Genetic Profiling in Children With Acute Lymphoblastic Leukemia Referred for Allogeneic Hematopoietic Stem Cell Transplantation

Kinga Kwiecinska, MD1, Wojciech Strojny, MD1, Miroslaw Bik-Multanowski, MD, PhD2, Michal Korostynski, PhD3, Marcin Piechota, PhD3, Walentyna Balwierz, MD, PhD1, and Szymon Skoczen, MD, PhD1

Abstract

Introduction: Hematopoietic stem cell transplantation (HSCT) is the essential and often the only curative therapeutic option in high risk and relapsed pediatric acute lymphoblastic leukemia (ALL).

Methods: The objective of the study was to investigate whole-genome expression in children with high risk or relapsed ALL referred for HSCT. Gene expression was assessed in 18 children with ALL referred for HSCT (10 high risk, 8 relapsed; median age of 9.4 years) and in a control group of 38 obese children (median age of 14.1 years). Whole-genome expression was assessed in leukocytes using GeneChip® HumanGene 1.0 ST microarray.

Results: The analysis of genomic profiles revealed a significantly lower expression of 21 genes with a defined function, involved in immunoglobulin production, lymphocyte function, or regulation of DNA processing in ALL patients referred for HSCT compared with the control group.

Conclusion: Genome expression of patients with ALL in remission referred to HSCT revealed deep immunosuppression of both B-cell and T-cell lineages, which may increase the probability of donor cell engraftment.

Keywords
acute lymphoblastic leukemia, genome expression profile, HSCT

Introduction

Hematopoietic stem cell transplantation (HSCT) is currently the treatment option associated with highest survival rates in high risk and relapsed pediatric patients with acute lymphoblastic leukemia (ALL).1,2 Patients are referred for transplantation after the first- or subsequent-line intensive chemotherapy, usually being in complete hematologic remission.3,4 Based on our experience with gene expression pathways5 and study of peptides regulating hematopoiesis,6 we designed a retrospective study of peripheral blood gene expression in children with ALL referred to HSCT. The hypothesis was that the gene expression of peripheral blood cells in patients referred to HSCT may

1Department of Oncology and Hematology, Institute of Pediatrics, Jagiellonian University Medical College, Krakow, Poland
2Department of Medical Genetics, Institute of Pediatrics, Jagiellonian University Medical College, Krakow, Poland
3Department of Molecular Neuropharmacology, Institute of Pharmacology of Polish Academy of Sciences, Polish Academy of Sciences Cracow Branch, Krakow, Poland

Corresponding Author:
Szymon Skoczen, MD, Department of Oncology and Hematology, Institute of Pediatrics, Jagiellonian University Medical College Wielicka St. 265, Krakow 30-663, Poland.
Email: szymon.skoczen@uj.edu.pl

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reflect their immune status after prior intensive treatment. The aim of our study was to assess potential usefulness of genome expression studies before HSCT.

Since the late 1990s, genome expressions in various malignancies (including ALL) have been studied. Such research is particularly important in patients with poor prognosis, who are classified as high-risk group, have suffered relapse, or are classified as low or intermediate-risk groups but have unfavorable prognosis due to genetic abnormalities. It may also help to identify the group of patients with resistance to specific cytotoxic agents. In future, the inhibition of specific genes may be used to reverse resistance to chemotherapy and improve the outcomes. The genomic studies may be essential for better stratification of risk groups, especially regarding the candidates for HSCT.

Some gene mutations or rearrangements are responsible for the development of aggressive and treatment-resistant types of leukemia. Patients with ALL should be screened for the following genetic aberrations: deletion of IKZF1, PAX5, CDKN2A, CDKN2B, P2RY8, CRLF2, EGR, rearrangements of ABL1, ABL2, CSF1R, PDGFRbeta, IGH, CRLF2, EPOR, NTRK3, BCR-ABL1. AFF1 (mll), AFF1-KMT2A (MLL-AF4), ETV6-RUNX1 (TEL-AML1), TCF3, mutation of JAK2, as well as hypo- or hyperdiploidy. Detection of the genetic background that contributes to poor prognosis remains a challenge and may be a basis for developing new therapeutic targets. On the other hand, the alterations in certain genes and their products may influence the development of complications of the treatment. The ST2 gene (Suppression of Tumorigenicity 2 gene) can serve as an example. Its increased expression can be observed in acute graft vs host disease.

Materials and Methods

Whole-genome expression has been assessed in the study group and in the control group. The study group consisted of 18 patients aged 2.2–19 (median 9.4) years, referred to the HSCT Center between August 27, 2009 and May 17, 2012. In 10 patients, the indication for HSCT was high-risk ALL (HR-ALL) in first complete remission, and in 8 children relapsed ALL in remission. At diagnosis, within the group of 10 HR-ALL patients, B-lineage ALL without coexpressions was found in 1 patient (10%), B-lineage ALL with coexpressions in 5 patients (50%), and T-ALL in 4 patients (40%). In the group of 8 relapsed patients, 7 (87.5%) were diagnosed with BCP-ALL (2 with coexpressions and 5 without coexpressions) and 1 with T-ALL (12.5%). First-line therapy for 17 patients was ALLIC-2002 protocol, for 1 patient with ALL/AML phenotype, it was AML-BFM-2004. All 8 patients with relapse were treated with ALL-Rez-BFM-2002 Protocol as second-line treatment. Minimal residual disease (MRD) analysis based on 6-color flow cytometry was performed in the bone marrow samples of 13 children. The results were positive (MRD ≥ 0.1%) in 5 patients, negative (MRD < 0.1%) in 4 patients, and unavailable due to poor quality of samples in 4 patients. Due to the small number of informative MRD results, we did not analyze its impact on the genome expression. The blood samples for whole-genome assessment were collected at admission to the HSCT Center, just before the introduction of the conditioning regimen, so the patients were free from infection.

The limitation of the study was that no power calculation was used for estimation of the sample size because the number of ALL patients referred to HSCT in our center is small (about 6 per year).

Characteristics of children referred for HSCT are presented in Table 1. The control group consisted of 38 children (17 boys and 21 girls) with obesity, aged 3.4–17.8 (median 14.1) years. According to the study schedule, initially, a group of healthy children was recruited. Unfortunately, the Affimetrix Chips of ALL patients and healthy control were bought in different series. As the Chip quality of healthy control was poor, we decided (as it was previously done in other published studies) to take children with obesity as a control. The chips used in obesity group were from the same series as in ALL patients, with good quality assessment. The obesity group was recruited from patients treated at the Pediatric and Adolescent Endocrinology Department. The inclusion criteria were obesity developing before puberty, negative medical history, and absence of any signs or symptoms of acute or chronic diseases. The exclusion criteria were secondary obesity due to other conditions (e.g., single gene mutations and endocrinopathy) or drug-related obesity (e.g., glucocorticoids).

Microarray Analysis

Blood samples (1.5 mL) were collected from each patient. Subsequently, mononuclear cells were separated, total RNA extraction was performed, and microarray analysis was conducted using the GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, USA) according to the manufacturer’s protocol. Microarray data were initially processed using GeneChip Operating Software. DTT data were transferred by Transfer Tool software (Affymetrix). The Chip quality was assessed according to the Affymetrix guidelines. Raw data were processed using model-based expression index implemented in dChip. After background subtraction, the data were normalized using quantile normalization. The signal was taken as the measure of mRNA abundance derived from the level of gene expression.

Statistical Analysis

The microarray data were preprocessed using the R/Bioconductor package. Robust Multiarray Average (RMA) was used for normalization. Quality control was performed by investigating Principal Component Analysis, Relative Log Expression, and Normalized Unscaled Standard Error plots. Moderated t tests were used to detect the probes with different expression in various groups; the data were RMA-normalized and presented as mean values and SDs.
Table 1. Characteristics of Studied Groups.

| Pre-HSCT group | Control group |
|----------------|---------------|
| **N (%)**      | 18            | 38            |
| Sex            | Boys 15 (83.3%), girls 3 (16.7%) | Boys 17 (44.73%), girls 21 (55.27%) |
| Age (years)    | 2.2–19 (median 9.4) | 3.4–17.8 (median 14.1) |
| B-lineage +/- coexpressions | 5 (50%) | 5 (50%) |
| T-lineage      | 4 (40%) | 4 (40%) |
| B-lineage      | 1 (10%) | 1 (10%) |

ALL = acute lymphoblastic leukemia; HSCT = Hematopoietic stem cell transplantation.

Results

A comparison of the whole-genome expression before HSCT and the control group revealed 34 genes having the highest expression differences ($P$-value <.01, expected false positive results >1.2). In the HR-ALL subgroup, gene activation was seen more frequently (5 genes) than gene inhibition (1 gene). The activated genes were STAC3, DHRS1, FAM103A1, TCEB2, and ALDH1A1, and the suppressed gene was RICS.

Discussion

We studied genome expression in 18 children referred for HSCT in the treatment of ALL. At diagnosis, most of the relapsed patients (88%) had B-lineage ALL, while the remaining 12% had T-lineage ALL. In HR-ALL group, the most frequent phenotype was B-lineage ALL (50% with coexpressions and 10% without), and T-lineage ALL was diagnosed in remaining 40% of patients.

The MRD results before HSCT were obtained in 9 patients, and due to the limited number, their interpretation was not informative. The outcome results are presented in Table 4.

The GeneCards - Human Genes Database was used to analyze the function of genes. The Permanent Ethical Committee for Clinical Studies of the Medical College of the Jagiellonian University approved the study protocol. All parents, adolescent patients, and adult patients signed written informed consent before enrollment in the study.

**Results**

A comparison of the whole-genome expression before HSCT and the control group revealed 34 genes having the highest expression differences ($P$-value <.0005, expected false positive results equal to 2) (Figure 1, Table 2). Based on search in the GeneCards - Human Genes Database and the literature, well-defined function was found in the following 21 genes: SNRPN, SNORA38B, SNORA60, IGI, IGHM, IGHA1, IGH@, IGHD, IGH@, IGKC(88937789), IGKC(89836211), LOC652493, FCRL1, TCL1A, CXCR5, CD22, CD79A, MS4A1, COBLL1, RALGPS2, and BANK1. All the above showed lower expression in the HSCT group.

A comparison of the HR-ALL and the relapsed ALL patients (Figure 2, Table 3) revealed 6 genes having the highest expression differences ($P$-value <.01, expected false positive results >1.2). In the HR-ALL subgroup, gene activation was seen more frequently (5 genes) than gene inhibition (1 gene). The activated genes were STAC3, DHRS1, FAM103A1, TCEB2, and ALDH1A1, and the suppressed gene was RICS.

The primary microarray data were submitted to Gene Expression Omnibus (GEO) public repository and are accessible using GEO Series accession number GSE69421.27 In our study, a part of the submitted microarray data was used. A summary of the differentially expressed genes is presented in Tables 2 and 3.

The outcome of the patients was as follows: out of 10 HR-ALL patients, 8 live in first complete remission 8–10 years after HSCT, and 2 patients died due to relapse. In the group of 8 relapsed children, 4 live in second or third remission 10–11 years after HSCT and remaining 4 died. The comparison between B and T-ALL subsets did not reveal significant differences.

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**Discussion**

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Therefore, B-cell ALL at diagnosis was found in 72% of patients and T-cell ALL in 28% of patients (Table 1). Most of the patients were boys (15/18; 83%), and the median age for the whole study group was 9.4 years (range 2.2–19 years). All children were heavily pretreated. A second-line chemotherapy was used in 8 patients and involved field radiotherapy in 7 patients.

At the time of HSCT, all patients were in complete remission, but positive MRD was detected in 5 children. The genome expression of the study group was compared with obese children with no comorbidity, with a median age of 14.1 years. The study revealed that 21 genes with well-defined function had lower expression in the pre-HSCT group compared with the control group. More than 50% of these genes were involved in immunoglobulin production. Three genes were related to regulation of DNA processing, and one gene was involved in T-cell regulation.

Overall, our results indicate deep immunosuppression of both B-cell and T-cell lineages after prior intensive chemotherapy. As approximately 70% of patients were cured from leukemia after HSCT (conditioning: 17-TBIC-VP, 1-BuCy2-ATG), the genome expression results suggest that previous treatment was successful in reducing the leukemia burden (no expression of genes responsible for leukemia development) and making space for donor immune system (inhibition of genes responsible for immune system regulation). In our previous
study, we have shown activation of genetic pathways regulating immune reactions after HSCT. In the other study showing the influence of visfatin secretion on hematopoietic reconstitution, we compared CBC before and after HSCT in almost the same group of patients as in current study. The lymphocyte count was significantly lower in pre-HSCT group comparing to the post-HSCT group. The expression results are in accordance with these results showing deep immunosuppression. For the reason explained...
Table 2. The Lower Expressed Genes in Pre-HSCT Group in Comparison With Control Group.

| Gene       | Function of gene/coded protein                                                                 | Genome localization                                      |
|------------|------------------------------------------------------------------------------------------------|----------------------------------------------------------|
| SNRPN      | Pre-mRNA processing, possibly tissue-specific alternative splicing events                      | Small nuclear ribonucleoprotein polypeptide N // 15q11.2 |
| SNORA38 B | Small Nucleolar RNA                                                                           | Small nucleolar RNA, H/ACA box 38B (retrotransposed) // 17q24.2 |
| SNORA60    | Small Nucleolar RNA                                                                           | small nucleolar RNA, H/ACA box 60 // 20q11.23            |
| IGJ        | Linker protein for immunoglobulin alpha and mu polypeptides                                   | Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides // 4q21 |
| IGHM       | •Encodes the C region of the mu heavy chain, which defines the IgM isotype                     | Immunoglobulin heavy constant mu // 14q32.33              |
| IGHA1      | •Immunoglobulin heavy constant alpha 1                                                         | Immunoglobulin heavy constant alpha 1 // 14q32.33         |
| IGHV@      | Immunoglobulin heavy chain variable region gene                                               | Immunoglobulin lambda joining 3 // 22q11.1-q11.2          |
| IGHD       | Immunoglobulin Heavy constant delta protein coding gene                                        | Immunoglobulin heavy constant delta // 14q32.33           |
| IGH@       | Gene for the heavy chains of human antibodies                                                 | Immunoglobulin heavy locus // 14q32.33                     |
| IGKC       | Immunoglobulin kappa constant                                                                  | chr2,"-","88937789","89400997","24",BC073791 // immunoglobulin kappa constant // 2p12 |
| IGKC       | Protein coding gene                                                                            | chr2,"+","89836211","89836503","29","ENST00000390276 // IGKC // immunoglobulin kappa constant // 2p12 // 3514 // ENST00000390251 |
| LOC652493  | Ig kappa chain V-I region                                                                      | Similar to Ig kappa chain V-I region HK102 precursor/chr 2 |
| FCRL1      | Immunoglobulin receptor superfamily                                                             | Fc receptor-like I // 1q21-q22                            |
| TCL1A      | •TCL1 functions as a coactivator of the cell survival kinase AKT                                | T-cell leukemia/lymphoma 1A // 14q32.1                     |
|            | They are expressed mainly in CD4-/CD8- immature T-cells, pre-B-cells and virgin B-cells.       |                                                           |
|            | Expression decreases significantly at more mature stages of B-cell development. Activation of TCL1 and/or TCL1b in mature T-cells causes T-cell leukemia in humans involvement in the enhancement of the PI3-K dependent anti-apoptotic function of Akt |
|            | This cytokine receptor binds to B-lymphocyte chemotactic (BLC), and is involved in B-cell migration into B-cell zones of secondary lymphoid organs as well as T-cell migration. It is expressed in mature B-cells, Burkitt’s lymphoma cells, subsets of CD4+ and CD8+ T-cells, and skin-derived migratory dendritic cells. Certain malignant cells take advantages of CXCL13/CXCR5 and CCL19/CCR7 for infiltration, resistance to apoptosis, and inappropriate proliferation | Chemokine (C-X-C motif) receptor 5 // 11q23.3 |

(continued)
Before, we choose the obesity group as a control. Interestingly, children with obesity show decreased function of lymphocyte B.28 As our study showed uniform suppression of genes regulating lymphocyte B function in children with ALL referred to HSCT compared to obese children, it proves substantial immunosuppression of B-line cells in studied group. Some of the genes analyzed in our study may promote the development of malignancies (like lung cancer, pancreatic cancer, colorectal cancer, and leukemia),10,29-31 while some are present in aggressive, metastatic, or resistant disease. All of them may potentially be indicators of relapse or second malignancies and become targets for novel therapies.17,32 They might also be responsible for leukemogenesis and treatment resistance in patients with leukemia.33-37 Further analysis of genomic profile of patients with ALL at diagnosis, at relapse, and before HSCT are necessary to understand the role of the altered activity of the genes and the respective enzymes.

An important limitation of our study was its retrospective design. It would be useful to perform prospective observation of the changes in genome expression in pediatric ALL patients at diagnosis and at the later phases of treatment. The next issue is that the difference in gene expression between the different ALL groups before transplantation was not quantified.

Conclusion: The genome expression in pediatric patients with ALL in remission referred for HSCT revealed deep immunosuppression of both B-cell and T-cell lineages, which may increase the probability of donor cell engraftment. In the future, the assessment of gene expression profile at diagnosis and at later phases of treatment might be one of predictors of long-term outcomes.

Table 2. (continued)

| Gene   | Function of gene/coded protein                                                                 | Genome localization                                                          |
|--------|---------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| CD22   | CD22 (CD22 molecule) is a protein coding gene                                              | CD22 molecule // 19q13.1                                                     |
| CD79A  | Encodes the Ig-alpha protein of the B-cell antigen component                                | CD79a molecule, immunoglobulin-associated alpha // 19q13.2                   |
| MS4A1  | Encodes a member of the membrane-spanning 4A gene family                                    | Membrane-spanning 4-domains, subfamily A, member 1 // 11q12                  |
| COBL1  | Cordon-bleu WH2 Repeat protein like 1 is a protein coding gene                              | COBL-like 1 // 2q24.3                                                        |
| RALGPS2| Ral GEF with PH domain and SH3 binding motif 2 is a protein coding gene                     | Ral GEF with PH domain and SH3 binding motif 2 // 1q25.2                     |
| BANK1  | B-cell-specific scaffold protein that functions in B-cell receptor-induced calcium mobilization from intracellular stores promote Lyn-mediated tyrosine phosphorylation of inositol 1,4,5-trisphosphate receptors | B-cell scaffold protein with ankyrin repeats 1 // 4q24                       |

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### Table 3. The Differentially Expressed Genes in Comparison Between HRG-ALL and Relapse ALL.

| Gene               | Function of coded protein                                                                 | Genome localization            |
|--------------------|-------------------------------------------------------------------------------------------|---------------------------------|
| **Activation of function in VHRG ALL subgroup**                                                                                   |
| STAC3              | Neuron-associated GTPase-activating protein                                                 | SH3 and cysteine rich domain 3 // 12q13.3 |
| DHRS1              | Encodes a member of the short-chain dehydrogenases/reductases (SDR) family constitute a large protein family of (NAD(P)(H)-dependent oxidoreductase). | Dehydrogenase/reductase (SDR family) member 1 // 14q12 |
|                    | Found in human fetal brain                                                                 |                                 |
|                    | IDH1 mutation is an oncogenic mutations and could contribute to the metastasis in melanoma |                                 |
| FAM103A1           | Family with sequence similarity 103 member A1 is a protein coding gene RAM/Fam103a1 is required for mRNA cap methylation which is essential for efficient gene expression in eukaryotes - for transcript expression, translation and cell proliferation. | Family with sequence similarity 103, member A1 // 15q25.2 |
|                    |                                                                                           |                                 |
| TCEB2              | Encodes the protein elongin B, which is a subunit of the transcription factor B (SIII) complex. | Transcription elongation factor B (SIII), polypeptide 2 (18 kDa, elongin B) // 16p12.3 |
| ALDH1A1            | The protein encoded by this gene belongs to the aldehyde dehydrogenase family. Increased expression of ALDH1A1 has been identified in a wide-range of human cancer stem cells and is associated with cancer relapse and poor prognosis, raising the potential of ALDH1A1 as a therapeutic target. | Aldehyde dehydrogenase 1 family, member A1 // 9q21.13 |
|                    |                                                                                           |                                 |
| **Inhibition of function in VHRG ALLSubgroup**                                                                                   |
| RICS               | Neuron-associated GTPase-activating protein                                                | Rho GTPase-activating protein // 11q24-q25 |
|                    | It play a prominent role in dendritic development, in the regulation of neural functions, including postsynaptic NMDA signaling and neurite outgrowth. Ectopic expression of p200 RhoGAP stimulates fibroblast cell proliferation and cell cycle progression, leading to transformation. |                                 |

### Table 4. Outcome of the Patients.

#### Pre-HSCT group (10 patients)

| Immunophenotype                      | No of patients | MRD results | Outcome |
|--------------------------------------|----------------|-------------|---------|
| BCP-ALL with T-coexpression          | 3              | 1 - positive | Alive   |
|                                      |                | 2 - without result | 1 alive, 1 died |
| BCP-ALL with myeloid coexpression    | 2              | 1 - negative | Alive   |
|                                      |                | 1 - without result | Alive |
| BCP without coexpression             | 1              | Negative    | Died    |
| T-ALL with myeloid coexpression      | 4              | 2 - negative | Alive   |
|                                      |                | 1 - positive | Alive   |
|                                      |                | 1 - without result | Alive |

#### Relapsed group (8 patients)

| Immunophenotype                      | No of patients | MRD results | Outcome |
|--------------------------------------|----------------|-------------|---------|
| BCP-ALL with T-coexpression          | 1              | Without result | Alive |
| BCP-ALL with myeloid coexpression    | 1              | Negative    | Alive   |
| BCP-ALL without coexpression         | 5              | 3 - positive | 3 - died |
|                                      |                | 2 - without result | 2 - alive |
| T-ALL                                | 1              | Positive    | Died    |

### Abbreviations

- **ALL**: acute lymphoblastic leukemia
- **DTT**: data transfer tool
- **FDR**: false discovery rate
- **HR-ALL**: high-risk acute lymphoblastic leukemia
- **HSCT**: hematopoietic stem cell transplantation
- **MRD**: minimal residual disease
- **NUSE**: Normalized Unscaled Standard Error
- **PCA**: Principal Component Analysis
- **RLE**: Relative Log Expression
- **RMA**: Robust Multiarray Average
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Not applicable.

Author Contributions
KK and SS designed and performed research, analyzed and interpreted data, and wrote the manuscript. MBM performed research, analyzed, and interpreted data. AG, MK, and MP performed research and collected data. WS analyzed and interpreted data and critically reviewed the manuscript. WB conducted the clinical protocols and interpreted data. All authors read and approved the final manuscript.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Availability of Data and Material
The datasets used and analyzed during the current study are available from the corresponding author on a reasonable request. All the primary microarray data were submitted to GEO public repository and are accessible using GEO Series accession number GSE69421.16 In our study a part of the submitted microarray data was used.

Trial Registration
Bioethics Committee of the Jagiellonian University approved the study protocol. Registration number: KBET/96/B/2008. The Permanent Ethical Committee for Clinical Studies of the Medical College of the Jagiellonian University approved the study protocol. Registration number: KBET/96/B/2008. Written informed consent for collecting the samples and for its genetic evaluation as well as for publishing was obtained from all parents or their guardians and from all patients ≥16 years of age. The study conforms with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (July 18, 1964).

Ethics Approval
The study was approved by the Ethics Committee of the Jagiellonian University (KBET/96/B/2008) and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Consent for Publication
Written informed consent was obtained from all parents and from all patients ≥16 years of age. An informed consent to publish was obtained from all patients or their guardians, where applicable. All authors consented for publication.

ORCID iD
Szymon Skoczen https://orcid.org/0000-0001-6867-2717

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