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

The antifolate methotrexate (MTX) continues to be a key component of contemporary ALL treatment.[1,2] It is routinely used during the consolidation (iv high dose-MTX) and maintenance (oral MTX) phase of ALL treatment, as well as in the prevention of central nervous system involvement.[1,3] As a folate antagonist, MTX blocks several folate-dependent enzymes, thereby impairing a number of crucial metabolic pathways including *de novo* biosynthesis of purines and thymidylate, amino acid conversion and mitochondrial protein synthesis.[4] Its cytotoxicity largely depends on the activity of folylpolyglutamate synthetase (FPGS), which catalyzes the stepwise addition of multiple glutamate residues (i.e., polyglutamylation) to both folates and antifolates.[5] This unique metabolic conversion ensures higher intracellular retention of MTX and enhances target enzyme inhibition. Consequently, the capacity of leukemic cells to accumulate high levels of MTX polyglutamates, as determined in *ex vivo* ALL specimens, was shown to be associated with increased clinical efficacy of MTX and better event free survival (EFS) of childhood ALL patients.[2,6,7]

We previously reported that aberrant FPGS splicing constitutes a plausible basis for the loss of FPGS activity.[8-10] The most prominent FPGS splicing alteration found in childhood ALL was intron 8 partial retention (intron 8 PR). The ratio of this splice variant over the wild type transcript markedly increased in response to treatment with antifolates and other chemotherapeutic agents in antifolate-resistant cells, but not in their parental antifolate sensitive counterparts.[10] Moreover, FPGS intron 8 PR was predicted to cause a premature stop codon insertion which resulted in dysfunctional FPGS protein as indicated by *in vitro* FPGS catalytic activity analysis.[10]

To date, the actual relevance of altered FPGS splicing for MTX resistance and treatment outcome of childhood ALL in the clinical setting remains unclear. Therefore, the aim of the current study was to explore the relationship between impaired FPGS splicing and MTX resistance as well as the long term clinical outcome in childhood ALL patients.

We have included in our analysis cryopreserved mononuclear cells of 91 (*Online Supplementary Table S1*) newly diagnosed, untreated pediatric ALL patients enrolled in Dutch Childhood Oncology Group protocols ALL6 – ALL9[11] or German Co-operative ALL protocols 92-97.[12] The treatment details have been described elsewhere.[11,12] We previously characterised a number of MTX-related parameters for these patients, including primarily the levels of total and long-chain MTX polyglutamates as well as a short-term thymidylate synthase inhibition assay (TSIA) – providing a reliable reflection of MTX efficacy.[7] Screening for FPGS splicing alterations was performed using the previously described comprehensive PCR-based assay combined with fragment analysis.[7] The identity of the obtained PCR fragments was previously confirmed by sequencing to represent FPGS splice variants.[10] For more details on patient characteristics, MTX related variables and splice variant analysis see *Online Supplementary Materials and Methods* and *Online Supplementary Table S1*. Of note, since not all variables characterising MTX sensitivity could be measured in all the patients in our cohort (due to limited number of leukemic blasts available as well as logistic reasons), the number of patients included in the various analyses varied.

We have previously shown that MTX sensitivity differs substantially between precursor B-cell and T-cell leukemia samples.[7,13,14] To assess if these differences are also reflected in FPGS splicing, we first compared the levels of FPGS splice variants of these two ALL subtypes. Interestingly, intron 8 PR, exon 6 skipping and intron 5 retention were found at higher levels in T-cell ALL (P=0.03, P=0.007 and P=0.04, respectively). Since, T-cell ALL was shown to display decreased FPGS activity,[13] it is conceivable that the higher levels of FPGS intron 8 PR found in the present study in this subtype of ALL could further contribute to this difference.

Subsequently, FPGS splicing alterations were correlated to MTX resistance-related variables in the total patient cohort. Two splice variants showed positive associations with the short-term TSIA: concurrent intron 5 and intron 6 retention (R=0.285, P=0.03, N=58) and intron 8 PR, although it did not reach statistical significance (R=0.22, P=0.1, N=58). No other significant correlations were found for this alteration (*Online Supplementary Table S2*).

Since aberrant splicing of FPGS may possibly be the result of a broader splicing defect, we also investigated whether FPGS splicing alterations are associated with resistance to other chemotherapeutics, as determined by *ex vivo* cytotoxicity (MTT) assays. Intriguingly, this analysis revealed that intron 8 PR was correlated with mitox-

Table 1. Difference in drug resistance between ALL patients displaying high and low levels of FPGS intron 8 PR.

	Intron 8 PR high		Intron 8 PR low		*P*
	Median (range)	N	Median (range)	N	
mitoxantrone	0.06 (0.01-0.1)	5	0.01 (0.01-0.01)	4	0.014
prednisone	24.4 (0.01-260)	11	0.5 (0.01-260)	17	0.019
dexamethasone	6.1 (0.2-6.1)	6	0.06 (0.01-0.73)	8	0.006
teniposide	0.3 (0.1-1.1)	6	0.1 (0.03-2.3)	10	0.065
6-mercaptopurine	424 (61-510)	6	90 (14-510)	10	0.127
doxorubicin	0.3 (0.06-0.5)	6	0.1 (0.1-0.4)	7	0.116
cytarabine	0.8 (0.1-11)	10	0.4 (0.03-1.9)	16	0.140

Drug resistance is expressed as LC50 values (g/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 that could be evaluated in that particular test.

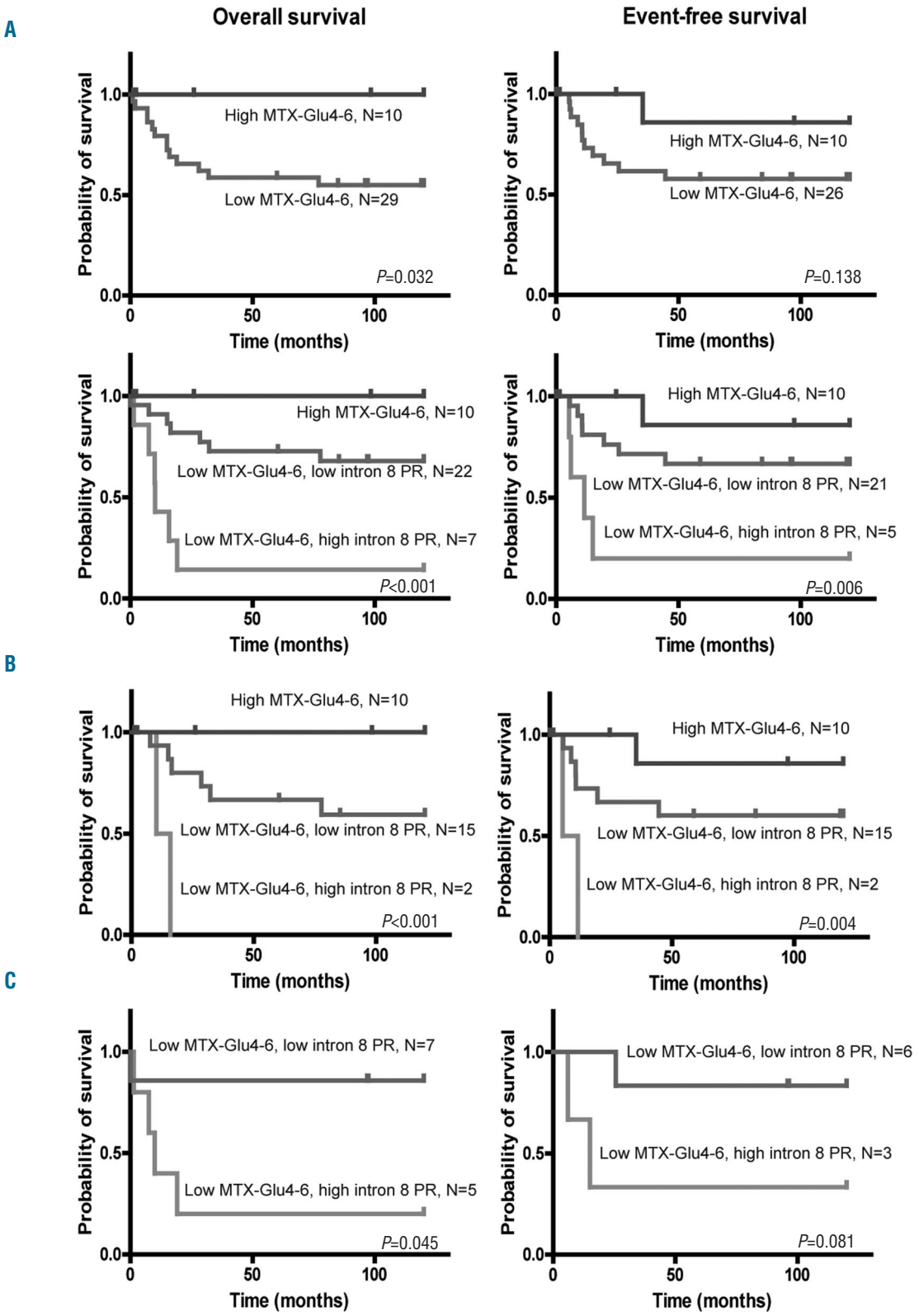

Figure 1. Kaplan-Meier analysis of the FPGS intron 8 PR in relation to overall and event-free survival in ALL patients with high and low concentration of long-chain MTX polyglutamates. (A) Association between the concentration of long-chain MTX polyglutamates with OS and EFS in the total cohort of ALL patients. Within the group of patients accumulating lower levels of long-chain MTX polyglutamates, the level of intron 8 PR discriminates between favourable and less favourable OS and EFS. This association is significant when analyzed in precursor B-cell ALL (B) and T-cell ALL (C) patients separately.

Table 2. Univariate and multivariate survival analysis of FPGS intron 8 PR expression in paediatric ALL patients accumulating suboptimal levels (< 1000 pmol/10^9 cells) of long-chain MTX polyglutamates.

Intron 8 PR	N	Univariate OS Hazard ratio (95%CI)	P	Multivariate OS Hazard Ratio (95%CI)	P
high	7	5.6 (1.8-17.3)	0.003	6.6 (1.7-25.6)	0.006
low	22	1.00		1.00	
Intron 8 PR	**N**	**Univariate EFS Hazard ratio (95%CI)**	**P**	**Multivariate EFS Hazard Ratio (95%CI)**	**P**
high	5	4.2 (1.2-14.9)	0.024	7.6 (1.7-33.3)	0.007
low	21	1.00		1.00	

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 beta coefficient test; N - the number of patients.

antrone (N=9, R=0.933, *P*<0.001) and dexamethasone resistance (N=14, R=0.650, *P*=0.01). A trend towards a positive association with resistance to doxorubicin was also observed, although it did not reach significance (N=13, R=0.500, *P*=0.08). Since the frequency distribution of intron 8 PR levels was bimodal, we selected a cut-off value which discriminated between the two subpopulations observed. Remarkably, high level of intron 8 PR was indicative of increased resistance to mitoxantrone, prednisone and dexamethasone (Table 1). These differences were present in the total patient cohort as well as in the precursor B-cell subtype of ALL (*Online Supplementary Table S3*).

To explore the association of FPGS splice variants with treatment outcome of paediatric ALL patients, we assessed their relation with overall survival (OS) and EFS (defined as time from complete remission to relapse or death). No associations were found in the total patient cohort. Since other mechanisms, such as high activity of reduced folate carrier (RFC), the influx transporter of MTX, or low expression of efflux pumps can contribute to high levels of MTX polyglutamates in leukemic cells, we hypothesized that FPGS-dependent MTX resistance may be more relevant in patients with suboptimal accumulation of these active metabolites. Intriguingly, we found that within the population of patients with relatively low accumulation of long-chain MTX polyglutamates, the presence of high levels of intron 8 PR (Figure 1A) was related to an inferior EFS and OS (Figure 1A). This association remained significant when analysed in precursor B-cell and T-cell ALL patients separately (Figure 1B and 1C), although the number of cases was rather low. In multivariate analysis of OS and EFS, including WBC, lineage and age, high intron 8 PR levels were still significantly associated with worse outcome in this subgroup of patients which accumulated low levels of the long-chain MTX polyglutamates (Table 2). In addition, in these ALL patients intron 8 PR was associated with higher MTX resistance as measured in the short-term TSIA (N=24, R=0.492, *P*=0.015), and with lower accumulation of long-chain MTX polyglutamates (N=38, R=-0.359, *P*=0.027, *Online Supplementary Figure S1*). When analysed separately within the precursor B-cell or T-cell ALL subgroups, most of these associations became insignificant, possibly due to low sample numbers. The only correlation to remain significant in this analysis was the association between intron 8 PR and short-term TSIA in T-cell ALL (N=6, R=0.886, *P*=0.019).

Low levels of long-chain MTX polyglutamates were previously shown to result from decreased mRNA levels of RFC and FPGS as well as decreased FPGS activity.[7,14,13] This suggests that, as we observe in this cohort, among patients with diminished accumulation of long-chain MTX polyglutamates, individuals displaying high levels of intron 8 PR might have particularly low activity of this enzyme and, consequently, very poor response to MTX. Furthermore, FPGS intron 8 PR was also associated with glucocorticoid resistance, which is a known predictor of the outcome in paediatric ALL. Hence, the relative attribution of this factor in our survival analysis is currently unclear.

Intriguingly, we previously found that FPGS-deficient MTX-resistant human leukemia cells displaying high levels of intron 8 PR were also highly resistant to dexamethasone (*Online Supplementary Figure S2*).[10] These observations imply that intron 8 PR presumably reflects a broader splicing defect resulting in resistance to multiple chemotherapeutics; most likely affecting other genes as well. These genes have not yet been identified, but could be involved either in drug metabolism or regulation of apoptosis.[15] The relation between intron 8 PR and resistance to chemotherapeutics other than MTX, as well as clinical outcome, should be further characterized in a larger cohort.

Despite an extensive cohort and comprehensive data, the current study has limitations related to low patient numbers; in particular subgroup analyses. Moreover, this patient cohort is not fully representative of the typical treatment outcomes achieved in childhood ALL on these protocols as the OS and EFS are lower than expected and relapses (and death after relapse) occur earlier than expected. This discrepancy is most likely due to the selection of individuals with sufficient leukemic blasts for comprehensive analyses. Since high blast count is a hallmark of more aggressive disease, the cohort used in the current study contains many patients with relatively poor outcome. For example, when compared with DCOG protocol ALL8 or ALL9, patients with high WBC or T-cell ALL in our cohort were overrepresented (*Online Supplementary Table S1*), while sex and age at diagnosis were comparable. Further validation of our main findings is warranted in unselected patient samples from more current treatment regimens.

Taken altogether, our findings suggest that FPGS intron 8 PR is related to MTX resistance and specific other drugs. This splice variant and/or its splice regulators are, therefore, interesting potential prognostic markers for future patient stratification and personalized medicine in ALL.

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