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CLINICAL TRIALS AND OBSERVATIONS

The Association of Reduced Folate Carrier 80G>A Polymorphism to Outcome in Childhood Acute Lymphoblastic Leukemia interacts with Chromosome 21 copy number

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Abstract

The reduced folate carrier (RFC) is involved in the transport of methotrexate (MTX) across the cell membrane. The *RFC* gene (*SLC19A1*) is located on chromosome 21, and we hypothesized that the *RFC* 80 G>A polymorphism would affect outcome and toxicity in childhood leukemia and that this could interact with chromosome 21 copy number in the leukemic clone. Five-hundred children with acute lymphoblastic leukemia treated according to the common Nordic treatment protocols were included and we found that the *RFC* AA variant was associated with a 50% better chance of staying in remission compared to the GG or GA variants (p=0.046). Increased copy numbers of chromosome 21 appear to improve the outcome also in children with GA or GG variant. In a subset of 182 children receiving 608 high-dose MTX courses, we observed higher degree of bone marrow toxicity in patients with the *RFC* AA variant compared to the GA/GG variants (platelet 73 versus 99/105 x10^9/L (p=0.004)) and a higher degrees of liver toxicity in patients with the *RFC* GG variant (ALAT 167 versus 127/124 U/L, p=0.05). In conclusion the *RFC* 80G>A polymorphism interacts with chromosome 21 copy numbers and affects both MTX efficacy and toxicity.

Introduction

Pharmacogenetics influence the risk of relapse in childhood acute lymphoblastic leukemia (ALL)[1]. Methotrexate (MTX) is one of the most widely used drugs in these patients[2-5] and many genetic polymorphisms may influence MTX pharmacokinetics and dynamics[6]. MTX is a folic acid antagonist with increased affinity for its target enzymes when polyglutamated intracellularly. Furthermore, the polyglutamated MTX (MTX-glu(n)) is intracellular retained far longer than the administered monoglutamated MTX. MTX and MTX-glu(n) exert their effect by inhibiting enzymes essential for thymidylate synthesis and de novo purine synthesis, which will affect DNA synthesis and cellular proliferation[7;8]. The first step, cellular uptake, involves the reduced folate carrier (RFC)[9;10]. A polymorphism, 80G>A (rs1051266, His27Arg), in the *RFC* gene (*SLC19A1*) has been identified with an allele frequency of 0.48[11;12]. Functional studies of the polymorphism have produced somewhat ambiguous results. Whetstine et al.[13] found no significant differences in MTX uptake between *RFC*-allele variants in transfected cells, whereas Baslund et al. demonstrated increased MTX uptake in cells from healthy persons with the AA variant[14]. The *RFC* gene is located at chromosome 21 and there appears to be a direct relationship between copy number of chromosome 21 and the risk of toxicity after MTX therapy; as patients with Down´s syndrome show more treatment-related toxicity. Furthermore, hyperdiploid B-precursor ALL patients which in >90% of the cases include three or four copies of chromosome 21 have a low relapse rate and they generate significantly higher intracellular levels of MTX-glu(n) in their leukemic cells compared to non-hyperdiploid patients[15-17]. These findings are likely to reflect enhanced intracellular transport of MTX, since it has been shown that the *RFC* gene is expressed at significantly higher levels in hyperdiploid lymphoblasts than in non-hyperdiploid lymphoblasts[18;19].

Studies of rheumatoid arthritis (RA) patients in MTX monotherapy have, like the functional study of MTX uptake by Baslund et al[14] demonstrated better MTX efficacy in AA variants[20;21]. However,

only two studies have previously investigated the effect on outcome in childhood ALL: Rocha et al.[22] could not demonstrate any significant effect on cure rate in 246 children with ALL, whereas Laverdiere et al[11] in a study of 204 patients found a reduced relapse rate for patients with the G-allele. However, different MTX-treatment strategy was used in the latter study, and neither of the two studies explored the impact of chromosome 21 copy number [11;22].

In this nation-wide study comprising 500 children with ALL, we have investigated the influence of *RFC* (*SLC19A1*) polymorphism on the risk of relapse and of post-HDMTX toxicity as well as the interactions with chromosome 21 copy number.

Patients, Materials, and Methods

Patients: 563 children aged 1-15 years were diagnosed with non-mature B cell or T cell leukemia in Denmark from January 1992 to January 2007 (246 girls, 317 boys, aged 1.1-14.9 - median 4.5). At a median follow-up time of 7.9 years (50% range: 4.1–12.1 years), 452 (80%) are still in 1st remission, whereas 74 (13%) patients have relapsed within 0.2–8.3 years from the diagnosis (median: 2.6 years). Seventeen of the 74 relapses involved the CNS, 12 of which were isolated. Five patients died in first remission (1%) and five developed a second malignancy (1%) (Table 1). Twenty-two patients died before the first HDMTX was given (4%), and five patients did not achieve remission during induction treatment and thus changed protocol before the first HDMTX (1%).

Sixty-three patients were excluded from the study due to a lack of DNA material or poor quality of DNA in the specimens (n=47), because of change of protocol (n=5) or death (n=11) before the first HDMTX course. All the remaining 500 patients included in this study, i.e. 89% of those potentially eligible in Denmark during the study period (Table 2), were treated according to the NOPHO-92 protocol (n=295) or NOPHO-2000 (n=205), either with a low-risk (n=350) or high-risk protocol (n=150). Less than 5% of the patients were of other ethnical origin than Nordic Caucasian race.

65% of samples were drawn at the time of diagnosis. To ensure that leukemic DNA did not differentiate from germ line DNA, we compared RFC genotypes in 42 patients with samples taken at time at diagnosis and after remission was achieved; 14 pair of samples (of which 4 samples were from patients with hyperdiploid clone involving chromosome 21) in each RFC variant group and found no mismatch. The Ethic´s Committee of Copenhagen (j.nr. 01-259108) as well as the Danish Data Protection Authority (j.nr. 2005-41-4808) approved the study design and protocol. Toxicity studies were conducted only in children treated at Rigshospitalet in Copenhagen, where HDMTX data were available. Blood samples from 202 healthy donors were used to compare *RFC* variant frequencies (Table 2).

Table 1. Patients and Studies

	Patients	No events	Relapse	Died before the first HDMTX	Change of protocol before the first HDMTX	SMN	Dead in CR
All patients	563	452	74	22	5	5	5
All patients treated at Rigshospitalet	238	192	31	6	3	3	3
Outcome study	500	422	70	0	0	5	3
Toxicity study first HDM course	123	110	15				1
Toxicity study all HDM courses	182	156	22			2	2

Therapy: The children were divided into treatment groups based on risk assessments according to the NOPHO protocol[23]. The children were considered high risk if any of the following parameters were present: white blood count (WBC) $\geq 50 \times 10^9$/L, T-lineage ALL, the presence of CNS or testicular leukemia, translocations t(9;22)(q34;q11) or t(4;11)(q21;q23), lymphomatous leukemia or mediastinal lymphoma, and/or a poor treatment response ($\geq 25\%$ blasts in BM day 15 or $\geq 5\%$ blasts in BM day 29).

Table 2. Patient and blood donor characteristics in relation to reduced folate carrier 80G>A polymorphism

	RFC GG variant	*RFC* GA variant	*RFC* AA variant
Patients (fraction)			
Male	90(0.3)	136(0.5)	60(0.2)
Female	70(0.3)	106(0.5)	38(0.2)
Median age (min-max)	4.5 (1.1-14.8)	4.5(1.1-14.9)	4.2(1.6-14.6)
B/T lineage	137(0.3)/23(0.4)	206(0.5)/36(0.5)	89(0.2)/9(0.1)
High/low risk	45(0.3)/115(0.3)	80(0.5)/162(0.5)	25(0.2)/73(0.2)
NOPHO-92 / NOPHO-2000	98(0.3)/62(0.3)	131(0.5)/111(0.5)	66(0.2)/32(0.2)
Chr 21 copy number >2/2	44(0.4)/92(0.3)	55(0.4)/152(0.5)	27(0.2)/59(0.2)
Relapse - CNS only/CNS+	23(0.3)-3(0.3)/2(0.4)	39(0.6)-7(0.6)/3(0.6)	8(0.1)-1(0.1)/0
Secondary malignancy	1(0.2)	3(0.6)	1(0.2)
Bone marrow transplant	6(0.4)	4(0.2)	6(0.4)
Died in remission	2(0.7)	1(0.3)	0
t(12;21) in high/low risk	3(0.3)/19(0.3)	7(0.6)/31(0.5)	2(0.1)/14(0.2)
Red cell transfusions patients/numbers (min-max)	51(0.3)/56 (1-147)	93(0.5)/64 (1-324)	35(0.2)/64 (24-416)
Platelets transfusion patient/numbers (min-max)	38(0.3)/41 (3-144)	68(0.5)/40 (2-216)	25(0.2)/64 (8-208)
Excluded patients with genotype			
Death before first HDMTX	3(0.3)	6(0.5)	2(0.2)
Change of protocol before the first HDMTX	2(0.4)	2(0.4)	1(0.2)

There was no difference in the distribution of gender and genotype (p=0.7). Among the Healthy blood donors there were 38(0.36), 54(0.51) and 14(0.13) men in the RFC GG, GA and AA variant groups, respectively and 22(0.23), 54(0.56) and 20(0.21) women, respectively.

High-dose MTX (HDMTX): Children with low-risk ALL (Figure 1) received 5g/m^2 HDMTX courses 3-4 times during the consolidation period, with an interval of 14-28 days, and 5 times during maintenance therapy with an interval of approximately 8 weeks. Leucovorine rescue 15 mg/m^2 was given at 6 hours intervals from hour 36 (NOPHO 2000: 42 hours) after the start of each HDMTX course until s-MTX<200nmol/L[24]
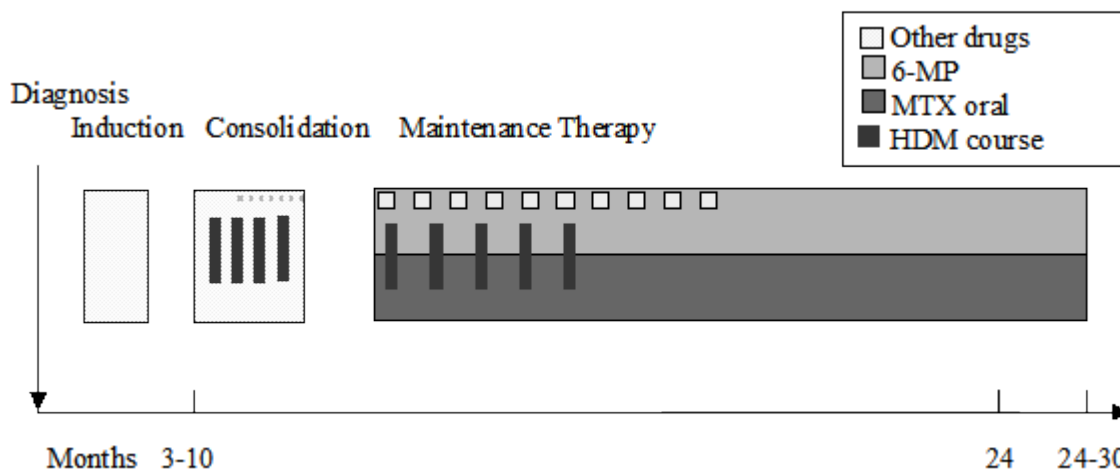

Figure 1. *Low-risk MTX therapy*
A punctured line indicates that not all patients had received this specific dose. In maintenance therapy 6-mercaptopurine (6-MP) and MTX are administered in low oral doses. 8-12 mg MTX was administered intrathecally 6 times before consolidation.

Children with high-risk ALL (Figure 2) received 8g/m^2 HDMTX courses 2-4 times during the consolidation period with an interval of at least 42 days. The initial leucovorine rescue dose at hours 36 was 50 mg/m^2 (NOPHO 2000: 15 mg/m^2) followed by leucovorine rescue (15 mg/m^2) at 6 hours intervals until s-MTX<200nmol/L[24]. Intrathecal MTX (8-12mg) was administered during all HDMTX courses in both low and high-risk leukemia protocols.

Methods: Genomic DNA was extracted and purified by NaCl- and ethanol-precipitation from 1-5 ml EDTA-stabilized blood. Allelic discrimination was carried out to detect the *RFC*80 G>A polymorphism using fluorogenic 3'-minor groove-binding probes in an end-point PCR assay on an

ABI 7500 Fast platform (Applied Biosystems, Denmark). Primers and probes were as described in an earlier study[14]. The method was validated using the BigDye terminator v1.1 cycle sequencing kit and Sanger sequencing on ABI 3730. All reagents were purchased from Applied Biosystems, Denmark. To measure MTX a photometric assay was used.
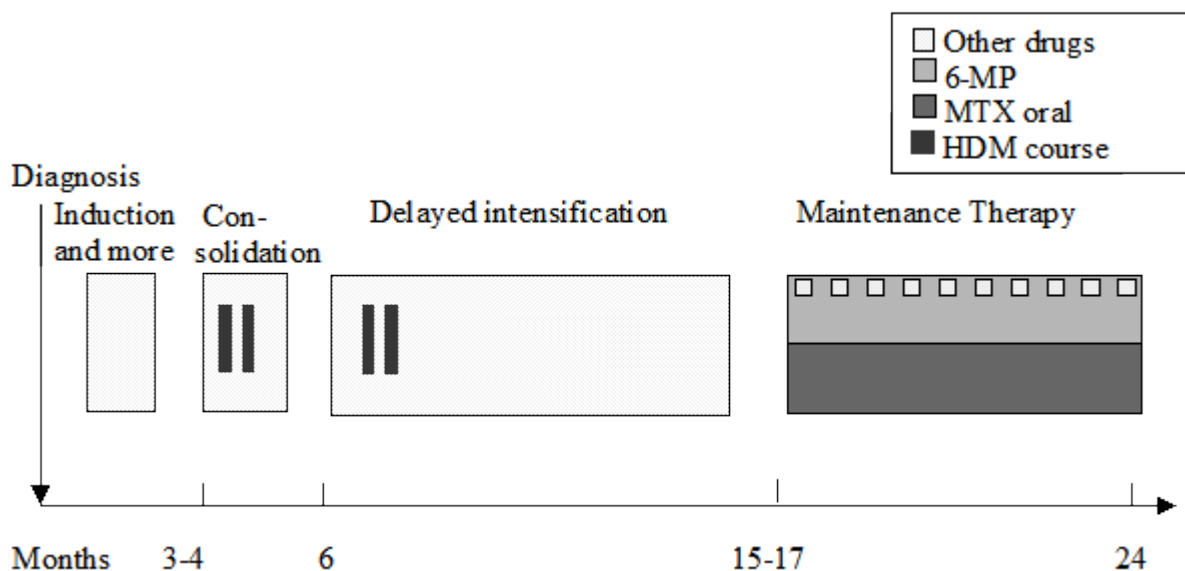

Figure 2. *High-risk MTX therapy*
A punctured line indicates that not all patients had received this specific dose. In maintenance therapy 6-mercaptopurine (6-MP) and MTX were administered in low oral doses. 8-12 mg MTX was administered intrathecally 6 times before consolidation.

Validation of Method: Blood donor *RFC* frequencies were in agreement with Hardy-Weinberg equilibrium and sequencing of 3 samples with each *RFC* variant (n=9) showed identical results with the allelic discrimination method.

Pharmacokinetic and toxicity after HDMTX: The lowest platelet and hemoglobin values within a month after HDMTX or before the next HDMTX treatment (if given earlier) were used to quantify the degree of myelosuppression. The highest value of plasma alanine aminotransferase (ALAT) during the same time period was used as a marker of liver toxicity. Samples drawn 20-24 hours after administration of HDMTX were used for plasma MTX measurements.

Karyotype results: Only G-band karyotyping was mandatory in the ALL-92 protocol, but the ALL-2000 protocol also required directed analysis by fluorescent in-situ hybridization and/or

reverse transcriptase PCR for translocation t(9;22)(q34;q11)[*BCR-ABL*] and for 11q23/*MLL* aberrations. Furthermore, many leukemic samples have been explored by comparative genomic hybridization and spectral karyotyping[25]. All cytogenetic results are scrutinized annually by the NOPHO cytogenetic working group and described according to ISCN 1995[26]. Karyotype results were available for 429 of the included patients and were used to classify patients according to their chromosome 21 copy number.

Statistics: SAS software version 9.1.3 was used for the statistical analysis. Two-sided p values <0.05 were considered significant. Survival analyses were performed with a basic time scale defined by the date of diagnosis. The duration of event-free survival (EFS) was defined as the time from diagnosis until the date of relapse, death, or the development of a second malignancy (whichever first) or the last known follow-up for event-free survivors. Since the RFC genotype may reflect both relapse, risk of toxic death and secondary malignancy (SMN), all these events are included in the survival analysis. Bone marrow transplantation and protocol failure or changes of protocol (NOPHO guidelines 1992 or 2000) were classified as censuring events. Patients in first remission were followed until July 30, 2008. Survival probabilities were estimated using the Kaplan-Meier method. Univariate Cox regression and multivariate Cox regression analysis with stepwise backwards selection (variables: phenotype, protocol and enhanced number of chromosome 21) with stratification by risk group were used to identify potential risk factors for an event. Model assumptions, including the proportionality assumption, were assessed using conventional methods A general linear model was used for pharmacokinetic analyses after the first HDMTX. Repeated measurements (mixed model analysis), with the order of HDMTX courses as a random effect, were used to explore toxicity and pharmacokinetic after all HDMTX courses. Each low risk patient had from one to nine HDMTX courses and each high risk patients one to three courses. In the statistical tests, all data was logarithmically transformed and adjustment for low/high risk group was applied.

Chi^2 test were used for tests of independence to determine *RFC* polymorphism and risk of developing leukemia.

Results

Risk of developing leukemia: We found no significant differences in the *RFC* allele frequencies between the 516 patients and the 200 blood donors (p=0.44) (Table 2) and patients *RFC* frequencies were in agreement with Hardy-Weinberg equilibrium.

Event risk by *RFC* genotype: The risk of developing an event was 50 % lower for the *RFC* variant AA patients than for the AG/GG variant patients (hazard ratio 0.5 [95%CI:0.25-1.00]; p=0.046) (Figure 3). Although not being statistically significant, patients with the AA variant had an approximately 75 % lower risk of developing a CNS relapse than *RFC* GA/GG variants when including all CNS relapses (n =16 relapses, p=0.20) and a 60% lower risk when including only isolated CNS relapses (n =11 relapses, p=0.38).
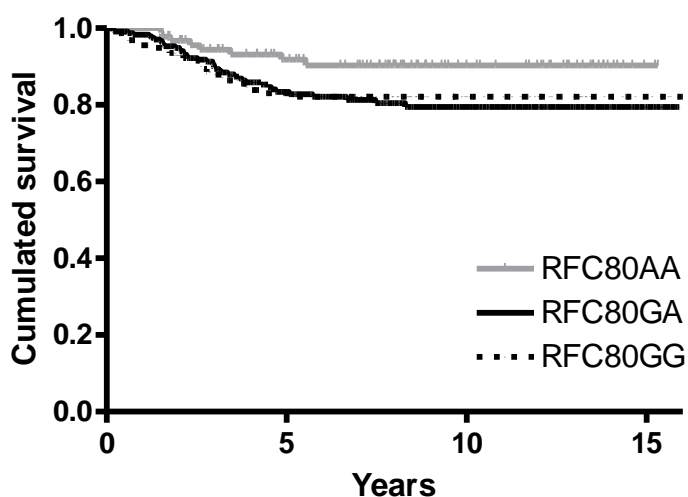

Figure 3. RFC polymorphism and outcome
The Kaplan-Meier survival curve is shown to visualize the difference between AA variant and GG/GA variants (log rank p=0.046).

RFC and chromosome 21 copy numbers: To investigate the interaction between the *RFC* variants and chromosome 21 copy numbers, 429 patients for whom cytogenetic information was available,

were divided into groups with a normal chromosome 21 copy number (n=303) or an increased copy number (n=126). Among patients with two copies of chromosome 21 in their leukemic clone, the 59 patients with the *RFC*80 AA variant had a 64% reduced risk of relapse compared to the 244 patients with the *RFC*80 GA/GG variants (p=0.048 hazard ratio 0.36 [95%CI:0.13-0.99]) (Figure 4). In contrast, the 126 patients with three or more copies of chromosome 21 in their leukemic clone showed no significant differences in the risk of relapse between the *RFC*80 variant groups (p=0.80), and exclusion of patients with Down´s syndrome (n=2) did not change this result.
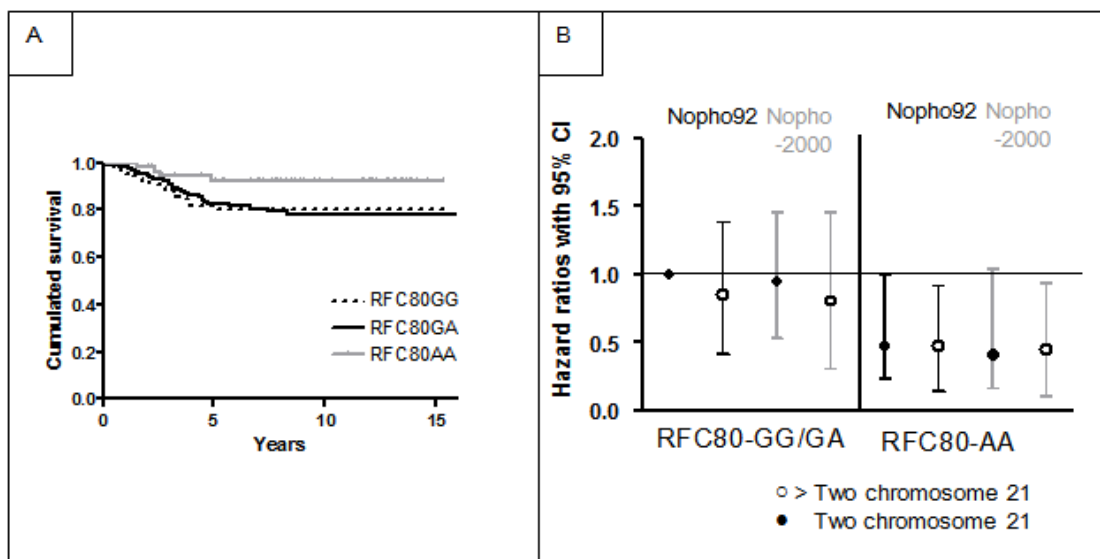

Figure 4. RFC polymorphism, outcome and relation to chromosome 21
A. Outcome in patients with a normal copy number of chromosome 21. Patients with an increased copy number of chromosome 21 (expected higher MTX influx) had no significant differences between RFC variants (p=0.94) (data not shown). B. Different hazard ratios compared to patients with a normal copy number of chromosome 21 and RFC80 GA/GG variant according to the NOPHO 92 protocol

Multivariate analysis: In a backward stepwise Cox regression model adjusted for risk group, phenotype, protocol and chromosome 21 copy numbers, the *RFC* AA variant still had a better prognosis (p=0.10), but only risk group (hazard ratio 2.83 [95%CI:1.83-4.47]) had a significant effect on outcome (Table 3).

Toxicity after high-dose methotrexate:

To investigate the impact of *RFC*80 polymorphism on toxicity after HDMTX, the highest level of ALAT and the nadir of neutrophils, platelets and hemoglobin within a month after HDMTX or

before the next HDMTX (if earlier) were available for 182 patients. Low risk patients in the AA variant group had significantly lower platelets (p=0.004/0.002) and hemoglobin nadirs (p=0.004/0.001) compared to the GA and GG variant groups and the levels of ALAT were significantly higher in the GG variant group compared to the GA and AA variant groups (p=0.030/0.049) (Table 4). The same trends were seen in high risk patients although it did not reach statistical significance.

Table 3. Cox regression analysis

	Univariate		Multivariate	
Covariate	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
RFC80 AA vs GA/GG	0.50 (0.25-1.00)	0.046	0.56 (0.28-1.13)	0.10[1]
Phenotype Pre B vs T [2]	0.97 (0.51-1.87)	0.94		0.94[3]
Copy nr of Chr21 >2 vs 2	0.80 (0.46-1.39)	0.44		0.71[3]
Risk group High vs low	2.97 (1.90-4.63)	<0.0001	2.86 (1.83-4.47)	<0.0001
Protocol 2000 vs 1992	0.90 (0.55-1.48)	0.68		0.32[3]

[1]If only relapse was considered as an event, then p=0.13.
[2]Low risk patients were excluded from the model, because no T-ALL were assigned to low risk therapy
[3]P-value of covariate if included in the final model

Pharmacokinetics: To explore the pharmacokinetic impact of the RFC80 polymorphism we analysed 182 patients, all treated at Rigshospitalet, Copenhagen, with plasma MTX levels measured at 20-24 hours after initiation of the HDMTX infusion (n=608 HDMTX courses). One houndred-and-twenty-three patients had MTX measurements available from their first HDMTX. These patients did not differ significantly from the remaining total study cohort with respect to gender, treatment protocol, immunophenotype or relapse rate. Patients with the GG-variant had lower end-

of-infusion plasma MTX-levels (Table 5) than the others variant groups both at the first HDMTX course (p=0.0365, 123 patients stratified for low/high risk ALL), and after all HDMTX courses (p=0.0122, 608 HDMTX courses, 182 patients).

Table 4. Nadir of platelets, hemoglobin and lymphocytes and highest ALAT value after high dose methotrexate and relation to *RFC*80 polymorphism in low risk ALL patients

Reduced folate carrier - genotype		AA	GA	GG	Ratio (95% CI)	p-values
Platelets (10^9/L)	Patients	29	72	39		
	HDMTX courses	116	279	153		
	Mean (geometric)	73	99	105		
	Confidence-interval	55-95	78-125	81-136		
	GG vs. AA				1.44[1.14-1.82]	0.002
	GA vs. AA				1.36[1.10-1.68]	0.004
	GG vs. GA				1.06[0.88-1.28]	0.546
	Time to count recovery (>150) after first HDMTX in days, geometric mean	3.4	2.4	2.4		
Hemoglobin (mmol/L)	Patients	29	72	39		
	HDMTX courses	116	279	153		
	Mean (geometric)	5.6	5.9	6.0		
	Confidence interval	5.4-5.8	5.7-6.1	5.8-6.2		
	GG vs. AA				1.06[1.02-1.10]	0.001
	GA vs. AA				1.05[1.01-1.08]	0.004
	GG vs. GA				1.01[0.98-1.04]	0.395
Neutrophiles (10^9/L)	Patients	28	72	38		
	HDMTX courses	111	262	149		
	Mean (geometric)	0.7	0.8	0.8		
	Confidence interval	0.6-0.9	0.7-1.0	0.7-0.9		
	GG vs. AA				1.07[0.86-1.32]	0.551
	GA vs. AA				1.11[0.92-1.35]	0.286
	GG vs. GA				0.96[0.81-1.14]]	0.652
	Time to count recovery (>0.5) after first HDMTX in days, geometric mean	4.4	3.4	2.4		
ALAT (U/L)	Patients	28	71	38		
	HDMTX courses	102	227	132		
	Mean (geometric)	123.8	127.0	167.1		
	Confidence interval	86-177	93-174	119-236		
	GG vs. AA				1.35[1.00-1.82]	0.049
	GA vs. AA				1.02[0.78-1.34]	0.851
	GG vs. GA				1.31 [1.03-1.69]	0.030

p values are adjusted for repeated measurements

Table 5. RFC80 polymorphism and plasma levels of Methotrexate after HDMTX (nmol/L)

	First HDMTX course, 123 patients. (GG vs. GA/AA, p=0.037, n=123)			All HDMTX courses, 182 patients. (GG vs. GA/AA, p=0.012, n=608)		
	GG (n=37)	GA (n=58)	AA (n=28)	GG (n=173)	GA (n=307)	AA (n=128)
Low risk	82207	89554	90300	67818	79585	79074
High risk	91524	122684	148458	100160	105373	157403

Discussion

This study not only indicates a significant impact of *RFC* 80G>A polymorphism on MTX treatment efficacy in childhood ALL, but as important emphasizes that these results can only be fully appreciated within the karyotypic background of the leukemic cells.

Other studies have explored variations in the *RFC* expression in leukemic cells[19] and demonstrated that low expression of *RFC* is related to a poor outcome in childhood leukemia[27]. Furthermore, Belkov et al showed that higher mRNA levels of *RFC* were related to the chromosome 21 copy number[18] and Jansen et al. suggested that higher mRNA levels was related to the *RFC*80 A variant and to the chromosome 21 copy number[28]. This is in conformity with both the results in Baslund´s study, where the *RFC*80 AA variant takes up MTX more efficiently, and the clinical results of the present study, where higher numbers of chromosome 21 seem to neutralize the effect of *RFC* variants.

In contrast to the present study, Laverdiere et al.[11] found an association between variant A alleles and a worse outcome in childhood leukemia. However, in their study only one 4 g/m^2 HDMTX course and 30mg/m^2 per week after induction was given instead of the four to nine courses of 5 or 8 g/m^2 HDMTX courses and 20mg/m^2 per week after induction used in the NOPHO protocols.

Furthermore, the present study includes twice as many patients and the median follow up time is much longer (7.9 versus 4 years), which is important since relapses tend to come late in many low risk ALL patients such as the high-hyperdiploid cases and those with t(12;21)[25].

However, we confirmed the association between the *RFC* GG variant and lower MTX plasma concentrations reported by Laverdiere et al[11]. Furthermore, we found that low risk patients had more post-HDMTX liver toxicity (higher ALAT levels) in the GG variant group compared to patients with GA/AA variants. The biological background for this association is unclear. However, under normal conditions high levels of folic acid polyglutamates are stored in liver cells. Since the gene dosage of A-alleles would be expected to correlate with increased levels of folic acid polyglutamates in liver cells, this could protect the cells from MTX toxicity. In addition, higher folic acid polyglutamate levels in liver cells in A-alleles may also decrease the influx of MTX into the hepatocytes, where a significant fraction of MTX during HDMTX is eliminated, which could explain that patients with A-allele have a slower elimination of MTX and thus higher MTX plasma concentrations.

A new study by De Jonge et al[29] has shown a correlation between variants in the *RFC*80 polymorphism and the etiology of leukemia and although it was not the purpose of this study, we looked at the correlation, but found no connection between the *RFC* allele frequencies and etiology of childhood leukemia.

Conclusion

This study emphasizes the need to add pharmacogenetic profiling to the already extensive biological exploration of childhood ALL, since both these and previously published data [30] support that individual variations in genes that influence the disposition of anticancer agents may be as important for the risk of relapse as the genetic aberrations of the leukemic clone. Although this

study showed significant association between RFC genotypes and outcome, these results need to be validated in an independent patient cohort. Further studies looking at the interaction between pharmacogenetic polymorphisms and risk of treatment failure are needed to determine whether adjusting the drug dosing according to genotype will increase the efficacy of the antileukemic treatment. Such studies are ongoing in the study group and some are now integrated into the NOPHO ALL2008 protocol.

Acknowledgements

Authorship

Jannie Gregers, Professor Kjeld Schmiegelow, Professor Curt Peterson, and Consultants Kim Dalhoff and Birgitte Lausen designed the study. Jannie Gregers performed experiments, analyzed results, performed statistics, and wrote the paper. Senior researcher and Statistician Ib Jarle Christensen performed/validated statistics, and Head Physicians Birgitte Lausen, Henrik Schroeder, Steen Roesthoej, and Niels Carlsen provided clinical data on the patients. All authors commented on and approved the final manuscript. The authors declare no competing financial interest.

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