SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Expression of coagulation factor XIII subunit A (FXIII-A) in childhood B-cell progenitor acute lymphoblastic leukemia

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DEBRECEN, 2022

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The PhD Defense takes place at the Lecture Hall of Pediatrics, Department of Pediatrics, Faculty of Medicine, University of Debrecen, at 13:00 pm, on of 25th January, 2023.

1. INTRODUCTION

Since the discovery of coagulation factor XIII (FXIII), researchers have been actively concerned about their role in the body. Its biological function in coagulation and wound healing processes has been elucidated in several respects. The study of the expression, production, and function of FXIII in the coagulation cascade in unexpected, unusual, healthy, and pathological tissues, including cornea and neoplastically transformed hematopoietic cells, is a relatively new area of FXIII research. Of the latter, the dissertation focuses on the expression of the FXIII A subunit (FXIII-A) in leukemia cells.

Leukemia is the most common malignancy in childhood. [1] Over the last 60 years, the recovery rate for childhood leukemia has improved dramatically. Almost without exception, the overall survival rate of fatal disease before the 1960s is almost 80% today. [2] The dynamic development was due to the increasing intensity of combination chemotherapy. This therapeutic strategy has evolved toward risk-proportionate treatments based on increasingly sophisticated prognostic estimates. Among the risk factors, genetic differences are becoming increasingly important, on the basis of which leukemia subgroups can be clearly classified. Even more accurate with risk assessment and targeted treatment methods, there is an increasing opportunity to apply a personalized therapeutic approach. Approximately 70% of childhood leukemia is associated with B-cell precursor acute lymphoblastic leukemia (BCP-ALL), with over 90% survival.

The expression of the FXIII-A subunit in BCP-ALL lymphoblasts was first described by our group in 2006. Expression was confirmed by flow cytometry (FC) in addition to molecular biological methods. The FC method can be used to determine the percentage of leukemic blast cells expressing the FXIII-A molecule, which allows patients to be grouped for FXIII-A expression. Considering the results, the FXIII-A expression rate observed in BCP-ALL cases

may have prognostic significance, which may be a factor influencing the outcome of recovery. In this dissertation, we summarize our results on the study of FXIII-A expression and the underlying genetic changes observed in childhood BCP-ALL cases.

2. LITERATURE REVIEW

2.1. The coagulation factor XIII and it's biological functions

László Lóránd and Kálmán Laki were the first to identify coagulation factor XIII as a "fibrin stabilizing factor". [3, 4] Based on the significant results of more than 70 years of research, the role of FXIII in coagulation has been clarified in almost every detail.

Circulating FXIII in plasma is a heterotetramer (FXIII-A2B2) composed of two A subunits (FXIII-A) and two B subunits (FXIII-B). A protein with transglutaminase activity stabilizes thrombin-induced fibrin monomers by cross-linking in the last step of the coagulation cascade. The B subunit functions as a career protein, the enzyme activity is exerted by the A subunit. [5]

In terms of its biological function, FXIII-A plays a role in processes such as wound healing, phagocytosis, and bone and connective tissue matrix remodeling. [6] Recognition in the last two decades that the FXIII-A subunit is also expressed in malignantly transformed cells and tissues. Thus, it has been identified in oral mucosal tumors [7], promyelocytic leukemia [8], acute myeloid leukemia [9], Hodgkin's lymphoma [10], and leukemic lymphoblasts. [11]

2.2. Expression of FXIII-A in leukemia

In 1992, R. Invernizzi and colleagues raised the possibility that FXIII-A may play a role in the characterization of acute leukemia. In their studies, they found that cFXIII-A expression in myelomonocytic and monocyte blasts, and cFXIII-A expression correlated with monocyte-specific antigens and cytochemical markers. [12] In 2005, J. Kappelmayer et al. examined the expression of FXIII-A in acute myeloid leukemia. In myelomonocyteic, monocytic, and megakaryoblastic acute myeloid leukemia, FXIII-A was expressed in more than 50% of the

cases. Thus, FXIII-A can be considered a reliable intracytoplasmic marker for monocyte and megakaryocyte types and its presence is highly predictive of mono- and megakaryocyte AML as well as CMML. [9]

3. OBJECTIVES

The aim of our research was to monitor the protein-level expression of FXIII-A in pediatric patients with BCP-ALL using FC and to study the genetic background of the identified FXIII-A groups with molecular genetic testing methods.

Our underlying questions for our hypotheses were:

- How does the level of FXIII-A expression relate to other prognostic factors in patients with BCP-ALL, known LAIPs, and overall and event-free survival?
- Can other genetic abnormalities related to *F13a1* gene expression be identified? If so, how does it affect patients' survival and response to therapy?
- Are there any among the FXIII-A-expressing subgroups that can be associated with the as yet unidentified ‘B-other’ genetic subgroup? If so, can it be defined as a separate subgroup within the ‘B-other’ cases?
- Can FXIII-A expression, defined by FC, be considered as a prognostic factor in childhood BCP-ALL cases?

4. MATERIALS AND METHODS

4.1. Retrospective study

4.1.1. Patients involved in the study, data collection and management

Our retrospective study included 55 patients diagnosed with BCP-ALL. Selected pediatric patients between 1 and 18 years of age were diagnosed with BCP ALL between 2003 and 2011, 48 patients at the University of Debrecen and 7 patients at the University Hospital of Borsod-

Abaúj-Zemplén County. They were treated according to the BFM ALL-IC 2002 protocol. Bone marrow samples were collected in tubes containing EDTA after aspiration according to a routine diagnostic procedure. Bone marrow samples collected at diagnosis were analyzed in all 55 cases, while follow-up was obtained in 42 of the 15-day bone marrow samples.

4.1.2. Immunophenotyping

Flow cytometry assays were performed on a FACSCalibur flow cytometry instrument (Becton Dickinson, San Jose, CA) using a four-color staining procedure. Cell surface and cytoplasmic staining procedures were performed with monoclonal antibodies according to standard protocols. Expression of FXIII-A was measured with a monoclonal antibody conjugated to flourescent isothiocyanate (FITC) (Sigma, St. Louis Mo). [13] A leukemia clone from each patient was considered positive for different immunophenotype markers if the marker was expressed in at least 20% of the cells. For MRD determination, 300,000 events were detected. [14] The collected FC datas were analyzed using CellQuest 3.2 (Becton Dickinson, San Jose, CA) and FACS Diva (Becton Dickinson, San Jose, CA) software.

4.1.3. Chromosome analysis and fluorescent in situ hybridization procedures (FISH)

G-banding was performed according to standard protocols. Karyotype was determined according to the “International System of Human Cytogenetic Nomenclature”. [15] FISH procedures were performed by chromosome preparation from cell suspension according to the manufacturer's instructions LSI *MLL* DC, BA; Using LSI *BCR/ABL* DC, DF, and *TEL/AML1* DC, SF, ES (Abbot / Vysis, Downers Grove, IL) translocation probes. Cells were stained with DAPI (4,6-diamidio-2-phenylindole). According to the protocol, 200 interphase cells were used as a basis for each patient. Images were taken with a fluorescence microscope (Zeiss Axioplan (Carl Zeiss, Zaventem, Brussels)) and the results were analyzed using ISIS software

(Metasystems, Altlussheim, Germany) and patients were classified according to WHO genetic groups.

4.1.4. Statistical analysis

Data were classified using the Shapiro-Wilk test. For the two groups, Student's t-test was used for parametric analysis and the Mann-Whitney U test was used for non-parametric studies. If the p-value was <0.05, the result was considered significant. Pearson's Chi-square test was used to determine variability and logistic regression between the two categories and to perform multivariate analysis.

Kaplan-Meier survival analysis was used to calculate survival rates, and survival curves were generated using the log-rank test. A Cox regression calculation was performed, in which case, in addition to the relative hazard value, the 95% confidence limit was also given. Statistical analyzes were performed using the SPSS 20.0 biostatistics program.

4.2. Prospective study

4.2.1. Patients involved in the study, data collection and management

For the prospective study, bone marrow samples and clinical data from 408 patients treated with BCP-ALL were collected from Polish (188), Hungarian (114), Slovak (13) centers participating in the ALLIC study and Austrian (93) (AIEOP-BFM) participants in the AIEOP-BFM study between 2011 and 2018. Patients with Down syndrome, t(9;22) genetic abnormality, and infant patients were excluded from the study. Immunophenotype was determined in all patients by FC. On day 15 of treatment, MRD measurement was performed on all patients by FC in bone marrow samples. Of the 408 patients, 310 were able to determine the complete genetic profile. These samples were included in the regression analysis. Fifty-nine patients treated in Debrecen and Budapest centers underwent multiple ligation-dependent probe

amplification (MLPA) to examine copy number differences (CNA). Among the patients, 6 Hungarian, 8 Polish and 2 Slovak patients underwent allogeneic bone marrow transplantation.

Polish, Slovak and Hungarian patients were treated according to the ALL IC-BFM 2009 study, while Austrian patients were treated according to the AIEOP-BFM 2009 study. Due to the different treatment protocols, samples from the Vienna center were used only to analyze the correlations between the initial differences and FXIII-A expression. Only patients treated with the same treatment protocol were studied to compare FXIII-A expression and survival data. A total of 21 relapsed cases were detected between Polish, Slovak and Hungarian cases. Patients with relapsed ALL were treated according to the ALL-REZ BFM 2002 protocol (ClinicalTrials.gov ID: NCT00114348). Diagnostic risk classification and prednisolone response from day 8 peripheral blood were performed based on the ALL IC-BFM 2009 study. Cases with low hypodiploid (<45 chromosome number) and iAMP21 genetic abnormalities were grouped into the high-risk group (BFM-HR) according to the principles of the ALL IC-BFM 2009 study. Bone marrow samples from day 15 were assayed for FC-MRD. Based on the MRD results, three risk groups were distinguished: low-risk group (FLR), medium-risk group (FMR), and high-risk group (FHR). Cases with FLR status and SR classification according to conventional risk factors were included in the standard risk group (BFM-SR). Patients with FMR or FHR status at the time of diagnosis or on the basis of a prednisolone response on day 8, from a possibly more favorable risk group to the intermediate risk group (BFM-IR) or by high risk (BFM-HR), respectively.

The “standard” therapeutic arms used in the ALL IC-BFM 2009 study were identical to those in the ALL IC-BFM 2002 study. Hungarian and Slovak patients were randomized. Patients in the BFM-IR and BFM-HR groups either received a standard early intensification according to the ALL IC-BFM 2002 study or received an “augmented” early intensification according to the protocol recommended by the BFM and the Pediatric Tumor Group (CCG). [16, 17] Patients

diagnosed with IR BCP-ALL received 2 g/m2 methotrexate (Mtx) according to the ALL IC-BFM 2002 study or 5 g/m2 methotrexate (Mtx) according to ALL -BFM 86 protocol in the consolidation phase. No randomization was performed for Polish patients: patients in the BFM-IR and BFM-HR groups received standard early augmented intensification. Patients with IR BCP-ALL received 2 g/m2 methotrexate (Mtx).

4.2.2. Immunophenotyping

Samples were analyzed by 5-8 color staining with FacsCantoII (Becton Dickinson, Franklin Lakes, NJ, USA) and Navios and FC-500 (Beckman Coulter, Brea, CA, USA) flow cytometry. The cell line was determined according to the EGIL criterion. [18] Cell surface and cytoplasmic staining procedures were performed with monoclonal antibodies according to standard protocols. Expression of FXIII-A was measured with a monoclonal antibody conjugated to fluorescent isothiocyanate (FITC). To test the sensitivity of the FXIII-A assay, three serial dilutions (10X, 100X, 1000X) were performed in the remaining lymphoblasts to determine the percentage of FXIII-A expression. The percentage of FXIII-A expression in the remaining lymphoblasts was clearly estimated when the proportion of lymphoblasts was above 0.04%.

All centers used the same tubes for FXIII-A determination. All immunophenotype markers, including FXIII-A labeling, are positive above a threshold of 20% or greater. To further investigate the significance of FXIII-A expression, three groups were identified: BCP-ALL with FXIII-A negative blasts (<20% FXIII-A positive lymphoblasts; FXIII-A negative group), BCP-ALL with FXIII-A moderately positive expression (20-79% FXIII-A positive lymphoblasts; FXIII-A dim group) and BCP-ALL FXIII-A with strong positive expression (>80% FXIII-A positive lymphoblasts; FXIII-A bright positive group). Normal lymphoblasts were used as controls. FC data were analyzed using FACSDiva (Becton Dickinson, Franklin Lakes, NJ, USA) or Kaluza (Beckman Coulter, Brea, CA, USA) software. Flow cytometers were controlled with fluorescent microbeads from Cytometer Setup & Tracking (Becton

Dickinson, Franklin Lakes, NJ, USA) or Flow Check Pro (Beckman Coulter, Brea, CA, USA). All laboratories in the study participated in the UK-NEQAS Leukocyte Immunophenotyping MRD Program and successfully completed the ALLIC Annual Ring Trials. [19]

4.2.3. Genetic investigations

Bone marrow samples were processed according to the standard protocol within 24 hours. Bone marrow samples used for flow cytometry assays and assays recommended by the manufacturers were used to determine FISH. Cases with t(12;21)/ETV6-RUNX1 rearrangement or high hyperdipoidy (chromosomes 51-65) were classified as low risk. The high-risk group included cases of near haploid (chromosome 23–29) or low hypodiploid (<45 chromosome) with MLL translocation, complex karyotype with iAMP21. The medium-risk group included cases of t(1;19) as well as genetic abnormalities that did not fit into either the low-risk or high-risk groups, including the 'B-other' genetic subgroup.

DNA was extracted from bone marrow samples using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). SALSA MLPA P335-B2 ALL-IKZF1 (MRC-Holland, Amsterdam, The Netherlands) probes were used for MLPA assays. In terms of copy number alterations, three groups were distinguished: those with no detectable deletion in the *IKZF1*, *CDKN2A/B*, *PAR1*, *BTG1*, *EBF1*, *PAX5*, *ETV6*, *RB1* genes or an isolated deletion in *ETV6*, *PAX5*, *BTG1*, *ETV6* genes and a single additional deletion was detected in the *BTG1*, *PAX5*, or *CDKN2A/B* genes were considered to have a good prognostic group; cases with a deletion in the *CDKN2A/B* gene or in combination deletion with the *CDKN2A/B*/*PAX5* gene were at intermediate prognostic group; evenings with deletions in *IKZF1*, *PAR1*, *EBF1*, *RB1* genes and all genetic alterations not included in the above groups were classified as poor prognostic groups. [20-23]

4.2.4. Statistical analysis

Data were analyzed using the Shapiro-Wilk test. Student's t-test was used for parametric analysis and Wilcoxon test was used for non-parametric studies. In the case where more than two groups were analyzed, the Kruskal-Wallis test was used. The Dunn's multiple comparison test was used as a post hoc test. Patients' initial parameters, such as age and white blood cell count, were transformed into categorical variables based on their prognostic effect. Dichotomous categorical variables were compared using Pearson's Chi-square test, and multivariate logistic regression methods were used to analyze multivariate cases. If the p-value was <0.05, the result was considered significant. Kaplan-Meier survival analysis was used to calculate survival rates. Cox regression calculations were performed at the Hazard value (HR) and the 95% confidence limit (CI). The endpoint of event-free survival analysis was determined by the time between the diagnosis of ALL and the first relapse or death. Statistical programs STATA / IC 14.2 (College Station, TX, USA), SPSS 20.0 (Chicago, IL, USA) and GraphPad Prism 6.0 (San Diego, CA, USA) were used for statistical analysis and image editing.

4.3. Gene expression studies

4.3.1. Patients included in the study, collection and treatment of samples for gene expression studies

In the course of our work, we collected bone marrow patient samples nationwide from 2015 to 2018 to perform gene expression studies. The approval of the study was granted by ETT TUKEB under number 43033-1 / 2014 / EUK (423/2014).

A total of 71 bone marrow samples from newly diagnosed BCP-ALL patients were selected for analysis. Exclusion criteria included BCP-ALL with cumulative Down syndrome and Ph+-ALL and in patients less than 1 year of age.

4.3.2. Processing patient samples for MicroArray analysis

Two ml of the bone marrow aspirate at the time of diagnosis was collected in PAXgene Blood RNA (PreAnalytix, Hombrechtikon, Switzerland) tubes, from which the qualitatively and quantitatively appropriate RNA extract was obtained using the PAXgene Blood miRNA kit (PreAnalytix, Hombrechtikon, Switzerland). [24] In 42 of the 71 patient samples (14 FXIII-A negative; 21 moderately FXIII-A positive (FXIII-A dim); 7 FXIII-A positive (FXIII-A bright)) were isolated and quality contoroled by Agilent Bioanalyzer (Agilent Technologies, La Jolla, CA, USA). Following analysis, samples with an RIN (RNA integrity number) of 8.0 or greater were applied to the MicroArray chips.

4.3.3. Immunophenotyping and chromosome analysis, as well as FISH studies for both retrospective and prospective studies, were performed using the methods described.

4.3.4. MicroArray and gene ontology analysis

The expression pattern of 28,869 well-defined genes was analyzed using the Affymetrix GeneChip Human Primeview array. The 3'IVT Expression Kit (Affymetrix) and the GeneChip WT Terminal Labeling and Control Kit (Affymetrix) were used to amplify and identify a 250 ng RNA sample. Samples were hybridized for 16 hours at 45 ° C and then prepared according to the standard wash protocol for loading onto the GeneChip Fluidics Station 450 (Affymetrix) gene chip. Samples were analyzed using a GeneChip Scanner 7G (affymetrix). The raw data obtained were uploaded to NCBI's Gene Expression Omnibus, where it is publicly available in the GEO database under identification number GSE134480 (https://ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134480).

Gene ontology analysis (GO) was performed using Cytoscape 3.4.0 software (cytoscape.org) via the ClueGo application. Based on the data analysis settings, the GO biological functions and the functions associated with the GO immunological system were set up. The two-way

hypergeometric test and the Benjamini-Hochberg FDR test were used for statistical analysis. Those with a p-value of <0.05 and a κ value of <0.4 were considered significant GO categories.

4.3.5. Validation of identified genes by real-time quality polymerase chain reaction (RT-Q-PCR) and those in silico analysis

Data from microarray assays were validated by RT-Q-PCR. A 384-well TaqMan low-density assay (ThermoFisher Scientific, Waltham, MA, USA) was used in duplicate for each sample. For validation, the genes were selected based on two criteria: 1) based on the fold-change values obtained during the microarray analysis, the genes with a fold-change> 2.0 were selected; 2) genes with a biological function identified from the GO database. For RT-Q-PCR reactions, normalization was examined for *B2M, GAPDH,* and *GUSB* reference genes. Gene expression values were normalized based on ΔCt values. Validated genes co-expressed with the *F13a1* gene are described in STRING v11. [25] and using GeneHancer [26] software.

4.3.6. Statistical analysis

In this study, microarray data were analyzed using Genespring GX14.9.1 software (Agilent Technologies, La Jolla, CA, USA). Statistically significant genes were selected by volcano plot analysis. Data from the affymetrix assay were converted to Robust MultiArray Average software for normalization. The identified differentially expressed (DE) genes were examined by ANOVA, Tukey post-hoc [27], moderate T-test and Benjamini-Hochberg FDR tests.

5. RESULTS

5.1. Results of retrospective studies

Of the 55 ALL patients enrolled in the study, 18 were found to be FXIII-A negative and 37 were found to be FXIII-A positive. The pattern of FXIII-A expression was different in the two groups. The cut-off for FXIII-A positivity was set to 20%, and patients were divided into 2

groups, FXIII-A positive and FXIII-A negative. FC risk groups were separated based on MRD status measured by FC on day 15. Genetic risk groups, the 'B-other' genetic subgroup, and risk groups were defined based on the ALL IC-BFM 2002 study.

In our studies, we found that classical prognostic markers such as age and initial white blood cell count did not correlate with FXIII-A expression. Although the response to prednisone was found to be much higher in the FXIII-A negative group than in the FXIII-A positive group (29% vs. 5.7%), it was not statistically significant.

We also examined the relationships between FXIII-A expression and other categories. Relationships between age, initial FVS, prednisone response, FC, and genetic risk groups alone showed a significant difference in the comparison of FXIII-A negative and FXIII-A positive groups only in the 'B-other' genetic subgroup (OR: 7.1; 95 %, CI: 1.7-29.1; p=0.006).

The association between the FXIII-A and 'B-other' genetic subgroups was also supported by multivariate logistic regression analysis (OR: 7.1; 95%, CI: 1.7–29.1; p=0.006). The difference between the two groups was maintained even when initial parameters such as age or FVS were added to the analysis (OR: 7.8; 95%, CI: 1.8–34.7; p=0.007).

Kaplan-Meier survival curves showed a significant difference between the FXIII-A positive and FXIII-A negative groups in both event-free survival (EFS) and survival (OS) (p=0.031 and p=0.008). 10-year EFS and OS were significantly higher in FXIII-A positive (84%, 95%, CI: 67.4-92.4 and 89%, 95%, CI: 73.7-95.8) patients than in FXIII-A negative (61%, 95%, CI: 35.3-79.2 and 61%, 95%, CI: 35.3-79.2) patients. A significant difference between EFS and OS was observed not only when based on FXIII-A expression, but also when the known genetic subgroups were compared with the 'B-other' genetic subgroup (EFS p=0.058, OS p=0.021).

Survival data were also examined by multivariate Cox regression analysis. When the initial prognostic parameters (age and FVS) were included in the Cox studies, FXIII-A expression was the strongest effect on OS (HR: 4.8; 95%, CI: 1.2–19.2; p=0.025).

The above result, according to which FXIII-A is the most significant of the examined parameters in terms of survival data, was also confirmed by another uni- and multivariate analysis. In this analysis, all clinical parameters, including known genetic subgroups, initial parameters such as age and initial FVS, FXIII-A expression, were examined for EFS and OS. The results showed a significant difference in both cases.

Based on our results, it can be said that FXIII-A expression may be a prognostic factor for BCP ALL. FXIII-A negativity is associated with a worse outcome, which is not only due to association with the 'B-other' genetic subgroup, but in itself suggests poorer survival.

For EFS and OS, the 15-day FC-MRD results support the above findings. For the three groups based on FC-MRD, the FLR and FMR groups were also significantly different from the FHR group for both EFS and OS: p=0.01 for both EFS and OS between the FLR and FHR groups; p=0.01 for EFS and p=0.004 for OS between the FMR and FHR groups.

5.2. Results of prospective studies

5.2.1. Clinical relevance of FXIII-A in childhood acute progenitor B-cell lymphoblastic leukemia

In contrast to our retrospective studies, we distinguished three groups for FXIII-A expression: FXIII-A negative (FXIII-A <20%), FXIII-A dim (FXIII-A 20-79%), and FXIII-A bright (FXIII-A 80%) groups.

The expression FXIII-A describes a process that makes it difficult to distinguish sharply from the FXIII-A negative and FXIII-A bright groups. Analyzing the dot plots, it can be said that in

the case of the negative expression pattern, the leukemic lymphoblasts overlapped with the FXIII-A-negative normal lymphocytes. In bright cases, the leukemic blast cell population is almost completely separated from normal lymphocytes. In the dim pattern, leukemic lymphoblasts appeared as a broad but homogeneous group, partially overlapping with normal lymphocytes. Of the 408 samples analyzed, 137 were negative, 189 were in the dim group, and 82 were in the bright group.

In thirty-six cases, FXIII-A expression was also examined on day 15 of diagnosis and treatment. Cases in the FLR group were excluded because it is difficult to determine the rate of FXIII-A expression at lymphoblast rates below 0.1%. In patients in the FMR and FHR groups, 15-day FXIII-A expression was significantly lower than baseline ($p<0.001$). FXIII-A expression in FXIII-A negative de novo cases did not change significantly by day 15. None of the FXIII-A de novo cases exceeded the 20% limit by day 15 and did not become positive.

We analyzed the possible correlations between FXIII-A expression, EFS, and OS using a Kaplan-Meier survival curve, and no significant difference was detected between FXIII-A positive and FXIII-A negative patients.

We examined the correlations between EFS and OS for the three FXIII-A groups. We found that there was a significant difference in EFS compared to the FXIII-A dim group compared to the FXIII-A negative group (p=0.012) and the FXIII-A bright group (p=0.001). Based on the Kaplan-Meier survival curve, it can be concluded that the five-year EFS of the FXIII-A dim group is significantly higher (93%) than the FXIII-A bright (61%) and FXIII-A negative (70%) groups. Five-year EFS in the dim, negative, and bright groups developed as follows: FXIII-A dim 93%, FXIII-A negative 70%, and FXIII-A bright 61%. For EFS, the mean follow-up time was 1736 days for the FXIII-A dim group (95% CI: 1675 and 1796 days), 1277 days for the FXIII-A bright group (95% CI: 1153 and 1400 days), and the FXIII-A negative group had 1588 days (95% CI: 1484 and 1692 days).

The five-year OS was 95% in the FXIII-A dim group, which was significantly higher than in the FXIII-A negative group (88% p=0.044). The difference between the FXIII-A dim and FXIII-A bright groups (87%) showed no significant difference. For OS, the mean follow-up time was 1755 days for the FXIII-A dim group (95% CI: 1699 and 1810 days), 1454 days for the FXIII-A bright group (95% CI: 1341 and 1567 days), and the FXIII-A negative group had 1661 days (95% CI: 1572 and 1749 days).

Known risk factors, known risk groups, were examined using a multivariate Cox regression analysis comparing two FXIII-A groups. In one case, the FXIII-A negative group was compared to the FXIII-A dim group, and in another case, the FXIII-A dim group was compared to the FXIII-A bright group. Categorical variables such as FXIII-A expression pattern, age, prednisone response, distribution of genetic risk categories, and distribution of the "B-other" genetic subgroup had a significant effect on five-year EFS and five-year OS data for dim vs. negative FXIII-A groups. For the risk groups in the ALL IC-BFM 2009 study, there was a significant difference in five-year OS data. In the case of multivariate analysis, where only the genetic risk groups (good vs. moderate) were compared, in which case the significance remained.

In the FXIII-A expression, there were significant differences in survival data between the dim and bright FXIII-A groups in the case of ALL BFM-IC 2009 risk categories (BFM-HR vs. BFM-IR), and genetic risk groups. In the multivariate Cox analysis, significance remained when comparing FXIII-A expression and genetic risk groups.

Based on our studies, we found that the FXIII-A negative and FXIII-A bright groups were associated with unfavorable results for EFS and OS. Comparing the two groups, the prednisone response and the genetic risk group were significantly different. Poor prednisone response was characteristic of the FXIII-A negative group, whereas high-risk genetic abnormalities were concentrated in the FXIII-A bright group.

Copy number alterations (CNA) were examined in 59 patients, of whom 20 were FXIII-A negative, 26 FXIII-A dim, and 13 FXIII-A bright. Poor prognostic CNA was detected in a slightly higher proportion (7/20) in the FXIII-A negative group than in the FXIII-A dim (4/26) and FXIII-A bright (1/13) groups. However, the differences were not significant.

5.2.2. Relationships between FXIII-A expression, minimum residual disease (MRD) and genetic risk groups

Correlations between FXIII-A expression and other known risk factors were examined using Pearson's Chi-square test and multinomial logistic regression models. The different FXIII-A expression patterns did not correlate significantly with either the ALL BFM-IC 2009 or FC MRD risk category groups. The moderate genetic risk group was significantly more common in the FXIII-A negative group than in the FXIII-A dim or FXIII-A bright group (p=0.009 and p=0.039). Correlation occurred to the same extent in the FXIII-A negative and FXIII-A positive groups and in the "B-other" genetic subgroup (p=0.008 FXIII-A negative and FXIII-A dim group; p=0.004 FXIII-A negative and FXIII-A bright group).

Patients with FXIII-A dim or FXIII-A bright lymphoblasts are less likely to have a genetic abnormality characteristic of the 'B-other' genetic subgroup (OR: 0.49 and 0.35). This correlation was seen when age, sex, and white blood cell count were considered among the categorical variables.

Genetic variation in iAMP21 was also significantly more common (p=0.029) in the FXIII-A negative group than in the other two FXIII-A groups. Other known genetic aberrations showed a similar distribution within the three FXIII-A expression groups.

5.3. Results of gene expression studies

5.3.1. Characteristic of BCP ALL samples

In our studies, RNA from 42 BCP ALL patient samples were used to analyze the gene expression pattern. Cytoplasmic FXIII-A expression was determined by the FC method. Consistent with our prospective studies, three groups were distinguished: FXIII-A negative, FXIII-A dim, and FXIII-A bright.

Considering the genetic groups, 27 patients belonged to the group of known genetic differences and 15 patients belonged to the 'B-other' genetic subgroup. Among patients in the 'B-other' genetic subgroup, the following distribution was observed: 7/12 FXIII-A negative, 6/21 FXIII-A dim, and 2/7 FXIII-A bright.

5.3.2. Expression pattern of BCP ALL samples

Differentially expressed genes (DE) were determined based on FXIII-A expression by the FC method and the 'B-other' genetic subgroup. DE genes were screened using the volcano plot method. Comparing the FXIII-A negative and FXIII-A bright groups, 26 DE genes were identified. Comparing the FXIII-A dim and FXIII-A bright groups, 155 DE genes were identified, while 88 DE genes were identified between the FXIII-A negative and FXIII-A dim groups. With the exception of one or two outliers, the heat map analysis revealed that the three FXIII-A groups are separated not only at the protein level but also at the gene expression level. During the heat map analysis, it was observed that the samples belonging to the FXIII-A negative and FXIII-A bright groups are grouped close to each other, but they are nicely separated from the FXIII-A dim group.

Comparison of the gene expression pattern of the 'B-other' genetic subgroup with the other patient samples designated the non-'B-other' subgroup identified 142 DE genes when the fold-

change value was set to 1.5. In the heat map analysis, the ‘B-other’ genetic subgroup was separated from the non-‘B-other’ genetic subgroup.

For the above results, we examined whether the DE genes identified for the ‘B-other’ genetic subgroup are associated with the FXIII-A subgroups. Comparing the FXIII-A negative group and the ‘B-other’ genetic subgroup and the FXIII-A negative group and the non-‘B-other’ genetic subgroup, 32 DE genes were identified. In the heat map analysis, we found that the expression pattern of DE genes in the ‘B-other’ genetic subgroup overlaps with the expression pattern of DE genes in the FXIII-A negative group.

5.3.3. Functional characterization of differentially expressed genes in BCP-ALL samples

DE genes identified based on the FXIII-A expression pattern extended with a functional category were involved in 156 gene ontology (GO) processes. Most DE genes, especially those with the strongest statistical p-values, are involved in epigenetic and/or gene expression regulatory processes such as histone modification, chromatin organization, RNA destabilization, post-transcriptional regulation of gene expression, etc. or other regulatory and cellular processes such as apoptosis and morphogenesis. In addition, we have identified biological processes that result in peptidyl lysine modification associated with the known physiological function of FXIII-A, which catalyzes the formation of g-glutamyl-ε-lysylamide crosslinks between fibrin monomers to form an insoluble blood clot.

In the ‘B-other’ status comparison of DE genes with the fold change value was> 2.0, much less GO processes were identified than according to FXIII-A status comparison. The *CCL5*, *CD3G*, *IL7R*, and *PLAC8* genes involved in lymphocyte and T-cell apoptotic processes were overexpressed, while the *CX3CR1* and *RORA* genes regulate macrophage migration. If the fold change was set to > 1.5, genes involved in other biological processes became significant. These

genes are *BCL10, CX3CR1, GNLY, PTPRC, STK4, TNFSF10, ATP2B1, DNAJC3, PLAC8, THRA, MAPKBP1, PER1, RORA, USP32, CCL5, GNG2, PLCB3, BCL10, CCL5, CD3G*.

5.3.4. Validation of global transcriptomics data

Of the DE genes identified by microarray according to FXIII-A expression status or 'B-other' genetic status, 45 genes were selected for RT-Q-PCR validation. Based on the fold change values, 13/45 genes and 32/45 genes with functional biological characteristics were selected. During RT-Q-PCR validation, the *RORA* gene alone could not be detected, which may be due to a technical error.

5.3.5. FXIII-A expression-based results

The *F13A1* gene was identified in all samples and validated by RT-Q-PCR. After RT-Q-PCR validation, we found that three different intensities were observed in the intensity of gene expression according to the three FXIII-A subgroups. For the *F13A1* gene, the lowest intensity was observed in the FXIII-A negative group and the highest in the FXIII-A bright group. This trend was also observed for the *ANGPTL2, RAPGEF5, SEMA6A, HAP1, NUCKS1,* and *TRH* genes. For the *FOXO1* gene, the FXIII-A bright group had the highest intensity and the lowest the FXIII-A dim group. For the *PLAC8* gene, the highest intensity was measured in the FXIII-A dim group and the lowest in the FXIII-A bright group.

Potential interactions could not be revealed between the protein products of validated genes and FXIII-A expression using STRING v11 functional protein association networks database. Nevertheless, an FXIII-A-dependent expression could be identified for the *FOXO1* gene. Using the GeneHancer database, we were able to identify in silico enhancer processes that may play a role in the upregulation between the *F13A1* gene and the validated genes. Transcription factor binding sites of *ATF7, POLR2A, RAD21, SMARCA5* gene products were identified for all of the 14 genes.

5.3.6. ‘B-other’ status-based results

Based on ‘B-other’ status, DE genes involved in biological processes (macrophage migration, lymphocyte apoptotic process, T-cell apoptotic process, and their regulation) were selected for validation. In this case, the GO analysis results were less diverse than the FXIII-A expression-based GO annotations.

We were able to validate the differential expression of five genes by RT-Q-PCR. *DFFA*, *GIGYF1*, *GIGYF2*, and *INTS3* genes were overexpressed in ‘B-other’ vs. ‘non-B-other’ samples and in turn, *CD3G* exhibited a relatively lower expression in the ‘B-other’ vs. ‘non-B-other’ samples. *RORA*, *IL7R*, *CCL5*, *PLAC8* and *CX3CR* genes selected on the basis of microarray analysis and biological functions could not be validated.

6. DISCUSSION

The normal expression sites for the coagulation factor XIII subunit A are monocytes, megakaryocytes, and platelets. [28] Our group identified the expression of FXIII-A on BCP-ALL samples by flow cytometry, immunoblotting, and laser scanning microscopy. [8, 29] Based on the expression of FXIII-A observed in BCP-ALL lymphoblasts, a new subgroup or subgroups can be defined. In our retrospective studies, we found that BCP-ALL cases in the FXIII-A positive group had significantly better survival data, both overall survival and event-free survival, than in the FXIII-A negative group.

The results of our retrospective studies may provide other explanations for the poor outcome of FXIII-A negative cases. FXIII-A-negative lymphoblasts were significantly more common in patients classified as ‘B-other’ using univariate and multivariate analyzes. Thus, the lack of FXIII-A expression is related to the unfavorable course of disease of ‘B-other’ genetic pattern. This “biomarker nature” also draws attention to the fact that FXIII-A negative cases require detailed molecular genetic evaluation. Determination of FXIII-A by flow cytometry proves to

be a cost- and time-efficient procedure that may have prognostic significance in patients with BCP-ALL. Our retrospective studies had several limitations. On the one hand, we were able to analyze relatively few (55) patient samples, and on the other hand, only DNA samples were available, and in the absence of RNA samples, we were unable to perform either gene expression analysis or MLPA assays for more accurate molecular genetic analyses.

We wanted to support the results obtained in our retrospective studies with a prospective study. In our prospective studies, we examined the potential utility of FXIII-A as a risk-associated biomarker in children diagnosed with BCP-ALL treated in the ALL BFM-IC 2009 clinical trial. Four international working groups belonging to two different childhood ALL treatment consortia within the International BFM Working Group (Polish, Hungarian, Slovak: ALLIC and Austrian: AIEOP-BFM) joined the study. The large number of cases included in the study (408) allowed for a more detailed examination of FXIII-A expression patterns.

In the prospective study, FXIII-A expression was divided into three groups instead of the retrospective study: FXIII-A negative (<20%), FXIII-A dim (20-79%), and FXIII-A bright (≥80%). Most children with BCP-ALL belonged to the FXIII-A dim group. This subpopulation showed better survival than both the FXIII-A bright and FXIII-A negative groups according to the univariate Kaplan-Meier analysis. The lower survival chances of the FXIII-A negative subgroup were influenced by the accumulation of patients in the medium genetic risk group and the 'B-other' genetic subgroup. Several studies have shown that patients with 'B-other' BCP-ALL have a higher risk of relapse. [30] Multivariate Cox regression analysis revealed that the 'B-other' genetic subgroup has significant prognostic value for EFS and OS for FXIII-A negative vs. FXIII-A dim group. We observed a tendency for poor prognostic CNAs to be associated with FXIII-A negativity; however, due to the small number of cases included in the MLPA study, the difference was not statistically significant.

Based on multivariate Cox analysis, we found that high- and medium-risk genetic groups had a significant effect on survival data compared with the FXIII-A bright group to the FXIII-A dim group. In a univariate Cox regression analysis, we found a significant association between poor EFS and OS data for the FXIII-A bright group and the BFM-HR category. These results suggest that adverse genetic alterations may overwrite the beneficial effects of FXIII-A expression in children with BCP-ALL.

There were several limitations to our prospective studies. Only children with BCP-ALL were included in the study. FXIII-A labeling and measurement were performed based on the availability of anti-FXIII-A monoclonal antibody. Patients in the Polish group were not randomized. Over the past four years, a significant number of patients have been included in the ALL BFM-IC 2009 study, which may change the final result for 5-year EFS and OS data. However, the follow-up time was sufficient to show statistically significant differences between the groups with different FXIII-A expression patterns. The complete genetic background was available from only 214/317 patients, and changes in MLPA copy number were studied in only a small proportion of patients.

In our gene expression studies, we wanted to support the presence of *F13A1* gene expression and to observe possible correlations between BCP-ALL patient samples, known genetic subgroups, 'B-other' genetic subgroup, and FXIII-A protein-level expression. The transcriptional regulation of the *F13A1* gene is relatively little known. Molecular studies of myeloid leukemia cell lines revealed binding sites (NF-1 and SP-1) and the presence of binding sites for myeloid-specific (MZF-1-like protein, GATA-1 and Ets-1) transcription factors in the promoter region of the gene. Binding sites for the transcription factors SP-1, GATA-1, and Ets-1 could not be identified in the ENCODE ChIP-seq database. Another study showed that FXIII-A is synergistically regulated by IL-4 and dexamethasone in alternatively activated macrophages, and these regulatory molecules are observed in both normal and BCP ALL. [32]

The transcriptional regulation of the *F13A1* gene in leukemic lymphoblasts has not been studied.

In our preliminary studies, we found that patients with the FXIII-A dim expression pattern had better survival rates than with FXIII-A-negative lymphoblasts. Gene expression studies were performed on all three FXIII-A expression groups, and it was found that all three groups differed significantly. GO analysis of the data resulted in a number of biologically relevant functional categories. The clinical significance of our results is supported by the fact that we have successfully identified the biological processes influencing peptidyl-lysine modification. One of the well-known functions of FXIII-A is to stabilize the fibrin monomers through γ-glutamyl-ε-lysyl crosslinks, and it is involved in intracellular processes that affect monocyte/macrophage, osteoblast, and osteoclast differentiation. [33] Thus, the question may arise that FXIII-A may also have such biological functions in BCP ALL lymphoblasts. The *EHMT1*, *ING5*, *MDM2*, and *NUP43* genes we validated are proteins belonging to the intracellular and intracellular methylation and acetylation lysyl groups that are able to regulate neoplastic cell survival. [34]

Fourteen genes were validated based on FXIII-A status. We found that the *F13A1* gene was expressed in all samples and its expression levels followed the expression levels of the three groups identified by the FC method. The *ANGPTL2, EHMT1, FOXO1, HAP1, NUCKS1, PIK3CG, RAPGEF5, SEMA6A, SPIN1, TRH*, and *WASF2* genes of the 14 identified genes, have biological and clinical functions that play a role in leukemia and in the development of other cancers. The *NUP43* gene we identified, in contrast to the NUP gene family, is not associated with any function typically characteristic of human tumors. [35] The *PLAC8* gene, which is a physiologically trophoblast cell line marker, is intensively expressed in the FXIII-A dim group. This gene has been shown to be activated aberrantly in mammals and mammalian tumor cell lines in a variety of tumor types, however, none of the human ALL subtypes have been

identified yet. [34, 36] Based on in silico studies, we could not reveal a direct relationship between the DE genes and the regulation of the three different FXIII-A expression levels and the regulation of the *F13A1* gene. The common enhancer elements of the validated DE genes and *F13A1* gene suggest that common transcription factors may regulate the expression of these genes in a similar manner.

The DE genes validated in the 'B-other' subgroup show overlap with the FXIII-A negative subgroup, which is presumably a new subgroup within the *BCP-ALL1-like/Ph-like B-ALL* subgroup. Based on our microarray results, 14 DE genes (*ANXA5*, *ARL4C*, *CD34*, *IFNGR1*, *MAGT1*, *MAPKBP1*, *MAP3K2*, *ME3*, *OSBPL8*, *PAPOLA*, *PTPRC*, *SUZ12*, *TACC1*, *TEMPO*) were identified within the 'B-other' genetic subgroup, which shows overlap with the results published by Den Boer et al. [37] All validated genes, such as *CD3G*, *DFFA*, *GIGYF1*, *GIGYF2*, and *INTS3*, are involved in neoplastic processes as well as in the development of leukemias. [38-42] To date, none of the genes have been identified/described in childhood BCP-ALL cases.

The limitations of our gene expression studies are the small number of samples and the very small number of validated genes. Clinically relevant survival rates could not be established and no significance could be determined. Nevertheless, a significantly higher proportion of patients in the FXIII-A negative group died from their disease: 5/14 (36%) than in the FXIII-A dim group: 5/21 (24%) and the FXIII-A bright group: 1/7 (14%). Although not significant based on the small number of cases, based on the results of our prospective studies, patients in the FXIII-A negative group are more likely to be classified in the 'B-other' genetic subgroup and have a poorer outcome in terms of survival rates.

7. SUMMARY

Understanding the biological function of intracellular FXIII-A is increasing concern to researchers. Our group studied the expression of FXIII-A in childhood BCP-ALL patient samples as well as its potential biological functions and clinical relevance.

In our retrospective studies, we found a correlation between overall survival and the FXIII-A positive phenotype of lymphoblasts, demonstrating that the FXIII-A expression character of lymphoblasts may be not only a useful LAIP but also a potential prognostic factor in childhood BCP-ALL. In addition, the expression FXIII-A may be associated with the 'B-other' genetic subgroup, so FXIII-A may help identify cases that may require further detailed genetic testing.

In our prospective studies, three distinct groups (FXIII-A negative, FXIII-A dim, and FXIII-A bright) were separated by flow cytometry, depending on the quantity of FXIII-A expression in BCP-ALL lymphoblasts. There were both statistically significant and clinically significant differences in the expression pattern of FXIII-A in pediatric patients with BCP-ALL, which could be incorporated into the risk assessment strategy of a new ALL BFM-IC clinical trial. FXIII-A negativity is a prognostic factor for disappointing survival data, and FXIII-A negativity correlates with the 'B-other' genetic subgroup, so in these cases more detailed, specific molecular genetic studies are needed to patients in this group should receive risk therapy.

In our gene expression studies, we were the first to identify the *F13A1* gene in pediatric samples from BCP-ALL. The intensity of *F13A1* gene expression follows the same trend as that observed by flow cytometry. The FXIII-A negative patients were accumulated in the 'B-other' genetic subgroup which was also observed in these studies. Validated genes have not only biological function but also clinical relevance. We were able to identify genes that have not yet been described in childhood patients with BCP-ALL. The appearance of these genes at the

protein level and their identification raise additional therapeutic options in pediatric BCP-ALL patients.

8. SPECIAL THANKS

I would like to express my gratitude to my supervisor, Prof. Dr. Csongor Kiss, who helped with the collection of samples, the planning and execution of the work, the evaluation of the results and the preparation of the dissertation with his outstanding professional knowledge and guidance.

I would like to thank my colleague Dr. Bettina Kárai, without whom the flow cytometric grouping of FXIII-A would not have been possible.

I am grateful to the heads and staff of all domestic and international Pediatric Hematology Centers and to the heads and staff of all domestic and international Flow Cytometry Laboratories for their participation in sample and data collection and data evaluation.

I would like to thank the staff of UD Genomed Kft for carrying out the processes related to gene expression studies.

I would like to thank Dr. Beáta Scholtz for teaching me the methodology for analyzing gene expression patterns.

I have much to be thankful to my Mother, Father, Brother, and most of all to my Child for guiding me through my patience, supporting and helping me achieve my goal.

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