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Gene expression of *ASNS*, *LGMN* and *CTSB* is elevated in a subgroup of childhood BCP-ALL with *PAX5* deletion

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Abstract. Resistance to L-asparaginase (L-asp) is a major contributor to poor treatment outcomes of several subtypes of childhood B cell precursor acute lymphoblastic leukemia (BCP-ALL). Asparagine synthetase (*ASNS*), legumain (*LGMN)* and cathepsin B (*CTSB*) serve a key role in L-asp resistance. The association between genetic subtypes of BCP-ALL and the expression of *ASNS*, *LGMN* and *CTSB* may elucidate the mechanisms of treatment failure. Bone marrow samples of 52 children newly diagnosed with BCP-ALL were screened for major genetic abnormalities and *ASNS*, *LGMN* and *CTSB* gene expression levels. The cohort was further divided into groups corresponding to the key genetic aberrations occurring in BCP-ALL: Breakpoint cluster region and Abelson murine leukemia viral oncogene homolog 1 fusion; hyperdiploidy, hypodiploidy, ETS variant 6 and runt-related transcription factor 1 fusion and other BCP-ALL with no primary genetic aberration identified. A subgroup analysis based on the differences in copy number variations demonstrated a significant increase of *ASNS*, *LGMN* and *CTSB* median expression in other BCP-ALL cases with paired box 5 (*PAX5*) deletion (P=0.0117; P=0.0036; P<0.0001, respectively) compared with those with wild-type *PAX5*. Patients with high *ASNS* expression exhibited longer relapse-free survival (RFS) compared with those with low *ASNS* levels (P=0.0315; HR, 0.19; 95% CI, 0.04-0.86); the 5-year RFS for patients in the high *ASNS* expression group was 90.15% (95% CI, 87.90-92.40%). Despite the impact on *ASNS*, *LGMN* and *CTSB* expression, *PAX5* deletion did not influence RFS in the other BCP-ALL group (P=0.6839). Therefore, the results of the present study revealed high levels of *ASNS*, *LGMN* and *CTSB* expression in the other BCP-ALL group with concomitant *PAX5* deletion and no subsequent deterioration in 5-year RFS. High *ASNS* expression level, as a single factor, was strongly associated with an improved outcome.

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Introduction

L-asparaginase (L-asp) is a fundamental drug in the systemic treatment of childhood B cell precursor acute lymphoblastic leukemia (BCP-ALL). The introduction of L-asp improved the 5-year event-free survival (EFS) by 20% and boosted treatment response rates by 60% (1,2). However, in genetic subtypes of ALL with a high risk of relapse, L-asp treatment did not meet expectations, with drug resistance being one of the major mechanisms contributing to treatment failure (2-6). Although L-asp resistance is multifaceted and still not fully explained, several potential mechanisms have been proposed, such as hydrolysis of asparaginyl bonds and enzymatic degradation of the drug particle itself, immunological response against the drug triggered by presentation of L-asp to dendritic cells through MHC class II complex and increased production of asparagine as a response to its depletion in the blast environment during L-asp treatment (7-9). Previous studies have suggested that the genes involved in the degradation of L-asp and subsequent minimization of its effectiveness, may be upregulated by primary genetic abnormalities and copy number variations (CNVs) identified in BCP-ALL, such as hypodiploidy, breakpoint cluster region and Abelson murine leukemia viral oncogene homolog 1 (*BCR-ABL1*) fusion, lack of ETS variant 6 and runt-related transcription factor 1 (*ETV6-RUNX1*) fusion, *BCR-ABL1*-like phenotype and ikaros family zinc finger protein 1 (*IKZF1*) deletion (1,10,11).

Of the genes identified to be involved in the mechanisms of resistance to L-asp treatment, three key examples are asparagine synthetase (*ASNS*), legumain (*LGMN*) and cathepsin B (*CTSB*). ASNS synthesizes asparagine to reverse the antineoplastic action of L-asp, which depletes the surrounding leukemic cells of asparagine (12,13). LGMN is a cysteine

protease that inactivates drugs by specifically hydrolyzing asparaginyl bonds; it is also involved in the major histocompatibility complex (MHC) class II antigen presentation process, which may account for the allergic reactions to L-asp (14). *CTSB* encodes a peptidase that degrades L-asp (9). Although the aforementioned genes act in opposition to L-asp, no data on functional associations between them are currently available.

The role of the aforementioned genes in high-risk genetic subtypes of childhood BCP-ALL has not been fully elucidated. Studies of *ASNS* expression in groups with a number of genetic alterations are ambiguous (2,5,15). In addition, poor response to L-asp treatment is associated with *ASNS* overexpression at the protein, but not mRNA level (5,12,15,16). *LGMN* has been demonstrated to be upregulated in the intrachromosomal amplification of chromosome 21 (iAMP21) subtype of ALL, which is associated with high risk of relapse when treated with a low or standard risk protocol (14). No studies on the relevance of *CTSB* expression levels in BCP-ALL patients are currently available in the literature to the best of our knowledge, and a key aim of the present study was to determine the expression levels of the selected candidate genes underlying L-asp resistance in patients with different genetic types of BCP-ALL .

Materials and methods

Study group. A group of 52 patients ≤18 years were recruited retrospectively from three pediatric oncology centers in Poland: Lodz, Zabrze and Lublin. The patients were diagnosed with BCP-ALL between December 2005 and January 2016 and treated with the Berlin-Frankfurt-Münster backbone protocols with L-asp as a core regimen (ALL IC, 2002 and 2009) (17,18). Matched DNA and RNA samples from the bone marrow aspiration at the point of diagnosis, as well as clinical data, were acquired. *BCR-ABL1* fusion, *ETV6-RUNX1* fusion, hypodiploidy and hyperdiploidy were assessed routinely by fluorescence *in situ* hybridization and karyotyping performed in a certified laboratory (Genetic Laboratory at Collegium Medicum; Bydgoszcz, Poland) at the time of diagnosis. Each patient and/or their parents provided signed informed consent in writing prior to inclusion in the study.

Multiplex ligation-dependent probe amplification (MLPA). For all collected bone marrow samples, MLPA analysis with P327-B1 iAMP21-ERG probe mix was performed according to the manufacturer's protocol (MRC-Holland BV). Additionally, MLPA P329 CRLF2-CSF2RA-IL3RA and P335 ALL-IKZF1 (MRC-Holland BV) probe mixes for cytokine receptor-like factor 1, *IKZF1*, *IKZF2*, *IKZF3*, cyclin-dependent kinase inhibitor (*CDKN*) *2A/2B*, retinoblastoma 1 (*RB1*), *PAX5* and *ETV6* amplification or deletion status detection were used. Absolute fluorescence was normalized by comparing peak patterns of DNA in the sample of interest with a DNA isolated from blood samples from age- and gender-matched healthy individuals who provided written consent to participate in the study. The relative probe ratio of the tested samples was compared with the average relative probe ratio in the reference samples to calculate the dosage quotient. Data analysis and interpretation were conducted using GeneMarker v2.7.4 software (Softgenetics, LLC) according to the manufacturer's protocol.

Gene expression measurement. To obtain cDNA for quantitative PCR, all available RNA samples that were extracted using the TRIzol reagent (Thermo Fisher Scientific, Inc.) were reverse transcribed using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). The thermal conditions were 30 min at 50˚C, 15 min at 95˚C, followed by storage at 4˚C. TaqMan Gene Expression assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) for *ASNS* (cat. no. Hs04186194_m1), *LGMN* (cat. no. Hs00271599_m1) and *CTSB* (cat. no. Hs00947433_m1) were used; *GAPDH* (cat. no. Hs02786624_g1) was selected as a control. The thermocycling conditions were as follows: 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. All samples were tested in duplicate and the $2^{-\Delta\Delta Cq}$ value was calculated (19) with *GAPDH* used for normalization. The relative mRNA expression was used for further comparisons.

Transcription factor binding sites search. The search for *PAX5* binding sites throughout the human and mouse genome matrix was conducted using the HOCOMOCO v11, TRANSFAC database version 2018.3 (GeneXplain, GmbH). This is an online engine for searches through multiple module databases and was used as previously described (20).

Statistical analysis. Statistica 12.0 software (TIBCO Software, Inc.) was used for all analyses. Nominal variables are presented as percentages; differences between the groups were evaluated using the χ^2 test. Continuous variables are presented as medians with interquartile range (IQR); differences between groups were evaluated using the Kruskal-Wallis test and post-hoc Tukey's HSD test or the Mann-Whitney U test for paired groups were used to establish groups with significant differences. Correlations between continuous variables were calculated with the Spearman's rank-correlation coefficient. Kaplan-Meier curves were used to present relapse-free survival (RFS) time, and hazard ratios (HRs) were calculated using Cox proportional hazards. $P<0.05$ was considered to indicate a statistically significant difference. For the graphical presentation of result, GraphPad Prism 7.05 software (GraphPad Software, Inc.) was used.

Results

Clinical and genetic characteristics. A total of 52 children with BCP-ALL were enrolled in the study with a median age at diagnosis of 6.54 years (IQR, 2.97-12.38 years). The group was predominantly male (60.40%). The median white blood cell (WBC) count at diagnosis was $1.40x10^4$ cells/μl (IQR, 0.41-9.68x10^4 cells/μl) and the median leukemic cell percentage in bone marrow collected at diagnosis assessed by flow cytometry was 93.10% (IQR, 83.50-96.70%). The mean follow-up time was 4.43 years (range, 0.24-9.6 years).

The patient cohort was divided into groups based on the major genetic abnormalities detected in leukemic cells that have prognostic value within the used treatment protocols: The *BCR-ABL1* fusion-positive group (n=5; 9.62% of all patients), hyperdiploid group (n=7; 13.46%), hypodiploid (n=2; 3.85%), *ETV6-RUNX1* fusion (n=3; 5.77%) and other BCP-ALL group with no clear primary genetic aberrations (n=35; 67.31%)

Table I. Clinical characteristics of the analyzed cohort of 52 patients with BCP-ALL.

Characteristic	*BCR-ABL1*, n=5	*ETV6-RUNX1*, n=3	Hyperdiploid, n=7	Hypodiploid, n=2	Other BCP-ALL, n=35	P-value
Male, %	60.00	66.67	71.43	50.00	57.14	0.89
Mean age at diagnosis, years	13.76	8.54	2.97	1.62	7.03	0.04
WBC, x10^4 cells/μl	5.27	0.81	0.62	5.70	2.35	0.33
Blast count, %	88.00	83.25	96.80	94.00	94.50	0.64
Poor steroid response, %	40.00	33.33	0	50.00	10.71	0.23
MRD at day 15, %	0.40	11.34	0.71	29.45	2.23	0.51
Relapses, %	0	0	0	0	22.86	0.04
Deaths, %	0	0	0	0	20.59	0.07

BCR-ABL1, breakpoint cluster region and Abelson murine leukemia viral oncogene homolog 1 fusion; *ETV6-RUNX1*, ETS variant 6 and runt-related transcription factor 1 fusion; BCP-ALL, B cell precursor acute lymphoblastic leukemia; WBC, white blood cell count at diagnosis; MRD, minimal residual disease.

(Table I). The subgroups differed significantly in age at diagnosis, with the *BCR-ABL1* fusion group being the oldest at diagnosis (median, 13.76 years; IQR, 6.76-13.90 years; P=0.04).

The incidence of CNVs frequently observed in childhood ALL varied between the groups. All patients in the *ETV6-RUNX1* fusion group carried *IKZF1* and *ETV6* deletions in the leukemic clone, and 2 out of the 3 patients displayed concomitant *CDKN2A/2B* deletion. The majority of patients with *BCR-ABL1* fusion exhibited *IKZF1* and *PAX5* deletions (both were present in 3 out of 5 patients) and *CDKN2A/2B*, *CRLF2*, *ETV6*, *RB1* and transcriptional regulator ERG (*ERG*) deletions were detected in 1 out of 7 patients each. In the patients from the hyperdiploid group, *IKZF1* (3 out of 7), *CRLF2* (2 out of 7), *CDKN2A/2B* (1 out of 7), *PAX5* (1 out of 7) and Janus kinase 2 (*JAK2*; 1 out of 7) deletions were identified. The other BCP-ALL group was the most heterogenic among all groups; all patients exhibited CNVs in at least one of the tested genes with an incidence of 29 for *IKZF1*, 2 for *IKZF2*, 2 for *IKZF3*, 17 for *CDKN2A/2B*, 8 for *PAX5*, 4 for *CRLF2*, 4 for *JAK2*, 9 for *ETV6*, 4 for *ERG* and 6 for *RB1* out of 35 patients in this group (Table SI).

Expression analysis. Expression levels of *ASNS*, *LGMN* and *CTSB* were measured in diagnostic bone marrow samples from the 52 patients. The median relative expression value for *ASNS* was 2.57 (IQR, 2.26-3.26), for *LGMN* was 1.48 (IQR, 1.21-2.14) and for *CTSB* was 1.14 (IQR, 0.79-1.77). The median relative mRNA expression of *ASNS* correlated significantly with *LGMN* and *CTSB* (r=0.59 and r=0.62, respectively; P<0.05, data not shown. No correlations were identified between *ASNS*, *LGMN* and *CTSB* expression levels and minimal residual disease (MRD) on day 15 of the treatment protocol or RFS time (data not shown). In addition, no relevant differences in the median relative mRNA expression levels of the evaluated genes between the distinct molecular groups of BCP-ALL were identified (Table SII; Fig. 1A-C).

Analysis of the gene expression profiles based on common CNVs revealed a significantly higher median *LGMN* relative expression level in patients with *PAX5* deletion compared with patients with wild-type *PAX5* in all BCP-ALL subgroups (median, 1.93 and 1.34, respectively; P=0.0282; Table SIII). Patients with *PAX5* deletion also exhibited a significant correlation of *CTSB* median expression level with *ASNS* and *LGMN* (r=0.73 and r=0.64, respectively; P<0.05, data not shown). By contrast, deletion of *IKZF1*, *IKZF2*, *IKZF3*, *CDKN2A/2B*, *CRLF2*, *JAK2*, *ETV6*, *RB1* or *ERG* did not exhibit predictive significance for *ASNS*, *LGMN* and *CTSB* median expression levels as a single factor (Table SIII).

Differences in *ASNS*, *LGMN* and *CTSB* expression levels in the other BCP-ALL group were identified when the patients were divided into subgroups based on *PAX5* deletion status; 8/35 (24%) patients carried *PAX5* deletions, whereas 27/35 (76%) did not. Patients with *PAX5* deletions exhibited a higher relative expression level of *ASNS* compared with the wild-type *PAX5* group (3.14 vs. 2.47; P=0.0117; Fig. 2A). Similar results were observed for *LGMN* expression levels, which were higher in patients with *PAX5* deletion compared with patients with wild-type *PAX5* (2.24 vs. 1.45; P=0.0029; Fig. 2A). In addition, median *CTSB* expression levels were higher in patients with *PAX5*-deletions compared with patients with wild-type *PAX5* (2.08 vs. 1.04; P<0.0001; Fig. 2A). No significant associations between the median expression levels of the assessed genes were identified when the other BCP-ALL patient group was divided based on *IKZF1* deletion status (82.86% of the group carried deletions; Fig. 2B). However, in cases with concomitant deletions of *IKZF1* and *PAX5* (14.29% of the group-5 patients carried both deletions), significant differences were observed in *ASNS* and *CTSB* expression levels (P=0.0334 and P=0.0358, respectively) but not in *LGMN* expression levels (P=0.0735) (Table SIV; Fig. 2C).

Of the 12 BCP-ALL patients with *PAX5* deletion, four deletions were partial and heterozygous and eight affected the whole gene: Seven were heterozygous and one was homozygous. Additionally, TRANSFAC software (GeneXplain, GmbH) was used to test the *PAX5* transcription factor binding sites (TFBS) for a possible association with *ASNS*, *LGMN* or *CTSB*. A search through human and mouse databases revealed that the three genes of interest were not directly regulated by *PAX5*.

Outcome description. In the analyzed cohort of 52 patients, eight relapsed. Median time to relapse was 2.27 years

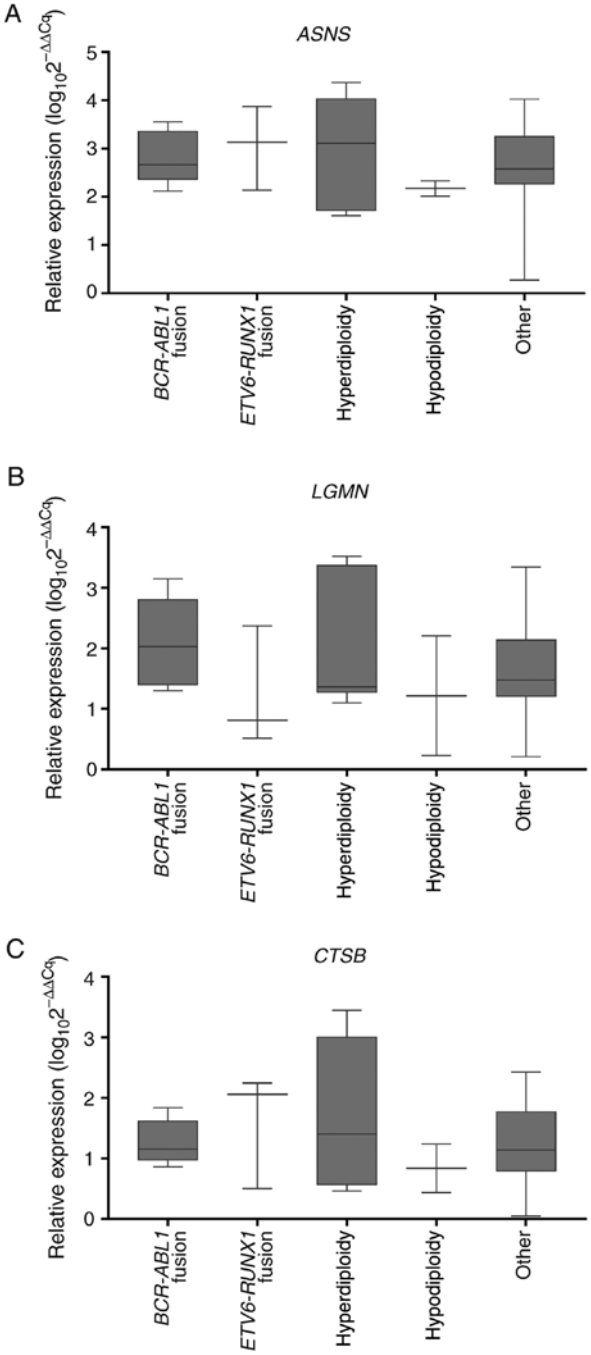

Figure 1. *ASNS*, *LGMN* and *CTSB* expression levels in patients with BCP-ALL with various genetic aberrations. Expression of (A) *ASNS*, (B) *LGMN* and (C) *CTSB* in every group according to the primary genetic abnormality identified in the leukemic clone of BCP-ALL. Sample sizes: *BCR-ABL1* fusion, N=5; hyperdiploidy, N=7; hypodiploidy, N=2; *ETV6-RUNX1* fusion, N=3; other, N=35. Data were obtained by reverse transcription-quantitative PCR and are presented as relative mRNA expression. Horizontal line, median; whiskers, minimum to maximum; boxes, interquartile range. ASNS, asparagine synthetase; *LGMN*, legumain; *CTSB*, cathepsin B; BCP-ALL, B cell precursor acute lymphoblastic leukemia; *BCR-ABL1*, breakpoint cluster region and Abelson murine leukemia viral oncogene homolog 1 fusion; *ETV6-RUNX1*, ETS variant 6 and runt-related transcription factor 1 fusion.

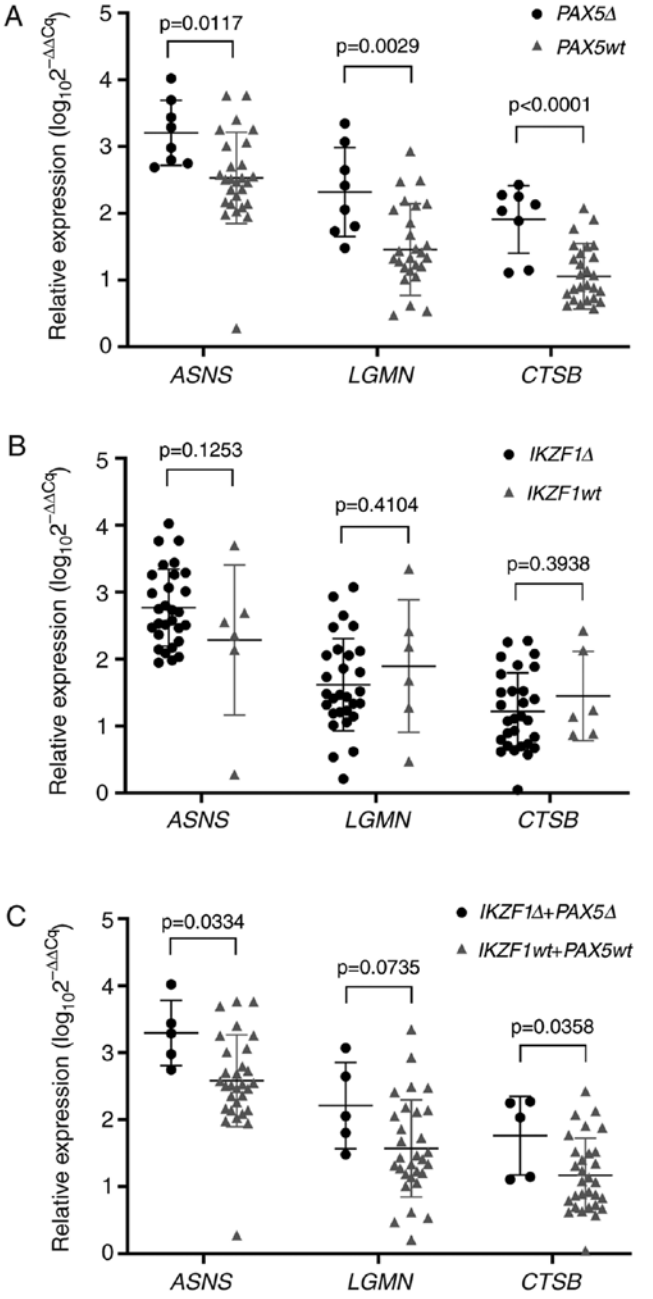

Figure 2. Expression of *ASNS*, *LGMN* and *CTSB* in patients carrying *PAX5* and *IKZF1* deletions. (A) Patients from the other BCP-ALL group with no identified primary genetic abnormality were separated into subgroups based on *PAX5* deletion (N=8) or wild-type (N=27). Relative expression of *ASNS* (3.21 vs. 2.50), *LGMN* (2.32 vs. 1.43) and *CTSB* (1.91 vs. 1.03) in patients with *PAX5* deletions was significantly higher compared with that in patients with wild-type *PAX5*. (B) Patients from the other BCP-ALL group were divided according to the *IKZF1* deletion status (*IKZF1* deletions, N=29; *IKZF1* wild-type, N=6). No significant differences were observed in the relative expression levels of ASNS, *LGMN* and *CTSB*. (C) Relative expression levels of *ASNS*, *LGMN* and *CTSB* in patients from the other BCP-ALL group with concomitant *IKZF1* and *PAX5* deletions (N=5). Middle horizontal line, median value; whiskers, interquartile range. *ASNS*, asparagine synthetase; *LGMN*, legumain; *CTSB*, cathepsin B; *PAX5*, paired box 5; *IKZF1*, ikaros family zinc finger protein 1; wt, wild-type; Δ, deletion; BCP-ALL, B cell precursor acute lymphoblastic leukemia.

(IQR, 1.37-3.04 years). All relapses occurred in patients in the other BCP-ALL group. RFS did not differ significantly when patients in the other BCP-ALL group were divided according to *PAX5* deletion status; median RFS was 5.14 in cases with *PAX5* deletion and 5.10 in *PAX5* wild-type cases (P=0.4540; HR, 1.56; 95% CI, 0.24-10.20). Out of the eight relapses, six (75%) occurred in patients with wild-type *PAX5*, whereas two (25%) occurred in patients with *PAX5* deletions

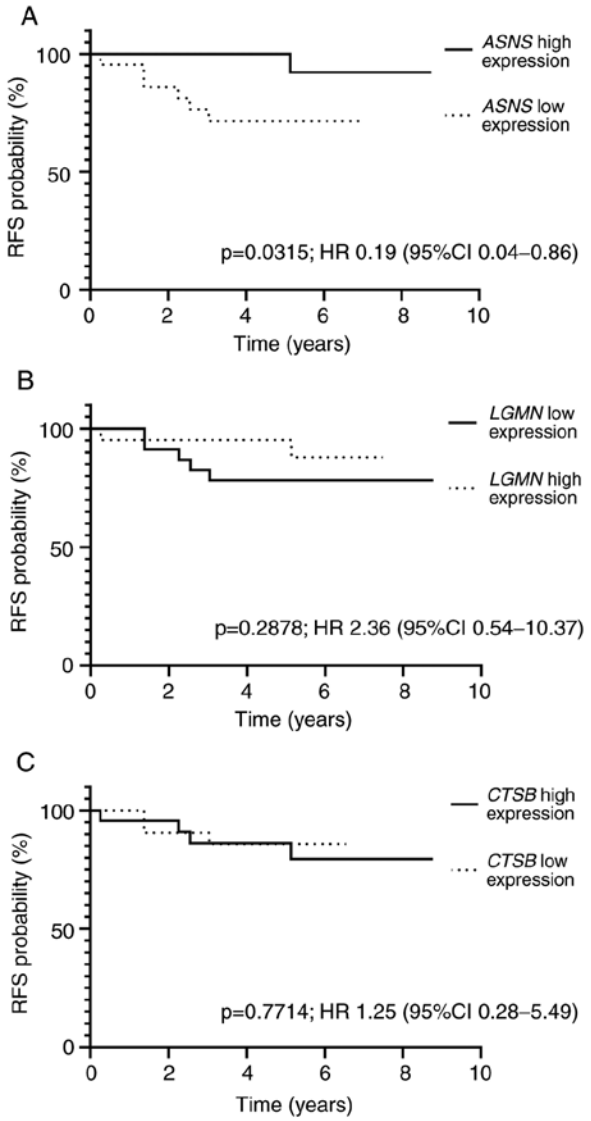

Figure 3. Kaplan-Meier curves for RFS based on *ASNS*, *LGMN* and *CTSB* expression levels. (A) For *ASNS*, the 5-year RFS rate was 90.15% (95% CI, 87.90-92.40%; N=24) in the high expression group and 73.34% (95% CI, 69.40-77.30%; N=25) in the low expression group. (B) For *LGMN*, the 5-year RFS rate in the high and low *LGMN* expression groups was 88.46% (95% CI, 85.70-91.20%; N=23) and 77.54% (95% CI, 74.30-80.80%; N=25), respectively. (C) In the high and low CTSB expression groups, 5-year RFS rate was 84.71% (95% CI, 81.70-87.70%; N=23) and 80.54% (95% CI, 77.40-83.70%; N=26), respectively. *ASNS*, asparagine synthetase; *LGMN*, legumain; *CTSB*, cathepsin B; RFS, relapse-free survival; HR, hazard ratio; CI, confidence interval.

(P=0.0002). When the impact of *PAX5* deletion on RFS was analyzed in the entire cohort, no significant differences were identified (median RFS for wild-type *PAX5*, 4.69 vs. 4.26 years in cases with *PAX5* deletion; P=0.7084; HR, 1.50; 95% CI, 0.22-10.03).

The cohort was divided based on the expression levels of *ASNS*, *LGMN* and *CTSB*, with the cut-off value set as the median expression for each gene (*ASNS*, 2.57; *LGMN*, 1.48; *CTSB*, 1.14). When median RFS was analyzed in patients with high and low *ASNS* expression, high *ASNS* expression was associated with a longer time to relapse (P=0.0315; HR, 0.19; 95% CI, 0.04-0.86; Fig. 3A). The 5-year RFS rate of patients with high *ASNS* expression was 90.15% (95% CI, 87.90-92.40%), and the 5-year RFS rate of patients with low *ASNS* expression was 73.34% (95% CI, 69.40-77.30%) (P=0.0164). No significant differences in RFS between patients with high and low *LGMN* or *CTSB* expression were observed (P=0.2878; HR, 2.36; 95% CI, 0.54-10.37; and P=0.7714; HR, 1.25; 95% CI, 0.28-5.49, respectively; Fig. 3B and C). Patients with high *LGMN* expression exhibited a 5-year RFS rate of 88.46% (95% CI, 85.70-91.20%), and those with low *LGMN* expression exhibited a 5-year RFS rate of 77.54% (95% CI, 74.30-80.80%). Similarly, the 5-year RFS rate was 84.71% (95% CI, 81.70-87.70%) for patients with high expression of *CTSB* and 80.54% (95% CI, 77.40-83.70%) for those with low *CTSB* expression.

Discussion

To evaluate the differences in *ASNS*, *LGMN* and *CTSB* gene expression in different types of BCP-ALL with possible additional effects of *PAX5* and/or *IKZF1* gene deletions, the patient cohort was grouped according to the major genetic abnormalities identified in leukemic cells such as *BCR-ABL1* fusion, *ETV6-RUNX1* fusion, hypodiploidy and hyperdiploidy (21). A large proportion of patients in the current study were classified into the other BCP-ALL group, in which none of the aforementioned aberrations were identified. However, considering the high incidence of *IKZF1* and *PAX5* deletions in these patients, it is likely that the group mostly comprised the *BCR-ABL1*-like type BCP-ALL (3). However, due to a lack of data on the gene expression profile, it is impossible to conclusively confirm this hypothesis, which is one of the limitations of the present study. Additionally, the small number of patients in the *ETV6-RUNX1* fusion group substantially limited the comparisons between the remaining groups; therefore, the clinical characteristics of the group may not fully reflect the favorable profile described in previous reports (21,22).

The median expression of *ASNS* did not significantly differ between the groups; however, relative mRNA expression of *ASNS* was the highest in the *ETV6-RUNX1* fusion group, which was consistent with previous observation (5). Previous studies have suggested the existence of an association between L-asp resistance and the upregulation of ASNS protein rather than mRNA (5,12,15,16); however, the data obtained in the present study were insufficient to verify this hypothesis, which was an additional limitation of this study. Despite this, longer RFS was observed in patients with high *ASNS* gene expression compared with those with low *ASNS* expression, which corresponded with the aforementioned findings (5).

The only previous study of *LGMN* gene expression in patients with BCP-ALL reported significantly higher expression in patients with iAMP21-positive BCP-ALL across all major genetic variants (14), which was associated with poor prognosis if not treated aggressively. Due to the lack of iAMP21-positive cases in the cohort analyzed in the present study, it was not possible to support those conclusions. No significant differences in *LGMN* expression were observed between the BCP-ALL subgroups; however, the lowest level of amplification was identified in patients with *ETV6-RUNX1* fusion.

To the best of our knowledge, the results of the present study are the first to describe the *CTSB* expression profile in

patients with childhood ALL. Despite the lack of statistical significance, the findings suggest that high *CTSB* expression may be associated with positive prognostic factors such as *ETV6-RUNX1* fusion and hyperdiploidy. These findings contradict our initial hypothesis of the association between high *ASNS*, *CTSB* and *LGMN* expression levels and an inferior outcome, although it is in line with the high *ASNS* expression observed in cases with *ETV6-RUNX1* fusion, as well as the longer time to relapse noted in a previous study (5).

The results of the present study also suggested an association between *PAX5* deletions and increased *ASNS*, *LGMN* and *CTSB* expression in the other BCP-ALL patient group, which has not previously been reported. *ASNS*, *CTSB* and *LGMN* expression was significantly higher in patients with *PAX5* deletion compared with those with concomitant *IKZF1* deletion, which suggested that *PAX5* may influence the expression of L-asp resistance genes as an independent factor. Although these comparisons were statistically significant, further experiments including *in vitro PAX5* silencing, as well as larger study cohorts are needed to support these results.

Although the outcome of *PAX5* deletion varies depending on its location in the front, middle or end of the gene (23), any *PAX5* deletion, regardless of its location and extent of deleted region, results in the dysfunction of the PAX5 protein. A small number of this subgroup enables to statistically connect the region of PAX5 deletion with gene expression levels or outcome. Thus, patients with *PAX5* CNVs were analyzed as a single cohort, irrespective of the type of *PAX5* deletion. This was a limitation of the present study, and *in vitro* tests (such as gene silencing) are required for further confirmation.

TRANSFAC analysis did not provide any information on the direct influence of *PAX5* TFBS on *ASNS*, *LGMN* or *CTSB* and could not explain the results of the present study. This suggested that *PAX5* may have an indirect effect on *ASNS*, *LGMN* or *CTSB* expression, and additional functional studies are required for clarification.

RFS was used for outcome assessment, as it is superior to overall survival when investigating the influence of gene expression on resistance to treatment; hence, relapse was the most accurate end point. High expression of the genes considered to be responsible for L-asp resistance did not correspond with shorter RFS or decreased 5-year RFS in this cohort, which rejected the initial hypothesis of the present study. In addition, higher 5-year RFS was observed in patients with high *ASNS* expression, although the results for *CTSB* and *LGMN* were not significant. Despite the differences in the median RFS in the subgroup analysis not being significant, patients in the other BCP-ALL group carrying *PAX5* deletions exhibited a longer time to relapse compared with those with wild-type *PAX5*.

The main limitation of the present study was a small number of patients and the lack of *BCR-ABL1*-like subtype confirmation, which resulted in challenges in achieving significant differences between variables. The present study is the first to report *CTSB* expression among different genetic subtypes of BCP-ALL, and the findings encourage further research. The results of the present study confirmed the association between *PAX5* deletions and the gene expression levels of *ASNS*, *LGMN* and *CTSB*, as well as between *ASNS* expression and 5-year RFS. The latter result, however, requires further study in a larger cohort for a more thorough explanation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EW, WM and AP designed the study. ML, JK, LS and TS obtained clinical data and bone marrow samples, and reviewed the manuscript. EW, JM and AP conducted the experiments. EW, JM, JJ and BP collected the data and interpreted the results. EW performed statistical analyses. EW and WM prepared a final manuscript for publication.

Ethics approval and consent to participate

All procedures performed in this retrospective study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments, and were approved by the Bioethical Committee of the Medical University of Lodz (approval no. RNN/155/13/KE). Informed consent was obtained from all individual participants and/or their parents included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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