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research article

Pharmacogenomic markers of glucocorticoid response in the initial phase of remission induction therapy in childhood acute lymphoblastic leukemia

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Background. Response to glucocorticoid (GC) monotherapy in the initial phase of remission induction treatment in childhood acute lymphoblastic leukemia (ALL) represents important biomarker of prognosis and outcome. We aimed to study variants in several pharmacogenes (*NR3C1*, *GSTs* and *ABCB1*) that could contribute to improvement of GC response through personalization of GC therapy.

Methods. Retrospective study enrolling 122 ALL patients was carried out to analyze variants of *NR3C1* (rs33389, rs33388 and rs6198), *GSTT1* (null genotype), *GSTM1* (null genotype), *GSTP1* (rs1695 and rs1138272) and *ABCB1* (rs1128503, rs2032582 and rs1045642) genes using PCR-based methodology. The marker of GC response was blast count per microliter of peripheral blood on treatment day 8. We carried out analysis in which cut-off value for GC response was 1000 (according to Berlin-Frankfurt-Munster [BFM] protocol), as well as 100 or 0 blasts per microliter.

Results. Carriers of rare *NR3C1* rs6198 GG genotype were more likely to have blast count over 1000, than the non-carriers ($p = 0.030$). *NR3C1* CAA (rs33389-rs33388-rs6198) haplotype was associated with blast number below 1000 ($p = 0.030$). *GSTP1* GC haplotype carriers were more likely to have blast number below 1000 ($p = 0.036$), below 100 ($p = 0.028$) and to be blast negative ($p = 0.054$), while *GSTP1* GT haplotype and rs1138272 T allele carriers were more likely to be blasts positive ($p = 0.034$ and $p = 0.024$, respectively). *ABCB1* CGT (rs1128503-rs2032582-rs1045642) haplotype carriers were more likely to be blast positive ($p = 0.018$).

Conclusions. Our results have shown that *NR3C1* rs6198 variant and *GSTP1* rs1695-rs1138272 haplotype are the most promising pharmacogenomic markers of GC response in ALL patients.

Key words: pharmacogenomics; childhood ALL; glucocorticoids; glucocorticoid receptor gene; glutathione S-transferase genes; multidrug resistance 1 gene

Introduction

Acute lymphoblastic leukemia (ALL) is the most common hematological and overall malignancy in pediatrics, accounting for around 30% of all childhood cancers and around 80% of all childhood leukemias. It is one of the pediatric malignancies with the highest cure rate, exceeding 80%, when treated

with standardized protocols like the European standard, the Berlin-Frankfurt-Munster (BFM) protocol.[1-3] However, there is still more than 10% of patients with unfavorable outcome. The treatment of childhood ALL is based on risk stratification. Patients can be classified into groups according to the features that have been shown to affect prognosis and risk of treatment failure. In time, more elements are considered in order to modulate the treatment protocols and make them more efficient. Implementation of pharmacogenomics in the childhood ALL therapeutic strategy is the most promising approach to improve the outcome of childhood ALL.[4]

The four main components of ALL therapy are remission induction, consolidation, maintenance, and central nervous system-directed therapy. According to the BFM protocol, in the initial phase of the remission induction treatment of childhood ALL, glucocorticoid (GC) monotherapy is administered during the first 8 days. Its goal is to lower the number of lymphoblasts since GC have the ability to induce apoptosis in leukemic cells mediated through the glucocorticoid receptor (GR).[5] The lymphoblast count on the day 8 is one of the stratification criteria important for therapy regime and survival.[6] If the blast count in blood is below 1000/microL, the patient is declared as a GC sensitive patient or a prednisone good responder (PGR). If the peripheral blast count of a patient remains over 1000/microL, the patient is declared as GC resistant patient or a prednisone poor responder (PPR) and this is associated with a poor prognosis.

The mechanism of GC resistance in childhood ALL is still poorly understood, but genetic factors might play an important role.[7-9] Therefore, it is of great importance for better treatment of childhood ALL to investigate, understand and overcome the problems related to pharmacogenomics profile of patients with a poor response to the initial GC treatment.

The glucocorticoid receptor gene (*NR3C1*) codes the GR, which is essential for the effects of glucocorticoids to manifest. Several *NR3C1* variants, leading to altered sensibility of GR to glucocorticoids have been studied in pediatric diseases. Most frequently studied variants, like rs6189/rs6190 (ER22/23EK) and rs56149945 (N363S) have not shown significant association with the response on the day 8, when it comes to the therapeutic response to glucocorticoids in ALL.[10,11] One extensively studied variant, rs41423247 (*BclI* polymorphism), has shown association with the therapeutic response.[12]

Three variants in the *NR3C1* gene, rs33389 (c.1185-6766C>T), rs33388 (c.1185-3562A>T) and rs6198 (c.*3833A>G) have not been widely studied as pharmacogenomics markers in childhood ALL. The first two variants are located in intron 2, where they can alter consensus recognition sites for RNA splicing factors.[13] If the minor rs33389 T allele and the major rs33388 A allele are present, alternate splicing occurs and an isoform of GR with lower affinity for glucocorticoids is expressed in a higher degree.[14] In the pediatric nephrotic syndrome, the steroid response was affected by the presence of these two alleles in intron 2.[15] The rs6198 variant is located in the 3' UTR region exon 9β, in the "ATTTA" motif of an isoform of GR with drastically lower affinity for glucocorticoids.[16] If the minor rs6198 G allele is present, the mRNA becomes more stable and it leads up to greater translation of the isoform of GR with lower affinity for glucocorticoids.[17]

Three glutathione S-transferase (GST) genes (*GSTP1*, *GSTT1*, and *GSTM1*) code the GST proteins, which are essential for GC elimination by making its first step, conjugation, possible.[18, 19] Null-allele variants of *GSTM1* and *GSTT1* caused by a deletion of the gene, result in the absence of activity of these enzymes. Additionally, it was reported that *GSTP1* gene variants rs1695 (c.313A>G, p.Ile105Val) and rs1138272 (c.341C>T, p.Ala114Val) influence the activity and the structure of GSTP1 and alter the efficiency of GC conjugation, if the minor alleles are present.[20] An association between the rs1695 variant and GC response was found in ALL.[21]

The multidrug resistance 1 gene (*MDR1*, also known as *ABCB1),* encodes for a membrane transporter P-glycoprotein (P-gp), responsible for the efflux of chemotherapeutic agents used in leukemia therapy.[22] Glucocorticoids are substrates of P-gp, which transports glucocorticoids out of cells. Overexpression of P-gp could mediate GC resistance.[23] When considering *ABCB1*, three variants were often analyzed as pharmacogenomics markers for GC response (rs1128503 (c.1236C>T, p.Gly412=), rs2032582 (c.2677G>A/T, p.Ser893Ala) and rs1045642 (c.3435TC>T, p.Ile1145=)). The rs2032582 variant is a missense mutation, while rs1045642 is a synonymous mutation which leads to decreased expression of *ABCB1* gene on the intestinal cell membranes.[24] It was found that the steroid response in children with nephrotic syndrome varied based on the expression of *ABCB1* gene.[25]

There have been a few reports which dealt with the topic of pharmacogenomics of GC resistance

in adult leukemias, but they lacked conclusive evidence of a single contributing mechanism.[26] The topic of pharmacogenomics of GC resistance in ALL, when it comes to the pediatric population, has not been sufficiently studied. In the reported results, only tendencies towards association with GC response for certain genotypes[27] have been found, while most of the genetic variants, shown to be relevant for GC response, have never been studied in childhood ALL.

The aim of this study is to investigate the association between variants in *NR3C1, GSTP1*, *GSTT1*, *GSTM1* and *ABCB1* genes and GC therapeutic response in the initial phase of remission induction therapy of pediatric ALL patients. Also, we aimed to investigate if the analyzed pharmacogenomics markers could be helpful to achieve improved personalization of GC therapy, leading to more individualized approach. Namely, other values than 1000 of blast number on day 8 might be potentially used as a marker of therapy efficacy. For example, it has been reported that childhood ALL patients who has zero blasts on day 8 (blast negative), have longer disease-free survival than patients with detectable blasts (blast positive).[28] In order to better characterize GC response on day 8 related to analyzed genetic variants, we carried out additional analysis in which cut-off value for GC response was 100 or 0 blasts in peripheral blood. By understanding the factors which contribute to GC resistance or good response, predictions could be made for an individual patient before the initial treatment, in order to use the adequate treatment regime and increase the chances of more efficient GC response.

Patients and methods

Patients

Peripheral blood samples (n = 122) have been collected from unselected patients with the diagnosis of childhood ALL from the University Children's Hospital in Belgrade. The samples for genetic analyses were collected on the day of the diagnosis. Childhood ALL patients were diagnosed, stratified in risk groups and treated according to Berlin-Frankfurt-Munster protocols: BFM ALL IC-2002 and BFM ALL IC-2009. All patients received induction therapy with prednisone. This study was approved by the Ethics Committee of the University Children's Hospital, University of Belgrade. The study was conducted according to the principles of Declaration of Helsinki.

DNA isolation

Genomic DNA was extracted from peripheral blood samples of the participants' using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and stored at - 20 °C until analysis.

GSTM1 and GSTT1 deletion detection

The detection of *GSTM1* and *GSTT1* homozygous deletions was performed using multiplex polymerase chain reaction (PCR), as previously described with modifications.[29] In the final reaction volume of 20 microL, 1x buffer were added, 3.875 mM of $MgCl_2$, 0.5 mM of dNTP, 0.3 microM of the forward and reverse primer for *GSTT1*, 0.25 microM of the forward and the reverse primer for *GSTM1*, 0.25 microM of forward and reverse primer for β globin gene segment (control PCR product), 1U of Taq polymerase (Hot Star polymerase, Qiagen, Hilden, Germany) and 60ng of DNA. After the initial denaturation at 95 C for 15', followed 35 cycles of 95 °C / 53 °C / 72 °C, lasting 30'', 45'' and 60'' respectively, ending with a final extension step at 72 °C lasting for 7'.

Genotyping of *ABCB1* variants

The variant rs2032582 of *ABCB1* gene was genotyped using the amplification-refractory mutation system polymerase chain reaction (ARMS PCR). A forward primer was designed for each allele specifically in order to pinpoint the exact genotype. The protocol was adapted from Kuzawski and coworkers.[30] For each patient's sample, 3 separate PCR mixes were prepared, each containing different allele specific primer. In the final reaction volume of 15 microL, 1x buffer were added, 3 mM of $MgCl_2$, 0.67 mM of dNTP, 0.3 microM of the forward (allele specific) and reverse primer, 1U of Taq polymerase (Hot Star polymerase, Qiagen, Hilden, Germany) and 60ng of DNA. The PCR program started with a 95 °C initial denaturation which lasted for 15', followed by 10 cycles of 95 °C / 60 °C / 72 °C lasting 30'', 30'' and 40'' respectively, followed by 30 cycles of 95 °C / 56 °C / 72 °C, lasting 30'', 30'' and 40'' respectively and the final step was an extension at 72 °C which endured for 5'.

Variants rs1045642 and rs1128503 of *ABCB1* were genotyped using the Kompetitive Allele Specific PCR genotyping system (KASP) (LGC, Teddington, Middlesex, UK), according to manufacturer's instructions.

Genotyping of *NR3C1* variants

Variants rs33389, rs33388 and rs6198 of *NR3C1* were genotyped using TaqMan® SNP Genotyping Assays (Thermo Fisher Scientific) according to manufacturer's instructions. The fluorofore VIC was used to detect the wild type allele, while FAM was used to detect the variant allele. For genotyping of rs33389, rs33388 and rs6198 variants, C___1032036_10, C___1046426_10 and C___8951023_10 assays were used, respectively.

Genotyping of *GSTP1* variants

Variants rs1695 and rs1138272 of *GSTP1* were detected using the KASP genotyping system according to manufacturer's instructions (LGC, Teddington, Middlesex, UK).

Statistical analysis

Hardy-Weinberg equilibrium conformance was examined using χ^2 test. Haplotype phases and frequencies were estimated using Arlequin software.[31] The associations between carrier status of specific allele or haplotype and the number of blasts at the day 8 have been analyzed in 2x2 contingency tables using the χ^2 test or the Fisher's exact test, when appropriate. Both dominant and recessive genetic model were applied when we considered single variant at the time, and stronger association with GC response was reported. Carriers of a specific haplotype were compared to all other patients with any other haplotype for each haplotype. Odds ratio with 95% confidence interval was used to assess the impact of clinical or genetic variable on GC drug response. The cut-off for statistical significance has been chosen at the value of p = 0.05, while the cut-off value for borderline significance has been chosen at the value of p = 0.07. To control for demographic and clinical difference between groups, multivariate analysis was performed using logistic regression. Correlation between continuous variables were estimated using Spearman's correlation coefficient (rs). The SPSS software package (IBM SPSS Statistics v.21) was used for statistical analyses.

Results

Demographic and clinical characteristics of childhood ALL patients on diagnosis

Out of 122 childhood ALL patients, there were 66 boys (54.1%) and the median age was 5.2 (interquartile range: 3.3–10.2) years. B-cell leukemia was represented with 108 (88.5%) cases and the rest of patients were diagnosed with T-cell leukemia. About 47% of patients had initially over 20,000 white blood cells (WBC) per microliter of blood, which is considered as unfavorable factor according to both BFM ALL IC-2002 and BFM ALL IC-2009 protocols (Table 1).

GC response on day 8

In our study, blast count per microliter of blood on day 8 was used as surrogate marker of GC response. There were thirteen patients (11%) with more than 1000 blasts/microL on day 8 of GC treatment in our cohort of patients. We have analyzed the correlation of clinical and demographic characteristics of patients with prednisone response. Namely, leukocyte count on diagnosis was positively correlated with absolute blast count on day 8 (rs = 0.44, p = 0.000001). In addition, patients suffering from T-cell leukemia were in greater risk to respond poorly to initiation GC treatment (≥ 1000 blasts/microL on day 8) (Fisher's exact test, p = 0.043) than B-cell leukemia patients. Furthermore, age and gender of childhood ALL patients showed borderline association with prednisone response (Table 1).

Association of gene variants with PGR and PPR according to BFM protocol

Two homozygous deletions in *GSTM1* and *GSTP1* genes were studied as well as 8 single nucleotide variants (SNV) in *NR3C1* (rs33389, rs33388 and rs6198), *GSTP1* (rs1695 and rs1138272) and *ABCB1* (rs1128503, rs2032582 and rs1045642) genes. Genotype frequencies of all analyzed SNVs conformed to HW equilibrium for the ALL cohort.

When we carried out analysis in which 1000 blasts/microL set the limit of PGR and PPR, we found some positive correlation of pharmacogenomic markers with GC response. Regarding *NR3C1* gene, our results have shown that *NR3C1* variants were associated with glucocorticoid response on day 8. Namely, rare *NR3C1* rs6198 GG genotype was associated with PPR (Fisher's exact test; p = 0.030) (Table 2). When estimated haplotypes of *NR3C1* gene were considered, it was found that CAA (rs33389-rs33388-rs6198) haplotype was associated with PGR (< 1000 blasts/microL) (Fisher's exact test; p = 0.030) (Table 3). Both associations remained significant or borderline significant when controlled for age, gender and ini-

TABLE 1. Clinical and demografic characteristics and their association with glucocorticoid (GC) response . The GC response is assesed by absolute number of blasts per mm3 of blood on day 8. Statistically significant associations (p < 0.05) were bolded

Patients characteristics	Group	Entire group	≥1000 blasts	100≤ blasts <1000	1≤ blasts <100	blast negative patients	GC response (cutoff=1000 blasts)[0]	GC response (cutoff=100 blasts)[1]	GC response (cutoff=0 blasts)[2]
		n (%)	n (%)	n (%)	n (%)	n (%)	OR [95%CI], p[0]	OR [95%CI], p[1]	OR [95%CI], p[2]
Age	≥1 and <6 (non-risk)	65 (53.3)	4 (30.8)	17 (54.8)	21 (55.3)	23 (57.5)	reference	reference	reference
	<1 or ≥6 (risk)	57 (46.7)	9 (69.2)	14 (45.2)	17 (44.7)	17 (42.5)	2.86 [0.83-9.85], 0.085	1.42 [0.68-2.98], 0.356	1.29 [0.60-2.76], 0.514
Gender	male	66 (54.1)	10 (76.9)	13 (41.9)	20 (52.6)	23 (57.5)	reference	reference	reference
	female	56 (45.9)	3 (23.1)	18 (58.1)	18 (47.4)	17 (42.5)	0.32 [0.083-1.26], 0.081	1.12 [0.54-2.35], 0.761	1.28 [0.57-2.63], 0.598
Initial WBC count*	<20,000/microL	64 (53.3)	1 (8.3)	13 (43.3)	21 (55.3)	29 (72.5)	reference	reference	reference
	≥20,000/microL	56 (46.7)	11 (91.7)	17 (56.7)	17 (44.7)	11 (27.5)	15.40 [1.92-123.6], **0.001**	3.57 [1.62-7.88], **0.001**	3.39 [1.49-7.72], **0.003**
Immunophenotype	B	108 (88.5)	9 (69.2)	27 (87.1)	35 (92.1)	37 (92.5)	reference	reference	reference
	T	14 (11.5)	4 (30.8)	4 (12.9)	3 (7.9)	3 (7.5)	4.40 [1.15-16.90], **0.043**[F]	2.67 [0.86-8.27], 0.081	1.91 [0.50-7.28], 0.546

[0] Association with prednosine response on day 8 according to Berlin-Frankfurt-Munster (BFM) protocol: prednisone poor responder (PPR) group (≥1000 blasts) *vs.* prednisone good responder (PGR) group (<1000 blasts)
[1] Association with number of blasts on day 8 with cut-off value of 100: higher (≥ 100 blasts) vs lower (< 100 blasts) number of blasts
[2] Association with blast status on day 8: blast positive vs blast negative patients.
[F] Fisher exact test
OR = Odds ratio between a group with higher number of blasts in comparison with a group with lower number of blasts. The group with lower number of blasts represents reference group.
CI = Confidence interval

tial WBC count (logistic regression, p = 0.036 and p = 0.052, respectively) (Tables 2 and 3).

When variants in *ABCB1* and *GST* genes were considered in relation to GC response, no significant association was found. However, when estimated haplotypes were considered, *GSTP1* GC (rs1695-rs1138272) haplotype was associated with PGR (χ^2 test, p = 0.036) (Table 3).

Additional analyses of GC response on day 8 in regard to genetic variants

Besides cut-off value of 1000 blasts/microL on day 8, used to delimit patients with good or poor GC response according to BFM protocol, other values of blast count on day 8 might be potentially used as a marker of GC response. In order to confirm importance of analyzed genetic variants to GC response, we carried out additional analyses in which cut-off value for prednisone response was 0 (blast negative) or 100 blasts in peripheral blood. In our group of childhood ALL patients, 40 (32.8%) were blast negative, while 38 (31.1%) patients had between 1 and 99 blasts/microL after 8 days of GC treatment. Initial WBC count was correlated with blast positive status and higher number of blasts (≥ 100 blasts/microL) (Table 1).

Regarding *NR3C1* gene, our results have shown that carriers of minor rs33389 T allele tended towards higher blast count (≥ 100 blasts/microL) (χ^2 test; p = 0.095), while carriers of minor rs33388 T allele tended towards lower blast count (< 100 blasts/microL) (χ^2 test; p = 0.098), but the results didn't reach statistical significance. When estimated haplotypes were considered, identical associations were obtained, because rs33389 T allele defines relatively rare TAA (rs33389-rs33388-rs6198) haplotype, while rs33388 T allele defines the most frequent CTA haplotype (Tables 2 and 3).

Additional analysis regarding *GSTP1* gene showed that carriers of minor *GSTP1* rs1138272 T allele were about 5 times more likely to be blast positive on day 8, when compared to carriers of CC genotype (χ^2 test; p = 0.024). Next, we analyzed estimated haplotypes of *GSTP1* gene consisting of rs1695 and rs1138272 variants. We found that *GSTP1* GC haplotype is associated not only with PGR, but also with lower blast count on day 8. Namely, this haplotype was associated with blast count below 100 (< 100 blasts/microL, χ^2 test; p = 0.028) and borderline associated with blast negative status (χ^2 test; p = 0.054). Also, it was shown that *GSTP1* GT haplotype is borderline associated with higher blast count (≥ 100 blasts/microL, χ^2 test; p = 0.062) and significantly associated with blast positive status (χ^2 test; p = 0.034). The majority of those associations remained significant or borderline significant when controlled for age, gender and initial WBC count employing logistic regression. Taken together, our results regarding *GSTP1* variants indicate that carriers of GC haplotype have better response to prednisone treat-

TABLE 2. Genotype frequencies and association with glucocorticoid (GC) response. The GC response is assesed taking into account absolute number of blasts per mm3 of blood on day 8. For univariate analysis, chi square test was used, unless differently stated. Dominant model was used unless differently stated. Statistically significant associations ($p < 0.05$) were bolded

Genotype	≥ 1000 blasts	100 ≤ blasts < 1000	1 ≤ blasts < 100	blast negative patients	GC response (cutoff=1000 blasts)[0]	GC response (cutoff=1000 blasts)[0, ADJ]	GC response (cutoff=100 blasts)[1]	GC response (cutoff=100 blasts)[1, ADJ]	GC response (cutoff=0 blasts)[2]	GC response (cutoff=0 blasts)[2, ADJ]
	n (%)	n (%)	n (%)	n (%)	OR[95%CI] P value	OR[95%CI] P value	OR[95%CI] P value	OR[95%CI] P value	OR[95%CI] P value	OR[95%CI] P value
NR3C1 rs33389										
CC	10 (76.9)	21 (67.7)	33 (86.8)	32 (80.0)	reference	reference	reference	reference	reference	reference
CT	3 (23.1)	9 (29.0)	4 (10.5)	6 (15.0)	1.12[0.29-4.41] 1[F]	1.10[0.21-5.92] 0.910	2.1[0.87-5.05] 0.095	1.85[0.73-4.71] 0.195	1.12[0.44-2.86] 0.805	0.89[0.32-2.44] 0.826
TT	0 (0.0)	1 (3.2)	1 (2.6)	2 (5.0)						
NR3C1 rs33388										
AA	4 (30.8)	11 (35.5)	7 (18.4)	9 (22.5)	reference	reference	reference	reference	reference	reference
AT	4 (30.8)	15 (48.4)	19 (50.0)	19 (47.5)	0.74[0.21-2.60] 0.737[F]	0.71[0.16-3.13] 0.658	0.5[0.22-1.15] 0.098	0.53[0.28-1.26] 0.148	0.79[0.32-1.92] 0.606	0.83[0.32-2.11] 0.699
TT	5 (38.5)	5 (16.1)	12 (31.6)	12 (30.0)						
NR3C1 6198										
AA	7 (53.8)	21 (67.7)	28 (73.7)	30 (75.0)	reference	reference	reference	reference	reference	reference
AG	4 (30.8)	10 (32.3)	9 (23.7)	10 (25.0)						
GG	2 (15.4)	0 (0.0)	1 (2.6)	0 (0.0)	19.64[1.65-234.32] **0.030**[R,F]	16.76[1.20-234.27] **0.036**[R]	1.66[0.75-3.68] 0.222[R]	4.01[0.34-47.4] 0.27[R]	1.04[0.99-1.08] 0.22[R]	- 1[R]
GSTP1 rs1695										
AA	7 (53.8)	15 (48.4)	16 (42.1)	16 (40.0)	reference	reference	reference	reference	reference	reference
AG	5 (38.5)	13 (41.9)	18 (47.4)	19 (47.5)	0.65[0.21-2.06] 0.46	0.91[0.26-3.25] 0.885	0.70[0.33-1.46] 0.338	0.73[0.34-1.58] 0.423	0.77[0.36-1.66] 0.508	0.84[0.37-1.9] 0.682
GG	1 (7.7)	3 (9.7)	4 (10.5)	5 (12.5)						
GSTP1 rs1138272										
CC	9 (69.2)	25 (80.6)	31 (81.6)	38 (95.0)	reference	reference	reference	reference	reference	reference
CT	3 (23.1)	6 (19.4)	7 (18.4)	2 (5.0)	2.79[0.76-10.20] 0.119[F]	3.17[0.76-13.28] 0.115	2.26[0.84-6.07] 0.122	2.23[0.81-6.15] 0.121	4.97[1.09-22.69] **0.024**	4.44[0.9-21.08] 0.060
TT	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)						
GSTM1 homozygous deletion										
WT	6 (46.2)	13 (41.9)	18 (47.4)	19 (47.5)	reference	reference	reference	reference	reference	reference
DEL	7 (53.8)	18 (58.1)	20 (52.6)	21 (52.5)	0.99[0.31-3.13] 0.99	0.96[0.25-3.70] 0.953	1.19[0.56-2.50] 0.707	1.03[0.47-2.27] 0.941	1.0[0.51-2.38] 0.805	0.96[0.43-2.18] 0.935
GSTT1 homozygous deletion										
WT	8 (61.5)	23 (74.2)	31 (81.6)	35 (87.5)	reference	reference	reference	reference	reference	reference
DEL	5 (38.5)	8 (25.8)	7 (18.4)	5 (12.5)	2.78[0.82-9.09] 0.138[F]	3.70[0.95-14.08] 0.058	2.33[0.94-5.56] 0.063	2.39[1.03-6.25] **0.044**	2.27[0.78-6.67] 0.127	2.06[0.67-6.29] 0.202
ABCB1 rs1128503										
CC	5 (38.5)	10 (32.3)	13 (34.2)	13 (32.5)	reference	reference	reference	reference	reference	reference
CT	6 (46.2)	16 (51.6)	21 (55.3)	17 (42.5)	0.79[0.24-2.59] 0.759[F]	0.74[0.20-2.76] 0.651	0.97[0.44-2.11] 0.932	0.89[0.40-1.99] 0.773	0.92[0.41-2.07] 0.857	0.84[0.36-1.97] 0.691
TT	2 (15.4)	5 (16.1)	4 (10.5)	10 (25.0)						
ABCB1 rs2032582										
GG	5 (31.3)	9 (24.3)	15 (30.6)	14 (27.5)	reference	reference	reference	reference	reference	reference
GT	4 (25.0)	16 (43.2)	17 (34.7)	16 (31.4)	0.86[0.26-2.80] 0.769[F]	1.08[0.29-4.05] 0.908	1.27[0.58-2.78] 0.693	1.27[0.56-2.88] 0.57	0.98[0.44-2.17] 0.968	0.9[0.39-2.1] 0.813
TT	3 (18.8)	5 (13.5)	6 (12.2)	9 (17.6)						
GA	1 (6.3)	1 (2.7)	0 (0.0)	1 (2.0)						
ABCB1 rs1045642										
CC	3 (23.1)	6 (19.4)	11 (28.9)	11 (27.5)	reference	reference	reference	reference	reference	reference
CT	7 (53.8)	16 (51.6)	19 (50.0)	18 (45.0)	1.15[0.30-4.49] 1[F]	1.47[0.31-6.35] 0.657	1.53[0.63-3.70] 0.393	1.92[0.74-4.98] 0.18	1.17[0.5-2.77] 0.711	1.31[0.52-3.28] 0.559
TT	3 (23.1)	9 (29.0)	8 (21.1)	11 (27.5)						

[0] Association with prednosine response on day 8 according to Berlin-Frankfurt-Munster (BFM) protocol: prednisone poor responder (PPR) group (≥1000 blasts) vs. prednisone good responder (PGR) group (< 1000 blasts)
[1] Association with number of blasts on day 8 with cut-off value of 100: higher (≥ 100 blasts) vs lower (< 100 blasts) number of blasts
[2] Association with blast status on day 8: blast positive vs blast negative patients.
[F] Fisher exact test
[R] Recessive model
[ADJ] Adjusted for age, gender and initial white blood cells (WBC) count using logistic regression
OR = Odds ratio between a group with higher number of blasts in comparison with a group with lower number of blasts. The group with lower number of blasts represents reference group.
CI = Confidence interval

TABELE 3. Haplotype carring status and association with glucocorticoid (GC) response. The GC response is assesed taking into account absolute number of blasts per mm3 of blood on day 8. For univariate analysis, chi square test was used, unless differently stated. Statistically significant associations ($p < 0.05$) were bolded

Haplotype (estimated frequency)	Carrier status*	≥ 1000 blasts	100 ≤ blasts < 1000	1 ≤ blasts < 100	blast negative patients	GC response (cutoff=1000 blasts)[2]	GC response (cutoff=1000 blasts)[2, ADJ]	GC response (cutoff=100 blasts)[1]	GC response (cutoff=100 blasts)[1, ADJ]	GC response (cutoff=0 blasts)[0]	GC response (cutoff=0 blasts)[0, ADJ]
		n (%)	n (%)	n (%)	n (%)	OR [95%CI] p value	OR [95%CI] p value	OR [95%CI] p value	OR [95%CI] p value	OR [95%CI] p value	OR [95%CI] p value
NR3C1 (rs33389-rs33388-rs6198) haplotypes											
CTA (51.2%)	absent	4 (30.8)	11 (35.5)	7 (18.4)	9 (22.5)	reference	reference	reference	reference	reference	reference
	present	9 (69.2)	20 (64.5)	31 (81.6)	31 (77.5)	0.71[0.21-2.60] 0.737[F]	0.72[0.16-3.13] 0.658	0.50[0.22-1.15] 0.098	0.53[0.22-1.26] 0.148	0.79[0.32-1.92] 0.606	0.83[0.32-2.11] 0.699
CAA (20.5%)	absent	12 (92.3)	15 (48.4)	25 (65.8)	25 (62.5)	reference	reference	reference	reference	reference	reference
	present	1 (7.7)	16 (51.6)	13 (34.2)	15 (37.5)	0.12[0.015-0.98] **0.030**[F]	0.12[0.013-1.02] 0.052	1.12[0.52-2.41] 0.763	1.27[0.57-2.80] 0.561	0.96[0.44-2.1] 0.922	1.22[0.53-2.80] 0.643
CAG (16.0%)	absent	7 (53.8)	20 (64.5)	28 (73.7)	29 (72.5)	reference	reference	reference	reference	reference	reference
	present	6 (46.2)	11 (35.5)	10 (26.3)	11 (27.5)	2.06[0.64-6.62] 0.222[F]	1.82[0.49-6.74] 0.372	1.71[0.78-3.75] 0.18	1.60[0.70-3.65] 0.262	1.29[0.56-2.97] 0.543	1.28[0.53-3.1],0.576
TAA (12.3%)	absent	10 (76.9)	21 (67.7)	33 (86.8)	32 (80.0)	reference	reference	reference	reference	reference	reference
	present	3 (23.1)	10 (32.3)	5 (13.2)	8 (20.0)	1.12[0.29-4.41] 1.000[F]	1.10[0.21-5.92] 0.91	2.10[0.87-5.05] 0.095	1.85[0.73-4.71] 0.195	1.12[0.44-2.86] 0.805	0.89[0.32-2.44] 0.826
GSTP1 (rs1695-rs1138272) haplotypes											
AC (66.4%)	absent	1 (7.7)	3 (9.7)	4 (10.5)	5 (12.5)	reference	reference	reference	reference	reference	reference
	present	12 (92.3)	28 (90.3)	34 (89.5)	35 (87.5)	1.49[0.18-12.45] 1.000[F]	0.99[0.11-9.21] 0.993	1.30[0.38-4.51] 0.768[F]	1.18[0.33-4.19] 0.797	1.32[0.4-4.33] 0.756	1.15[0.33-3.93] 0.825
GC (25.4%)	absent	11 (84.6)	20 (64.5)	21 (55.3)	18 (45.0)	reference	reference	reference	reference	reference	reference
	present	2 (15.4)	11 (35.5)	17 (44.7)	22 (55.0)	0.22[0.045-1.01] **0.036**	0.27[0.054-1.38] 0.117	0.42[0.19-9.20] **0.028**	0.42[0.19-0.96] **0.041**	0.47[0.22-1.02] 0.054	0.55[0.24-1.23] 0.149
GT (7.8%)	absent	9 (69.2)	25 (80.6)	32 (84.2)	38 (95.0)	reference	reference	reference	reference	reference	reference
	present	4 (30.8)	6 (19.4)	6 (15.8)	2 (5.0)	3.02[0.82-11.12] 0.101[F]	3.41[0.81-14.34] 0.094	2.57[0.93-7.11] 0.062	2.61[0.93-7.37] 0.069	4.6[1.00-21.12] **0.034**	4.33[0.91-20.62] 0.065
ABCB1 (rs1128503-rs2032582-rs1045642) haplotypes											
CGC (45.9%)	absent	4 (30.8)	10 (32.3)	8 (21.1)	12 (30.0)	reference	reference	reference	reference	reference	reference
	present	9 (69.2)	21 (67.7)	30 (78.9)	28 (70.0)	0.85[0.25-2.98] 0.754[F]	0.74[0.19-2.81] 0.654	0.74[0.33-1.67] 0.465	0.73[0.32-1.69] 0.461	1.17[0.5-2.7] 0.714	1.43[0.58-3.53] 0.432
TTT (36.9%)	absent	6 (46.2)	12 (38.7)	17 (44.7)	16 (40.0)	reference	reference	reference	reference	reference	reference
	present	7 (53.8)	19 (61.3)	21 (55.3)	24 (60.0)	0.82[0.26-2.60] 0.772	0.85[0.24-3.08] 0.805	1.06[0.50-2.24 0.88	1.13[0.52-2.47] 0.76	0.89[0.41-1.93] 0.778	0.91[0.4-2.05] 0.821
CGT (8.6%)	absent	10 (76.9)	24 (77.4)	30 (78.9)	38 (95.0)	reference	reference	reference	reference	reference	reference
	present	3 (23.1)	7 (22.6)	8 (21.1)	2 (5.0)	1.62[0.40-6.52] 0.446[F]	2.29[0.50-10.59] 0.289	2.00[0.76-5.27] 0.156	2.34[0.86-6.33] 0.095	5.34[1.17-24.31] **0.018**	7.56[1.6-35.82] **0.011**

[0] Association with prednosine response on day 8 according to Berlin-Frankfurt-Munster (BFM) protocol: prednisone poor responder (PPR) group (≥ 1000 blasts) vs. prednisone good responder (PGR) group (< 1000 blasts)
[1] Association with number of blasts on day 8 with cut-off value of 100: higher (≥ 100 blasts) vs lower (< 100 blasts) number of blasts
[2] Association with blast status on day 8: blast positive vs blast negative patients.
[F] Fisher exact test
[ADJ] Adjusted for age, gender and initial white blood cells (WBC) count using logistic regression
OR = Odds ratio between a group with higher number of blasts in comparison with a group with lower number of blasts. The group with lower number of blasts represents reference group.
CI = Confidence interval

ment, while carriers of GT haplotype have poorer response to prednisone treatment.

Regarding *GSTT1* gene, our results have shown that carriers of null genotype are more likely to have blast count over 100 (≥ 100 blasts/microL) (χ^2 test; p = 0.063), in comparison with carriers of at least one functional *GSTT1* gene copy. Interestingly, when controlled for age, gender and initial WBC count, this association turned out to be statistically significant (Logistic regression, p = 0.044)

Regarding *ABCB1* gene variants in relation to blast count, no association was found. However, when estimated haplotypes were considered, it was found that carriers of relatively rare CGT (rs1128503-rs2032582-rs1045642) haplotype had been 5 times more likely to be blast positive (χ^2

test; p = 0.018), than the non-carriers. This association remained significant when controlled for age, gender and initial WBC count employing logistic regression.

Discussion

Pharmacogenomics is dealing with the fact that the efficacy of the drug depends on the patient's ability to absorb and metabolize the drug, which influences the effectiveness of the treatment. Furthermore, the toxicity of drug depends on the patient's genome. Pharmacogenomics testing is already incorporated as a dosage-calibrating tool in the maintenance phase of childhood ALL treatment in order to minimize the occurrence of serious toxicities during 6-MP treatment.[4, 32]

Glucocorticoids are an essential component to induction remission phase of childhood ALL therapy. A poor response to the standard initial GC treatment and the persistence of blast count over 1000 per microliter on the day 8, puts a patient in a higher risk group with a poor prognosis. The following phases of treatment are dependent on risk-directed stratification of patients. However, many children experience severe toxicity associated with treatment with dangerous side effects, while some of them are not cured.[33] So, it could be argued that these groupings are not yet comprehensive enough.[34] As for induction remission phase of ALL treatment, it is essential to find as many potential markers of GC resistance as possible. By analyzing the associations between the pharmacogenetic variants and GC resistance or good response, this study was meant to contribute to individualization of GC treatment, so that the patients could be in future adequately treated according to their genetic background.

A few studies dealt with variants in *NR3C1*, *GST*s and *ABCB1* gene in relation with GC toxicity or disease-free survival in childhood ALL patients, often with conflicting results.[10, 35-38] Although toxicity and survival are the most important therapy outcome signifiers, still, they cannot be associated solely with GC response. On the contrary, we believe that GC response on day 8 assessed by blast count in blood is probably the best measure of GC efficacy in childhood ALL, because no other chemotherapeutic drug is given systemically beforehand. Low blast count (< 100 blasts/microL) or blast negative status could also be important to reveal patients with particularly good response to GC therapy. Those patients might require adjustment of GC dose to achieve remission.

In this study we focused on variants in non-coding region of *NR3C1* gene, rather than on the most extensively studied variants of *NR3C1* gene that were earlier analyzed in regard to GC response on day 8 in childhood ALL. One of those studies found only *BclI* variant to be associated with GC response in relatively small Chinese cohort of ALL patients.[12] However, other studies did not find significant association with GC response on day 8.[10, 11]

Concerning the variant rs6198 in the *N3RC1* gene, we have found an increased risk of PPR (> 1000 blasts/microL) in the initial stage when the carrier has the rare GG genotype. This variant is important for GRβ mRNA stabilization. Moreover, the GG genotype leads to greater expression of the GRβ isoform.[17] And the increased level of GRβ isoform leads to the dominant negative inhibition of the GRα isoform.[39] The GRβ isoform provides enhanced resistance to the biological and pharmacological effects of glucocorticoids.[14, 16, 40, 41] The level of isoform GRβ was shown to influence glucocorticoid response in childhood ALL. Namely, glucocorticoid sensitivity was negatively correlated with GRβ/GRα ratio in leukemic blast cells.[42] Our study is the first to report any result concerning association between rs6198 variant and response to GC treatment on day 8. The association of this variant and glucocorticoid response was shown in patients suffering from other diseases. In the pediatric nephrotic syndrome, it was found that carriers of the GG genotype had a worse treatment outcome,[43] which is in line with our findings. Also, in the major depressive disorder, a haplotype (rs10482605-rs6198) containing the G allele of rs6198 was associated with GRβ mRNA stability. This haplotype contributed to the hyperactivity of the hypothalamus-pituitary-adrenal axis.[44]

Our results have shown that carriers of minor *NR3C1* rs33389 T allele tended towards higher blast count (≥ 100 blasts/microL), while carriers of *NR3C1* rs33388 T allele tended towards lower blast count (< 100 blasts/microL) at day 8 of GC treatment. It has been shown that the variants rs33389 and rs33388, T and A alleles respectively, are located in intron 2 of *NR3C1*, in a region where alternate splicing occurs, resulting in increased expression of isoform GRγ.[14] GRγ has an affinity for the ligand similar to the standard isoform GRα, but it lacks the stability of GRα in binding to the glucocorticoid response element.[45] On the other hand, rs33389 C allele and rs33388 T allele are

parts of ACT (rs41423247-rs33389-rs33388) haplotype which is strongly associated with glucocorticoid sensitivity.[46] Also, in the pediatric nephrotic syndrome, a significant association was shown between this haplotype and a good response to GC treatment.[15] Moreover, CTA (rs33389-rs33388-rs6198) haplotype consisting of alleles found to be favorable for GC response on day 8 in our study, was associated with longer survival time in acute leukemia patients who underwent hematopoietic stem cell transplantation.[47] Interestingly however, in our cohort, CAA haplotype was associated with PGR. This result further points out favorable association of rs33389 C and rs6198 A alleles with lower blast count.

Carriers of *GSTP1* GC (rs1695-rs1138272) haplotype had decreased risk of PPR, were more likely to have low blast count (< 100 blasts/microL) and to be blast negative on day 8 of GC treatment. It was shown, while investigating the activity and the structure of GSTP1, that this haplotype codes the substrate binding region, H-site, of the GSTP1 protein, turning it into a protein with a much smaller Michaelis constant, leading to less efficient conjugation of agents.[20] Consequently, glucocorticoid agents are capable of acting for a longer period of time. Our results have also associated *GSTP1* rs1138272 T allele carriers and *GSTP1* GT haplotype with blast positive status. Two studies that dealt with variants in *GSTP1* gene and GC response on day 8 of ALL treatment, did not find significant association, but they enrolled relatively small number of patients.[21, 48]

Carriers of the *GSTT1* null-genotype were more likely to have higher blast count on day 8 in our childhood ALL cohort. In contrast to our result, one study did not find any association,[21] while the other observed statistical trend towards a PGR in childhood ALL.[48] Also, Meissner and coworkers found that in subgroup of childhood ALL patients who were in higher risk for PPR, *GSTT1* null allele is correlated with decreased risk of PPR.[27] When it comes to risk of relapse and outcome in relation with *GSTT1* null genotype, conflicting results were noted in two studies that enrolled large number of childhood ALL patients.[36, 38]

Regarding *ABCB1* gene, we found that carriers of rare CGT (rs1128503-rs2032582-rs1045642) haplotype are more likely to be blast positive. Higher expression of *ABCB1* was associated with steroid resistance.[25, 49] Mayor alleles were found to lead to higher *ABCB1* expression or higher ABCB1 activity,[50] making them more likely to be associated with poor GC response.[51] Our study is the first to deal with GC response on GC treatment day 8 of childhood ALL patients regarding *ABCB1* haplotypes. In a large cohort of idiopathic thrombocytopenic purpura patients, various haplotype combinations of the same variants we analyzed were associated with GC response.[51] However, no association was found in regard to CGT haplotype.

Despite the promising results, the limitations of the study need to be affirmed. The sample size is not big, since this is a single centric study enrolling patients suffering from a rare disease. Moreover, certain alleles of genetic variants we studied are not frequent, meaning that in some cases there are only a few carriers of certain genotypes. As a consequence, conclusions drawn analyzing such small groups of patients need to be taken with caution. Considering the shortcomings mentioned, it would be of great benefit to validate the results gained in this study on a larger sample preferably using prospective approach.

Association studies on the pharmacogenomic profile of patients and data on the toxicity of drugs are the most promising directions on the road to personalized medicine. The ultimate goal of the ongoing multicentric clinical trials is to optimize the use of known antileukemic drugs in the context of individual pharmacogenomic profile of each patient and molecular markers of the leukemic cells and modulate the treatment resulting in less toxicity and adverse reactions, and a higher survival rates.[52] Personalized medicine approach of tailoring treatment to the individual characteristics of each patient has been a great success in several diseases. One thing that we have learnt from those successful examples is that a personalized childhood ALL approach implementation may be difficult. Our study pointed out the association between several variants in *NR3C1, GSTP1, GSTT1, GSTM1* and *ABCB1* genes and GC therapeutic response in the initial phase of remission induction therapy of pediatric ALL patients. We have shown that *NR3C1* rs6198 variant and *GSTP1* rs1695-rs1138272 haplotype are the most promising pharmacogenetic markers of GC response in ALL patients. However, studies including more childhood ALL patients, as well as more comprehensive analysis of personal "pharmacomics" profiles are needed for discovery of novel potential genetic markers for targeted therapy[53] and for a design of modulations of the existing treatment protocols, leading to more individualized and more successful childhood ALL treatment.

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