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High-dose methotrexate in Egyptian pediatric acute lymphoblastic leukemia: the impact of *ABCG2* C421A genetic polymorphism on plasma levels, what is next?

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Abstract
Purpose High-dose methotrexate (HD-MTX) is a cornerstone antineoplastic drug in most treatment protocols of pediatric acute lymphoblastic leukemia (ALL). Among the membrane efflux transporters of MTX, the human breast cancer resistant protein is the second member of the G subfamily of ATP-binding cassette (ABC) efflux pump (*ABCG2*). A single-nucleotide polymorphism (SNP) in *ABCG2,* the exchange of C to A at position 421, represents 13 % in the Middle Eastern population. We studied the effect of this SNP on the plasma levels of HD-MTX in Egyptian pediatric ALL.
Methods Two hundred ALL patients were recruited from Children's Cancer Hospital Egypt-57357, and all were treated according to the St Jude Total XV protocol. Determination of plasma MTX levels was done at 23, 42 and 68 h. Genotyping of C421A of *ABCG2* was done by polymerase chain reaction-restriction fragment length polymorphism.
Results We found 14.5 % of the variant allele of the *ABCG2* C421A SNP. The statistical association between *ABCG2* 421C>A SNP and the cutoff toxic plasma level of 24 h HD-MTX infusion at different time points tested was not statistically significant. There was no statistical significance between steady-state plasma concentration in patients with and without with this SNP.
Conclusion To date, this is the largest study on Egyptian ALL patients for this SNP. This study shows that there is no effect of *ABCG2* 421C>A on plasma concentrations of HD-MTX. Replacing candidate gene association studies with genome-wide studies of HD-MTX is now mandatory and is part of our research blueprint.

Keywords High-dose methotrexate · Egyptian pediatric acute lymphoblastic leukemia · *ABCG2* C421A genetic polymorphism · Plasma level

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Introduction

Acute lymphoblastic leukemia (ALL) represents 80 % of childhood leukemia (Metayer et al. 2013). At Children's Cancer Hospital Egypt-57357 (CCHE-57357), ALL constitutes 69.5 % of all leukemia pediatric patients according to the hospital-based cancer registry.

High-dose methotrexate (HD-MTX) is a very important antineoplastic drug in most contemporary treatment protocols for pediatric ALL (Silverman et al. 2001; Igarashi et al. 2005; Pession et al. 2005; Pui and Evans 2006). It is used in the consolidation phase of ALL protocols with other antineoplastic drugs, as it improves the outcome in both intermediate and high-risk ALL patients (Pui et al. 2012). MTX enters the cell by reduced folate carrier and also by the folate receptors, which act as the second conduit for MTX influx. With the presence of high extracellular concentrations of MTX, the influx of MTX is through passive diffusion and this is an important property of the HD-MTX (Ackland and Schilsky 1987; Stamp et al. 2009). HD-MTX behaves pharmacologically

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differently from low-dose methotrexate (LD MTX) (Malaviya et al. 2010).

HD-MTX acts by inhibition of the critical enzyme in the folate metabolism; dihydrofolatereductase (DHFR) leading to depletion of the intracellular folate pool and inhibits de novo synthesis of the nucleoside thymidine that is required for DNA synthesis. It also inhibits purine-based synthesis (Schmiegelow 2009). Therefore, HD-MTX acts as antifolate by inhibiting DNA, RNA, and thymidylate synthetase, as well as protein synthesis (Devita et al. 2008; Stamp et al. 2009). It mainly acts at the S-phase of the cell cycle (Malaviya et al. 2010).

Risk-adapted therapy represents one of the most important advances in childhood ALL treatment. Focusing on pharmacogenetic markers of the relevant antileukemic drugs will refine risk stratification and lead to optimal treatment with dose individualization in children with ALL (Pui et al. 2012). Among the membrane efflux transporters of MTX, the human breast cancer resistant protein (*BCRP*) is the second member of the G subfamily of ATP-binding cassette (ABC) efflux pump (also called *ABCG2*) (Abbott 2003; Doyle and Ross 2003; Gervasini 2009; Ni et al. 2010). The *ABCG2* gene is located on chromosome 4q22, spans over 66 Kb, and consists of 16 exons (Bailey-Dell et al. 2001). It is highly expressed in primitive stem cells and in organs responsible for absorption (small intestine), distribution (blood brain and placenta barriers), and elimination (liver and kidney) of many drugs and xenobiotics (Maliepaard et al. 2001). Many clinical studies have documented the possible role of this transporter for drug resistance in leukemia (Benderra et al. 2004; Wilson et al. 2006; Robey et al. 2007). Out of more than 80 single-nucleotide polymorphisms (SNPs) in the *ABCG2* gene, the C421A (Q141K) nonsynonymous polymorphism located on exon 5 is the most extensively studied SNP. Many studies demonstrated its association with the inter-individual variation in the pharmacokinetics, efficacy, and toxicity of drugs (Woodward et al. 2009; Keskitalo et al. 2009; Sissung et al. 2010). The allelic frequency of C421A (Q141K) varies between different populations; it is present in approximately 13 % of Middle Eastern people (Zamber et al. 2003).

Although many studies have shown that the efficacy and toxicity of MTX could be modified by the presence of genetic alterations involved in its metabolism, transport, and function, results were conflicting due to differences in the ethnicity of the population under consideration and differences in treatment protocols. Individualization of HD-MTX to achieve predefined steady-state plasma concentrations (Cpss) will allow optimization of the antileukemic effect and reduction of its toxicity (Pui et al. 2012). Here, we have investigated the effect of genetic polymorphism of *ABCG2* C421A on plasma levels at 23, 42, and 68 h after 24 h infusion of HD-MTX in Egyptian pediatric ALL patients, depending on the fact that MTX plasma level was actually used in previous studies as a quantifiable measure of toxicity (Lopez-Lopez et al. 2011).

Materials and methods

Patients

Two hundred ALL patients were recruited from CCHE-57357. Patients were included if they were newly diagnosed Egyptian patients with ALL, age <18 years, and homogeneously treated according to St. Jude Total XV protocol (Pui et al. 2009) without the up-front window phase. Patients were excluded if they were nonEgyptian ALL patients, age <1 year, had Down's syndrome, were seropositive for hepatitis B or hepatitis C, or had renal impairment (Grade I or higher).

Ethics statement The ethical committee of CCHE approved the study and a written informed consent was obtained for each patient's guardian according to the guidelines of the Helsinki Declaration.

The treatment protocol started with remission–induction phase (42 days) in which patients took prednisone, vincristine, doxorubicin, asparaginase (*E. coli*), cyclophosphamide, 6-mercaptopurine, and cytarabine. The patient also received triple intrathecal therapy with hydrocortisone, cytarabine, and MTX as a central nervous system-directed treatment based on the patient's central nervous system status. According to patient initial characteristics and response at remission date (day 42), each patient was assigned to low-risk, standard-risk, or high-risk at the end of induction (Pui et al. 2009). Consolidation phase (8 weeks) followed the induction period. It consisted of four cycles of HD-MTX given every other week. Low-risk or standard-/high-risk patients received 2.5 or 5 g MTX/m^2/24 h, respectively. Along with HD-MTX, oral mercaptopurine (50 mg/m^2/day) was given for 8 weeks to all risk groups. Triple intrathecal administration was given on the day of HD-MTX administration. For HD-MTX administration, one-tenth of the total HD-MTX dose (loading dose) was given over 1 h infusion and the remaining nine-tenths of the dose given via continuous infusion over 23 h. Before HD-MTX administration, intravenous prehydration crystalloid infusion (at 100 or 125 ml/m^2/h for low-risk or standard-/high-risk, respectively) with sodium bicarbonate was given over 12 h. Patients started HD-MTX if urine pH was ≥6.5 (Pauley et al. 2013).

Leucovorin rescue was given at 42 h from the start of the HD-MTX administration. The low-risk group received 10 mg/m^2 of leucovorin every 6 h for 5 doses, while the standard- and high-risk groups received 15 mg/m^2 every

6 h for 5 doses. The leucovorin dose was adjusted if the methotrexate plasma concentration was >1 μM at 42 h and/or >0.1 μM at 68 h (Pauley et al. 2013).

Genotyping for *ABCG2* by RFLP

Genomic DNA (gDNA) was extracted from peripheral blood specimens using salting-out procedure (Miller et al. 1988). Briefly, 200 ng of gDNA was amplified using primers flanking the regions of BCRB C421A polymorphism (rs2231142, Gln 141 Lys).

Primers sequences were:

BCRP Ex 5 f TGT TGT GAT GGGCAC TCT GATG
BCRP Ex 5 r ATCAGAGTCATTTTATCCACAC

A 25-μl PCR containing 0.5 μM of each primer, 50 ng of gDNA, and 12.5 μl AmpliTaq Gold® PCR master mix (Applied Biosystems, Branchburg, NJ, USA) was subjected to cycling conditions of 10 min at 95 °C and 3 steps of 30 s 94 °C, 30 s 53 °C, and 30 s 72 °C for 35 cycles, followed by final extension 7 min. at 72 °C. The expected product was a 222-bp fragment containing the 421C>A variant in exon 5. About 18 μl of the PCR product (222 bp) was digested by TaaI (HpyCH4 III) overnight. Digestion of the PCR product for *ABCG2* 421 C/A gene polymorphism yielded bands of 39 and 183 bp in CC wild type, 222 bp in AA homozygotes, and all 3 bands (222, 183, and 39 bp) in CA heterozygotes; digested fragments were visualized after electrophoretic separation (100 V for 40 min.) on a 3 % agarose gel using ethidium bromide stain (Meissner et al. 2006).

Serum concentration of MTX and toxicity level

MTX serum concentration at 23, 42, and 68 h after the start of its infusion was routinely determined daily by a fluorescent polarization immunoassay (Hayashi et al. 2008) on a TDX system (Abbott Laboratories, Abbott Park IL 60064, USA) and was followed until concentration was below 0.05 μM. A total number of 2,032 MTX measurements in 800 cycles of HD-MTX therapy were examined in this study. The cutoff toxic plasma level of HD-MTX, according to protocol definition of the MTX concentrations at each time measured, was (a) at 42 h: (≥1 μM) for all risk groups and (b) at 68 h: (≥0.1 μM) for all risk groups.

MTX concentration at 23 h was taken as an indicator of Cpss, which was 33 μM and 65 μM for low-risk and standard-/high-risk, respectively.

Statistical analysis

Data management and analysis were performed using the statistical package for social sciences (SPSS) version 17 software packages (IBM Corp., USA, 2010). Comparisons between groups with respect to numeric variables were done using the nonparametric Mann–Whitney test. The chi-square test or the Fisher's exact test for small sample size was used to compare between groups with respect to categorical data (Dawson and Trapp 2000). All *P* values are two-sided. *P* values ≤0.05 were considered significant.

Table 1 Clinical characteristics of 200 studied subjects

Characteristics	Subcategory	Result (%)
Age[a]		(1–18, 4.5 years)
Age group	<10 years	157 (78.5)
	≥10 years	43 (21.5)
Sex	Male	115 (57.5)
	Female	85 (42.5)
Immunophenotyping	B-lineage	168 (84)
	T-lineage	32 (16)
Risk	Low-risk	85 (42.5)
	Standard-risk	97 (48.5)
	High-risk	18 (9)
MTX dosage	2.5 g/m^2	85 (42.5)
	5.0 g/m^2	115 (57.5)
Genotype	CC	171 (85.5)
	CA	28 (14.0)
	AA	1 (0.5)

[a] Age expressed as (range, median)

Results

Patients' clinical characteristics

Clinical characteristics of patients participating in the study are summarized in Table 1. Patients' age ranged from 1 to 18 years (median age 4.5) where 78.5 % of patients were younger than 10 years old.

Genotyping analysis for *ABCG2* C421A genetic polymorphism and MTX plasma measurements

Wild type (*CC*) was detected in 171 patients (85.5 %), and 28 patients (14.0 %) were heterozygous type (*CA*) as shown in Fig. 1a. Out of the 200 patients, only one (0.5 %) was homozygous type (*AA*) as shown in Fig. 1b.

For low-risk Group: the percentage of patients who achieved Cpss (33 μM) at 23 h was 18.0 % out of 82 patients.

For standard- and high-risk groups: the percentage of patients who achieved Cpss (65 μM) at 23 h was 13.4 % of 112 patients. In six patients, the 23-h readings were not collected.

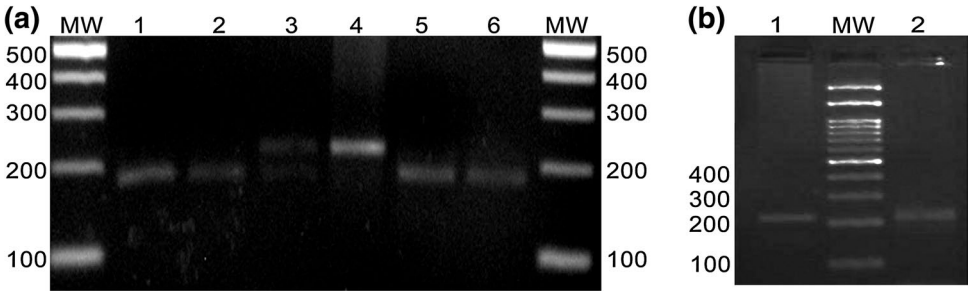

Fig. 1 Detection of the 421C/A polymorphism of *ABCG2* gene using PCR–RFLP. **a** Bands of 39 and 183 bp in CC homozygote wild type (lanes *1*, *2*, *5,* and *6*) and all 3 bands (222, 183, and 39 bp) in CA heterozygote (lane *3*), lane (*MW*) molecular weight ladder standard (100–500 bp), and undigested control (lane *4*). **b** Undigested control (lane *1*), lane (*MW*) molecular weight ladder standard (100–400 bp), and band of 222 bp in AA homozygote (lane *2*)

Table 2 MTX toxic levels at different time points tested and the *ABCG2* 421C>A genetic polymorphism

Hours/level	Low-risk (2.5 g/m^2)		*P* value	Hours/level	Standard-/high-risk (5.0 g/m^2)		*P* value
	Wild *n* (%)	Variant allele *n* (%)			Wild *n* (%)	Variant allele *n* (%)	
42/(≥1)	56 (70)	2 (40)	0.321	42/(≥1)	85 (93.4)	22 (91.7)	0.672
42/(<1)	24 (30)	3 (60)		42/(<1)	6 (6.6)	2 (8.3)	
68/(≥0.1)	73 (91.2)	4 (80)	0.398	68/(≥0.1)	89 (97.8)	23 (95.8)	0.508
68/(<0.1)	7 (8.8)	1 (20)		68/(<0.1)	2 (2.2)	1 (4.2)	

Data were compared using the chi-square or Fisher's exact test

As shown in Table 2, for the *low-risk group*: 58 patients had plasma MTX level (>1 μM) at 42 h of HD-MTX administration but two patients were with variant allele of *ABCG2* 421C>A genetic polymorphism. At 68 h, there were 77 patients who had delayed clearance (plasma MTX level >0.1 μM) with 4 patients of variant allele.

*For standard- and high-risk gro*ups: at 42 h, there were 107 patients had plasma MTX level (>1 μM) with 22 patients of variant allele. While at 68 h, there were 112 patients with 23 patients of variant allele.

We tested the association between the *ABCG2* 421C>A genetic polymorphism and the plasma toxic levels of MTX at 42 and 68 h after the intravenous infusion. We found no statistical significance between *ABCG2* C421A polymorphism and the number of patients who had MTX levels in the toxic range for *low-, standard-, or high-risk groups* at the various time points tested as shown in Table 2.

Furthermore, Table 3 summarizes that there was no statistical significance between *ABCG2* 421C>A SNP and HD-MTX Cpss at 23 h in the risk groups.

Table 3 Association between *ABCG2* 421C>A genetic polymorphism and steady-state plasma concentration (CPss)

Risks	*ABCG2* (421C>A)	(CPss = 33 μM)		*P* value
Low-risk (2.5 g/m^2)		Yes	No	
	Wild *n* (%)	14 (17.9)	64 (82.1)	0.57
	Variant allele *n* (%)	1 (25)	3 (75)	
Standard-/high-risk (5.0 g/m^2)	*ABCG2* (421C>A)	(CPss = 65 μM)		*P* value
		Yes	No	
	Wild *n* (%)	10 (11.4)	78 (88.6)	0.31
	Variant allele *n* (%)	5 (20.8)	19 (79.2)	

Data were compared using the chi-square or Fisher's exact test

Discussion

In the present work, we evaluated the correlation between 421C>A in the *ABCG2* gene, that is, involved in the cellular outward transport of MTX, with HD-MTX plasma toxic levels in 200 Egyptian children with newly diagnosed ALL. It is well known that most contemporary protocols for ALL treatment use a combination of chemotherapy, generally imparting a survival rate of above 90 % (Pui et al. 2009). HD-MTX is a critical antileukemic drug that does its job by killing rapidly dividing cells and does not differentiate between cancer and healthy cells. As a result, HD-MTX may lead to toxicity that can prolong hospitalization and increase the economic burden of ALL treatment, which overall lasts from 2.5 to 3 years. Therefore, continued research is needed to determine how best to reduce side

effects and toxicities from HD-MTX, which are currently reported to be about 5 % (Schmiegelow 2009).

Previous reports on 421C>A SNP in *ABCG2* gene revealed that its frequency was within broad range across different ethnic populations. In these reports, the allelic variant of this SNP was found to have low frequency in African Americans (2–5 %). Moderate frequency was detected in European populations (11–14 %), Hispanic (10 %), and those of Middle Eastern (13 %) descent, while high frequency (>30 %) was observed in those of Chinese and Japanese ancestry (Zamber et al. 2003; Lepper et al. 2005). In line with these reports, we found the allelic variant of the *ABCG2* 421C>A SNP to be (14.5 %) in our institution (Zamber et al. 2003; Lepper et al. 2005).

Vlaming et al. (2009) showed the functional overlap or complementary effect of the multidrug transporters *ABCC2*, *ABCC3,* and *ABCG2* in the rapid elimination of MTX and its toxic metabolite 7-hydroxymethotrexate. They explained that decreased expression of one of these transporters does not affect MTX clearance, as it requires the deficiency of expression of all three proteins. Other studies, contradictory to this one, showed that certain genetic mutations of *ABCC2* gene had an impact on the cellular outward transport and elimination of MTX (Hulot et al. 2005; Rau et al. 2006; Simon et al. 2013). But regarding the multidrug transporter *ABCG2* gene, only Imanishi et al. (2007) studied the correlation between HD-MTX toxicity (3 g/m^2) and the genetic polymorphism C421A in *ABCG2* gene for 26 patients (20 ALL patients, 6 malignant lymphoma patients). They did not find a significant correlation between MTX serum concentration at 48 h after the start of MTX infusion and this SNP. As the number of patients was very small, a definitive conclusion regarding this SNP is difficult to ascertain.

We tested the toxicity of HD-MTX at two time intervals 42 and 68 h after HD-MTX administration over 24 h infusion in correlation with the *ABCG2* C421A genetic polymorphism in 200 Egyptian ALL patients. Out of the 29 patients with variant allele, there were 5 patients in *low-risk* group and 24 patients in *standard-/high-risk* group.

There is only one patient in low-risk group who is homozygous (*AA*) for *ABCG2* C421A genetic polymorphism. His plasma levels at the two time intervals 42 and 68 h after HD-MTX administration were higher than predefined cutoff. Given the extremely low incidence of homozygous patients, a statistical significance cannot be concluded.

As shown in Table 2, although the number of patients with variant allele experiencing toxicity in standard-/high-risk group was higher compared with the low-risk group, we did not find a statistically significant association of the genetic polymorphism C421A in *ABCG2* gene and the HD-MTX plasma level. This is in line with previously described findings (Imanishi et al. 2007). We also investigated the association between *ABCG2* 421C>A SNP and Cpss of HD-MTX at 23 h of its administration in both groups but we found no statistical association (Table 3). Moreover, we did not find any statistical significant association between Cpss at 23 h of HD-MTX and age or sex of patients.

Apart from this SNP, we observed an increase in the number of patients who had toxicity and delayed excretion in both low-risk group and standard-/high-risk group at 68 h. This may be explained based on the posthydration or pH of urine but in general, it needs more investigation as it leads to prolonged hospitalization.

In recent years, the introduction of the science of "personalized medicine" and the fact that one dose does not fit all shed light on more progress in HD-MTX pharmacogenomic research worldwide. But there are small number of Egyptian studies that fill the gap between genotype and phenotype to personalize treatment and refining the use of HD-MTX for optimum cure with minimum toxicity in the treatment protocols used for Egyptian children with ALL (Tantawy et al. 2010; El-Khodary et al. 2012). It was proved that the infusion of HD-MTX over 24 h has a great impact on the antileukemic effect of this drug (Mikkelsen et al. 2011). In our study, we measured Cpss at 23 h to identify the percentage of patients who achieved target value of Cpss in the risk groups. We found that 17.6 % of the low-risk group (85 patients) achieved the target level (33 μM). On the other hand, 13 % of the standard-/high-risk group (115 patients) achieved the target level (65 μM). Based on the recently published work from St. Jude Children's Research Hospital (Pauley et al. 2013) that recommended adjusting HD-MTX dose of subsequent cycles based on measuring patient's MTX clearance at the first cycle, we are working to individualize the administration of HD-MTX, especially for the standard-/high-risk group. Although this will add to the economic burden of ALL treatment, especially in a charity hospital such as ours, however, it will optimize efficacy of HD-MTX given to ALL patients, ultimately decreasing the costs of treating relapsed disease, and achieve the mission of the hospital.

Blueprint for future direction(s)

We are working to individualize HD-MTX administration. Also, as the science moves faster from single-candidate gene association studies to genome-wide studies, we are aiming of performing genome-wide studies for HD-MTX in Egyptian ALL patients to fill the gap between genotype and phenotype. Finally, we seek try to establish a National Childhood Leukemia Genetics Consortium (NCLGC) that

includes professionals with expertise in pediatric leukemia all over Egypt to encourage more detailed large studies and give recommendations of dose modifications and apply results into practice based on our own data. The need of this is increasing due to the high number of leukemia patients diagnosed every day. For example, according to the data from CCHE-based cancer registry, the percentage of newly diagnosed ALL patients is between 220 and 280 patients per year.

Conclusion

To our Knowledge, this study is the largest study performed on Egyptian pediatric patients with ALL. We found no effect of *ABCG2* 421C>A on the toxic plasma level of HD-MTX. We are working to individualize HD-MTX administration to comply with recent practice. We are aiming of performing genome-wide studies for HD-MTX. It is included in the blueprint research at CCHE as this will pave the way to give recommendation of dose modification based on our own data.

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Conflict of interest None.

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