The association of aberrant folylpolyglutamate synthetase splicing with *ex vivo* methotrexate resistance and clinical outcome in childhood acute lymphoblastic leukemia

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Supplemental materials and methods

ALL patient specimens

This study included 91 newly diagnosed, untreated paediatric ALL patients treated either with the Dutch Childhood Oncology Group protocols (DCOG) ALL6 – ALL9[1,2] or the German Co-operative ALL (COALL) protocols 92-97.[3,4] The treatment details have previously been described elsewhere.[1–4] The studies have been approved by the local medical ethical committees. All patients (or parents/legal guardians of patients) have provided a written informed consent.

Leukemic cell specimen preparation (including density gradient centrifugation), assessment of blast percentages, immunophenotyping and DNA index flow cytometry were performed as described previously.[5] Cryopreserved mononuclear (bone marrow or peripheral blood) cells of childhood ALL patients, including precursor B-cell and T-cell ALL at diagnosis were used in this analysis.

Isolation of RNA

After thawing cryopreserved ALL patient samples, total RNA was extracted using the AllPrep DNA/RNA/Protein mini kit (Qiagen, Venlo, The Netherlands). Total RNA was subsequently treated with DNaseI (Roche, Basel, Switzerland), followed by reverse transcription of 1 µg of the isolated RNA to cDNA using Moloney Murine Leukemia Virus (M-MLV; Invitrogen) in a reaction buffer containing random hexamer primers (Roche), dNTPs (Roche), and a ribonuclease inhibitor Rnasin (Promega, Madison, WI, USA).

FPGS splice variants

ALL patient samples was screened for FPGS splicing alterations using the previously described[5] comprehensive polymerase chain reaction (PCR)-based assay, with 9 primer pairs (Supplemental Table 4). Since PCR was combined with fragment analysis, the reverse primers were fluorescently labelled at their 5' end with 6-carboxyfluorescein. PCR was carried out using 2x Reddy Mix PCR master mix (Thermo Fisher Scientific Inc., Waltham, MA, USA). The obtained PCR products were used in fragment analysis: for semi-quantification of FPGS splice variants, each PCR product was diluted in HiDi^{TM} Formamide (Applied Biosystems Inc., Foster City, CA, USA) and subsequently resolved in capillary electrophoresis using the Genetic Analyzer 3500 system (Applied Biosystems Inc.). Data were analyzed using GeneMapper® Sofware v4.1 (Applied Biosystems Inc). The ratios (percentages) of expression of particular FPGS splice variants over the wild type FPGS fragment were calculated based on the height of peaks corresponding to each PCR product.

Ex vivo MTX sensitivity using in situ Thymidylate Synthase inhibition assay

Inhibition of TS was determined in freshly isolated whole cells using the *in situ* Thymidylate Synthase (TS) inhibition assay (TSIA), which assesses the TS-catalysed conversion of [^{3}H]-dUMP to dTMP with the release of ^3H_2O, as described previously.[6–8] A short 3 h exposure to MTX was applied, followed by an 18 h drug free period, completed by assessing the aqueous phase for ^3H_2O-radioactivity. Data are expressed as the concentration of MTX necessary to inhibit 50% of TS activity (TSI_{50}) compared to the controls (in triplicate) incubated without MTX.

MTX polyglutamylation

To assess the cellular levels of MTX polyglutamates, freshly isolated leukemic cells were incubated for 24 h with 1 µM [3', 5', 7-^{3}H]-MTX (Moravek Biochemicals; final specific activity 2 Ci/mmol). Subsequently, radioactivity, cell number and viability levels were assessed together with separation of the polyglutamates by high performance liquid chromatography using an anion exchange column as described previously.[5,9] The data are expressed as pmol MTX-Glu_n/10^9 cells.

FPGS activity assay

The FPGS activity assay was carried out in crude cell extracts upon a 2 h incubation with [^{3}H]-glutamic acid and MTX. The resulting MTX-[^{3}H]-Glu_2 formed was separated from unreacted [^{3}H]-glutamic acid by reverse phase column chromatography and quantified as indicated previously.[9]

MTT assay

Cytotoxicity of selected chemotherapeutics, including dexamethasone (Brocacef, Maarssen, Netherlands), prednisone (Brocacef), doxorubicin (Farmitalia, Catania, Italy), cytarabine (Mack), teniposide (Bristol-Myers, Wallingford, USA), 6-mercaptopurine (Sigma-Aldrich, St. Louis, USA) and mitoxantrone (Lederle, Wayne, USA), in ALL patient samples was determined after a continuous 96 h incubation using the colorimetric MTT dye reduction assay as described previously.[8,10] Results are expressed as LC_{50} (lethal concentration 50) – the drug concentration that kills 50% of the cells as compared to viable untreated control cells.

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics 20 software. Associations between FPGS splicing alterations and variables related to drug resistance (including MTX) were analysed using Spearman's Rho test. The Mann-Whitney U test was used to compare the variables related to MTX resistance as well as resistance to other chemotherapeutics, between ALL patients displaying high and low levels of particular FPGS splicing alterations. The cut-off values used to dichotomize variables were based on the frequency distribution among patients. In case of a bimodal distribution, the border was based on separating these two populations. For all other parameters we used median values as cut-off levels, while cut-offs for clinical factors were selected based on historical values. Kaplan-Meier analysis and Cox regression were used in the univariate analysis of EFS (defined as time from complete remission to an event) and OS in relation to the cellular levels of FPGS splice variants. Events included in EFS were relapse and death. MTX related variables, which showed significant impact on survival in the univariate analysis, were tested using the multivariate Cox regression model in the context of clinical factors with a known prognostic significance.

Reference

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Supplemental Table S1. Characteristics of the ALL patient cohort used in this study including reference to the patient characteristics of the protocols our patients were selected from.

Feature	Current cohort (N=91)	DCOG-ALL8 (N=467)	DCOG-ALL9 (N=859)
WBC (cells x 10^9/L)			
< 50	53 (58.2%)	385 (82.8%)	664 (77.6%)
≥ 50	38 (41.8%)	80 (17.2%)	192 (22.4%)
Age at diagnosis (years)			
< 1	6 (6.6%)	13 (2.8%)	-*
≥ 1	83 (91.2%)	454 (97.2%)	859 (100%)
Lineage			
precursor B-cell	72 (79.1%)	396 (87.6%)	701 (88.6%)
T-cell	19 (20.9%)	56 (12.4%)	90 (11.4%)
Sex			
female	39 (42.9%)	212 (45.4%)	331 (38.5%)
male	52 (57.1%)	255 (54.6%)	528 (61.5%)
DNA index			
≤1.16	59 (64.8%)	286 (79.0%)	642 (79.4%)
>1.16	13 (14.3%)	76 (21.0%)	167 (20.6%)
Survival			
Overall survival at 120 months	73.5%	79.6%	-**
Event free survival at 120 months	70.4%	70.0%	-**

*- only children below 1 year of age were included in this study; **- currently unknown

Supplemental Table S2. Correlations between MTX resistance-related variables and intron 8 PR.

	$TSIA_{long}$	**$TSIA_{short}$**	**MTX-Glu1-6**	**MTX-Glu4-6**	**$FPGS_{act}$**
FPGS Intron 8 PR					
Correlation coefficient	0.037	0.219	-0.058	-0.125	-0.192
P-value	0.781	0.099	0.66	0.394	0.328
Number of patients	60	58	60	49	28

$TSIA_{long}$ – TSIA assay utilizing a continuous 18 h MTX exposure; $TSIA_{short}$ – TSIA assay utilizing a short 3 h MTX exposure followed by a 15 h drug-free period, both expressed as the concentration of MTX (in µM) necessary to inhibit 50% of the TS activity (TSI_{50}) compared to the controls incubated without MTX (in triplicate); MTX-Glu1-6 – concentration of total MTX polyglutamates; MTX-Glu1-6 – concentration of long-chain MTX polyglutamates (pmol MTX-Glu_n/10^9 cells); $FPGS_{act}$ – FPGS activity assay (pmol MTX-Glu_2 formed/h/10^6 cells); intron 8 PR is expressed as a ratio relative to the WT variant (FPGS alternative variant/FPGS WT variant*100%); P-value was determined by Spearman's Rho test;

Supplemental Table S3. Difference in drug resistance between precursor B-cell ALL patients displaying high and low levels of FPGS intron 8 PR.

	Intron 8 PR high		Intron 8 PR low		p-value
	Median (range)	**N**	**Median (range)**	**N**	
mitoxantrone	0.05 (0.01-0.11)	4	0.01 (0.01-0.01)	3	**0.034**
prednisone	24.4 (0.01-260)	9	0.32 (0.01-260)	16	**0.039**
dexamethasone	6.1 (0.21-6.1)	6	0.05 (0.01-0.7)	7	**0.006**
teniposide	0.3 (0.1-1.1)	6	0.1 (0.03-2.3)	9	0.099
6-mercaptopurine	424 (61-510)	6	91 (14-510)	9	0.193
doxorubicin	0.3 (0.06-0.5)	5	0.1 (0.1-0.4)	6	0.273
cytarabine	0.7 (0.1-11)	8	0.5 (0.03-1.9)	15	0.366

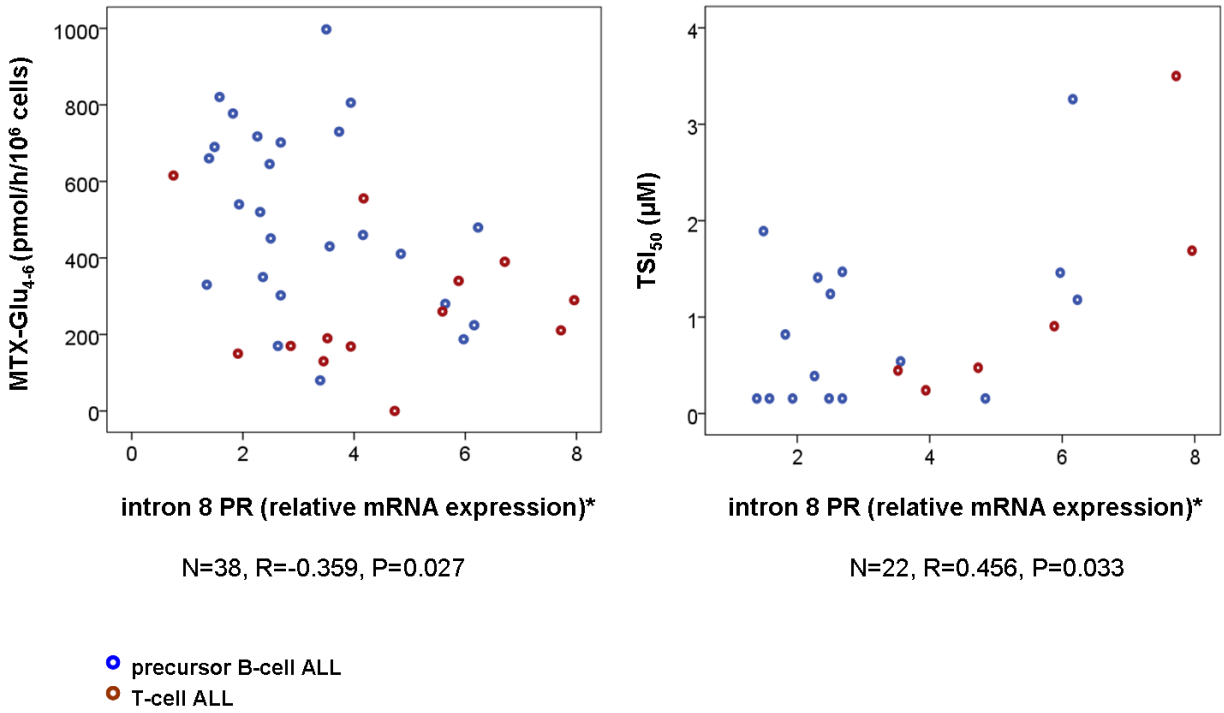
Drug resistance is expressed as LC_{50} values (μg/ml) determined by the MTT assay; intron 8 PR is expressed as relative mRNA level of the splice variant to the wild type FPGS (cut-off 5); p-value was determined by the Mann-Whitney U test; N - the number of patients evaluated per particular test.

Supplemental Table S4. Primers used in the RT-PCR based screen for FPGS mRNA splicing alterations (previously published [9]).

Primer set	Primer name	Primer sequence	Binding site cDNA
1	EX1-up	5'-CGCGGCATAACGACCCAG-3'	108-125
	EX3-dw*	5'-TTCCCCTTCGTCCCAGTGAC-3'	351-370
2	EX3-up	5'-CCGGCTGAACATCATCCA-3'	332-349
	EX10-dw*	5'-AGCATCGGACACAGGTATAGA-3'	878-898
3	EX3_F	5'-AGGACTTGGACCGGCTGAACA-3'	322-342
	EX6_R*	5'-GCCATGAGTGTCAGGAAGCGGA-3'	583-604
4	EX4_F	5'-GCTCCACCTGTGCCTTCACG-3'	373-392
	EX7_R*	5'-CCGCCAATGCCCACCTCCAC-3'	645-664
5	EX5_F	5'-CGCCTCTACCACCGGCTGGA-3'	522-541
	EX9_R*	5'-GCTCGGTCCCTCAGCACTGC'-3'	840-859
6	EX6_2F	5'-CCGCTTCCTGACACTCATGGC-3'	584-604
	EX9_2R*	5'-CTGCTGGGCTCGGTCCCTCA-3'	847-866
7	EX8-up	5'-TCTCCTCTCTTGGCATCGA-3'	712-730
	EX13-dw*	5'-CGGTCCCCGGTAGCATTGA-3'	1288-1306
8	EX9_2F	5'-GTGTCCCTGCCTTCACTGT-3'	799-817
	EX13_3R*	5'-AAGAGCAAGACTCGAACCTC-3'	1269-1288
9	EX11-up	5'-CAAAGGCATCCAGGCCAGG-3'	1033-1051
	EX15-dw*	5'-TGCTCTTCGTCCAGGTGGTT-3'	1473-1492

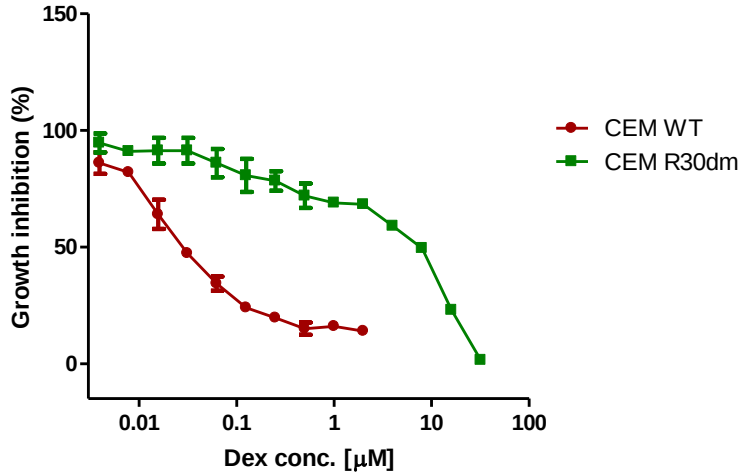
*- primers labelled with 6-FAM used in fragment analysis

Supplemental Figure S1



Supplemental Figure S1. Associations between intron 8 PR levels and MTX resistance in ALL patients displaying low levels of long-chain MTX polyglutamates.
The figure depicts the associations between the levels of intron 8 PR and the accumulation of long-chain MTX polyglutamates as well as the results of short-term TSIA in ALL patients accumulating low levels of long-chain MTX polyglutamates. The correlations were assessed with Spearman's Rho test. Precursor B-cell (blue circles) and T-cell (brown circles) ALL patients are indicated. Most correlations became insignificant when assessed in precursor B-cell (accumulation of long-chain MTX polyglutamates: N=25, R=-0.337, P=0.099; short-term TSIA: N=16, R=0.308, P=0.247) and T-cell ALL (accumulation of long-chain MTX polyglutamates: N=13, R=0.247, P=0.415; short-term TSIA: N=6, R=0.886, P=0.019), separately. *- ratio of mRNA expression of intron 8 PR to the WT variant (FPGS alternative variant/FPGS WT variant*100%).

Supplemental Figure S2



Supplemental Figure S2. Sensitivity of parental CCRF-CEM and CEM/R30dm cells to dexamethasone as determined by MTTcell growth inhibition assay. Cells were incubated with the drugs for 72 h. The mean (±SD) IC50 (drug concentration, which inhibits cell growth by 50%) values for FPGS activity defective CEM/R30dm were markedly elevated when compared to the parental CCRF-CEM cells (8.31 µM and 0.023 µM, respectively).