Analysis of the rRNA methylation complex components in pediatric B-cell precursor acute lymphoblastic leukemia: A pilot study

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Conflict of interest

None declared

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Abstract

Background. Dysregulation of ribosome biogenesis and alteration of ribosome composition, including alteration in ribosomal RNA (rRNA) 2'-O-ribose methylation, can play a role in malignant transformation and cancer progression. Several studies recently reported that components of the rRNA methylation complex are associated with leukemogenesis. However, no study ever investigated the alteration of ribosome biogenesis factors in the most common pediatric malignancy – B-cell precursor acute lymphoblastic leukemia (BCP-ALL).

Objectives. The objective of this study was to examine the expression of factors building the rRNA methylation complex, either the protein components (1 methyl-transferase (*FBL*), *NOP56*, *NOP58*, *NHP2L1*) or some RNA components (box C/D snoRNAs: *SNORD35B*, *SNORD65*, *SNORD46*, *SNORD50A*, *SNORD38B*), as well as *CMYC*, and nucleolin (*NCL*) – a protein involved in rRNA synthesis. Clinical effects in children with BCP-ALL were also investigated.

Material and methods. The factors involved in ribosome biogenesis were studied in 28 children with BCP-ALL with the use of real-time polymerase chain reaction (RT-PCR) using the BioMark HD System (Fluidigm, San Francisco, USA) in cDNA prepared from the bone marrow samples collected at diagnosis.

Results. Strong correlations were observed between *NOP56*, *NOP58* and *NCL*, and multiple weaker correlations were observed in the box C/D snoRNA category, and between box C/D snoRNA and transcripts coding proteins of the rRNA methylation complex. The expression of analyzed transcripts did not correlate with the initial white blood cells count (WBC) or with bone marrow blast percentage. Ribosome biogenesis upregulation with overexpression of *FBL* and *NOP56*, and *CMYC* was found in patients who subsequently relapsed and the upregulation signature was not associated with known risk predictors.

Conclusions. This is the first report on the clinical aspect of ribosome biogenesis in pediatric BCP-ALL, and it shows that overexpression of *CMYC* and C/D box nucleoproteins *FBL* and *NOP56* is an antecedent event in patients who subsequently relapse. The dysregulation pattern is different from the previous reports in acute myeloid leukemia (AML), suggesting that dysregulation of ribosome biogenesis is specific for BCP-ALL.

Key words: pediatric, methylation, relapse, acute lymphoblastic leukemia (ALL), ribosome

Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood with an incidence of 46.7 cases per million children under 15 years of age per year.¹ The ALL is a group of various subtypes with the most common being the B-cell precursor acute lymphoblastic leukemia (BCP-ALL). Although the majority of children with ALL are cured with chemotherapy, up to 20% of patients succumb to the disease mostly due to resistant or relapsing leukemia.² From a clinical perspective, pediatric ALL is a highly heterogeneous entity and early identification of patients with suboptimal response is necessary for therapy intensification.

Among the genes associated with leukemogenesis and clinical response in leukemia, those involved in ribosome biogenesis are promising and only now beginning to be studied.^{3–8} In addition, several studies reported that the high proliferation rate of tumor cells is sustained by an increased ribosome biogenesis due to hyperactivation of RNA polymerase I (RNA pol I) associated with an increase in protein synthesis.⁹ This view is sustained by the recent discovery that inhibiting RNA pol I activity specifically kills cancer cells in lymphoma without damaging non-cancer cells.¹⁰

Ribosomes are essential machinery in all cells, as they translate mRNA into protein. Due to their conserved sequence, their analysis was helpful in establishing evolutionary relationships between different organisms.¹¹ Moreover, ribosomes showed a conserved structure through evolution, which is the basis of the conserved intrinsic activity of the ribosome in the tree of life. In eukaryotes, the ribosomes consist of a small subunit containing one 18S ribosomal RNA (rRNA) and 33 ribosomal proteins (RPS), and a large subunit comprised of the 28S, 5.8S and 5S rRNAs as well as 47 ribosomal proteins (RPL). Production of such complex ribonucleoprotein machinery takes place in the nucleolus and up to 4,500 nucleolar factors have been identified so far.¹² Ribosome biogenesis requires fine regulation of rRNA synthesis using RNA pol I to give rise to a pre-rRNA, a step that is regulated by several factors, including nucleolin (NCL). A recent study reports that NCL is a marker of prognosis in acute myeloid leukemia (AML).⁵ The pre-rRNA is then subjected to numerous cleavages, specific folding and extensive nucleotide modifications, which are necessary to maintain the conserved ribosome structure and thus its intrinsic translational activity.¹³ However, recent findings showed that alterations of rRNA chemical modifications occur during tumor initiation and tumor progression, directly affecting the translation of some mRNAs coding oncogenes or tumor suppressors.^{14–18} Among rRNA chemical modifications, the 2'-O-ribose methylation of rRNA is the most abundant, representing more than 50% of rRNA chemical modifications. They are catalyzed by a box C/D small nucleolar ribonucleoproteins (snoRNPs) complex

that contains 3 proteins involved in snoRNP structuration and assembly (NOP56, NOP58, NHP2L1), 1 methyl-transferase (FBL) and 1 box C/D small nucleolar RNA (snoRNA or SNORD), which pairs with the target RNAs determining the specific nucleotide to modification.^{18–21} About 250 different box C/D snoRNAs that are necessary to methylate the 106 rRNA sites at their 2'-O ribose residues have been identified. Several box C/D snoRNAs have been shown to promote leukemogenesis, including SNORD50 in T-ALL or SNORD35 in AML.^{3,6} Although many snoRNAs and factors involved in ribosome biogenesis have been identified to be associated with leukemogenesis or clinical outcome in different leukemia types, no study has ever investigated their alteration of expression and their clinical value in pediatric BCP-ALL. Here, we have investigated the significance of factors involved in ribosome biogenesis in the proliferative capacity and relapse propensity.

Material and methods

Human samples

The study group consisted of 28 consecutive children diagnosed with BCP-ALL treated at Department of Pediatric Hematology and Oncology (Wrocław Medical University, Poland). The clinical characteristics of the analyzed group are presented in Table 1. The patients were treated within the ALL IC-BFM 2009 study and according to biological and clinical factors were stratified into 3 chemotherapy arms – standard risk group (SRG), intermediate risk group (IRG) and high risk group (HRG).²² The response to the initial 7-day long therapy with glucocorticosteroids, defined

Table 1. Patient characteristics

Category	Parameter	Value
Sex	male	22
	female	6
Age at diagnosis in months	median	40.5
	range	7.3–198.1
Initial peripheral blast count per μ L	median	8,456
	range	0–18,7680
Initial bone marrow blast percentage	median	56
	range	0–95
Prednisone response	prednisone good response	25
	prednisone poor response	3
Risk group	standard	7
	intermediate	16
	high	5
Relapse	yes	3
	no	25
Follow-up in months	median	61
	range	43–84

as good (prednisone good response (PGR)) with the blast count below 1,000/ μ L or poor (prednisone poor response (PPR)) with blast count $\geq$ 1,000/ μ L was used as a stratification factor. During the observation period, 3 patients showed bone marrow relapse, and none of the studied patients died. The parents gave their written informed consent for the treatment and analysis of clinical data and the study was approved by the Bioethical Committee at Wrocław Medical University.

Quantification of RNA using medium throughput RT-qPCR

Initial diagnostic bone marrow samples were collected and suspended in Trizol Reagent (Thermo Fisher Scientific, Waltham, USA) for frozen conservation. Ribonucleic acid was then purified by adding chloroform to separate nucleic acids from proteins, before it was precipitated using isopropanol and 70% ethanol wash. Reverse transcription was performed in duplicate using the PrimeScript RT kit (Takara, Kusatsu, Japan). Briefly, 200 ng of total RNA were retrotranscribed into cDNA using a mix of 2 kind of primers: 2.5 M oligo dT primers for mRNAs, which contain polyA at their 3'-end; and 5 μ M random 6-mer primers, which allow reverse transcription of all RNAs, containing or not a poly-A, including mRNAs and snoRNAs. RNA expression levels of genes of interest were finally quantified in triplicate with real-time quantitative polymerase chain reaction (RT-qPCR) using the microfluidic medium-throughput BioMark HD System (Fluidigm, San Francisco, USA). Utilizing the 48.48 Dynamic ArrayTM IFC (Fluidigm), this system allowed us to perform RT-qPCR on 48 samples by using up to 48 couples of primers. As recommended by the supplier, cDNA was subjected to an initial step of pre-amplification for 10 cycles using the PreAmp Master Mix (Fluidigm) and a mix of all the primers of interest to increase the quantity of initial cDNA matrices since each well of the 48.48 Dynamic ArrayTM IFC corresponds to only 6 nL. Residues of primers were then degraded by incubation with the Exonuclease I (New England Biolabs, Ipswich, USA). Pre-amplified cDNAs were then 1:10 diluted in 1X TE buffer prior to the addition of 1X GE Sample Loading Reagent (Fluidigm) and 1X SsoFastTM EvaGreen[®] Supermix with Low Rox (BioRad, Hercules, USA). In parallel, 2.5 M of each couple of primers was mixed with 1X Assay Loading Reagent (Fluidigm) and 1X DNA Suspension Buffer. After the priming of the 48.48 Dynamic ArrayTM IFC in the IFC controller to prepare the microfluidic system to receive fluids, samples and assays were loaded on the chip by the IFC controller using controlled pressure, this loading method improving the technical reproducibility of the RT-qPCR. The loaded 48.48 Dynamic ArrayTM IFC was then introduced in the BioMark system (Fluidigm) to measure fluorescent EvaGreen at each end of PCR cycle. Raw data were analyzed using the Fluidigm Real-Time PCR Analysis software (Fluidigm).

Each couple of primers was introduced in triplicate to obtain 3 cycle thresholds (CT) values per sample. In addition to *CMYC*, 10 genes involved in the ribosome biogenesis were quantified: the 4 genes coding proteins involved in rRNA 2'-O-ribose methylation complex (*FBL*, *NOP56*, *NOP58*, *NHP2L1*); 5 C/D snoRNAs or SNORDs (*SNORD35B*, *SNORD65*, *SNORD46*, *SNORD50A*, *SNORD38B*); and 1 gene involved in rRNA synthesis (*NCL*). Quantification of RNA was first normalized using mean of 6 housekeeping genes (*ACTIN*, *GAPDH*, *HPRT1*, *PPIA*, *PGK1*, *RNU6B*) then using Human XpressRef Universal Total RNA (Qiagen, Hilden, Germany) for inter- and intra-run normalization, respectively, to calculate the relative fold-change using the $2^{-\Delta\Delta C_t}$. Expression of ribosome biogenesis factors and snoRNAs was presented as mRNA relative levels.

Statistical analysis

Statistical analysis and data presentation were performed using GraphPad Prism computer software (GraphPad Prism v. 6.07 for Windows; GraphPad Software, La Jolla, USA; www.graphpad.com). Associations between the mRNA relative levels were analyzed using Spearman's rank correlation. The Mann–Whitney U test was applied for the comparison of 2 groups, and Kruskal–Wallis test for 3 or more groups. Survival curves were estimated using the Kaplan–Meier method and the differences in leukemia-free survival (LFS), defined as time from diagnosis to relapse, were analyzed between subgroups using log-rank test. A p-value less than 0.05 was considered significant.

Results

The values of the mRNA relative levels were first tested for correlation with peripheral blood absolute blast count and with bone marrow blast percentage, but no statistically significant results were observed. Results are presented in Table 2. The RNA levels of all analyzed genes were analyzed and several two-by-two correlations were identified (Table 3). To determine whether correlations occur between the different gene categories (i.e., box C/D snoRNAs, proteins involved in the rRNA methylation complex or rRNA synthesis), graphical representation of correlation data was drawn (Fig. 1). Correlations were observed within the box C/D snoRNA category, and separately in the group containing the mRNA transcripts of the genes encoding the C/D box snoRNPs and *NCL*. The strongest correlations were observed between *NOP56*, *NOP58* and *NCL*. In addition, multiple weaker correlations were observed in the box C/D snoRNA category, and between box C/D snoRNA and transcripts coding proteins of the rRNA methylation complex. For example, the *SNORD35B* expression correlated with *NOP56/NOP58/NCL* expression, while the *SNORD46/SNORD38B* correlated in turn with

Table 2. Results of Spearman's rank correlation coefficient between mRNA relative levels and blast count in peripheral blood (PB) or bone marrow (BM)

Variable		<i>FBL</i>	<i>Nop56</i>	<i>Nop58</i>	<i>NHP2L1</i>	<i>NCL</i>	<i>CMYC</i>	<i>SNORD35B</i>	<i>SNORD65</i>	<i>SNORD46</i>	<i>SNORD50A</i>	<i>SNORD38B</i>
PB blast count vs RNA levels	Spearman's r	−0.34	−0.11	0.067	0.21	0.27	−0.36	0.14	−0.13	−0.081	0.062	−0.073
	95% CI	−0.64 to 0.051	−0.48 to 0.28	−0.32 to 0.44	−0.19 to 0.55	−0.12 to 0.59	−0.65 to 0.027	−0.26 to 0.49	−0.49 to 0.27	−0.45 to 0.31	−0.33 to 0.43	−0.44 to 0.32
	p-value (two-tailed)	0.0778	0.5673	0.7365	0.2882	0.1612	0.06	0.489	0.5057	0.6818	0.7544	0.7125
	p-value summary	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
BM blast percentage vs RNA levels	Spearman's r	−0.26	−0.22	−0.21	0.029	−0.19	−0.039	−0.2	0.12	−0.028	−0.23	−0.057
	95% CI	−0.58 to 0.14	−0.56 to 0.18	−0.55 to 0.19	−0.36 to 0.41	−0.54 to 0.20	−0.42 to 0.35	−0.54 to 0.19	−0.27 to 0.48	−0.41 to 0.36	−0.56 to 0.17	−0.43 to 0.33
	p-value (two-tailed)	0.1879	0.2608	0.2817	0.8832	0.3219	0.8446	0.2965	0.5389	0.8888	0.2483	0.7715
	p-value summary	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

95% CI – 95% confidence interval.

Table 3. Results of Spearman's rank correlation coefficient between mRNA relative levels of the analyzed transcripts

Gene	<i>FBL</i>	<i>NOP56</i>	<i>NOP58</i>	<i>NHP2L1</i>	<i>NCL</i>	<i>CMYC</i>	<i>SNORD35B</i>	<i>SNORD65</i>	<i>SNORD46</i>	<i>SNORD50A</i>	<i>SNORD38B</i>
<i>FBL</i>		p = 0.008	p = 0.21	p = 0.15	p = 0.4	p = 0.1	p = 0.36	p = 0.087	p = 0.23	p = 0.98	p = 0.59
<i>NOP56</i>	0.49*		p < 0.001	p = 0.04	p < 0.001	p = 0.7	p < 0.001	p = 0.51	p = 0.25	p = 0.65	p = 0.21
<i>NOP58</i>	0.24	0.68*		p = 0.02	p < 0.001	p = 0.67	p < 0.001	p = 0.86	p = 0.06	p = 0.45	p = 0.027
<i>NHP2L1</i>	0.28	0.38*	0.42*		p = 0.007	p = 0.7	p = 0.29	p = 0.25	p = 0.3	p = 0.12	p = 0.42
<i>NCL</i>	0.17	0.74*	0.65*	0.5*		p = 0.31	p = 0.002	p = 0.8	p = 0.82	p = 0.11	p = 0.88
<i>CMYC</i>	0.31	0.07	−0.08	−0.07	−0.2		p = 0.75	p = 0.46	p = 0.88	p = 0.69	p = 0.96
<i>SNORD35B</i>	0.18	0.65*	0.67*	0.21	0.55*	−0.06		p = 0.37	p = 0.02	p = 0.14	p = 0.009
<i>SNORD65</i>	−0.32	−0.13	−0.03	−0.22	−0.05	−0.14	−0.18		p = 0.26	p = 0.4	p = 0.38
<i>SNORD46</i>	0.23	0.22	0.35	0.2	−0.04	0.03	0.43*	−0.22		p = 0.007	p = 0.012
<i>SNORD50A</i>	0	−0.1	0.15	−0.38	−0.31	−0.08	0.28	−0.16	0.5*		p < 0.001
<i>SNORD38B</i>	0.1	0.24	0.42*	−0.16	−0.03	−0.01	0.48*	−0.17	0.47*	0.61*	

Lower part of the table shows Spearman's rho and upper part the p-values. Statistically significant results are shown in bold and marked with an asterisk.

Fig. 1. The correlations between mRNA relative levels of analyzed transcripts. The lines represent the values of Spearman's rank correlation coefficient

Fig. 2. The mRNA relative levels of analyzed transcripts among patients within different risk stratification groups (high risk group (HRG), intermediate risk group (IRG) and standard risk group (SRG))

Bars and whiskers on the plot represent mean and standard deviation (SD).
* $p < 0.05$.

Fig. 3. The RNA relative levels in patients with prednisone good response (PGR) and prednisone poor response (PPR)

Bars and whiskers on the plot represent mean and standard deviation (SD).
* $p < 0.05$.

SNORD35B. RNA relative levels of all transcripts were analyzed for correlation with initial peripheral blast count, initial marrow blast percentage and factors used to establish the different risk stratification groups (HRG, IRG and SRG) (Fig. 2). The RNA levels of the *NOP58* were associated with risk stratification ($p = 0.04$). The response to the initial steroid therapy was assessed as PGR in 25 children and PPR in 3 children. The mRNA relative levels for all tested genes were compared between groups with PGR and PPR (Fig. 3). In the PGR group, only the *NOP56* RNA levels were lower ($p = 0.03$). During the observation period, 3 patients relapsed, all in the IRG, and all showed PGR. We then evaluated the association of expression of ribosome biogenesis factors with the later stage of the BCP-ALL disease. The groups of patients who either remained in complete remission (CR) or relapsed were compared for the mRNA relative levels of analyzed transcripts (Fig. 4). Initial RNA levels of *FBL*, *NOP56*, *CMYC*, *SNORD35B*, and *SNORD46* were significantly higher in the patients who subsequently experienced the leukemia relapse than in those who remained in CR. For each factor identified as significant (*FBL*, *NOP56*, *CMYC*, *SNORD35B*, and *SNORD46*), the patients were grouped based on the RNA

Fig. 4. The mRNA relative levels at first diagnosis of analyzed transcripts between patients who maintained complete remission (CR) and those who relapsed

Bars and whiskers on the plot represent mean and standard deviation (SD).
* $p < 0.05$; ** $p < 0.005$.

levels – 1st group 1–3Q – below the 3rd quartile, and the 2nd – 4Q – above the 3rd quartile (Fig. 5). The survival analysis revealed inferior probability of LFS (pLFS) in the *FBL*, *CMYC*, *SNORD35B*, and *SNORD46* 4Q groups (1–3Q vs 4Q, 100% vs 57%, $p = 0.0011$), and in all relapsing patients the analyzed RNA levels were above the 3rd quartile threshold.

Overall, these data show that expression of some ribosome biogenesis factors at diagnosis might be associated with patient relapse.

Discussion

The association between ribosomal dysregulation and tumorigenesis is indisputable, as proven by increased carcinogenesis risk in constitutional diseases caused by ribosomal gene mutations.²³ In T-cell acute lymphoblastic leukemia (T-ALL), the ribosome function is indeed deregulated through mutations in RPL genes, leading to impaired ribosome biogenesis and translational fidelity.^{24–26} Homeostasis of ribosomal machinery thus appears crucial for the preservation of steadfast protein synthesis and there are observations that during tumorigenesis, including leukemogenesis, these mechanisms are flawed.

Our research on expression of different ribosomal biogenesis factors in the analyzed BCP-ALL group showed multiple positive correlations between mRNA expression levels. The correlation of *NOP56/NOP58/NCL* points to common regulation mechanisms responsible for ribosome biogenesis dysregulation in leukemic cells. The up-regulation of box C/D snoRNP components can be caused by the dysregulation of the intracellular genetic homeostasis enacted by upstream regulators like *TP53* and *CMYC*, both of them reported as strong regulators of box C/D snoRNAs, and other proteins involved in the rRNA methylation complex.^{6,16,27,28}

Interestingly, we observed the signature of ribosome biogenesis upregulation at initial diagnosis in BCP-ALL

Fig. 5. The probability of leukemia-free survival (pLFS) in patients grouped according to RNA levels of *FBL* (A), *NOP56* (B), *CMYC* (C), *SNORD35B* (D), and *SNORD46* (E). Groups: 1–3Q – below 3rd RNA level quartile, 4Q – above the 3rd RNA level quartile

patients who subsequently relapsed. The increased expression levels of *CMYC* and the components of the rRNA methylation complex, such as *FBL* and *NOP56*, were identified in patients, even though leukemic clone in 3 out of 3 children did not show PPR or resistance to chemotherapy with delayed remission. The role of *FBL* and *NOP56* proteins can be pivotal in the enhancement of ribosomal biogenesis. Animal studies showed that *FBL* regulates stem cell pluripotency, and gene knockdown led to significant delays in rRNA processing, growth inhibition and apoptosis.²⁹ Thus, the upregulation of *FBL* can protect leukemic cells from self-elimination and sustains mechanism leading to further evolution. The *FBL* was shown to be responsible for the decrease in translational quality control in response to p53 inhibition.¹⁶ The *FBL* overexpression promoted in vitro MCF7 breast cancer cells proliferation and protected cells from doxorubicin, and was associated with poor survival in tumor patients.¹⁶ The lack of correlation of RNA expression with peripheral blast count and tumor burden suggests that the leukemic cells do not require the upregulation of protein synthesis machinery to maintain massive proliferation. The increased initial peripheral blast count in ALL is considered an unfavorable prognostic factor and is included into the stratification of therapeutic protocol with a chance of chemotherapy intensification. In the analyzed cohort, the *NCL* gene – direct enhancer of ribosomal production – was not overexpressed. The normal expression of *NCL*-1 in *Caenorhabditis elegans* (*C. elegans*) is correlated with the repression of rRNA synthesis and decreased cell, and loss of function mutations in the nucleolin gene leads to increased protein and rRNA production with increased nematodes body size.³⁰ In AML, the mutations of *NPM1* lead to loss

of interaction with *NCL* protein contributing to leukemogenesis.³¹ Nucleolin was found to be overexpressed in AML blasts and a high *NCL* mRNA expression level was associated with poor overall survival, particularly in elderly patients.⁵ The prognostic role of the *NCL* expression was not identified in our cohort and the *NCL* upregulation did not identify the patients at risk of relapse, suggesting that the intensified chemotherapy in pediatric ALL can compensate for this effect.

Only the *NOP56* RNA levels were significantly higher in the PPR subgroup, but *NOP56* RNA level did not affect LFS. Steroid resistance in ALL is only partially elucidated by the presence of complex mechanisms related to intracellular expression of the glucocorticosteroid receptor itself, signaling pathways modulating its function and enzymes modifying the availability of its ligands.^{32–34} This effect emphasizes the complexity of leukemic transformation with multiple dysregulated mechanisms of cellular homeostasis.

Conclusions

This is the first report on the clinical aspect of ribosome biogenesis in pediatric BCP-ALL identifying the overexpression of the rRNA methylation complex components, *FBL*, *NOP56*, *CMYC*, and *SNORD46* as an antecedent event in patients who subsequently relapse. The results may suggest that the analyzed ribosome biogenesis factor expression levels are not related to known initial risk predictors. Due to the low number of analyzed patients, the study lacks the power to identify all factors associated with leukemia relapse. In order to validate the presented

findings and to determine the value of ribosomal profiling as potential new risk factors, studies in larger groups are necessary. In conclusion, the observed dysregulation pattern in BCP-ALL is different from the reports in AML, suggesting a specificity in ribosome biogenesis defects in BCP-ALL compared to other leukemias.

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