THE INFLUENCE OF MTHFR C677T POLYMORPHISM ON METHOTREXATE TOXICITY IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

ВЛИЈАНИЕТО НА Ц677Т ПОЛИМОРФИЗМОТ НА ГЕНОТ ЗА МТХФР ВРЗ ИНЦИДЕНЦИЈАТА НА ТОКСИЧНИТЕ ЕФЕКТИ ОД ВИСОКИТЕ ДОЗИ МТХ КАЈ ДЕЦА СО АКУТНА ЛИМФОБЛАСТНА ЛЕУКЕМИЈА

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Abstract

In current protocols for treatment of acute lymphoblastic leukemia in childhood methotrexate (MTX) is one of the crucial cytostatics. The occurrence of MTX toxicity is still a great problem because of the interpatient differences in drug metabolism. These differences may be due to polymorphisms of genes involved in the folate metabolism. The present study was carried out to determine the prevalence of MTHFR C677T polymorphism in children with acute lymphoblastic leukemia (ALL). Also the effect of the genotype on the toxic effects during therapy with high doses of MTX in 45 patients with ALL treated in accordance with the protocol ALL BFM 95 and ALL BFM 2000 was evaluated. All 45 patients with ALL were genotyped for MTHFR C677T polymorphism. Correlation with the presence of a certain polymorphism and toxic effects of the chemotherapy with high doses of MTX was made in the Department for hematology and oncology at the University Clinic for children's diseases – Skopje. The control group included 32 healthy patients. In the study group 24 (53.3%) children had a wild type of polymorphism (CC), 15 (33.33%) children were heterozygous (CT) for MTHFR C677T polymorphism, and 6 (13.33%) children were homozygous for the variant type of the polymorphism (TT). The correlation of the genotype with MTX toxicity indicated a statistical significance only for oral mucositis, while for the other toxic effects there was no statistically significant correlation. In our study the results indicated that oral mucositis was statistically significantly more frequently identified in the case of the variant carriers for this polymorphism. For the other toxic effects caused by the therapy with high doses of MTX no statistically significant correlation with MTHFR C677T polymorphism was identified. This occurrence maybe due to the small number of patients analyzed in this study and the possible protective influence of other genetic polymorphisms included in the folate metabolism, which were not subject to consideration of this study.

Key words: acute lymphoblastic leukemia, methotrexate, toxic effects, MTHFR, polymorphisms

Извадок

Во актуелните протоколи за третман на акутна лимфобластна леукемија во детската возраст метотрексатот (МТХ) е еден од круцијалните ци-

тостатици. Предвидувањето на појавата на токсични ефекти во тек на терапијата со МТХ претставува сè уште голем проблем заради индивидуалните разлики во метаболизмот на лекови кај пациентите. Овие разлики можеби се должат на присуство на полиморфизми на гените вклучени во фолатниот метаболизам. Целта на студијата беше да се одреди инциденцијата на МТХФР Ц677Т полиморфизмот кај децата со акутна лимфобластна леукемија (АЛЛ) и да се анализира влијанието на генотипот врз манифестацијата на токсичните ефекти во тек на терапија со високи дози МТХ кај пациенти со АЛЛ третирани по протоколот АЛЛ БФМ 95. Беше вклучена и контролна група од 32 здрави испитаници. Беше направена генотипизација за полиморфизмот Ц677Т кај 45 пациенти со АЛЛ и негова корелација со токсичните ефекти од хемотерапијата со високи дози МТХ анализирани од болничките истории на пациенти со АЛЛ лекувани на Одделот за хематологија и онкологија при ЈЗУ Универзитетска клиника за детски болести - Скопје. Во студиската група 15 (33,33%) пациенти беа хетерозиготи (ЦТ) за полиморфизмот Ц677Т на генот МТНФР, а 6 (13,33%) пациенти хомозиготи (ТТ). Во контролната група 10 испитаници (31,25%) беа хетерозиготи за полиморфизмот (ЦТ), а 6 испитаници (18,75%) хомозиготи (ТТ). Корелацијата на генотипот со токсичните ефекти од високите дози на МТХ покажа статистичка сигнификантност само за орален мукозит, додека беше статистички несигнификантна за останатите токсични ефекти. Оваа појава можеби се должи на малата група пациенти којашто беше анализирана во оваа студија и можното протективно влијание на други генски полиморфизми вклучени во фолатниот метаболизам, а кои не беа предмет на оваа студија.

Клучни зборови: акутна лимфобластна леукемија, метотрексат, токсични ефекти, МТХФР, полиморфизми

Introduction

The acute lymphoblastic leukemia (ALL) is the most common type of cancer in childhood and it accounts for almost one third of all malignant diseases in children. The optimization of the chemotherapy regimens in the treatment of ALL led to complete remission in 95% of patients during treatment, and in 80% thereof a total success of the treatment is noted. Once the main study groups reached comparable results in the treatment of childhood ALL with current protocols, the focus of their attention is the reduction of acute and late toxic effects of chemotherapy, as well as the improvement of the outcome in case of patients with poor prognosis[1]. Predicting the toxic effects of chemotherapy is difficult taking into consideration the large differences between patients in terms of pharmacokinetics and pharmacodynamics of anti-leukemic agents. This diversity can, to some extent, be linked to sequence variations in genes involved in drug absorption, excretion, cellular transport and effector targets or target pathways.[2] Pharmacogenetics, the study of genetic variations in drug processing genes, may be used as a tool to further improve the treatment of childhood ALL as well to predict toxic effects of chemotherapy.

In all protocols for treatment of ALL, methotrexate (MTX) is one of the crucial cytostatics. It inhibits the function of the enzyme dihydrofolate reductase (DHFR), inhibiting the folate metabolism and indirectly interrupting the function of the enzyme methylenetetrahydrofolate reductase (MTHFR). MTHFR enzyme catalyzes the reduction of 5,10-methylenetetrahydrofolate required for purine and thymidine synthesis to 5- methylenetetrahydrofolate which is required for protein synthesis and nucleic acid methylation. Alterations in reduced folate pools, as a consequence of changes in MTHFR activity, may have a significant effect on the responsiveness of malignant and non-malignant cells to MTX. It has been proposed that alterations in intracellular folate pool could increase the toxic effects of MTX [3].

One of the most common polymorphisms of the MTHFR gene is the C677T polymorphism. MTHFR C677T polymorphism is characterized by replacement of the amino acid alanine with valine leading to reduced activity and thermolability of the enzyme[3]. The activity of MTHFR enzyme in the variant carriers of this polymorphism (TT) is 30%, and in heterozygous cases (CT) is 60% of the normal activity of the enzyme [4,5].

The frequency of MTHFR C677TT genotype is often reported to be high in European, Asian Central and South American (10-32%) populations, low in different African populations (0-3%) and also showing geographical gradients among Chinese Han populations [4].

Several studies in ALL have suggested that variations in single nucleotide polymorphisms of genes involved in folate metabolism contribute to the inter-individual variation in MTX toxicity [6,7]. The results are still controversial.

The aim of this study was to identify the distribution of MTHFR C677T polymorphism in children with ALL and to analyze the influence of this polymorphism over the manifestation of toxic effects during therapy with high doses of MTX in patients with ALL treated according to ALL BFM 95 and ALL BFM 2000 protocols.

Material and methods

This was a retrospective study comprising 45 children (stratified in standard and medium risk groups) treated with high doses of MTX(5g/m^2) for ALL at the University Children's Hospital, Department of Hematology and Oncology in Skopje as a study group and 32 healthy volunteers as a control group. The study was approved by the National Ethics Committee of the Republic of Macedonia and informed consent was obtained by parents and patients before inclusion in the study.

Toxicity assessment

The data were collected from medical records of patients with ALL treated according to ALL BFM 95 and ALL BFM 2000 protocols. To evaluate the occurrence of toxic effects during therapy with high doses of MTX a total number of 180 cycles of high doses MTX were analyzed. Every patient has received four cycles of high doses MTX, each separated by a period of two weeks. The toxic effects were analyzed according to the toxicity criteria from the protocol ALL BFM 95 and ALL BFM 2000. We collected the following data for each course of the treatment: presence of mucositis (oral and intestinal), hemoglobin level, leukocyte and thrombocyte count, presence of neurotoxicity signs (peripheral and central), skin changes and level of liver enzymes. Subsequently, the toxic effects were analyzed in correlation to the genotype.

Isolation of DNA

From each patient 3 ml peripheral blood with EDTA as anti-coagulant were taken. Subsequently, a standard salting – out protocol with 5M NaCl was used to isolate genomic DNA from peripheral leukocytes obtained from venous blood draws.

Genotyping of the C677T polymorphism in the MTHFR gene

Genotyping was conducted in line with the following procedure:
The region of the MTHFR gene was amplified by polymerase chain reaction using appropriate oligonucleotide primer pair (according to Bagheri et al., 2010, ordered from Sigma-Genosys); thermostable Taq polymerase, PCR buffer factory prepared with magnesium ions, a mixture of deoxynucleotides (dNTP) and a sample of DNA from a subject in reactive test tubes with thin walls. Amplification program is used in PCR-machine (Perkin-Elmer GeneAmp System 2400). The success of the amplification was verified by horizontal agarose electrophoresis and fluorescence staining of the gel with ethidium bromide. The gel is photographed under UV - light (312 nm) with a digital camera (Canon).
The digital analysis (identification of electrophoretic bands and determining their length in base pairs) was carried out by option for analysis of one-dimensional gels software Image J of NIH. The amplification product has a length of 265 base pairs (bp). Amplified products were digested with restriction endonuclease Hinfl under optimal conditions in order to determine the genotype of the C677T polymorphism (wild type – CC, heterozygous - CT and variant type – TT).
For the purposes of the statistical data analysis, the software SPSS for Windows 13,0 was used. Fisher's exact test was used for testing the significance of the differences.

Results

After performed genotyping in both groups and after analysis of the toxicity occurrence in 45 patients with ALL and correlation thereof with the genotype, the following results have been obtained.
The prevalence of the polymorphism in the study and control group is provided in Table 1.
The results from the analyzed distribution of MTHFR C677T polymorphism in the study group indicated that 24 (53.33%) patients were carriers of the wild genotype (CC), 15 (33.33%) were heterozygous (CT), and the remaining 6 (13.33%) were homozygous for the variant type of the polymorphism (TT).
In the control group of 32 healthy analyzed subjects, the following results have been obtained: 16 persons (50%) were carriers of the wild genotype (CC), 10 persons (31.25%) were heterozygous for the polymorphism (CT), and 6 persons (18.75%) were homozygous for the variant type (TT).
Toxic effects were analyzed in all four phases of the M protocol in correlation to the genetic profile of the patients who had shown toxic effects. The results obtained are presented in Tables 2, 3, 4, 5.

MTHFR C677T polymorphism	Study group	Control group
CC genotype (wild)	24 (53.3%)	16 (50%)
CT genotype(heterozygous)	15 (33.33%)	10 (31.2%)
TT genotype(variant)	6 (13.33%)	6 (18.75%)

Table 1: Prevalence of the MTHFR C677T polymorphism

Toxic effects Cycle No. I	MTHFR n=24 (wild polymorphism CC)	MTHFR n=15 (heterozygote CT)	MTHFR n=6 (variant TT)	p-value
Neutropenia	7 (29.17%)	1 (6.67%)	2 (33.33%)	p=0.19
Peripheral neuropathy	1 (4.17%)	0	0	
Allergy	3 (12.5)	0	0	
Thrombocytopenia	6 (25%)	1 (6.67%)	0	p=0.21
Anemia	5 (20.83%)	2 (13.33%)	1 (16.67%)	p=0.86
Hepatotoxicity	11 (45.83%)	7 (46.67%)	4 (66.67%)	p=0.65
Intestinal mucositis	2 (8.33%)	0	1 (16.67%)	p=0.24
Oral mucositis	7 (29.17%)	5 (33.33%)	3 (50%)	p=0.62

p (Fisher exact test for 3 x 2 groups)

Table 2: Toxic effects in correlation with genotype (after first dose MTX)

Toxic effects Cycle No. II	MTHFR n=24 (wild polymorphism CC)	MTHFR n=15 (heterozygote CT)	MTHFR n=6 (variant TT)	p-value
Neutropenia	7 (29.17%)	2 (13.33%)	1 (16.67%)	p=0.68
Skin toxicity	0	1 (6.67%)	0	
Thrombocytopenia	5 (20.83%)	2 (13.33%)	0	p=0.62
Anemia	5 (20.83%)	2 (13.33%)	0	p=0.62
Hepatotoxicity	8 (33.33%)	7 (46.67%)	3 (50%_	p=0.63
Intestinal mucositis	3 (12.5%)	1 (6.67%)	0	p=1.0
Oral mucositis	1 (4.17%)	3 (20%)	3 (50%)	**p=0.018**
Cardiotoxicity	1 (4.17%)	0	0	

p (Fisher exact test for 3 x 2 groups)

Table 3: Toxic effects in correlation with genotype (after second dose MTX)

Toxic effects Cycle No. III	MTHFR n=24 (wild polymorphism CC)	MTHFR n=15 (heterozygote CT)	MTHFR n=6 (variant TT)	p-value
Central neurotoxicity	1 (4.17%)	0	0	
Skin toxicity	1 (4.17%)	0	0	
Thrombocytopenia	7 (29.17%)	4 (26.67%)	1 (16.67%)	p=1.0
Neutropenia	9 (37.5%)	4 (26.67%)	3 (50%)	p=0.63
Anemia	7 (29.17%)	4 (26.67%)	3 (50%)	p=0.61
Hepatotoxicity	10 (41.67%)	6 (40%)	2 (33.33%)	p=1.0
Intestinal mucositis	1 (4.17%)	0	0	
Oral mucositis	3 (12.5%)	3 (20%)	2 (33.33%)	p=0.36

p (Fisher exact test for 3 x 2 groups)

Table 4: Toxic effects in correlation with genotype (after third dose MTX)

Toxic effects Cycle No. IV	MTHFR n=23 (wild polymorphism CC)	MTHFR n=15 (heterozygote CT)	MTHFR n=6 (variant TT)	p-value
Neutropenia	11	7	1 (16.67%)	p=0.45
Anemia	3 (13.04%)	2	0	p=1.0
Hepatotoxicity	10	4	3	p=0.48
Intestinal mucositis	1 (4.35%)	0	0	
Oral mucositis	1 (4.35%)	1 (6.67%%)	2	p=0.11

p (Fisher exact test for 3 x 2 groups)

Table 5: Toxic effects in correlation with genotype (after fourth dose MTX)

The results of the analysis indicated that the oral mucositis was more often in carriers of the variant type for this polymorphism, but it was statistically significant more often only after the application of the second dose of MTX (p=0.018).
As regards to the correlation of the other toxic effects in correlation to the genotype, no statistical significance was identified among the three groups.

DISCUSSION

High doses of MTX are effective in the treatment of many malignant diseases, especially leukemias and lymphomas. MTX after polyglutamation in the cell by the enzyme folylpolyglutamate synthetase in the form of MTX polyglutamate blocks the DHFR enzyme, which catalyzes the conversion of folate into its active form tetrahydrofolate. In addition, MTX polyglutamates inhibits the activity of other enzymes involved in folate metabolism including MTHFR. The incidence of toxic effects during treatment with MTX is characterized by interindividual and interethnic variations and may influence the clinical course of the disease[8]. Due to the established prognostic factors in children with ALL, the number of relapses and fatal outcomes in these patients is limited. The gene polymorphisms involved in the metabolism of MTX are reported as one of the possible predictive factors for the

manifestation of toxic effects due to the application of this agent.
C677T polymorphism is one of the most investigated polymorphisms of genes involved in folate metabolism. Its association with cardiovascular diseases, dementia, neural tube defects, autism, recurrent spontaneous abortion, etc, has been subject to research. In many studies related to the pediatric oncology, its association with the occurrence of ALL, with the manifestation of the toxic effects of high doses of MTX, with the risk of relapse as predictive for outcome of therapy, has been studed.
In the meta-analysis of Lin Yang it is reported that MTHFR C677T polymorphism is associated with a significantly increased risk of manifestation of toxicity during therapy with high doses of MTX, especially hepatotoxicity, myelosuppression, oral mucositis, gastrointestinal toxicity and skin toxicity. During stratification of patients according to their ethnicity they identified that among African and White population the association between this polymorphism and hepatotoxicity was more common, while it was not present in the Asian population. On the other side, oral mucositis was more common in patients - carriers of this polymorphism in African and Asian populations[9]. In a meta-analysis of nine studies conducted by Lopez-Lopez E. an association of this polymorphism with the toxic effects of high doses of MTX was identified. In three of them the association of this polymorphism with hepatotoxicity, renal toxicity and intestinal mucositis was determined, while in two studies the association of this polymorphism with thrombocytopenia, neutropenia and oral mucositis was reported[4].
On the other side, the studies of Shimasaki and Seidemann failed to confirm the association between MTHFR C677T polymorphism and the toxic effects of high doses of MTX [10,11].
In our study the results showed that oral mucositis was more common in carriers of the variant type (MTHFR 677TT) of this polymorphism, but this was statistically significant only after the application of the second dose of MTX ($p = 0.018$). This results are in agreement with the results of the Faganel's study, which showed significant association between the MTHFR C677T polymorphism and mucositis [12]. For the other toxic effects caused by the treatment with high doses of MTX no statistically significant correlation with genotype was identified. This phenomenon might be due to the small group of patients that were analyzed in this study and the possible protective influence of other gene polymorphisms involved in folate metabolism, which were not the subject of this study.
Regarding the prevalence of the C677T polymorphism our results were similar to the prevalence of this polymorphism reported for the European population in the literature [4], both in the group of patients with ALL and the control group.
Several authors discussed that the discrepancy between studies regarding the influence of polymorphisms on the incidence of toxic effects might be due to the influence of external factors, nutrition (folate status) of the patients and the concurrent medications[12,13]. In addition, the importance of the interaction between many genes involved in folate metabolism and their impact on the modification of the sensitivity of high doses of MTX is being emphasized. A number of studies have been dedicated to the study of gene-gene interaction, including two or more genes in association with the toxic effects of MTX, the risk of relapse and event-free survival in patients with ALL.
It is considered that the genotyping of polymorphisms involved in folate metabolism is necessary in order to optimize MTX therapy resulting in minimization of the toxic effects of treatment. This will lead towards improvement of the tolerance of the agent and achieving better results in terms of survival[9].

Conclusions

Nowadays almost 90% of children with ALL can be cured with the existing therapy protocols. However, better treatment for all children, 10% of patients who cannot be cured and 90% of those treated with cytotoxic drugs is still subject to research. The genotyping of polymorphisms involved in folate metabolism is considered to be necessary in the future before initiating therapy in any patient with ALL. It will open possibility of individual drug dose adjustment: patients who have lower chemotherapy toxicity and lower event free survival might benefit from increase of the drug dosage. But, it seems that genotyping of only one polymorphism is not sufficient; more and more emphasis is dedicated to the importance of the interaction between genes involved in folate metabolism and their impact on the modification of the sensitivity of high doses of MTX.
Genetics has played an important role in the last 25 years in the treatment of ALL and it is promising an opportunity for a radical change in the treatment of ALL in the next decade by providing diagnostics that will lead to individualization of therapy - an opportunity for each patient to have an individual dosage and pattern of specific

combination of chemoterapeutics. Each patient will receive the necessary treatment in accordance to his/her own genotype, with minimal risk of adverse toxic effects, failure of therapy, risk of relapse of the disease and an opportunity for good quality of life in the future.

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