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Research paper

Ex vivo resistance in childhood acute lymphoblastic leukemia: Correlations between BCRP, MRP1, MRP4 and MRP5 ABC transporter expression and intracellular methotrexate polyglutamate accumulation

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ABSTRACT

Chemoresistance is an important factor in the treatment failure of childhood acute lymphoblastic leukemia (ALL). One underlying mechanism of chemoresistance involves (over)expression of ATP-dependent drug efflux transporters such as multidrug resistance protein 1–5 (MRP1–5) and breast cancer resistance protein (BCRP), which can extrude the important antileukemia drug methotrexate (MTX). Survival of childhood ALL critically depends on the leukemic blasts' capacity for intracellular retention of MTX and MTX-polyglutamates. This pilot study assessed whether expression of MRP1, MRP4, MRP5 and BCRP (real-time PCR) in primary childhood ALL blasts (n = 23) correlated with ex vivo resistance to MTX (assayed by in situ thymidylate synthase inhibition assay (TSIA)), ex vivo accumulation of (radioactive) MTX polyglutamates, and patient survival. Results show that high MRP4 expression is correlated with ex vivo MTX resistance assayed by TSIA (P = 0.01). Moreover, elevated MRP4 and BCRP expression correlated with lower accumulation of MTX-PGs (P = 0.004 and P = 0.03, respectively). Combined high expression of BCRP and MRP4 even further impacted reduced MTX-PG accumulation (P = 0.02). Overall survival was lower (P logrank = 0.04) in children with ALL cells which featured a relatively high expression of both BCRP and MRP4 transporters. These results underscore the impact of high drug efflux transporter expression, notably MRP4 and BCRP, in diminished MTX response in childhood ALL.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent type of childhood malignancy [1]. Current treatment protocols are successful in achieving 5 years overall survival rates of 83–94% [2]. However, approximately 20% of children with ALL will relapse and face a poor survival (30–50%) [3]. One important factor contributing to relapse is the onset of multi-drug resistance (MDR) [4], mediated by the upregulation of specific drug efflux transporters of the ATP-binding cassette (ABC) transporter family [4–7]. Active extrusion of cytotoxins by ABC transporters results in lower intracellular concentration of chemotherapeutics and confers chemoresistance.

Methotrexate (MTX) is the most widely used anchor drug for central nervous system prophylaxis and in the maintenance therapy for childhood ALL, but emergence of resistance to MTX remains an issue of concern for which several mechanisms have been hold accountable [8,9]. With respect to ABC transporters, multidrug resistance protein 1–5 (MRP1–5, ABCC1–5) and breast cancer resistance protein (BCRP, ABCG2) have been implicated in cellular extrusion of MTX, thus constituting a critical factor in the intracellular accumulation of MTX

Abbreviations: ALL, Acute lymphoblastic leukemia; FPGS, folylpolyglutamate synthetase; MRP 1-5, multidrug resistance protein 1-5; BCRP, breast cancer resistance protein; MTX, methotrexate; MTX-PG, methotrexate polyglutamates; TS, thymidylate synthase; TSIA, thymidylate synthase inhibition assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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[10–14]. Intracellular accumulation of MTX and its active metabolites, i.e. MTX-polyglutamates (MTX-PGs), is crucial for treatment efficacy in leukemia, as it correlates with event-free survival (EFS) [15–19]. Upon intracellular entry of MTX via the reduced folate carrier (RFC), MTX is metabolized into PG forms via the enzyme folylpolyglutamate synthetase (FPGS) which adds 2–6 glutamate residues to the γ-carboxyl group of MTX [20]. These polyanionic active metabolites are prevented from efflux by ABC transporters [10–14] although BCRP has the ability to extrude short chain polyglutamates (up to MTX-PG3) [11,21]. *Ex vivo* assessments of MTX sensitivity in primary leukemia samples by commonly used MTT-based cell growth inhibition assays have been hampered by the release of nucleosides from dying cells which rescue leukemic cells from the effects of MTX [22,23]. To this end, alternative methods based on *in situ* inhibition of TS by MTX-(PGs) have proved useful to distinguish variable MTX sensitivity profiles in primary leukemia samples [23–25]. By examining short-term (3 h) versus long-term (21 h) MTX drug exposure periods, the *in situ* TS inhibition assay (TSIA) provides insights in the kinetics and dynamics of MTX-PG formation reflected by the capacity to inhibit TS [24,26], which can be complemented with actual analysis of MTX-PG levels [16,19,23].

In the present pilot-study, we explored the association between the *ex vivo* MTX drug sensitivity by TSIA, gene expression of ABC transporters *MRP1, 4, 5* and *BCRP*, and intracellular MTX-PG accumulation with survival in a small cohort of newly diagnosed childhood ALL patients.

2. Materials & methods

2.1. Reagents

RNAzol was purchased from Campro Scientific (Veenendaal, The Netherlands); for reverse transcriptase, Moloney murine leukemia virus reverse transcriptase (M-MLV-RT) and RNase inhibitor were obtained from Promega (Madison, WI, USA). Deoxyribonucleotides (dNTPs), random hexamers and Taq polymerase (5 IU/μl) were obtained from Pharmacia Biotech (Roosendaal, The Netherlands). RESponse Research agarose was purchased from Biozym (Landgraaf, The Netherlands). [3,5,7-[^{3}H]-MTX (20 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA).

2.2. Patient samples

Diagnostic samples were collected from children with B-cell precursor ALL (n = 17) and T cell-ALL (n = 6) after obtaining informed consent. Patients were enrolled in ALL-8 and ALL-9 study protocols [16], coordinated by the Dutch Childhood Oncology Group. Induction and consolidation regimens with MTX were comparable among the different treatment groups. A detailed description of MTX drug schedules and doses has been reported elsewhere [27]. A description of the patient demographics is presented in Table 1. Because the various assays required relatively large amounts of cells, sample selection was based on availability of white blood cell (WBC) concentration in primary diagnostic bone marrow (BM) or peripheral blood (PB) from patients.

Table 1
Patient characteristics.

	B-cell precursor ALL	T-cell ALL	Total cases
Sample size	18	5	23
Median Age (years)	6.1 (0-14)	6.4 (1-11)	6.1
Sex (male/female)	11/7	3/2	14/9
Treatment protocol:			
ALL 8, 9	10	4	14
Other	3	1	4
N/A	5		5
Relapse rate	8 (44%)	2(40%)	1 (43%)
Complete remission	10 (56%)	2 (40%)	12 (52%)

B-cell precursor acute lymphoblastic leukemia (ALL) includes common-precursor and pre-B cell ALL lineages.

Mononuclear cells were isolated through Ficoll-Paque density gradient centrifugation, following manufacturer's recommendations, within 24 h after collection essentially as described by Rots et al [23]. Samples with < 80% leukemic cells were enriched for blasts by removing non-leukemic cells using magnetic bead bound monoclonal antibodies (Dynabeads M-450; Dynal Inc, Oslo, Norway) as described elsewhere [28]. Samples were washed twice with RPMI-1640 medium + 2% fetal calf serum (FCS) and resuspended in culture medium consisting of RPMI-1640 (Dutch modification; GIBCO, Uxbridge, UK) supplemented with 20% FCS and antibiotics (all obtained from Flow Laboratories, Irvine, UK). For RNA isolation, 5×10^6 cells were lyzed in 1 ml RNAzol and stored at −80 °C.

2.3. mRNA extraction and real time-PCR

For RNA extraction procedure and Real Time-PCR methods, please refer to the supplementary material section.

2.4. Ex vivo drug sensitivity

Sensitivity of primary leukemia cells was assessed *ex vivo* by the *in situ* TSIA which measures the amount of tritiated water (^3H_2O) released from the conversion of [5-^{3}H]-2′-deoxycytidine to [5-^{3}H]-2′-deoxyuridine monophosphate (^{3}H-dUMP) to deoxythymidine monophosphate (dTMP) in the reaction catalyzed by TS. MTX, and in particular its polyglutamate forms, can effectively inhibit dTMP synthesis and thus block the release of tritiated water from [5-^{3}H]-2′-deoxycytidine. The TSIA has been used previously as a validated tool for *ex vivo* assessment of (inhibition of) intracellular TS activity in primary cells and cell lines [24–26]. Moreover, TSIA enabled generation of dose-response curves and detected differential MTX sensitivity patterns in T-cell versus common/pre-B cell ALL patient-derived cells, in accordance to clinical observation in patients [23,29]. To examine the impact of MTX-polyglutamylation, leukemic blast cells were exposed to MTX short-term, i.e. 3 h followed by an 18 h drug-free period, or continuously for 21 h, after which drug sensitivity was evaluated by calculation of the concentration of the drug needed to inhibit 50% of the TS activity, depicted as $TSI_{50,short}$ and $TSI_{50,cont}$, respectively [23].

2.5. MTX accumulation and polyglutamylation analysis

For this analysis 10^7 cells in 10 ml RPMI-1640 medium supplemented with 10% FCS were incubated for 24 h at 37 °C with 1 μM [^{3}H]-MTX (2 Ci/mmol). After this period, cells were washed three times with 10 ml of ice-cold phosphate-buffered saline (PBS) and after cell counting and determining total accumulated [^{3}H]-MTX, analysis of intracellular distribution of [^{3}H]-MTX-PGs was performed by high-performance liquid chromatography (HPLC) coupled to a radioactivity detector, as described elsewhere [16,23].

2.6. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

For assessment of drug sensitivity of drugs other than MTX, the MTT colorimetric assay was performed on cells suspended in 96-well round bottom plates at a concentration of 2×10^6 cells/ml in RPMI-1640 cell culture medium containing 20% FCS, 200 μg/ml of gentamycin, 2 mM glutamine, and 0.5 ml ITS (insulin: 5 μg/ml, transferrin: 5 μg/ml, sodium selenite: 5 ng/ml) [22]. Treatment wells contained 80 μl of cell suspension and 20 μl of the drugs of interest (concentration ranges are indicated in supplementary Table A.1). Control wells contained 20 μl of RPMI-1640 medium and 80 μl of cell suspension, while blank controls

had 100 µl of RPMI-1640 medium. The MTT assay was performed in duplicates and plates were incubated at 37 °C, in a humidified 5% CO_2 incubator for 4 days. At the end of the incubation period, 10 µl of MTT dye (5 mg/ml) was added to each well and placed in the 37 °C incubator for an additional 6 h, followed by the addition of 100 µl isopropanol. After 5 min, the microplate optical density (OD) was measured at 562 nm. For the analysis, the average OD of the blank wells is subtracted from the average OD of the control wells or the treatment wells. The lethal concentration 50 (LC_{50}) was then determined by calculating cell survival with the following equation [22]: mean OD treatment well (minus blank)/mean OD control well (minus blank) x100.

2.7. Statistical analysis

The Mann-Whitney *U* test was used to analyze differences in the subtypes of childhood ALL (common/B-cell ALL vs. T-ALL, diagnosis vs. remission). The Spearman rank correlation test was used to determine associations between mRNA expression (of MPR1, MPR4, MRP5 and BCRP) with *ex vivo* sensitivity, and to detect correlations between the mRNA expression-drug sensitivity ratios. Significance level was set at two-sided P value of < 0.05. For survival analysis, patients were classified as therapy responders when complete remission (CR) was achieved. The time between the initial treatment date until the end point (death or censoring) was considered as the overall survival (OS). Kaplan-Meier analysis was used to plot OS, and the log-rank test to compare differences between survival curves. Statistical calculation was performed with SPSS software (SPSS, Inc., IL, USA) and GraphPad Prism version 7.0 for Windows (GraphPad Software, CA, USA).

3. Results

3.1. Patient samples

Patients were enrolled in several treatment protocols which are listed in Table 1 along with the outcome data, remission rate and relapse rate. Due to logistic reasons and leukemic blast number limitations, not all of the MTX-related parameters could be measured and therefore the number of cases included in the statistical analysis varied between parameters (as listed in the various tables).

3.2. ABC transporter expression in childhood leukemia

Notwithstanding inter-patient variabilities, mean ABC transporter (*MRP1, MRP4, MRP5* and *BCRP*) expression values did not vary significantly amongst childhood precursor-B cell (n = 18) and T-cell (n = 5) ALL samples. These expressions were also not different between relapsing (n = 10) and non-relapsing patients (n = 11), regardless of ALL subtypes (Fig. 1A and 1B). Due to the limited cytogenetic/molecular data available, further risk stratification could not be performed in relation to ABC transporter expression. Spearman rank analysis was performed to detect a pattern of correlated expression amongst ABC transporters in all leukemia samples (n = 23). Significant correlations were found between *BCRP* and MRP1 expression (R = 0.44; $P = 0.035$), *BCRP/MRP4* (R = 0.53; $P = 0.009$), *BCRP/MRP5* (R = 0.42; $P = 0.047$) and *MRP5/MRP1* (R = 0.51 $P = 0.013$). Based on these observations, we analyzed the intracellular MTX-PG accumulation, *ex vivo* MTX resistance, and survival of patients with a combination of high or low expression of drug efflux transporters.

3.3. Association of intracellular accumulation of MTX polyglutamates and ABC transporter expression ex vivo

To examine the impact of ABC transporter expression on *ex vivo* MTX-PG accumulationin in the cohort of ALL patients (n = 20), we quantified the intracellular accumulation of total MTX-PGs (1–6 glutamate residues) and long-chain MTX-PGs (4–6 glutamate residues) after 24-hour exposure to 1 µM [^{3}H]-MTX (Fig. 2 and supplementary Table A2 and A3). High *BCRP* and *MRP4* expression correlated with significantly lower accumulation of total MTX-PGs (MTX-Glu1-6) ($P = 0.027$ and 0.004, respectively, Supplementary Table A.2). Significantly lower accumulation of long chain MTX-PGs (MTX-Glu4-6) correlated with higher *MRP4* expression ($P = 0.003$) and trended with higher *BCRP* expression ($P = 0.064$, supplementary Table A.3). Spearman rank correlation analysis revealed that *BCRP* and *MRP4* mRNA expression significantly inversely correlated with total MTX-PGs ($P = 0.04$ and 0.02, respectively) (Table 2). In addition, high *MRP4* expression was significantly associated with lower intracellular concentration of long-chain MTX-PGs ($P = 0.01$, Table 2). From all combined correlation analysis, the most significant effect in total and long-chain MTX-PG accumulation was seen with the *MRP4* high */BCRP* high group ($P = 0.005$). These results concord with previous published results that confirm correlations between transporter mRNA and its correspondent protein levels [30,31].

3.4. ABC transporter expression and cellular MTX sensitivity ex vivo

The TSIA has previously served as a suitable method to generate MTX-dose response curves in intact primary leukemia cells. TSIA data available from a former study were used here to correlate MTX sensitivity to ABC transporter expression in childhood ALL specimens [23]. The short-term, 3-hour MTX exposure ($TSI_{50,short}$) and the continuous 21-hour exposure ($TSI_{50,cont}$) are representative of the leukemic cells' capacity to efflux and to accumulate MTX, respectively. These parameters can be used as indirect indicators of the polyglutamylation capacity of leukemic cells and reliably reflect MTX sensitivity *ex vivo,* as shown previously [23].

Mann-Whitney U analysis between high and low expression of drug efflux transporters *MRP1, MRP4, MRP5* and *BCRP* showed that high *MRP4* expression is significantly related to diminished MTX sensitivity in both $TSI_{50,cont}$ ($P = 0.01$) and $TSI_{50,short}$ ($P = 0.05$). Notably, an elevated BCRP expression was not significantly related to MTX efflux ($P = 0.08$) but was related to MTX accumulation, as shown by the $TSI_{50,cont}$ exposure ($P = 0.01$, Fig. 3A and 3B).

Next, the impact on MTX resistance was evaluated in relation to all possible paired combinations of ABC transporter expression values. In these analyses, higher LC_{50} values (i.e. increased resistance) were observed in $TSI_{50,cont}$ ($P = 0.02$) and $TSI_{50,short}$ ($P = 0.03$) in leukemia samples with *BCRP*-high/*MRP4*-high expression when compared with *BCRP*-low/*MRP4*-low cells (Fig. 3C and D).

3.5. Relation between ABC-transporter expression and the sensitivity to other chemotherapeutics

The sensitivity of cells for anti-leukemic drugs other than MTX was measured using MTT analysis. Remarkably, the strong relation between *MRP4* and *BCRP* expression and sensitivity to MTX was not as pronounced for the other anti-leukemic drugs (Table 3). Increased *MRP1* expression showed a significant relationship to resistance to numerous drugs, such as mitoxantrone ($P = 0.026$), doxorubicin ($P = 0.002$), vincristine ($P = < 0.001$), daunorubicin ($P = < 0.001$), teniposide ($P = 0.001$), thiotepa ($P = 0.033$) and cladribine ($P = 0.049$). MRP4 expression only showed a significant relationship to cladribine resistance ($P = 0.049$), while *MRP5* expression was related to thiotepa resistance ($P = 0.033$) and showed a tendency to teniposide resistance ($P = 0.052$). *BCRP* expression was not related to drug sensitivity in the MTT assay (Table 3).

3.6. ABC transporter expression and survival

Despite the small sample number, the influence of ABC transporter expression on survival was examined by Kaplan Meier analysis. A relatively poor outcome was observed for patients with elevated *BCRP*

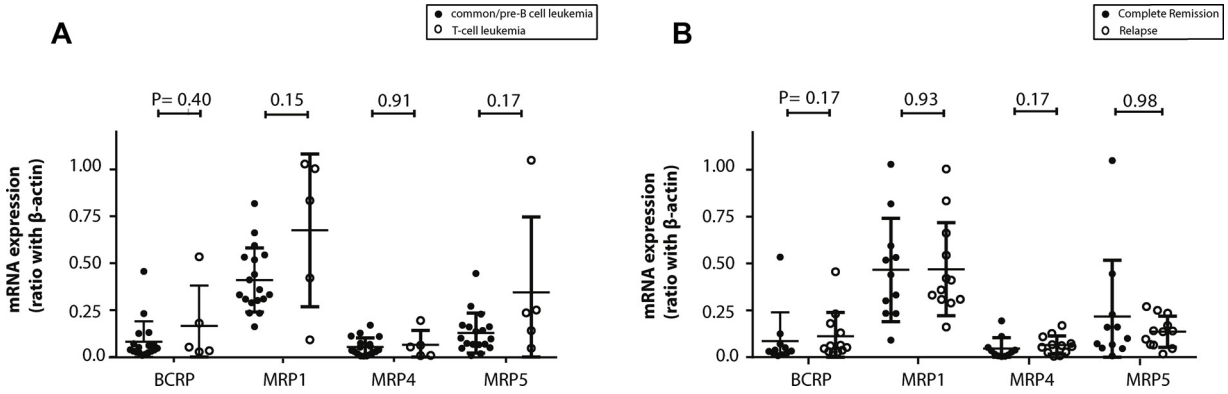

Fig. 1. (A): mRNA expression levels (relative to β-actin) of *BCRP, MRP1, MRP4* and *MRP5* in common/pre-B cell (closed symbols) and T-cell childhood leukemia cells (open symbols). (B): mRNA expression levels (relative to β-actin) of *BCRP, MRP1, MRP4* and *MRP5* in patients who underwent relapse (open symbols) compared to patients who remained in complete remission (CR) during treatment (closed symbols). Each symbol represents an individual patient. Data is depicted as mean ± standard deviation. The median mRNA expression level for ABC transporters relative to the mRNA expression of β-actin was used as cut-off point [*BCRP* (0.04), *MRP1* (0.38), *MRP4* (0.04) and *MRP5* (0.14)] to differentiate between high and low expression of ABC transporters. Mann Whitney U test was performed to analyze differences between means.

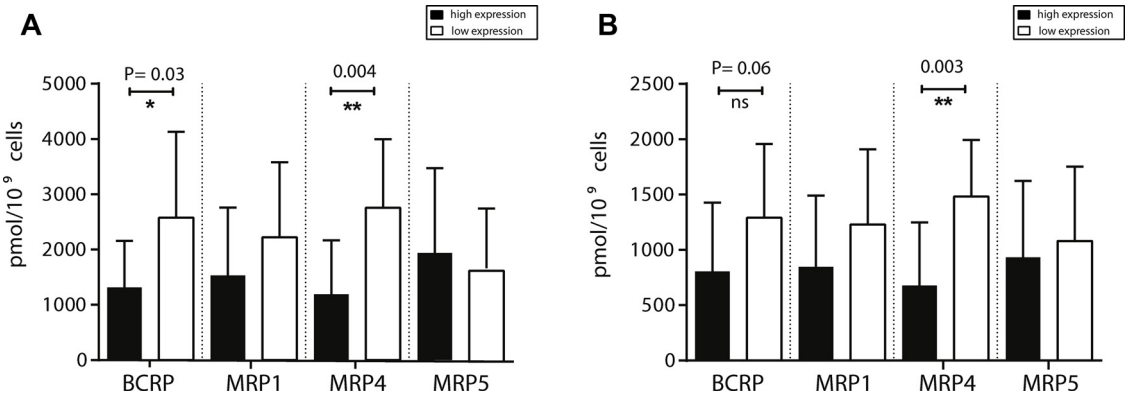

Fig. 2. (A): Total MTX-PG accumulation and (B) long-chain MTX-PG accumulation in childhood ALL samples with high (black bars) or low (white bars) expression of *BCRP, MRP1, MRP4* and *MRP5* ABC transporters. Data are depicted as mean ± standard deviation (SD) and expressed as pmol/10^9 cells. Mann Whitney U test was used to analyze differences between means.

Table 2
Correlation between MTX-PG accumulation and BCRP and MRP4 expression.

(n = 20)	Total MTX -PG	LC MTX-PG
BCRP	−0.5; $P = 0.04^*$	−0.4; $P = 0.1$
MRP4	−0.5; $P = 0.02^*$	−0.6; $P = 0.01^*$

P- value (*P*), total (total MTX-PG) and long-chain methotrexate polyglutamates (LC MTX-PG).
* = statistically significant (Spearman's correlation coefficient).

expression compared to low expression, which did not reach statistical significance ($P_{logrank} = 0.30$; Fig. 4A). Notably, the combined expression of *BCRP* high/*MRP4* high had a negative impact on OS ($P_{logrank} = 0.04$; Fig. 4B) compared with the rest of *BCRP/MRP4* combinations together. Due to the relatively small number of samples and the fact that treatment regimens were comparable, further stratified survival analysis was not performed.

4. Discussion

This study shows that ABC drug efflux transporters MRP4/BCRP contribute to MTX accumulation and polyglutamylation, and the clinical outcome data suggest an association with response to treatment including MTX in pediatric ALL patients.

Intracellular accumulation of MTX and its conversion to MTX-PGs is well recognized as a predictive factor for response and favorable outcome in childhood leukemia [15–19]. Consequently, deficient MTX-polyglutamylation due to loss of FPGS activity has been associated with MTX resistance [16,20,32–34] as non-polyglutamated MTX is prone to be extruded from cells by several family members of the ABC, including MRP1–5 [12–14] and BCRP [10,11,21], rather than by P-gp which effluxes primarily non-hydrophilic substrates [10,14]. To fully unravel the role of MTX polyglutamylation in *ex vivo* assessments of MTX sensitivity in leukemic specimens, short incubation periods (e.g. 3 h) are more informative than extended (72–96 h) incubation periods [23–25,29]. Moreover, *ex vivo* testing of leukemia specimens for MTX sensitivity is hampered by the release of nucleosides and bases from dying cells which rescue the cytotoxic effect of MTX [23]. In the present study, these considerations and limitations were met by applying the *in situ* TSIA for MTX in a time and MTX-PG dependent manner. Additional analysis of RNA expression levels of *MRP1, MRP4, MRP5* and *BCRP* mRNA levels, and analysis of (long-chain) MTX-PG accumulation, and outcome allowed assessments of correlating these parameters with *ex vivo* MTX sensitivity.

A considerable inter-patient variability of *MRP1, MRP4, MRP5* and *BCRP* mRNA expression levels was observed in childhood ALL samples (Fig. 1A), being consistent with previously reported data [31,35–39]. In normal peripheral blood lymphocytes, MRP4 and BCRP expression is rather low as compared to other drug efflux transporters [40,41]. Elevated expression levels of *MRPs* and *BCRP* in leukemia cells have been reported of clinical relevance and associated with worsened prognosis and relapse of treatment [38,39,42–44]. However, various studies

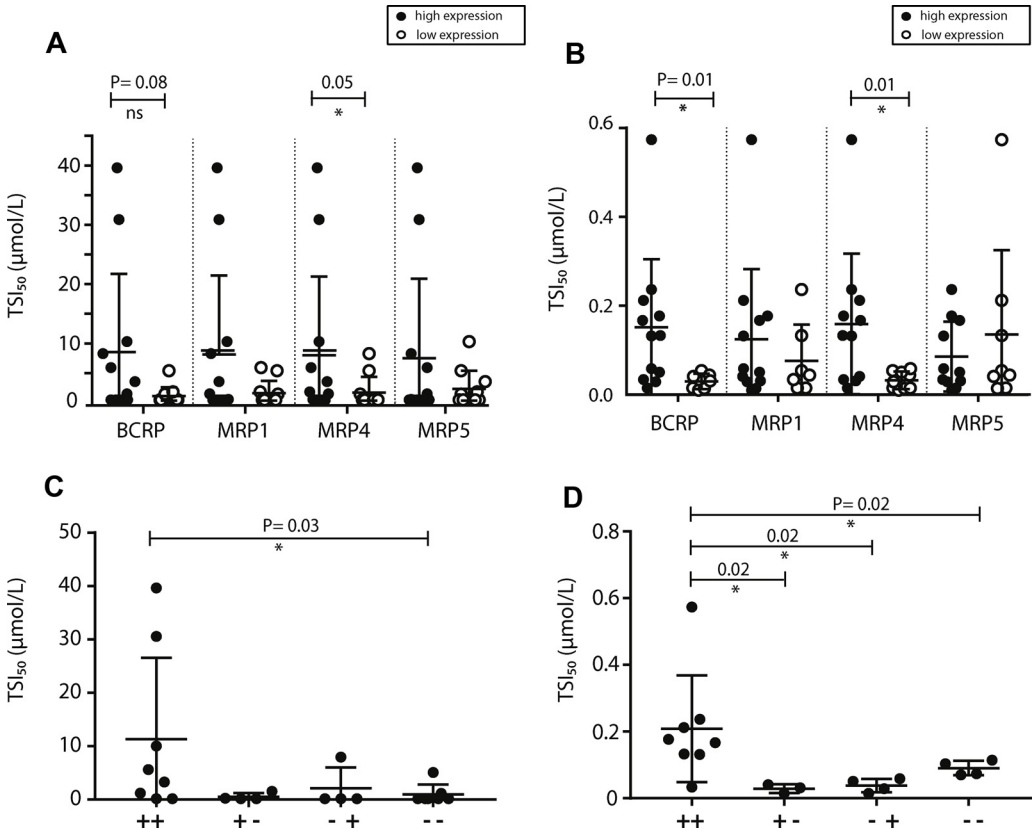

Fig. 3. MTX sensitivity and ABC transporter expression in childhood ALL samples as represented by the (A) short term (3 h) MTX exposure and (B) continuous (21 h) MTX exposure in the TSIA assay. ABC transporters expression is divided in patient groups with either high (black bars) or low (white bars) expression. TSIA results are represented as TSI_{50} concentration, the concentration of MTX needed to inhibit 50% of thymidylate synthase (TS) activity. (C) TSI_{50} values following the short-term (3-hr) exposure to MTX and (D) continuous (21 h) MTX exposure for patients with *MRP4/BCRP* double high (+ +), high/low (+ -) low/high (- +) and low/low (- -) expression. Data are depicted as mean ± SD and expressed as µmol/L. Mann Whitney U test was performed to analyze differences between means.

employing 3–4 days MTT-based drug testing did not reveal significant correlations with *MRPs* and *BCRP* expression levels in childhood ALL cells and *in vitro* drug resistance to drugs being bona fide substrates for MRPs and BCRP [37,42,45,46]. Of note, these drug testing studies did not include MTX. In the current study we show that *MRP1* expression in childhood ALL cells was associated with in MTT-*in vitro* drug resistance to known MRP1 substrates doxorubicin, daunorubicin, vincristine and mitoxantrone, but not for drugs being substrates for BCRP [7] and MRP4/5 (e.g. thiopurines and cytarabine) [13,47]. The latter may be explained by the fact that in long term drug testing conditions these prodrugs are converted to their phosphorylated metabolites which are not effluxed by MRP4/5. Previous studies reported monitoring of intracellular accumulation of daunorubicin [48], ara-CTP [47] and thioguanine nucleotides [49], but sample size limitations in the present study did not allow us to assess intracellular accumulations of drugs other than MTX.

Many in vitro cell lines studies underscored that overexpression of *MRP1–5* and *BCRP* confers MTX resistance after short term drug exposure during which time MTX has not been sufficiently converted to long chain polyglutamates which lack substrate affinity for MRPs and BCRP [11,21]. Additionally, decreased RFC mediated MTX uptake or diminished FPGS activity can impact to reduced polyglutamylation,

Table 3
Correlates of antileukemic drug sensitivity [a] and ABC transporter expression[b].

DRUG		MRP1		MRP4		MRP5		BCRP	
	n	rs	*P*-value	rs	*P*-value	rs	*P*-value	rs	*P*-value
Asparginase	17	−0.24	0.347	−0.19	0.467	−0.22	0.399	−0.49	0.356
Carboplatin	4	−0.40	0.750	−0.40	0.750	−0.80	0.333	−0.80	0.333
Cisplatin	5	−0.70	0.233	−0.60	0.350	−0.70	0.233	−0.30	0.683
Cladribine	10	−0.65	0.049*	−0.67	0.039*	−0.61	0.067	−0.07	0.865
Cytarabine	16	−0.33	0.213	−0.34	0.200	−0.10	0.704	−0.11	0.680
Daunorubicin	16	−0.78	0.00*	−0.33	0.189	−0.24	0.362	−0.28	0.284
Dexamethasone	16	−0.43	0.100	−0.19	0.471	−0.19	0.489	−0.08	0.768
Doxorubicin	14	−0.73	0.002*	−0.28	0.334	−0.17	0.553	−0.12	0.692
Etoposide	12	−0.52	0.080	−0.22	0.485	−0.08	0.795	−0.22	0.484
Ifosfamide	16	−0.02	0.940	0.08	0.770	−0.07	0.787	−0.09	0.754
Mitoxantrone	11	−0.66	0.026*	−0.18	0.593	−0.15	0.670	−0.03	0.937
Prednisolone	15	−0.39	0.151	0.24	0.398	−0.11	0.685	−0.17	0.541
Teniposide	15	−0.78	0.001*	−0.47	0.076	−0.51	0.052	−0.16	0.576
Thiotepa	6	−0.89	0.033*	−0.77	0.103	−0.89	0.033*	−0.49	0.356
Vincristine	17	−0.78	0.00*	−0.33	0.189	−0.24	0.362	−0.28	0.284
6-Mercaptopurine	16	−0.30	0.264	0.30	0.261	−0.12	0.652	−0.22	0.415
6-Thioguanine	14	−0.18	0.537	0.25	0.396	0.03	0.917	−0.04	0.905

a = as determined by MTT assay b = as determined by real-time PCR.
* = statistically significant (Spearman's correlation coefficient).

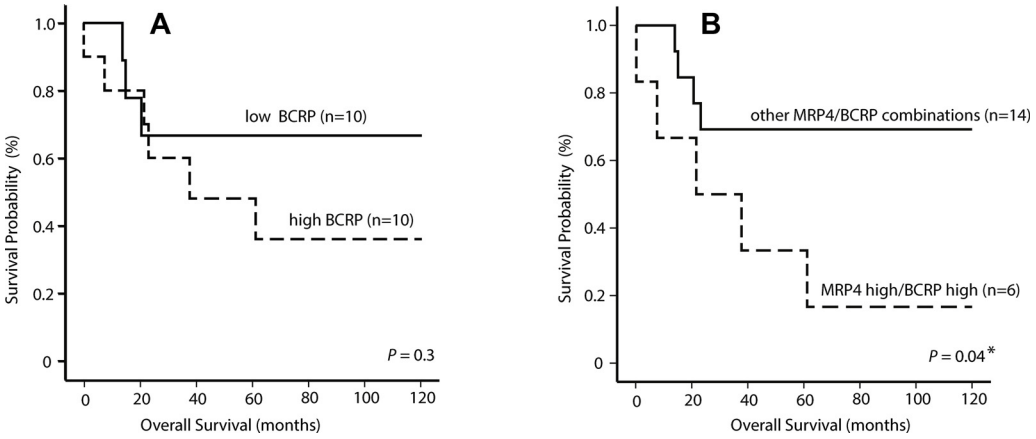

Fig. 4. (A): Kaplan-Meier analysis of BCRP high (dashed line) and BCRP low (continuous line) expression and overall survival (OS) in childhood ALL. **(B):** Kaplan-Meier analysis of the combined expression of *MRP4/BCRP* and OS in childhood ALL. *MRP4/BCRP* survival curves are labeled with the mean OS estimate (in months), the percentage of censored patients and the number of patients in each subgroup. The continuous line in Fig. 4B represents the Kaplan-Meier OS curve of patients with low/high, high/low, low/low combinations of *MRP4* and *BCRP* expression, the dashed line represents the *MRP4* high/*BCRP* high group of patients. Statistical evaluation is expressed as P-values analyzed by log-rank test.

facilitating extrusion of non-polyglutamated MTX by MRPs and even short chain MTX-PGs by BCRP [10]. The current study is the first to corroborate that lowered ex vivo accumulation of MTX-PGs in childhood ALL cells is significantly correlated with increased expression of *MRP4 and BCRP*, and *in vitro* MTX drug resistance determined by TSIA. Moreover, combined $MRP4^{high}/BCRP^{high}$ expression in childhood ALL samples proved to be a good indicator of worsened clinical outcome as compared to patients with $MRP4^{low}/BCRP^{low}$ expression. Earlier studies by Bartholomae et al. [50] also showed that in childhood AML dual high expression of *BCRP* and *MRP3* was strongly associated with therapy response, underscoring the impact of coexpression of multiple MDR transporters in drug resistance. Although this study investigated multiple parameters contributing to MTX resistance, one limitation is its small sample size. Confirmatory studies with greater number of patients are warranted and should include transporter protein detection (e.g. with flow cytometry), follow-up at different time points and cytogenetic/molecular phenotypes. Suggested additional analysis may include polymorphic variants of BCRP and MRP4, which impact their functional activity and were implicated in unfavorable clinical outcome [51–55]. Interestingly for MRP4, a specific polymorphic variant was associated with aberrant splicing of *MRP4* pre-mRNA [54] and thus expression. Consistently, recent studies by Wojtuszkiewicz et al [19,53] identified aberrant pre-mRNA splicing of FPGS in childhood ALL as a molecular basis of reduced FPGS activity and consequently diminished MTX-polyglutamylation. Altogether, a combined approach of selected ABC transporter gene expression analysis, assessment of aberrant FPGS pre-mRNA splicing, MTX-PG analysis and TSIA-based MTX drug sensitivity monitoring could guide a more personalized medicine of MTX in childhood ALL patients.

5. Conclusions

The thymidylate synthase inhibition assay (TSIA) proved a valuable tool to screen for MTX drug resistance in primary childhood ALL cells. ABC transporters BCRP and MRP4 were identified as determinants associated with reduced MTX accumulation and polyglutamylation, potentially impacting outcome of childhood ALL patients.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.02.008.

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