidy t(1;19)/*TCF3-PBX1* *MLL* translocation t(9;22)/*BCR-ABL1*	 16 (30%) 17 (32%) 5 (9%) 2 (4%) 1 (2%)
'B-other'	12 (23%)
FC risk categories (FLR/FMR/FHR/NA)	17/19/6/13

Between 2011 and 2018, we collected clinical data of 317 children with BCP-ALL treated by centers of the Polish (188), Hungarian (116) and Slovak (13) national groups of the ALLIC consortium. All patients were analyzed for FXIII-A expression by FC. All patients were screened for FXIII-A expression by FC. Of the 116 Hungarian patients with BCP ALL DNA and RNA samples of 71 patients were collected from excess diagnostic BM aspirates for targeted exon sequencing and MicroArray analyses from 5 Pediatric Hematology and Oncology Centers between 2014 and 2018. Patients were treated according to the prospective multi-center ALL IC-BFM 2009 protocol. The prospective study was approved by the Scientific Research Ethical Committee Medical Research Council of Hungary (no 43033-1/2014/EUK(423/2014)) and was performed according to the 2008 Declaration of Helsinki. Written informed consent was obtained from legal guardians of participating patients. Clinical characteristic of patients is shown in Table 2 [***Kárai B. Gyurina K., Ujfalusi A., Sedek L. Barna G., Jáksó P., Svec P., Szánthó E., Nagy AC., Kowalczyk JR., Kolenova A., Kovács TG., Szczepanski T., Kapplemayer J., Hevessy Z., Kiss C. Expression patterns of leukemic lymphoblasts for coagulation factor XIII subunite A correlate with clinical outcome and genetic subtypes in childhood acute B-cell progenitor lymphoblastic leukemia. (Submitted for publication)***].

Table 2.

Variables	*N*	*%*
Gender		
Male	163	51.4
Female	154	48.6
Age		
<6 years	220	69.4
≥6 years	97	30.6
WBC count		
<20,000 cells/µl	227	71.6
≥20,000 cells/µl	90	28.4
FXIII-A expression ratio		
Negative (<20%)	112	35.3
Positive	205	64.7
moderate positive (20-79%)	148	46.7
strong positive (≥80%)	57	17.9
Genetic risk categories*		
Good	106	33.4
Intermediate	90	28.4
Poor	18	5.7
N/A	103	32.5
„B-other" genetic risk category		
Yes	124	39.1
No	90	28.4
N/A	103	32.5
Prednisone response on day 8		
Good	290	91.5
Poor	21	6.6
N/A	6	1.9
FC-MRD (day15)		
FLR: <0.1%	66	20.8
FMR: 0.1 - <10%	208	65.6
FHR: ≥10%	42	13.2
N/A	1	0.3
iBFM ALL-IC 2009 risk groups		
Standard risk	53	16.7
Intermediate risk	196	61.8
High risk	62	19.6
N/A	6	1.9

Evaluation of sensitivity and specificity of FC in quantitative evaluation of circulating tumor cells

The ALL-IC network has developed a powerful method for detecting FC-MRD [*Mejstrikova E et al. Pediatr Blood Cancer 2010;54:62-70.*]. Within the frame of BFM ALL-IC 2002 we have

investigated the MRD load in the day 15 BM sample at the 10-3 sensitivity level. Samples were analyzed by 4 color labeling procedure using Becton Dickinson (FacsCanto-II) and Beckman Coulter (Navios and FC-500) flow cytometers. Lineage assignment was determined according to EGIL criteria [*Bene MC et al. Leukemia 1995; 9:1783-1786.*]. Surface and cy staining was performed according to standard protocols, with the ALLIC-2002 suggested panel of monoclonal antibodies. Generation and fluorescent isotihocyanate (FITC) labeling of mouse monoclonal antibody against FXIII-A was carried out as previously described [*Katona EE. et al. J Immunol Methods 2001; 258:127-35*]. We used a single tube to assess FXIII-A labeling: cyFXIII-A(FITC)–CD10(PE)–CD45(PerCP-Cy5.5)–CD19(APC). The threshold of positivity was set to 20% positive leukemic cells for a certain immunophenotype marker. For MRD detection 300,000 events were acquired. FC data were analyzed by FACSDiva (Becton Dickinson, Franklin Lakes, NJ) and Kaluza (Beckman Coulter, Brea, CA) softwares. Flow cytometers were subjected to daily performance checks, using Cytometer Setup&Tracking (Becton Dickinson, Franklin Lakes, NJ) and Flow Check Pro (Beckman Coulter, Brea, CA) fluorescent microbeads. Our laboratory participated in the UK-NEQAS Leukocyte Immunophenotyping MRD program and in the ALLIC Annual Ring Trials successfully. FC-MRD successfully separated patients with flow low-risk (FC-MRD<0.1%), flow medium-risk (FC-MRD 0,1-<10%), and flow high-risk (FC-MRD >/=10%) patients with ALL. We found significant differences in event-free survival (EFS) and overall survival (OS) of children with different risk categories [***Kárai B., Hevessy Z., Szánthó E., Csáthy L., Ujfalusi A., Gyurina K. Szegedi I., Kapplemayer J., Kiss C. Expression of coagulation factor XIII subunite A correlates with outcome in childhood acute lymphoblastic leukemia. Pathol. Oncol. Res. 2018; 24:345–352***]. A similar FC-MRD approach has been utilized in the BFM ALL-IC 2009 protocol. However, final results of MRD analysis have not yet been released, and our group is not permitted to present such results before the publication of the final results of BFM ALL-IC 2009.

We evaluated the sensitivity and specificity of FC in detecting circulating tumor cells using and additional model. We have labeled disseminated tumor cells in the BM and body fluids of patients with solid tumors including 36 samples of 16 children with neuroblastoma by FC and immunohistochemistry (IHC). The neuroblastoma panel was a 4-color combination of CD81-FITC (clone: JS-81; Becton Dickinson Pharmingen, San Diego, CA), CD117-PE (clone: 104D2; Becton Dickinson Biosciences), CD45-PerCP (clone: 2D1; Becton Dickinson Biosciences), and CD56-APC [clone: N901 (NKH-1); Immunotech—Beckman Coulter]. In 17 cases results were concordant (13 positive and 4 negative), whereas in 9 cases discordant results

were obtained. Among the discordant cases, 2 cases were FC-negative/IHC-positive, both from the same patient. The 7 FC-positive/IHC-negative discordant samples were from 6 different patients, and these were all follow-up samples with a ratio of the residual tumor cells below 1%. Altogether we found 65% (17/26) concordance in the neuroblastoma group between IHC and FC. For neuroblastoma cell detection, FC was more sensitive than IHC, especially in MRD detection (90% vs. 65%). FC and IHC were both 100% specific in neuroblastoma cell detection. Detection of disseminated tumor cells in BM specimens was found to be more effective with FC than with IHC in the diagnostic neuroblastoma samples (100% vs. 86%) [***Szánthó E. Kárai B., Ivády G., Bedekovics J., Szegedi I., Petrás M., Ujj G., Ujfalusi A., Kiss C., Kapplemayer J., Hevessy Z. Comparative analysis of multicolor flow cytometry and immunohistochemistry for the detection of disseminated tumor cells. Appl Immunohistochem Mol Morphol. 2018;26:305-315.***]. As a further utilization of our results, we have reviewed the role of MRD assessment in pediatric non-Hodgkin lymphoma. Circulating lymphoma cells detected by FC have a significant prognostic effect in NHL and can be used for fine-tuning of risk-tailored therapeutical interventions [***Szegedi I., Gáspár I., Gyurina K., Zele Z., Kiss C. Principles, practice and perspectives of the management of children and adolescents with non-Hodgkin lymphoma. Survey of the literature and retrospective analysis of the results of a single center at the University of Debrecen. (Article in Hungarian; submitted for publication)***].

Investigation of the value of FXIII-A expression by FC in BCP ALL as a diagnostic marker

Each two set of our investigations (the retrospective analysis of BFM ALL-IC 2002 data and the multicentric study performed by three national groups participating in BFM ALL-IC 2009) confirmed our original findings when we described first BCP ALL blats as a novel expression site for FXIII-A that FXIII-A is expressed in a certain part of leukemic lymphoblast of children with BCP ALL but are not expressed either in normal lymphocytes, or normal BM lymphocyte progenitors or mature-B leukemia/lymphoma cells [*Kiss F et al. Thromb Haemost 2006;96:172-82.*]. In course of the retrospective analysis 37/55 patients had FXIII-A positive BCP ALL and 18 exhibited FXIII-A negative lymphoblasts. FXIII-A positivity of the blasts was declared based on the 20% cut-off limit of labeling [***Kárai B., Hevessy Z., Szánthó E., Csáthy L.Ujfalusi A., Gyurina K. Szegedi I., Kapplemayer J., Kiss C. Expression of coagulation factor XIII subunite A correlates with outcome in childhood acute lymphoblastic leukemia. Pathol. Oncol. Res. 2018; 24:345–352***]. In the multicentric study we examined the

significance of FXIII-A expression in more detail. Patients were assigned to three groups: BCP-ALL with FXIII-A negative blasts (<20% FXIII-A positive lymphoblasts; FXIII-A negative group), BCP-ALL with moderate FXIIII-A expression (20%-79% FXIII-A positive lymphoblasts; moderate FXIII-A positive group), and BCP-ALL with strong FXIII-A expression (≥80% FXIII-A positive lymphoblasts; strong FXIII-A positive group). In case of the negative expression pattern, leukemic lymphoblasts overlapped with the residual normal lymphocytes. In strong positive cases, the leukemic blast cell population separated almost completely from residual normal lymphocytes. In patients with moderate positive cases leukemic lymphoblasts appeared as a broad but homogenous group with a partial overlap with residual normal lymphocytes. The histogram analysis of FXIII-A moderate expression cases showed that the leukemic cell population exhibited a single group with a continuously increasing fluorescence intensity and excluded the existence of two distinct subpopulations, i.e. a negative and a strong positive one with respect to FXIII-A expression. Of the analyzed 317 cases, there were 112 negative, 148 moderate, and 57 strong FXIII-A positive [***Kárai B. Gyurina K., Ujfalusi A., Sedek L. Barna G., Jáksó P., Svec P., Szánthó E., Nagy AC., Kowalczyk JR., Kolenova A., Kovács TG., Szczepanski T., Kapplemayer J., Hevessy Z., Kiss C. Expression patterns of leukemic lymphoblasts for coagulation factor XIII subunite A correlate with clinical outcome and genetic subtypes in childhood acute B-cell progenitor lymphoblastic leukemia. (Submitted for publication)***]. Both set proved that FXIII-A is a suitable marker of leukemia-associated immunophenotype. Once FXIII-A expression can be detected in a cell appearing in the blat cell window by the FC, the leukemic nature of the given cell can be ascertained. However, FXIII-A, as a cytoplasmic marker is less suitable for MRD detection because exact ratio of FXIII-A positive lymphoblast below 0.1°% cannot be precisely estimated.

Interrelationships between FXIII-A expression and response to prednisolone treatment

The relationship between FXIII-A expression and prednisolone response was examined in the retrospective analysis. The ratio of poor prednisolone response (absolute blats cell count <1 G/L after 7 days prednisolone monotherapy) was slightly higher in the FXIII-A negative group than in the FXIII-A positive one (29% vs. 6%) although this difference was not statistically significant. Using multivariate logistic regression analysis we were not able to prove an effect of FXIII-A expression on prednisolone response [***Kárai B., Hevessy Z., Szánthó E., Csáthy L., Ujfalusi A., Gyurina K. Szegedi I., Kapplemayer J., Kiss C. Expression of coagulation factor***

XIII subunite A correlates with outcome in childhood acute lymphoblastic leukemia. Pathol. Oncol. Res. 2018; 24:345–352]. In the multicentric prospective study the impact of prednisolone response on two pairs of FXIII-A expression groups, i.e. the moderate FXIII-A positive vs. FXIII-A negative groups and the moderate FXIII-A positive vs. strong FXIII-A positive groups was analyzed with the multivariable Cox regression model. Prednisolone response had significant effects on 5-year EFS and OS figures of the moderate positive vs. FXIII-A negative groups. Possible causal relationships between the FXIII-A expression pattern and prednisolone response were investigated with multivariate logistic regression models. Prednisolone response differed significantly between the FXIII-A combined positive (moderate positive plus strong positive) and negative groups. This association however, this appeared in the adjusted model. In 36 patients FXIII-A expression was evaluated in parallel at diagnosis and in the day 15 BM sample. We excluded patients of the FLR category because exact ratio of FXIII-A positive lymphoblasts below 0.1% could not be estimated due to the cy expression of FXIII-A. The ratio of FXIII-A positive leukemic lymphoblast was significantly lower in Day 15 than in Day 0 ($p<0.001$). FXIII-A expression of FXIII-A negative *de novo* cases did not change significantly by Day 15. Of the *de novo* FXIII-A negative cases, none surpassed the cut-off value of 20% for FXIII-A positivity by Day15. The significant decrease in the ratio of FXIII-A positive lymphoblasts in day 15 BM samples of FXIII-A positive vs. negative BCP-ALL suggested a preferential clearance of FXIII-A positive blast cells over negative ones. This finding was in accordance with the results of logistic regression analysis. Patients with FXIII-A negative lymphoblasts were three times more likely to exhibit a poor prednisone response than patients of the FXIII-A positive group [***Kárai B. Gyurina K., Ujfalusi A., Sedek L. Barna G., Jáksó P., Svec P., Szánthó E., Nagy AC., Kowalczyk JR., Kolenova A., Kovács TG., Szczepanski T., Kapplemayer J., Hevessy Z., Kiss C. Expression patterns of leukemic lymphoblasts for coagulation factor XIII subunite A correlate with clinical outcome and genetic subtypes in childhood acute B-cell progenitor lymphoblastic leukemia. (submitted for publication)***].

In addition, response to glucocorticoid (GC) treatment was investigated by expression profiling of glucocorticoid sensitive and resistant BCP ALL cells. Gene expression data, obtained from Affymetrix Human Genome U133A Array, for 27 non-infant children (>1 yr of age) diagnosed with BCP ALL either sensitive (14 samples with LC50 values <0.1 lg/mL prednisolone) or resistant (13 samples with LC50 values >150 lg/mL prednisolone) to in vitro prednisolone

treatment were retrieved from public Gene Expression Omnibus repository (series GSE19143). For quality control (QC), we have applied the instructions of the http://www.arrayanalysis.org portal. We chose those CEL files for analysis which were in the normal range by the 2D virtual, MA plot, RLE, and box plot of row intensities after normalization. Dataset files were imported into GeneSpring v12.6. Expression values for each probe sets were compared between prednisolone sensitive and resistant groups and log of fold change was calculated. The significance was tested with moderated t-test adjusted by Benjamini–Hochberg correction for multiple testing. Corrected p-value cutoff was set to 0.05, and the fold change cutoff was 2.0. A list containing 721 autophagy-related genes was generated using GeneOntology Biological Process: Autophagy (GO006914), and gene lists downloaded from Autophagy Database [*www.tanpaku.org/autophagy/index.html, http://autophagy.lu/index.html*]. The combined autophagy list and the list containing transcripts that were differently expressed between prednisolone sensitive and resistant groups were imported into Microsoft Access, and query was designed to identify overlapping entities between the two sets of genes. To gain insight in the biological function of the given transcripts, canonical pathways and biological processes with overrepresentation of differently expressed transcripts were identified using g:Profiler application [*http://biit.cs.ut.ee/gprofiler/index.cgi*] relying on GeneOntology (GO) Biological Process and KEGG pathway databases. We have identified 723 transcripts which were differently expressed, based on at least a twofold difference, between GC-sensitive and GC-resistant BCP ALL samples. Functional profiling using GeneOntology Biological Process revealed that genes from the 723 transcripts showed statistically significant overrepresentation in 961 GO terms. Some of the relevant overrepresented GO terms were the following ones: cell differentiation (153 genes); programmed cell death (102 genes); cell proliferation (99 genes); regulation of cell death (88 genes); and response to GC hormone (22 genes). Pathway analysis revealed 22 canonical pathways which were significantly enriched for differently expressed genes. Of the 22 pathways, 6 were related to viral, bacterial or parasitic infectious diseases; another 5 to various cancers. Six canonical cell signaling pathways were identified as well. Among these, we can find the PI3K-Akt (with 30 genes) and TNF terms (with 11 genes), two signaling pathways regulating cell survival, growth and death. We have identified 36 autophagy-associated genes which were differently expressed, based on at least a twofold difference. Of the 36 genes, 10 were downregulated and 26 upregulated in the GC-resistant group. Our data implied that GC sensitivity might depend on the expression of several genes involved in regulation and execution of autophagy in a way that key autophagy inducers are

downregulated while inhibitors of autophagy are upregulated in GC-resistant cells [***Sarang Z., Gyurina K., Scholtz B., Kiss C., Szegedi I. Altered expression of autophagy-ralted genes might contribute to glucocorticoid resistance in precursor B-cell-type acute lymphoblastic leukemia. Eur J Haematol. 2016;97:453-460***].

FXIII-A as a new independent prognostic factor in childhood BCP ALL

In the retrospective cohort investigating children with BCP ALL of the Debrecen and Miskolc centers treated according to the BFM ALL-IC 2002 protocol we demonstrated a significant EFS and OS advantage in favor of FXIII-A positive patients. Traditional risk factors, such as age, initial WBC, risk groups as defined according to the BFM ALL-IC 2002 clinical trial did not differ significantly between FXIII-positive and negative groups of patients. Only the prevalence the "B-other" genetic group exhibited a significantly different distribution. This genetic category was significantly more frequent among patients with FXIII-A negative BCP ALL. The multivariate logistic regression analysis confirmed association between the FXIII-A characteristics and the "B-other" group. This association persisted after adjusting for initial diagnostic risk parameters, such as age, WBC [***Kárai B., Hevessy Z., Szánthó E., Csáthy L., Ujfalusi A., Gyurina K. Szegedi I., Kapplemayer J., Kiss C. Expression of coagulation factor XIII subunite A correlates with outcome in childhood acute lymphoblastic leukemia. Pathol. Oncol. Res. 2018; 24:345–352***].

Investigating a much larger number of patients (317) prospectively in the multi-centric study associated with the BFM ALL-IC 2009 clinical trial we have described a more complex association. EFS and OS of patients with FXIII-A positive BCP-ALL were not significantly different from that of patients with FXIII-A negative BCP-ALL. Investigating the three different FXIII-A expression patterns separately, a significant EFS advantage of the moderate FXIII-A positive group was demonstrated when compared both with the FXIII-A negative ($p=0.010$), and with the strong FXIII-A positive group ($p<0.001$). The 5-year EFS of patients with moderate positive, negative, and strong positive FXIII-A expression was 92%, 70%, and 61%, respectively. The 5-year OS of the moderate FXIII-A positive group (93%) was significantly higher than that of the FXIII-A negative group (76%; $p=0.041$). The difference between the 5-year OS of the moderate FXIII-A positive and the strong FXIII-A positive group (87%) was not significant. The impact of categorical variables representing known risk factors on the two pairs of groups, i.e. the moderate FXIII-A positive vs. FXIII-A negative groups, and the moderate FXIII-A positive vs. strong FXIII-A positive groups, was analyzed with the multivariable Cox regression model. Categorical variables, such as FXIII-A expression pattern,

age, distribution of genetic risk categories, and distribution of the "B-other" genetic subgroup had significant effects on 5-year EFS and 5-year OS figures of the moderate positive vs. negative FXIII-A groups. The effect of ALL BFM-IC 2009 risk groups was significant on the 5-year OS. In the multivariate analysis model, only genetic risk category (good vs. intermediate) remained significant. Significant differences between survival figures of the moderate positive vs. strong positive FXIII-A groups were seen in case of FXIII-A expression pattern, ALL BFM-IC 2009 risk categories (BFM-HR vs. BFM-IR) and the genetic risk categories. In the multivariate Cox analysis, FXIII-A expression pattern and genetic risk categories remained significant. Possible causal relationships between the FXIII-A expression patterns and other known risk factors were investigated with multivariate logistic regression models. The different FXIII-A expression patterns did not correlate significantly with either risk stratification according to ALL BFM-IC 2009 or FC MRD risk categories. The intermediate genetic risk category was significantly more prevalent (p=0.007) in the FXIII-A negative group than in the FXIII-A positive group. Correlation between negative and positive FXIII-A expression patterns and the "B-other" subgroup was equally strong (p=0.005). Patients with FXIII-A negative lymphoblasts had twice as much chance for having "B-other" alteration (OR: 2.33, 95% CI: 1.29-4.2). Patients with FXIII-A negative expression had 50% lower chance of having low instead of intermediate genetic risk compared with FXIII-A positive patients (OR: 0.53, 95% CI 0.19-1.42). This association persisted after adjusting for categorical variables, as sex, age, WBC, and ALL BFM-IC 2009. Analyzing moderate and strong FXIII positive subgroups separately, the accumulation of cases with intermediate genetic risk, and the "B-other" category persisted in the FXIII-A negative group.

We have performed a pilot analysis in 59 patients of the Debrecen center. Copy number alterations (CNA) were investigated using DNA extracted from the excess of diagnostic BM samples (QIAmp DNA Blood Mini Kit, Quiagen, Hilden, Germany). SALSA multiplex ligand-dependent probe amplification (MLPA) P335-B2 ALL-IKZF1 probe mix was used to perform MLPA analysis (MRC-Holland, Amsterdam, The Netherlands). The kit includes probes for IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1 and the PAR1region (CRLF 2, CSF2RA, IL3RA). No deletion of IKZF1, CDKN2A/B, PAR1, BTG1, EBF1, PAX5, ETV6 or RB1, isolated deletions of ETV6, PAX5, or BTG1, ETV6 deletions with a single additional deletion of BTG1, PAX5 or CDKN2A/B were considered as good; single deletions of CDKN2A/B, and combined deletions of CDKN2A/B/PAX5 were considered as intermediate; and any deletion of IKZF1, PAR1, EBF1 or RB1, and all other CNA profiles not mentioned above were considered as poor prognostic factors according to CNA. Poor prognostic CNA

were detected in a slightly higher ratio (7/20) in the negative FXIII-A group than in the moderate (4/26), and the strong FXIII-A positive (1/13) groups (Table S2). The differences were not significant, probably due to the limited samples size. We have demonstrated the usefulness of the investigation of CNA by MLPA and we would like to carry on and to extend these investigations in pediatric BCP ALL within the frames of a new multi-centric prospective BFM ALL-IC clinical trial [***Kárai B. Gyurina K., Ujfalusi A., Sedek L. Barna G., Jáksó P., Svec P., Szánthó E., Nagy AC., Kowalczyk JR., Kolenova A., Kovács TG., Szczepanski T., Kapplemayer J., Hevessy Z., Kiss C. Expression patterns of leukemic lymphoblasts for coagulation factor XIII subunite A correlate with clinical outcome and genetic subtypes in childhood acute B-cell progenitor lymphoblastic leukemia. (Submitted for publication)***].

FXIII-A expression status of leukemic lymphoblast can be easily and accurately determined by FC and as such, the method can be applied by moderate income countries of the ALL-IC consortium. FXIII-A negative expression pattern is a prognostic indicator of dismal survival in children with BCP ALL. However, FXIII-A is only one of the ever-growing numbers of recently discovered genetic and proteomic biomarkers. It may prove difficult to determine the place of FXIII-A as a prognostic biomarker among the gigabytes of genomic and proteomic information burden. We have recently reviewed the methodology of providing guidance for physicians who would like to offer personalized treatment to children with cancer, including ALL. We proposed formation of consortiums (out of which ALL-IC network could be one example) providing standardized approaches for selection of diagnostic and therapeutic targets aided by computer-assisted information processing and facilitated through an online tumor board review [***Klement GL, Arkun K, Valik D, Roffidal T, Hashemi A, Klement C, Carmassi P, Rietman E, Slaby O, Mazanek P, Mudry P, Kovacs G, Kiss C, Norga K, Konstantinov D, André N, Slavc I, van Den Berg H, Kolenova A, Kren L, Tuma J, Skotakova J, Sterba J. Future paradigms for precision oncology. Oncotarget. 2016;7:46813-46831.***].

FXIII-A expression defines a new subpopulation of pediatric BCP ALL with an overlap with the "B-other" genetic subtype

We have searched the public databases in order to assess associations between the expression of *F13a1* gene with other genes characteristically deregulated in childhood ALL. We have used the same methods as described for the investigation of gene expression profiles (GEPs) associated with glucocorticoid resistance and sensitivity [***Sarang Z., Gyurina K., Scholtz B., Kiss C., Szegedi I. Altered expression of autophagy-ralted genes might contribute to glucocorticoid resistance in precursor B-cell-type acute lymphoblastic leukemia. Eur J***

Haematol. 2016;97:453-460]. Gene expression data, obtained from Affymetrix Human Genome U133A Array, for 405 samples were retrieved from public Gene Expression Omnibus (GEO) repository (series GSE13351, GSE13425 and GSE47051). A wide variation of *F13a1* expression levels were observed. In the GeneSpring analysis, patients were separated into two groups: *F13a1* low expression, and *F13a1* high expression group, with at least 3-fold difference in their *F13a1* expression level. *F13a1* low and high expression groups exhibited a characteristically different GEP (at least 2-fold difference, $p \leq 0.05$, t-test, Benjamini-Hochberg correction for FDR). Low *F13a1* expression level was prevalent among "B-other" samples, high *F13a1* expression level was associated with t(1;19) genetic subgroup of childhood ALL. We found eight genes that were significantly down-regulated, and one that was up-regulated in the *F13a1* low expression group in all three datasets, and 59 similarly dysregulated genes in at least two of the three datasets. Moreover, we identified two chromosomal loci, 19p13.3 and 16q22, where 11 and 2 genes, respectively, that were dysregulated within the *F13a1* low expression group. Using Ingenuity Pathway Analysis, we identified a network of genes participating in B cell development that were downregulated in the *F13a1* low expression group. Using the Ingenuity upstream regulator analysis function, aberrant activation of NUPR1, and aberrant suppression of TCF3 and IKZF1 was predicted in the *F13a1* low expression group. [***Gyurina K, Csáthy L, Bresolin S, Kronnie G, Hevessy Z, Szegedi I, Scholtz B, Basso G, Kiss C, Kappelmayer J. Subunite A of Coagulation Factor XIII as a New Biomarker In Childhood Acute Lymphoblastic Leukemia? Blood 2014;124:5346***].

As a pilot to the prospective multi-center BFM ALL-IC 2009 clinical trial we have investigated RNA samples obtained from 71 children with BCP ALL treated by the centers of the Hungarian national group. 45/71 children had sufficient and qualitatively suitable RNA for Affymetrix analysis. Affymetrix GeneChip Human Primeview array was used to analyze global expression pattern of 28869 well-annotated genes. 3'IVT Expression Kit (Affymetrix) and GeneChip WT Terminal Labeling and Control Kit (Affymetrix) were used for amplifying and labeling 250 ng of RNA samples. Samples were hybridized at 45°C for 16 hours and then standard washing protocol was performed using GeneChip Fluidics Station 450 and the arrays were scanned on GeneChip Scanner 7G (Affymetrix). Microarray data were analyzed by Genespring GX14.9.1 software (Agilent Technologies). Affymetrix data files were imported using the RMA algorithm and median normalization was performed. To identify differentially expressed genes between clinical conditions, statistical analysis was performed using ANOVA with Tukey post hoc test and moderated T-test, Benjamini-Hochberg FDR was used for multiple testing corrections, p value <0.05 was considered as significant difference. First, differentially

expressing (DE) genes were identified using two distinctive features: FXIII-A protein expression determined by FC and "B-other" status determined by genetic tests. Samples according to FXIII-A protein expression were divided into three different groups as strong and moderate FXIII-A positive, and FXIII-A negative groups, while in the other setting as "B-other" and non-"B-other" groups. After statistical analysis genes were filtered to 1.5 fold change and overlapping DE genes were identified in negative vs. moderate positive, negative vs. strong positive and strong positive vs. moderate positive DE gene groups. Interestingly, the least number of DE genes were found between the strong positive and negative groups (26) while between strong positive and moderate positive groups there were 155 DE genes. Venn diagram showed that 66 DE genes were found exclusively in the strong positive versus moderate positive groups while 69 genes overlapped between the negative vs. moderate positive and the strong positive vs. moderate positive DE genes implying that these genes can be specific for the moderate FXIII-A positive status. This distinction was confirmed by heat map analysis showing a specific pattern for the moderate FXIII-A positive status compared to the strong positive and to the FXIII-A negative groups. Comparing "B-other" and non-"B-other" groups 142 DE genes were found after filtering to 1.5 fold change. Investigating how these genes were related to DE genes of subgroups of moderate positive + "B-other", moderate positive + non-"B-other", negative + "B-other" and negative + non-"B-other", a substantial overlap could be identified between moderate positive + "B-other" vs. moderate positive + non-"B-other" and "B-other" vs. non-"B-other" DE genes. There were 23 DE genes found exclusively in the FXIII-A negative +" B-other" vs. negative + non-"B-other" and not in "B-other" vs. non-"B-other" groups. Samples were clustered primarily by FXIII-A protein expression and only secondarily by "B-other" status although there was no complete separation between the four groups. Recapitulating, our results showed that though "B-other" status conferred a specific pattern for BCP ALL samples, FXIII-A protein expression level seemed to have a stronger effect on the gene expression pattern.

Gene Ontology (GO) analysis was performed using Cytoscape 3.4.0 software (cytoscape.org) with ClueGO application. The settings were the following: GO biological process, GO immune system process, and KEGG human diseases pathways. For statistical analysis two-sided hypergeometric test and Benjamini-Hochberg FDR were used. Significantly enriched GO categories were considered to p value <0.05 and κ score <0.4. DE over-presented genes in biological processes were identified using Gene Onthology analysis. In the "B-other" group the following genes were over-represented with a fold change of >2.0: apoptotic process genes (*CCL5, CD3G, IL7R, PLAC8*), genes participating in lymphocyte and T-cell regulation of

apoptosis, and two other genes, *CX3CR1, RORA* corresponding to macrophage migration process. *IL7R* is also one of the genes characteristically dysregulated in the recently defined *BCR-ABL1*-like genetic subgroup.

When changing filtering to fold change >1.5, additional biological processes were revealed (Table 3) containing the following genes: *BCL10, CX3CR1, GNLY, PTPRC, STK4, TNFSF10, ATP2B1, DNAJC3, PLAC8, THRA, MAPKBP1, PER1, RORA, USP32, CCL5, GNG2, PLCB3, BCL10, CCL5, CD3G*.

Table 3. Over-represented GO biological processes in the "B-other" group

GOID	GOTerm	Term P Value Corrected
GO:0006968	cellular defense response	1,32E-02
GO:2001238	positive regulation of extrinsic apoptotic signaling pathway	3,46E-03
GO:0009409	response to cold	1,91E-03
GO:0043124	negative regulation of I-kappaB kinase/NF-kappaB signaling	3,43E-03
GO:1905517	macrophage migration	2,15E-03
GO:0048246	macrophage chemotaxis	4,73E-03
GO:1905521	regulation of macrophage migration	3,13E-03
GO:0007223	Wnt signaling pathway, calcium modulating pathway	4,72E-03
GO:0070227	lymphocyte apoptotic process	2,21E-03
GO:2000106	regulation of leukocyte apoptotic process	3,06E-03
GO:0070231	T cell apoptotic process	2,87E-03
GO:2000107	negative regulation of leukocyte apoptotic process	1,01E-02
GO:0070228	regulation of lymphocyte apoptotic process	9,96E-04
GO:0070229	negative regulation of lymphocyte apoptotic process	4,18E-03
GO:0070232	regulation of T cell apoptotic process	4,76E-03

Biological processes among DE genes identified according to FXIII-A expression were much more diverse, 173 GO processes were revealed. Most of these genes, in particular those with the strongest statistical p values were related to epigenetical and/or gene expression regulatory processes, such as histone modification, chromatin organization, RNA destabilization,

posttranscriptional regulation of gene expression, or other regulatory and cellular processes, particularly apoptotic and morphogenesis ones (Table 4).

Table 4. Over-represented GO biological processes according to FXIII-A status comparison having lowest corrected p values

GOID	GOTerm	Term P Value Corrected
GO:0016569	covalent chromatin modification	3,20E-05
GO:0016570	histone modification	3,59E-05
GO:0006325	chromatin organization	1,07E-04
GO:0050779	RNA destabilization	7,55E-04
GO:1900118	negative regulation of execution phase of apoptosis	1,14E-03
GO:0034248	regulation of cellular amide metabolic process	1,29E-03
GO:0010608	posttranscriptional regulation of gene expression	1,93E-03
GO:0010608	posttranscriptional regulation of gene expression	1,93E-03
GO:0006417	regulation of translation	1,97E-03
GO:0097284	hepatocyte apoptotic process	1,99E-03
GO:0061038	uterus morphogenesis	2,07E-03
GO:0071478	cellular response to radiation	2,10E-03
GO:0061157	mRNA destabilization	2,35E-03
GO:0031330	negative regulation of cellular catabolic process	2,52E-03
GO:0071482	cellular response to light stimulus	2,76E-03
GO:0016571	histone methylation	3,34E-03
GO:0033169	histone H3-K9 demethylation	3,59E-03
GO:0104004	cellular response to environmental stimulus	4,76E-03
GO:0071214	cellular response to abiotic stimulus	4,76E-03
GO:2000767	positive regulation of cytoplasmic translation	5,63E-03
GO:0048048	embryonic eye morphogenesis	5,65E-03
GO:0032074	negative regulation of nuclease activity	6,76E-03
GO:0043487	regulation of RNA stability	7,30E-03
GO:0009895	negative regulation of catabolic process	7,49E-03
GO:0048596	embryonic camera-type eye morphogenesis	9,13E-03
GO:0018205	peptidyl-lysine modification	9,39E-03

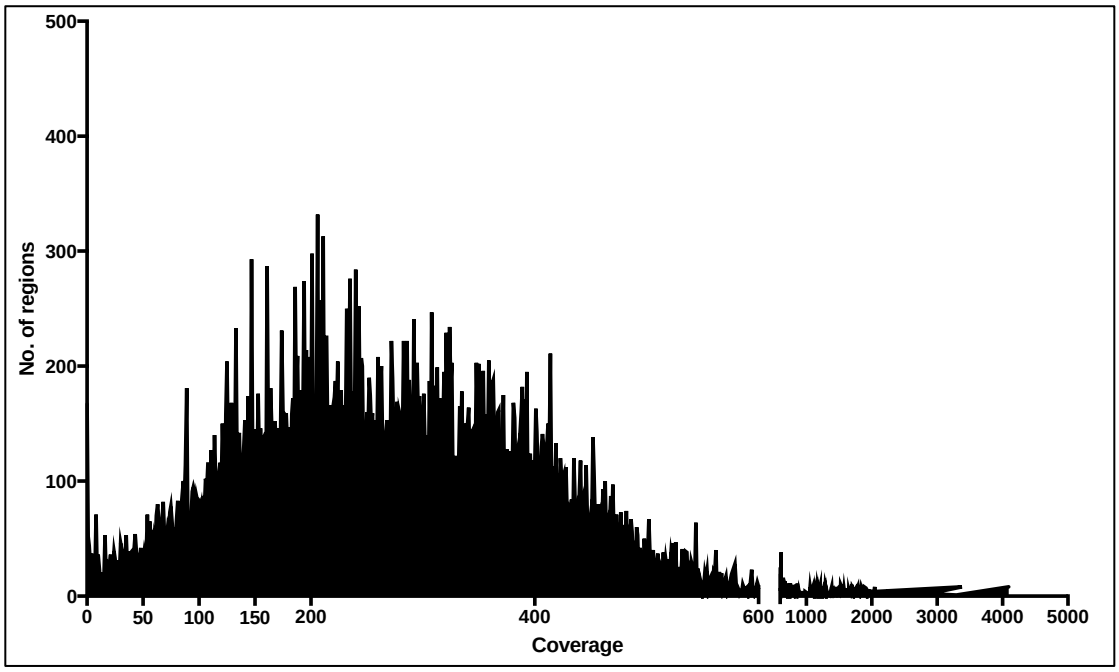
Validity of our data was confirmed not only by the normalized gene expression levels of *F13a1* microarray probes that correlate with FXIII-A protein expression but also by the identified biological process. Peptidyl-lysine modification is in well-known relation with the physiological function of FXIII-A, member of the FXIII-A_2B_2 transglutaminase heterotetramer forming γ-glutamyl-Є-lysyl amide cross links between fibrin molecules to form an insoluble clot [***manuscript in preparation – to be submitted before November 1, 2018***].

We have performed targeted exon sequenation in 10-10 strong FXIII-A positive and FXIII-A negative samples. Genes were selected for targeted capture from three sources:

- All genes were included from the NimbleGen Comprehensive Cancer Gene List (2015. February version), 497 human genes altogether.
- Genes characteristically mutated in childhood ALL were identified by literature search: 62 genes.
- Genes with significantly altered expression between FXIII-A negative and FXIII-A postive childhood ALL patients, based on several independent microarray experiments, with the following GEO identifiers: GSE47051, GSE13425, GSE13351, GSE10255 and GSE28497. This resulted in a list of 476 genes.

The non-redundant combination of the lists resulted in 1022 genes for targeted exon-capture followed by next generation sequencing. Exon coordinates for these genes were downloaded from the UCSC Genome Browser database – the targeted region was 6 392 558 basepairs. The custom exon capture probe panel for the Roche NimbleGen SeqCap EZ Library SR kit was designed by Roche NimbleGen, based on the exon coordinates of the final gene list. Genomic DNA isolated from leukemic blasts of the selected patients was used for exon-capture, performed by UD-Genomed, Debrecen. Next generation sequencing was done on the Illumina MiSeq platform, using paired-end 125 bp sequencing, aiming for at least 100X coverage per base. Primary data analysis was performed on the Galaxy platform (usegalaxy.org), with the Variant Effect Predictor-Ensembl (https://www.ensembl.org/vep) and with the Integrated Genomics Viewer (Broad Institute). Basic quality assessment was done with the FASTQC program. After adapter removal and preprocessing with the Trimmomatic program, reads were aligned to the hg19 human genome reference sequence with BWA-MEM. Post-aligment quality asessment indicated that the majority of the targeted regions were captured successfully, and the coverage was at least 100X for most regions (exons) (Figure 1).

Figure 1. Coverage of the captured regions in a representative sample.



We used two approaches to detect variant sequences in the aligned sequence data. The first approach included MarkDuplicates, Mpileup and Varscan steps, the first two with default settings. For the Varscan analysis, we used three different parameter settings, to identify SNP variants present in most leukemic blasts, as well as variants present in a minor subpopulation of blasts (Table 5). A non-redundant SNP variant list was produced from the combination of the three Varscan analyses.

Table 5. Parameter settings of the Varscan analysis.

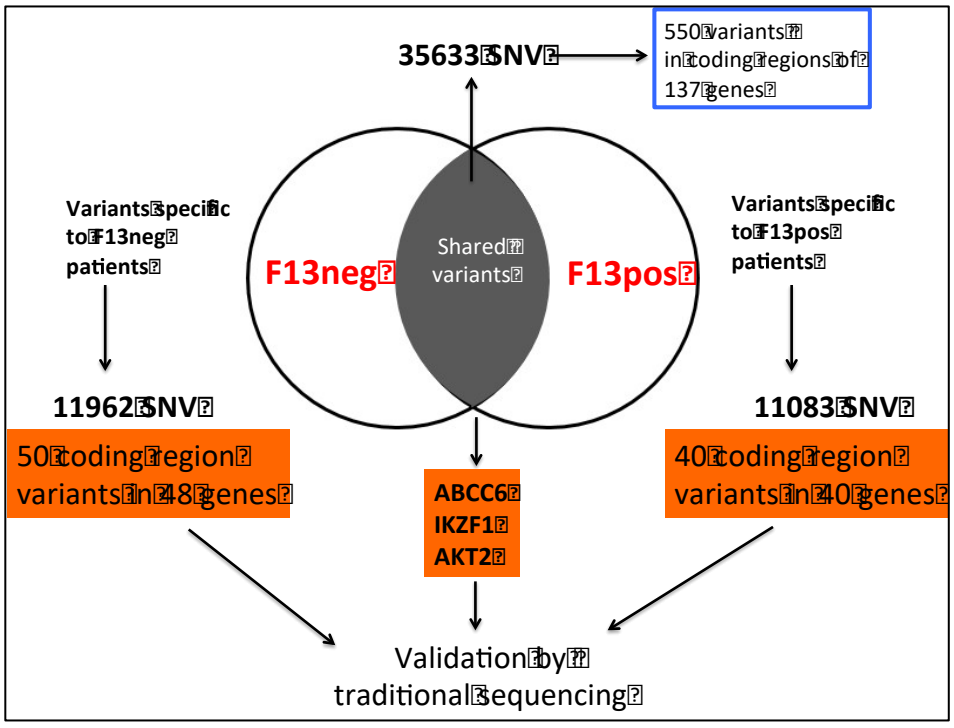
	Varscan 1	Varscan 2	Varscan 3
Minimum read depth	10	50	100
Minimum supporting reads	4	20	10
Minimum base quality at a position to count a read	20	20	20
Minimum variant allele frequency threshold	0,3	0,1	0,05

The second approach followed the GATK pipeline, including Indel realignment and Base recalibrations steps, ending with either Varscan analyses (3 settings as above), or with Haplotype Caller analysis. Both Varscan and Haplotype Caller were set to identify SNPs and Indels as well.

A non-redundant list of predicted sequence variants was produced for each sample from the combined results of the previous steps. Sequence variants were annotated with Variant Effect Predictor-Ensembl. Know SNPs (with a dbSNP ID) were excluded from further analysis. Novel variants in the coding region of genes were selected for further quality assessment with the Integrated Genomics Viewer. Variants with the following characteristics were considered as probable sequencing errors, and were excluded from our list of candidate mutations:

- Variant overlaps with or is close to a homopolymer run: 6 bp long, and within 10 bp and/or overlapping with 4 bp
- 2 or more variants close to each other: More than three SNV calls within a given window (10 bp) around the site. SNV closer than a given distance (10 bp) from a predicted indel of a certain quality (≥ 50)
- Strand imbalance: variant allele is detected on forward (or reverse) reads mostly
- Variant allele is close to the end of the reads: within 5 bps of the fragment end
- Variant allele is in a repetitive region

Figure 2. summarize our findings:



Altogether, 50 coding region variants were identified only in the FXIII-A negative samples, 40 variants only in the strong FXIII-A positive samples, and 3 genes (*ABCC6, IKZF1* and *AKT2*) carried putative mutations in both sample sets, although in different positions. After quality filtering, 550 other variants in 137 genes were also identified, which were present in both

sample sets (Figure 2.). These, however, were excluded from the following analysis because they could be detected in all samples as characteristics of novel polymorphism, or technical error of sequencing or data analysis. Table 6 and 7 provide the list of genes with predicted mutations.

Table 6. Predicted coding region mutations in FXIII-A negative patients

Gene	Location of mutation	ALL_01	ALL_02	ALL_03	ALL_04	ALL_05	ALL_06	ALL_07	ALL_08	ALL_09	ALL_10
IKZF1	7:50450288-50450288	1									
KMT2D_1	12:49424990-49424990				1		1				
KMT2D_2	12:49420696-49420696										
ETV6	12:11992105-11992105		1								
TET2	4:106155204-106155204		1								
KDM6A	X:44896924-44896924			1							
ATRX	X:76938292-76938292										1
SH2B3	12:111885560-111885560	1									
SLFN11	17:33679440-33679440			1						1	1
DUSP5	10:112269955-112269955							1			
MN1	22:28193705-28193705				1						
DPEP1	16:89696834-89696834	1									
FOLR1	11:71907063-71907063	1									
IKZF3	17:37947702-37947702				1						
PRKDC	8:48826505-48826505							1			
SOCS1	16:11349179-11349179						1				
ZBTB21	21:43413375-43413392								1		
MAX	14:65543340-65543340							1			
EBF1	5:158500590-158500590									1	
MAP2K4	17:12032517-12032517		1								
ATF7IP	12:14631289-14631289							1			
DICER1	14:95574226-95574226			1							
ABCC6	16:16315528-16315528						1				
TRIM33	1:114968208-114968208	1									
PIK3R1	5:67591127-67591127		1								
CD79A	19:42385019-42385019					1					
BCOR	X:39913548-39913548						1				
TAL1	1:47697834-47697834						1				
JAK3	19:17936118-17936118		1								
EP300	22:41558788-41558788										1
AKAP13	15:86125010-86125010										1
COL6A3	2:238283510-238283510				1						
MXRA7	17:74684530-74684530		1								
PKP3	11:397191-397191			1							
LILRB1_1	19:55144623-55144623			1							
LILRB1_2	19:55144208-55144208	x			x		x	x	x		x
RANBP2	2:109384848-109384848		1								
ZRSR2	X:15841230-15841230		1								
CHD5	1:6188639-6188639								1		
KIAA0226L	13:46946603-46946603						1				
LILRA2	19:55086249-55086249	1									
MLLT3	9:20414044-20414044						1				
SEMA6A	5:115814334-115814334										1
SLC27A3	1:153747794-153747794				1						
USF2	19:35760851-35760851			1							
AKT2	19:40741849-40741849			1							
AFF1	4:88036282-88036282										1
SRGAP3	3:9121778-9121778								1		
ZMYM5	13:20437589-20437589								1		
LILRA1_1	19:55106663-55106663			1					1		
LILRA1_2	19:55106865-55106865			x					x		
UTY	Y:15447843-15447843							1			
EIF2AK1	7:6089634-6089637								1		

Table 7. Predicted coding region mutations in strong FXIII-A positive patients

Gene	Location of mutation	ALL_11	ALL_12	ALL_13	ALL_14	ALL_15	ALL_16	ALL_17	ALL_18	ALL_19	ALL_20
EPOR	19:11492635-11492635				1						
KRAS	12:25398281-25398281		1								
TCF7L2	10:114849290-114849290				1						
WRN	8:30933755-30933755					1					
IKZF1	7:50450291-50450291	1									
KMT2D	12:49423257-49423257					1	1				
ATM	11:108098516-108098520						1				
MECOM	3:168838939-168838939				1						
STK39	2:169038498-169038498										1
XPO1	2:61719472-61719472									1	
ZMYM2	13:20635284-20635284							1			
AKT2	19:40740417-40740417						1				
CHCHD7	8:57125432-57125432					1					
MLLT4	6:168276073-168276073		1								
NRAS	1:115252276-115252276		1								
NUP98	11:3735173-3735173				1						
ROS1	6:117681565-117681565	1									
SARDH	9:136529036-136529036		1								
TYK2	19:10464712-10464712								1		
TP53	17:7578406-7578406		1								
ARID5B	10:63851925-63851925										1
ELL2	5:95297333-95297333					1					
FANCG	9:35075062-35075062						1				
KMT2C	7:151879672-151879672		1								
PCLO	7:82784833-82784833		1								
ABCC6	16:16302586-16302586						1				
MAX	14:65472929-65472929	1									
BCL9	1:147096131-147096131							1			
CRAMP1L	16:1705915-1705915						1				
CYB5R2	11:7686742-7686742					1					
HSPA6	1:161494927-161494927								1		
INTS3	1:153745403-153745403				1						
LAMA4	6:112450185-112450185		1								
NFATC1	18:77246718-77246718				1						
PLXNB2	22:50722186-50722186								1		
PRKCZ	1:1990973-1990973	1									
STXBP2	19:7709794-7709794							1			
TRPM4	19:49692024-49692024		1								
FH	1:241672039-241672039		1								
TPBG	6:83074778-83074778								1		
MYH9	22:36681219-36681219										1
TBC1D3F	17:36288204-36288204									1	
CREB3L2	7:137565254-137565254			1							
NINL	20:25457273-25457273						1				
SLTM	15:59182553-59182553	1									
NOC2L	1:892471-892471									1	

17-17 predicted mutations from both sample groups were selected for validation with traditional sequencing. The sequencing will be done using gDNA of leukemic blasts as well as normal gDNA. The region around the mutation will be amplified with PCR, then sequenced. We designed the PCR primers and are currently optimizing the PCR reactions. Table 8. summarizes the selected genes/mutations and the PCR primers.

Table 4. Mutations selected for validation and the PCR primers

FXIII-neg	Mutation location	Left primer	Right primer
ABCC6	16:16315528-16315528	GACCTCTGTAGCCTTTCTAA	CCTGTGCTTCCTGAGAAC
AKT2	19:40741849-40741849	ACACTGCGACCCTACAAG	CTTCTACAACCAGGACCAC
ATRX	X:76938292-76938292	TGCTGTGTTTCTCATCTTC	AAAGTTCTACATCTGGCTCA
DPEP1	16:89696834-89696834	ACCTGTCATCACTTCCAC	CAGTCCTGGTTCCCTAAG
DUSP5	10:112269955-112269955	CTTATGAAGACCAAGCAGTT	ATGTCAGGGCTCAGTGTC
ETV6	12:11992105-11992105	GGGACTCAGAGTTGAACATA	TGTTAAAGACCAACCACTAAC
FOLR1	11:71907063-71907063	CCTCTCTACAGGGTTTAACA	TTTCCAGGTATCAGAAGGTA
IKZF1_1	7:50450288-50450288	AACAACTTTCTCGTAGCATC	AGAAGCAACAGCTCACAC
IKZF3	17:37947702-37947702	GTAACCCTTCAGAACAAGAA	CCCATTCCAGTGTAATCA
KDM6A	X:44896924-44896924	TGTGATAATGTTTGATGCTT	TAAGAAGTAACGCCCAGA
KMT2D_1	12:49424990-49424990	CTCATCTCTTCTGTCTGACC	GCAAAGAATGTGCAACTCA
MAX	14:65543340-65543340	CTCTACCAACGAACTGAAAG	CCAGAAGGCAGTAACACA
MN1	22:28193705-28193705	GCGAACTACTGTCCGACTT	CGGATTACTTCCCAGGAG
PRKDC	8:48826505-48826505	CAGCCCAATTACCTATTAAG	TTTCTAACAGTCACGTCCTC
SH2B3	12:111885560-111885560	GGAGTTCAGGGTCCTAGAG	CTCTACCCTCTACCCAGTG
SLFN11	17:33679440-33679440	GTTTCTACCATCAGCAGACT	CATGTGATATGTTGGGTGA
TET2	4:106155204-106155204	CTGATGGAACAGGATAGAAC	GTCAGGACTCACACGACTAT
FXIII-pos			
AKT2	19:40740417-40740417	TCTCTGTATGAAACCCTTCC	GGACACAGCACTGATTGAG
CHCHD7	8:57125432-57125432	AAATTGACATTTCCTCTTCA	TTAAGTTTCGTGCCTTACAT
IKZF1_2	7:50450291-50450291	AACAACTTTCTCGTAGCATC	CAAAGAAGCAACAGCTCAC
MECOM	3:168838939-168838939	AAGAGACAGATGTGGGAAG	AATTTCTTCTTGAGTCATCC
MLLT4	6:168276073-168276073	GTACAGATGTAGCCAAATAGC	TAGTTAAAGCAAGTCTCACG
STK39	2:169038498-169038498	CACAATCATAAGGTACAGTCC	TGAATACGCTGAGTAGAAGA
XPO1	2:61719472-61719472	ATCAATAAATGGCATCACTT	AGAGGCAAAGATAATAAAGC
EPOR	19:11492635-11492635	ACGTCATGGGTGTCTCAG	GTGTCACACATACACACTGC
ABCC6	16:16302586-16302586	GGGGTTTCACTATGTTGG	CAGGGTCACACAGCTACT
KTM2D_2	12:49423257-49423257	TGAATAGATGTCTTACTTGAAATC	GCAGGACAGACTCAATAAAT
ATM	11:108098516-108098520	AAATTGTGAACCATGAGTCT	GCATAATGATATATAGGAAGCA
KRAS	12:25398281-25398281	CTGTATCAAAGAATGGTCCT	TTTCATATAAAGGTGAGTTTGT
ZMYM2	13:20635284-20635284	CAAAGTAGATCATGTGTCAGA	TGATGTTGGTTGTCTCTGTT
WRN	8:30933755-30933755	GAATATCTCTGCTTTGTGGT	ACCTTTGGATTCACTATTCA
TCF7L2	10:114849290-114849290	CGAGCCTTACTCTGTTCC	ACAGTGACCCAACAAGAAT
NRAS	1:115252276-115252276	CAAAGAATATGAATATGGATCA	CAGGCTAATCTCAAACTCC
MAX	14:65472929-65472929	AATCCTAGCAAGTCAGTCTC	TTTATTGAGGTCAGAGCATT

Our previous results, as reported, revealed the importance of the moderate FXIII-A positive subgroup which exhibited different clinical outcome and gene expression profile than either the strong FXIII-A positive or the FXIII-A negative subgroup. Therefore, with the help of a novel financial support we would like to include 10 samples of the moderate FXIII-A subgroup in the next generation sequencing experiments. These investigations will be performed before the end of 2018. Final analysis of the data and preparation of the manuscript is to be expected in the second semester of the 2018/2019 academic year.

Conclusions

The grant support provided and excellent opportunity to investigate the role of FXIII-A expression in children with BCP-ALL using a complex set of methods, i.e. precision multicolor FC, conventional genetic methods (G-banding, fluorescence in situ hybridization), CNA by MLPA, gene expression profiling and next generation DNA sequencing. Laboratory data were analyzed in the context of demographic and clinic-pathological data and clinical treatment outcome measures. Complex analysis of FXIII-A expression in BCP ALL has not been published previously. We have demonstrated a significant effect of FXIII-A expression on clinical outcome measures. Moreover, the type of FXIII-A expression has been shown to define new homogeneous molecular subgroups. Incorporation of FXIII-A determination by FC in a new pediatric ALL protocol of the BFM ALL-IC network is feasible. Accurate determination of FXIII-A expression by FC is easy-to-perform and is a non-expensive technic, which can be afforded by medium income countries participating in the ALL-IC cooperation. FXIII-A negative samples should be investigated further with more sophisticated and expensive molecular and genetic methods.

Outstanding the new results of this project have been published in four original research articles and in 8 conference abstracts, one of them an abstract of the American Society of Hematology published in Blood. Two additional manuscripts have been submitted (enclosed to this report as supplementarity material) and two additional manuscripts will be submitted by the end of October 2018 and in the second semester of the 2018/2019 academic year, respectively. Results was also disseminated 8 poster presentations in Hungarian and international conferences. Based on the results of this project one PhD student, a participating researcher of this project (KG) is about to prepare and to defend her PhD Thesis in 2019.

Summary

We have investigated 55 and 317 children with B-cell progenitor (BCP) ALL in a retrospective and a prospective cohort associated to BFM ALL-IC 2002 and 2009 clinical trial respectively. We have proven the high sensitivity and specificity of validated, quality controlled multicolor flow cytometry (FC) in the quantitative evaluation of tumor cells. The method was applied to detect the expression of subunit A of coagulation factor XIII (FXIII-A) in leukemic lymphoblasts. FXIII-A proved a leukemia specific marker in BCP ALL. FXIII-A expression pattern defined subgroups of children with BCP ALL characterized by significantly different event-free and overall survival. FXIII-A negativity correlated with poor prednisolone response and with the “B-other” genetic category. Patients with moderate FXIII-A positivity had a significant survival advantage vs. patients with either strong FXIII-A positive or FXIII-A

negative expression. These expression patterns at the protein level define three characteristic subgroups at the RNA level. Heat map analysis of our Affymetrix MicroArray investigations exhibited specific gene expression patterns for the three subgroups as defined by FC. Differentially expressed genes participating in biologically relevant processes were identified using Gene Ontology analysis. We have identified 50 coding region variants exclusively in the FXIII-A negative samples, 40 variants only in the strong FXIII-A positive samples by targeted exon sequencing analysis.

Enclosures

Copies of two manuscripts submitted for publication.